Source of Prolactin in Human Follicular Fluid

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Abstract. To analyze whether prolactin (PRL) in human follicular fluid (FF) is synthesized locally or derived from the circulation, PRL concentrations of plasma and FF were determined in the patients after ovarian stimulations. The amounts of PRL messenger ribonucleic acid (mRNA) in the follicular tissues during different menstrual phases were also determined. The FF PRL concentration was correlated positively with plasma PRL and highest estradiol levels during the stimulatory cycle. No PRL mRNA sequence was detected in the RNAs extracted from follicles at any stage in the menstrual cycle, although β-actin mRNA was detected in all samples. In a comparison with pituitary RNA, the PRL mRNA concentration in ovarian follicular tissues seemed to be 10,000 times less than that in the pituitary. These results suggest that FF PRL may not be synthesized locally, but derived from the pituitary via the circulation through passive diffusion, and thus regulated by estrogen.

Key words: PRL, Follicular fluid, Messenger ribonucleic acid, Estrogen.

PROLACTIN (PRL) is a peptide hormone synthesized in mammatrophs in the anterior pituitary [1–3], and it stimulates the mammary glands [4] and regulates ovarian functions [5]. Although ovulatory and/or luteal functions are disturbed in patients with hyperprolactinemia [5], it has not been determined clearly whether PRL acts on the central gonadotropin regulatory regions such as hypothalamus and pituitary, on peripheral organs such as the ovary directly, or on both. McNatty et al. [6] reported that human follicular fluid (FF) contained a significant amount of PRL, and recent in vivo [7] and in vitro [8] studies indicated that PRL in FF might play important roles in luteal function.

On the other hand, it has been reported that some tissues other than pituitary also synthesize PRL locally. For example, PRL synthesis has been detected in decidual tissues of the placenta by a cell culture system [9] or measurement of its messenger ribonucleic acid (mRNA) [10]. The high PRL concentration in the amniotic fluid [11] seems to be maintained by secretion from decidua. Ectopic PRL synthesis and secretion have been observed also in some neoplastic tissues [12]. These results suggest the possibility that PRL is synthesized not only in the pituitary but in other tissues also. And the following questions have not been answered: (1) Is the FF PRL synthesized locally in the ovarian cells such as granulosa or theca cells, or derived from circulating PRL secreted by the pituitary gland?, and (2) How is the level of PRL in FF regulated?

In the present study, we analyzed the correlation between plasma and FF PRL concentrations to answer the first question. Moreover, PRL mRNA

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levels in ovarian follicles at various menstrual and reproductive stages were determined. In answer to the second question, estradiol (E2) and progesterone levels in plasma and FF were measured, and the relationships between the levels of these hormones and the levels of FF PRL were analyzed. The results of these experiments indicated that PRL gene expression in the granulosa and luteal cells is extremely low. FF PRL could be derived from the secretion from the pituitary via the systemic circulation.

Materials and Methods

Patients

Thirteen patients with normal ovulatory cycles were enrolled in the in vitro fertilization (IVF) and embryo transfer (ET) program because of either tubal sterility or idiopathic infertility. All clinical and experimental procedures were performed following informed consent of the patients and permission of the University Committee.

Stimulation regimens and FF aspiration

Development of multiple follicles in the patients was stimulated in the following way. One hundred mg of clomiphene citrate and 150 IU of human menopausal gonadotropin (hMG; Pergonal®, Teikoku-zoki Co., Tokyo, Japan) were given from day 3 to day 7 of the menstrual cycle. The same dose of hMG was administered for a further 3 to 4 days depending on the individual response, which was monitored by daily E2 determination and ultrasonography. Five thousand IU of human chorionic gonadotropin (hCG; HCG®, Mochida Pharmaceutical Co., Tokyo, Japan) was administered intramuscularly when the dominant follicle was at least 18 mm in diameter and plasma E2 levels were above 300 pg/ml. Ultrasonographic aspiration of the follicles was performed 36 h after hCG administration. The aspirated oocytes were identified and fertilized in vitro. The FF was centrifuged and the cell free supernatants were stored at −20°C until analysis of the hormones. Blood samples for PRL, E2 and progesterone assays were obtained just prior to aspiration of the FF. The plasma E2 level for monitoring hMG treatment as described above.

Hormone determination

PRL in plasma and FF were determined with a PRL radioimmunoassay (RIA) kit, and E2 and progesterone were measured with a RIA kit obtained from Daiichi Radioisotope Co., Tokyo, Japan.

RNA extraction from ovarian tissues

The ovarian tissues were obtained from six patients (two in follicular phase, three in the luteal phase and one 9 weeks pregnant), who received operations for myoma uteri or adenomyosis. Immediately after excision of the ovaries, tissues surrounding the dominant follicle or corpus luteum were excised, frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Total RNA was extracted by the acid guanidium thiocyanate/phenol/chloroform method reported by Chomczynski et al. [13]. Poly(A) RNA was isolated by affinity chromatography with oligo(dT) cellulose [14]. The amount of RNA was determined by optical density at 260 nm. Total RNA was also extracted from a pituitary obtained from a 63-year-old male patient who died of non-endocrine diseases.

Analysis of PRL and β-actin mRNAs

PRL and β-actin mRNAs in ovarian and pituitary tissues were analyzed by Northern transfer and hybridization with their complementary deoxyribonucleic acids (cDNAs), as previously described [15]. In brief, 1.5 μg of follicular poly(A) RNAs and 1.5 μg of pituitary total RNA were electrophoresed on 1.0% agarose gel. The RNAs were transferred to nylon paper (Gene Screen Plus, New England Nuclear, Boston, MA), and hybridized with 32P-labeled cDNA probes for PRL [16] or β-actin [17]. The size of the hybridized mRNAs was analyzed by RNA marker (Boehringer Mannheim GmbH, Germany). The PRL and β-actin cDNAs were kindly provided by Dr. Martial and Dr. Singer, respectively.
Results

Concentrations of PRL, E2 and progesterone in plasma and FF in individual patients

The number of follicles aspirated from the 13 patients and hormonal levels in FF and plasma are summarized in Table 1. The number of follicles aspirated from one patient was averaged 4.2, but it ranged from 1 to 10. A total of 55 follicles which contained from 1.3 to 13.5 ml fluid were used for hormone determinations.

The PRL concentrations in FF ranged from 30 to 137 ng/ml. Although the follicular size differed, PRL concentrations in multiple FFs obtained from one patient showed very little variation. FF E2 and progesterone concentrations varied from 123 to 1,450 ng/ml and from 2.1 to 47.8 µg/ml, respectively. Coefficients of variation of both hormones were more than that of PRL.

The PRL concentration in plasma from 10 patients just prior to the FF aspiration was 60±11 ng/ml (mean ± SEM), which is higher than that during the normal menstrual cycle. Concentrations of plasma E2 in 12 patients and progesterone in 11 patients were 941±171 pg/ml and 5.7±1.1 ng/ml, respectively. The highest E2 levels observed 1 to 3 days before FF aspiration were 2,026±309 pg/ml. These peak values induced by ovarian stimulations were much higher than those in the normal menstrual cycle reported previously [18].

Correlations of FF and plasma concentrations of PRL, E2 and progesterone

A significant positive correlation was observed between PRL concentration in FF and plasma (r=0.94, P<0.01) (Fig. 1). It should be noticed that PRL in FF (X) and in plasma (Y) were very similar (Y= 1.05X + 1.77).

There were no significant correlations between the follicular fluid PRL concentration and FF

![Fig. 1. Correlation between follicular fluid and plasma PRL concentrations. The plasma concentration of PRL was determined in the sample obtained just prior to aspiration of the FF.](image)

Table 1. Follicular fluid and plasma hormones in individual patients

<table>
<thead>
<tr>
<th>Patient number</th>
<th>Numbers of aspirated follicles</th>
<th>Follicular fluid</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume (ml)</td>
<td>PRL (ng/ml)</td>
<td>E2 (ng/ml)</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>2.6±1.2</td>
<td>38±3</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>5.4±1.4</td>
<td>12±3</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>5.1±1.7</td>
<td>46±4</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>4.2±0.7</td>
<td>49±2</td>
</tr>
<tr>
<td>5</td>
<td>9</td>
<td>5.3±0.9</td>
<td>47±2</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>2.7±0.5</td>
<td>53±2</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>3.3±0.3</td>
<td>94±6</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>2.3</td>
<td>30</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>3.0</td>
<td>34</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>3.5±0.2</td>
<td>35±1</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>3.2±0.2</td>
<td>52±2</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>3.1±1.0</td>
<td>46±4</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>3.5±0.5</td>
<td>35±3</td>
</tr>
</tbody>
</table>

The results were expressed as the mean ± SEM. Plasma concentrations of PRL, E2, and progesterone were determined in the samples obtained just prior to aspiration of the follicular fluid except "E2 in peak" which was the highest E2 concentration during the ovarian stimulatory cycle.
volume, FF progesterone or plasma progesterone levels. Although FF PRL did not correlate with E2 levels in FF or in plasma collected just before FF aspiration, a positive but weak correlation was observed between the levels of FF PRL and plasma E2 at its peak level (r = 0.60, P < 0.05).

**Relationship of oocyte recovery and successful IVF with hormones in FF**

As shown in Table 2, oocyte was recovered from 37 follicles (67.3%). There was no difference in the hormone levels in FF whether the oocyte was retrieved or not. Successful IVF was achieved in 31 oocytes (83.8%). There was no difference in PRL and progesterone levels between the follicles whose oocytes were fertilized and those of unfertilized ova. However, the E2 level was significantly lower in the follicles whose oocytes were fertilized.

**Detection of PRL mRNA in ovarian follicles**

Table 3 shows the recovery rates of total RNAs from follicular and pituitary tissues, and poly(A) RNAs from total RNAs of follicles. The total RNA recovery was 1.98 ± 0.24 and 1.96 mg/g wet tissue of ovaries and a pituitary, respectively. The recovery rates of poly(A) RNAs were 0.72 to 1.26% of total RNA.

To evaluate whether PRL mRNA could be detected in granulosa cells or luteal cells, analysis by Northern blot and hybridization with PRL cDNA was performed. No PRL mRNA sequence was observed in any of RNAs obtained from ovarian tissues while a single PRL mRNA band at 1.0 kb was observed in a pituitary RNA sample (Fig. 2-A). Ovarian RNAs electrophoresed on the gel contained about 100 times more mRNAs than that from pituitary tissue, because the mean poly(A) RNA recovery from total RNA of follicu-

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**Table 2.** Follicular fluid volume and hormone concentrations in follicles in which oocyte was recoverd or not, and fertilized in *vitro* or not

<table>
<thead>
<tr>
<th>Oocyte recovery</th>
<th>In <em>vitro</em> fertilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes (n=37)</td>
<td>Yes (n=51)</td>
</tr>
<tr>
<td>No (n=18)</td>
<td>No (n=6)</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>PRL (ng/ml)</td>
<td>56.1 ± 4.5</td>
</tr>
<tr>
<td>E2 (ng/ml)</td>
<td>630 ± 57</td>
</tr>
<tr>
<td>Progesterone (μg/ml)</td>
<td>11.1 ± 1.5</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SEM. *P<0.05 vs. follicles in which oocyte was fertilized.

**Table 3.** RNA recovery from human ovarian and pituitary tissues

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Menstrual phase and reproductive stage</th>
<th>Total RNA (mg/g wet tissue)</th>
<th>Poly (A) RNA (% of total RNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>1.96</td>
<td></td>
</tr>
<tr>
<td>Follicle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Luteal</td>
<td>1.98</td>
<td>0.88</td>
</tr>
<tr>
<td>3</td>
<td>Pregnancy (9 weeks)</td>
<td>1.80</td>
<td>1.26</td>
</tr>
<tr>
<td>4</td>
<td>Follicular</td>
<td>3.04</td>
<td>0.74</td>
</tr>
<tr>
<td>5</td>
<td>Follicular</td>
<td>1.56</td>
<td>0.72</td>
</tr>
<tr>
<td>6</td>
<td>Luteal</td>
<td>1.33</td>
<td>0.98</td>
</tr>
<tr>
<td>7</td>
<td>Luteal</td>
<td>1.87</td>
<td>1.12</td>
</tr>
</tbody>
</table>

1.98 ± 0.24* 0.95 ± 0.09*

Total RNA was extracted with acid guanidium thiocyanate/phenol/chloroform method. Poly (A) RNA was isolated by oligo (dT) cellulose chromatography. *mean ± SEM.
Fig. 2. Detection of PRL mRNA in follicular tissues. 1.5 μg of poly(A) RNAs from human follicular tissues in different phases of the menstrual cycle (lanes 4 and 5: follicular; lanes 2, 6 and 7: luteal) and pregnancy (lane 3), and 1.5 μg of total RNA from human pituitary RNA (lane 1) were electrophoresed, Northern transferred, hybridized with cDNA probes of PRL (a) or β-actin (b), and exposed to X-ray films for 7 days and 48 h, respectively. Although a PRL mRNA band could be detected only in RNA from the pituitary at the 1.0 kb position, β-actin mRNAs were observed in all lanes at 2.1 kb.

lar tissues was 0.95%. The PRL mRNA band could be detected even when the pituitary RNA was diluted 100 times (data not shown).

When the same sheet was hybridized with β-actin cDNA, all the RNA preparations gave single mRNA species 2.1 kb in size (Fig. 2-B). Thus, the inability to detect PRL mRNA in ovarian samples could not be due to RNA degradation.

Discussion

In each individual patient after ovarian stimulation with clomiphene citrate, hMG and hCG administrations, PRL concentrations in different FFs obtained from follicles of various sizes showed no difference, and were very similar to the level of PRL concentration in plasma (Table 1). A significant positive correlation was observed between PRL concentrations in FF and those in plasma (Fig. 1). No correlation was observed between the size of follicles and the PRL levels in FF. These results suggest that PRL in FF might reflect a passive diffusion from plasma into the follicular compartment.

McNatty et al. [6] also indicated that the PRL concentration in FF did not change with respect to the size of the follicle in the natural menstrual cycle. However, in contrast with our observations, they reported that the mean concentration of PRL in FF (20 ± 5 ng/ml) was much lower than that in plasma. PRL concentrations in the plasma and FF in our study were much higher than those in the natural cycle. Laufer et al. [19] also reported very high concentrations of PRL in FF, with a mean value of 114 ng/ml after hMG/hCG stimulation. These results indicate that the rate of PRL exchange between plasma and FF could be altered by medical ovarian stimulation. Moreover, McNatty et al. [8] reported that the plasma PRL concentration did not decrease during the late follicular phase, while the PRL concentration in FF during the same period was lower than that at any other time of the cycle with the exception of the early luteal phase. Thus, during normal menstrual cycles, the transport of PRL from the circulation could be low in the late follicular phase, or its degradation may be high in that period.

Recently, Khan-Dawood [20] demonstrated the presence of PRL in the corpus luteum in the human ovary, and Einspanier et al. [21] detected low but significant levels of PRL mRNA in porcine luteal cells. To analyze the local PRL synthesis in human ovarian tissues, PRL mRNA in ovarian follicles during various phases of the menstrual cycle and reproductive stage was measured. No PRL mRNA was detected in any follicular tissues from different menstrual phases of pregnancy, while a single PRL mRNA band was observed in pituitary RNA (Fig. 2-A). Thus, the PRL mRNA concentration in follicles during any stage of the cycle and gestation seemed to be at least 10,000 times less than that in the pituitary for the following reasons: (1) the recovery rates for total RNA were almost the same from both ovaries and pituitary, (2) about 1% of poly(A) RNA was recovered from the total RNA of follicular tissues, and (3) pituitary PRL mRNA could be detected even after dilution 100-times. These results indicated that expression of the PRL gene in granulosa and luteal cells is extremely low, if any. When the same sheet was hybridized with cDNA to β-actin mRNA, a single mRNA species was observed in follicular poly(A) RNAs. The amount of β-actin mRNA was greater in follicular poly(A) RNA than in the same amount of pituitary total RNA (Fig.
This finding indicates that the inability to detect PRL mRNA in follicular tissue was not due to RNA degradation, and supports the idea that the amount of PRL mRNA in ovary is very small or that there is none.

Since no PRL synthesis was observed in follicles in any stages of the menstrual cycle, FF PRL could be supplied through the circulation even in physiological conditions. PRL permeability from plasma into FF may change during the menstrual cycle.

How is the concentration of FF PRL regulated? In the present study, the PRL concentration in FF did not correlate with E2 or progesterone levels in FF. However, it significantly correlated with the highest level of plasma E2 observed 1 to 3 days before the collection of FF. It is well-known that estrogens are potent stimulators of PRL synthesis and secretion [22]. High plasma PRL levels have been found in patients receiving ovarian stimulation with hMG [23], and the increase in PRL occurred in parallel with an increase in E2 [23, 24]. Moreover, we [18] as well as other investigators [25, 26] reported that a plasma PRL peak in the ovulatory phase in the natural cycle was observed 1 to 3 days after the estrogen peak during the late follicular phase. These data also indicate that PRL in FF could be derived from the pituitary via plasma.

It has been indicated by the results of many studies that a follicular microenvironment is important for the maturation of the oocyte and for steroid biosynthesis. McNatty et al. [8] have postulated a direct effect of PRL on ovarian function, since low PRL levels were essential for progesterone production by preovulatory human granulose cells cultured in vitro. Hunter [27] reported that PRL alone had no effect on steroidogenesis in human luteal tissue in vitro, but it significantly enhanced hCG stimulation of progesterone and E2 synthesis. Hamada et al. [28] indicated the inhibitory effects of PRL on ovulation with an in vitro perfused rabbit ovary preparation. In the present studies, however, no correlations of FF PRL were observed with E2 or progesterone in FF.

Moreover, FF PRL concentrations showed no differences in follicles from which oocytes were recovered or not, or fertilized in vitro successfully or not (Table 2). On the other hand, several investigators have examined FF PRL levels in women undergoing ovulation induction for IVF-ET and have found that lower mean levels are positively associated with oocyte fertilization [29]. In contrast, other studies found that the higher FF PRL levels in pre-ovulatory follicles highly correlated with successful oocyte fertilization [19, 30]. However, Dodds et al. [31] failed to show a positive role of murine PRL in affecting mature oocyte fertilization or subsequent embryo cleavage in vitro.

The direct effect of FF PRL on steroid synthesis and oocyte maturation could not be assessed from the present study. Future studies will clarify the role of human follicular PRL in vivo.

Acknowledgements

We are indebted to Dr. J. A. Martial (University of Liege, Liege, Brussels) and Dr. R. H. Singer (University of Massachusetts Medical School, USA) for the gift of human PRL and β-actin cDNA clones.

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