Reduction of progesterone receptor expression in human cumulus cells at the time of oocyte collection during IVF is associated with good embryo quality

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BACKGROUND: It has been reported that the progesterone receptor (PR) level is transiently increased within the follicle by LH stimulation and controls cumulus cells in follicles and oocyte maturation. The purpose of this study was to predict developmental competence of human oocytes during IVF via analysis of PR in cumulus cells surrounding mature oocytes. METHODS: Prior to oocyte retrieval, the follicular diameter was measured and follicular fluid was collected from each mature follicle. Cumulus cells were manually separated from the oocyte–cumulus complex under a microscope. PR and PR mRNA were assessed by immunohistochemistry and real-time quantitative polymerase chain reaction (RT–PCR) measurement in human cumulus cells. RESULTS: Immunoreactive PR-A was mainly localized in the cytoplasm and PR-B was localized in the nuclei. There was no significant relationship between PR expression and follicular diameter, follicular fluid concentration of steroids, or LH. There was no significant relationship between expression of PRs and fertilization or cleavage rate. However, PR expression was lower in the good morphology group (blastomeres ≥7 cells with fragmentation ≤5% on day 3) when compared to the other groups (P < 0.05). CONCLUSIONS: These results suggest that follicular LH or steroids do not affect PR expression, and full reduction of total PR expression on cumulus cells at the time of oocyte collection is associated with good morphology in human oocytes.

Key words: cumulus cells/follicular fluid progesterone/human embryo morphology/IVF/progesterone receptors

Introduction

Progesterone plays a central role in the reproductive events associated with pregnancy establishment and maintenance. The physiologic effects of progesterone are known to be mediated by the interaction of the hormone with specific intracellular proteins, termed progesterone receptors (PRs). The interaction between progesterone and its receptors induces specific structural and functional changes in PR that communicate in an association of ligand receptor complexes with target genes to regulate their transcription (Conneely et al., 2000, 2002). The expression of PR mRNA can be induced by either LH or FSH through a cAMP-mediated pathway in pre-ovulatory follicles. Evidence that ovary-derived progesterone may participate in autocrine regulation of ovarian function first emerged when it was demonstrated that LH can stimulate transient expression of PR mRNA and protein in granulosa cells isolated from pre-ovulatory follicles (Park and Mayo, 1991; Natraj and Richards, 1993; Park-Sarge and Mayo, 1994). Shimada et al. and Okazaki et al. reported that high concentrations of progesterone secreted by cumulus cells accelerated meiotic resumption in oocytes and improved the rate of early embryonic development to the blastcyst stage after IVF. Moreover, the progesterone bound newly synthesized PR in cumulus cells was associated with reduced proliferative activity of cumulus cells and cumulus–oocyte–complex (COC) expansion, and closed the gap junctional communication with in cumulus cells(Shimada et al., 2002, 2004a; Shimada and Terada, 2002a; Okazaki et al., 2003). Meiotic resumption is induced by the disruption of gap junctions within cumulus cells, which block the conduction of meiosis inhibitory signals from the outer cumulus cells to the oocyte (Isobe et al., 1998), and is associated with the reduction of gap junctional protein connexin-43 in the outer layers of cumulus cells (Shimada et al., 2001). It has been recognized that cAMP transferred from the cumulus cells via gap junction is an inhibitory factor involved in the meiotic resumption and the regulation of meiotic progression beyond the metaphase I (MI) stage (Rose-Hellekant and Bavister, 1996; Shimada and Terada, 2002b). However, it has been reported that progesterone down-regulates the expression of the gene (Zhao et al., 1996). Additionally, progesterone induced a reduction of
connexin-43 expression via the PR-mediated pathway in the outer layers of cumulus cells, resulting in meiotic resumption (Shimada and Terada, 2002a). A recent study reported that PR-B reached its maximum at 4–12 h in cumulus cells cultured with FSH and LH, whereas PR-A predominated at 20 h. However, the level of PR-B dropped significantly at 12 h. Accompanying the decrease of PR-B levels, increase of progesterone production, loss of proliferative activity, decrease in connexin-43 gene expression, meiotic resumption of oocytes and cumulus cell expansion were also observed (Shimada et al., 2004b). Treatment with anti-progesterone suppressed these developments (Mori et al., 1983; Shimada et al., 2004b). According to the previous reports, it has been suggested that the induction of PR isoforms in cumulus cells and their binding to progesterone appeared to impact on proliferation and differentiation in a time-dependent manner, and the shift from PR-B to PR-A may help mediate certain events (Shimada et al., 2004b).

Zhang and Armstrong reported that only about 20% of oocytes from untreated rats were fertilized in vitro, in contrast to 70% of oocytes fertilized from FSH-treated rats, whereas 80% of oocytes underwent germinal vesicle breakdown, regardless of the treatments to donor rat. The addition of progesterone during in vitro maturation period duplicated the beneficial effect of FSH on fertilization rate. Although having no apparent effect on nuclear maturation of the oocyte, it is possible that gonadotrophin and ovarian steroids are important regulators, either directly and indirectly, of some of the cytoplasmic changes in oocytes that lead to the normal fertilization of the oocyte. Moreover, Mori et al. suggested that gap junction communications between the oocyte and cumulus cells might play an important role in regulating cytoplasmic maturation (Mori et al., 2000).

The recognition of oocyte maturation and regulation of cumulus cells, and the harvesting and culturing of numerous good-quality oocytes has clinical significance for improving fertility treatment. The aforementioned studies led to the objective of this study: developmental competence of human oocytes in IVF can be predicted from analysis of PR in cumulus cells surrounding mature oocytes. We examined the localization and expression of PR in human cumulus cells at the time of oocyte collection during IVF cycles via immunohistochemistry and real-time quantitative polymerase chain reaction (RT–PCR).

Materials and methods

Subjects

The subject group comprised 44 women undergoing IVF. Their ages ranged from 28 to 44 years (mean 35.7 years), and the indications for IVF included tubal absence or occlusion, ovulation disorders, endometriosis, oligozoospermia, and idiopathic infertility. All patients gave written informed consent to participate in this study.

IVF procedure

The ovaries were stimulated with clomiphene citrate (Clomid®; Shionogi, Osaka, Japan), human menopausal gonadotrophin (HMG; HMG Injection TEIZO®, Teikoku-zouki, Tokyo, Japan), pure FSH (Fertinorm P®, Serono, Tokyo, Japan), or a combination of the three; the stimulation was done after pituitary desensitization with gonadotrophin-releasing hormone agonist (GnRH-a; Suprecure; Aventis Pharma, Tokyo, Japan) according to the long protocol. Ovarian follicle diameter was assessed by transvaginal sonography, and gonadotropins were administered daily until the second largest follicle reached a diameter of 18 mm. When the follicle grew beyond that diameter, (HCG; Gonatropin®, Teikoku-zouki, Tokyo, Japan) 10 000 IU or GnRH-agonist 600 μg was administered; 35 h later, oocytes were retrieved under ultrasonographic guidance. Prior to oocyte retrieval, the follicular diameter was measured by ultrasonography and follicular fluid was carefully collected from each mature follicle without any contamination with flushing medium. Serum and follicular fluid samples were stored at −20°C prior to hormone measurements. Cumulus cells were manually separated from COC under a microscope after oocyte retrieval.

Oocyte and embryo culture

Oocytes were cultured in dishes with 500 μl P-1™ medium (preimplantation-1 medium; Irvine Scientific) under mineral oil with 10% CO2, 5% O2, and 90% N2. Oocytes underwent conventional IVF or ICSI, depending on the patient’s specific needs.

Embryo quality assessment

Three days after insemination, embryo quality (EQ) was investigated under a microscope with a micro-manipulator. EQ was categorized as good morphology group if the number of blastomeres was greater than seven cells with less than 5% fragmentation.

Serum and follicular fluid analysis

Follicular fluid concentrations of estradiol (E2), progesterone and LH were determined by a commercial enzyme-linked immunosorbent assay kit (AIA-600I, TOSOH, Tokyo, Japan). After determining the follicular fluid steroid concentration, the follicular fluid was diluted 1:1000 in steroid-free serum before the measurements.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue was cut into 3 μm sections. The sections were then deparaffinized in xylene three times for 5 min each, and fixed in 100% ethanol three times for 5 min each, and then washed in water for 5 min. Endogenous peroxidase activity was blocked with a methanol solution, which included 3% H2O2, for 20 min at room temperature. To activate antigens, the sections were autoclaved in 10 mM sodium citrate buffer (pH 6.0) at 121°C for 15 min. The sections were rinsed in phosphate-buffered saline (PBS) at room temperature and incubated with primary antibodies at 4°C overnight. We used primary antibodies as follows: anti-human PR-A mouse monoclonal antibody (PR Ab7; Neo Markers, Foremont, CA); and anti-human PR-B mouse monoclonal antibody (PR Ab2; Neo Markers). The following day, the slides were washed in PBS for three times for 5 min each and treated for 30 min at room temperature with Histofine Simple StainPO(M) (Nichirei Corporation, Tokyo, Japan), which is a labelled polymer prepared by combining amino acid polymers with peroxidase and goat anti-mouse immunoglobulin reduced to Fab’ fragment. The slides were washed in PBS for three times for 5 min each, and AEC (3-amino-9-ethyl carbazole) was used for colour development. Nuclei were counterstained with Mayer’s hematoxylin. Normal proliferative endometrium was used as a positive control for PR-A and PR-B. Negative control sections, which had not been exposed to

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primary antibody, were included in all staining runs. Staining was assessed using a consulting microscope (Model BX51; Olympus, Tokyo, Japan).

**RNA preparation and reverse transcription, and real-time quantitative TaqMan PCR**

Total RNA was isolated from cumulus cells by phenol–chloroform extraction. The cDNAs were synthesized from 5 μg of total RNA using random hexamer and Superscript II reverse transcriptase at 55°C for 30 min and 99°C for 5 min. Five microlitres of this reaction were analysed by RT–PCR.

RT–PCR was performed using TaqMan PCR® (ABI PRISM 7700 Sequence Detection System; Perkin–Elmer Applied Biosystems, CA, USA). Because PR-A has no specific sequence to distinguish it from PR-B mRNA, PR-A mRNA cannot be distinguished from PR-B by RT–PCR. Therefore, we used an intersectional primer (Hs00172183_m1, Assay on demand Gene Expression probes; Applied Biosystems, CA) of PR-A and PR-B. glyceraldehyde-3-phosphatedehydrogenase (GAPDH) cDNA fragments were amplified as positive controls. Two RT–PCR reactions were performed with each sample.

**Results**

A total of 135 samples of cumulus cells in each follicle from the 44 IVF patients were analysed for PR expression. Among the samples, 35 (25.9%) oocytes were inseminated by conventional IVF, and 19 (54.3%) were fertilized and cleaved. A total of 100 (74.1%) were inseminated by ICSI, and 66 (66.0%) of were fertilized; 62 (93.9%) of fertilized oocytes cleaved. Five (11.4%) patients became pregnant after IVF and embryo transfer.

**Immunohistochemistry**

In cross-section, immunoreactive PR-A was mainly localized in the cytoplasm of 58 (75%) of 77 human cumulus cells (Figure 1a). Immunoreactive PR-B was localized in the nuclei of 44 (62%) of 71 human cumulus cells in cross-section. No cytoplasmic staining was found for PR-B (Figure 1b).

**Quantitative RT–PCR**

The baseline expression of PR mRNA in cumulus cells was detected in all samples (Figure 2). Amplification of increasing amounts of PCR reagent (diluted 1:10, 100, 1000, 2196
10,000), containing the target sequence was performed. Fluorescence intensity was plotted against the logarithm of the reagent concentration to construct a standard curve. The mRNA integrity was controlled by amplification of the GAPDH housekeeping gene.

**PRs and follicular diameter**

There was no significant relationship between PR/GAPDH and follicular diameter (Figure 3).

**PRs and follicular fluid concentrations of steroids; LH**

There was no significant relationship between PR/GAPDH and follicular fluid concentrations of E₂, progesterone, and LH, respectively (Figure 4a–c).

**Progesterone concentrations in serum and follicular fluid**

There was no significant relationship between serum and follicular fluid progesterone concentration (Figure 5).

**PRs and embryo development**

The expression of PRs and follicular fluid concentration of progesterone did not significantly differ between the fertilized group and the unfertilized group (Table I). The expression of PRs and follicular fluid concentration of progesterone were not significantly different between the group that cleaved, and the group, that did not cleave (Table II). The expression of PRs was significantly lower and follicular fluid concentration of progesterone was significantly higher in the good morphology group (per EQ evaluation) than in the other groups (P < 0.05; Table III). PR expression did not significantly differ between low and high follicular fluid concentrations of progesterone in the good morphology group or the other group (Table IV).

**Discussion**

Follicular diameter and either E₂ or progesterone levels are deemed to be the parameters for follicular maturation. Because serum concentrations of these substances are affected by the degree of ovarian stimulation and the number of the follicles, we examined the follicular fluid concentration of steroids and LH to assess the follicular environment. In our study, there was no significant relationship between the serum and follicular fluid concentrations of...
progesterone (Hasegawa et al., 2003). Each follicular fluid steroid sample retrieved from the same patient was markedly similar in concentration (data not shown). In the present study, follicular diameter, follicular fluid LH, E₂ or progesterone concentration did not affect PR expression. These results suggest that there is no correlation between PR expression in cumulus cells 35 h after HCG stimulation and the LH or steroid level in the follicular fluid.

It is known that premature luteinization is related to low ovarian reserve (Younis et al., 2001). Premature luteinization is defined as an early rising of the serum concentration of progesterone (progesterone/E₂ > 1) before the LH surge. In our study, because follicular fluid concentrations of progesterone at the time of oocyte collection were fully affected by LH, we were unable to determine a correlation between premature luteinization and follicular fluid progesterone levels.

In this study, follicular fluid concentrations of progesterone were higher in the good morphology group than the other group (Table III). Chian et al. reported that during in-vitro meiotic maturation of COC in humans, progesterone was produced by the cumulus cells, and that the level of progesterone was increased by stimulation with LH, FSH, or forskolin (Chian et al., 1999). Moreover, it has been demonstrated that significantly increased concentrations of progesterone were observed in COCs cultured with LH and FSH for 28 h (Shimada and Terada, 2002a). Other researchers have compared the follicular fluid steroid concentrations during the maturation of the morphological COCs and reported that the follicular fluid concentrations of progesterone in the mature COCs were significantly higher than they were in the immature stage (Botero-Ruiz et al., 1984; De Sutter et al., 1991). Furthermore, it has been reported that progesterone/E₂ ratios were higher in follicles whose oocytes were fertilized (Enien et al., 1995), that higher progesterone values associated with oocyte capable of fertilization by ICSI (Mendoza et al., 1999), and that two pronuclei zygotes that failed to undergo cleavage developed from oocytes that were harvested from follicles with lower concentrations of progesterone (Mendoza et al., 2002). However, it has been reported that there was no significant difference between the follicular fluid concentration of progesterone and either EQ at day 3 of an IVF (Hasegawa et al., 2003) or the pregnancy rate (Andersen, 1993). It is questionable whether higher follicular fluid concentrations of progesterone accurately reflect either oocyte quality or the capacity for embryo development. Differences in ovarian stimulation protocols also may introduce increased variability (Mendoza et al., 1999). However, regardless of progesterone concentration, good embryos were retrieved from COCs whose PR expressions were lower than the others (Table IV). It has been reported that the luteal phase of the cycle is marked by a sharp decrease in PR-B expression that precedes the decrease in PR-A by several days (Brandon et al., 1993). PR-A mRNA appears to persist longer than PR-B mRNA for 24 h in LH-stimulated rat granulosa cells (Natraj and Richards, 1993). A recent study also reported that the level of PR B mRNA in cumulus cells was up-regulated by FSH and LH during the first 8 h of cultivation and then significantly decreased at 12 h; furthermore, PR-A predominated at 20 h, and high level of total PR.

![Figure 5. Correlation of progesterone concentration in serum and follicular fluid. There was no significant relationship between serum and follicular fluid progesterone concentration.](image)

**Table I.** Correlation between PR/GAPDH, follicular fluid progesterone concentration, and fertilization

<table>
<thead>
<tr>
<th>PR/GAPDH</th>
<th>Progesterone (µg/ml)</th>
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<tbody>
<tr>
<td>Fertilized (n = 85)</td>
<td>0.027 ± 0.007</td>
</tr>
<tr>
<td>Unfertilized (n = 50)</td>
<td>0.023 ± 0.010</td>
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</tbody>
</table>

Data are the mean ± standard error. There were no statistically significant differences.

**Table II.** Correlation between PR/GAPDH, follicular fluid progesterone concentration, and cleavage

<table>
<thead>
<tr>
<th>PR/GAPDH</th>
<th>Progesterone (µg/ml)</th>
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<tbody>
<tr>
<td>Cleaved (n = 81)</td>
<td>0.028 ± 0.008</td>
</tr>
<tr>
<td>Not cleaved (n = 4)</td>
<td>0.014 ± 0.010</td>
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</table>

Data are the mean ± standard error. There were no statistically significant differences.

**Table III.** Correlation between PR/GAPDH, follicular fluid progesterone concentration, and embryo quality

<table>
<thead>
<tr>
<th>PR/GAPDH</th>
<th>Progesterone (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good morphology group (n = 19)</td>
<td>0.006 ± 0.001*</td>
</tr>
<tr>
<td>Other groups (n = 62)</td>
<td>0.035 ± 0.010*</td>
</tr>
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Data are the mean ± standard error. *P = 0.0181. **P = 0.0027.

**Table IV.** Correlation of PR/GAPDH and embryo quality stratified by progesterone follicular fluid concentration

<table>
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<tr>
<th>Low progesterone (&lt;7 µg/ml)</th>
<th>High progesterone (≥7 µg/ml)</th>
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<tbody>
<tr>
<td>Good morphology group (n = 19)</td>
<td>0.007 ± 0.003</td>
</tr>
<tr>
<td>Other groups (n = 62)</td>
<td>0.036 ± 0.010</td>
</tr>
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</table>

Data are the mean ± standard error.
mRNA was maintained up to a cultivation period of 20 h in porcine oocytes (Shimada et al., 2004b). In the present study, PR expression was neither significantly different between the fertilized group and the unfertilized group nor between the group which cleaved and group which did not cleave. However, PR expression was lower in the good morphology group than in the other group. In view of these results and previous studies, we suggest that full reduction of PR expression in cumulus cells at the time of oocyte collection (which may be transiently stimulated by LH) may be essential to acquire developmental competence of the human oocyte, and that oocyte maturation is controlled by the expression and the reduction of PRs in humans just as it is in other mammalian oocytes.

It has been reported that progesterone-induced Xenopus oocyte maturation is mediated via an extranuclear receptor, and is independent of gene transcription (Bayaa et al., 2000). Other investigators have reported that PRs were increased mainly in the cytoplasm during the proliferative phase. In the early luteal phase, PRs decreased in the cytosol; however, they remained high in the nuclei (Bayard et al., 1978; Follow et al., 1981). In the present study, we found for the first time that PR-A was localized in cytoplasm and PR-B was localized in nuclei of human cumulus cells by the technique of immunohistochemistry in IVF cycles. Shimada et al. suggested that the induction of PR isoforms in cumulus cells and their binding to progesterone appear to have an impact on proliferation and differentiation in a time-dependent manner, and a shift from PR-B to PR-A may help mediate certain events (Shimada et al., 2004b). These previous studies and our results support the theory that time-dependent expression of PR isoforms and the distinction of localization between PR-A and PR-B affect follicular growth, oocyte maturation, and early embryo development.

In conclusion, regardless of follicular fluid steroid or LH concentration at the time of oocyte collection, good morphology embryos were retrieved from COCs whose PR expressions were lower than the others. Our findings indicate that the full reduction of total PR expression may be essential to acquire developmental competence in the human. Although it could not be specifically demonstrated in this study, selective contribution of the two PR isoforms to progesterone action might affect oocyte maturation. It is possible that expression and reduction of PRs and localization of PR isoforms transiently stimulated by LH might regulate oocyte maturity and embryo development.

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