Amphiregulin is much more abundantly expressed than transforming growth factor-alpha and epidermal growth factor in human follicular fluid obtained from patients undergoing in vitro fertilization–embryo transfer

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Objective: To identify the most important epidermal growth factor (EGF) receptor ligand in the LH or hCG signal pathway in human ovary.

Design: A retrospective clinical study.

Setting: Tertiary university hospital.

Patient(s): Ninety-eight infertile patients who underwent IVF–embryo transfer.

Intervention(s): Sera and follicular fluid were collected at the time of oocyte retrieval. The levels of EGF, transforming growth factor-alpha (TGFα), and amphiregulin (AR) were measured in follicular fluid and sera by using ELISA.

Main Outcome Measure(s): The relationships between the level of AR and level of hCG, fertilization rate, and embryo quality.

Result(s): Amphiregulin was abundantly expressed in follicular fluid after hCG stimulation. Although large differences were found between AR and both EGF and TGFα in follicular fluid, no significant difference was detected in the levels of the three EGF receptor ligands in sera. The level of AR was inversely correlated with the fertilization rate and hCG level, whereas little significant association was observed between the level of AR and embryo quality.

Conclusion(s): Amphiregulin was expressed most dominantly among EGF receptor ligands tested and may mediate the hCG signal in human oocyte maturation. Elaborate interaction between AR and hCG may be required for an optimal oocyte maturation. (Fertil Steril 2009;91:1035–41. ©2009 by American Society for Reproductive Medicine.)

Key Words: Amphiregulin, epidermal growth factor, transforming growth factor-alpha, human follicular fluid, IVF-ET

The midcycle surge of LH in human beings and other mammals initiates a cascade of events, including the resumption of meiosis of the oocyte, cumulus expansion, and oocyte maturation, which culminates in the extrusion of a fertilizable oocyte and remodeling of the follicle into a functional corpora lutea. In IVF–embryo transfer (ET) to treat infertile patients, hCG usually is used instead of LH to induce such a cascade. Traditionally, studies on the control of ovarian follicular development and oocyte maturation have focused mainly on endocrine regulation of the hypothalamic-pituitary-ovarian axis, and it is well known that the growth of follicles depends on gonadotropins after the preantral stage. Epidermal growth factor (EGF) receptor ligand is a large group of closely related proteins that includes EGF, transforming growth factor-alpha (TGFα), amphiregulin (AR), epiregulin, betacellulin, epigen, neuregulins, and heparin-binding EGF-like growth factor (1). These EGF-like growth factors are synthesized as integral membrane precursors. The ligands are shed from the cell surface by proteolytic cleavage of the ectodomain and bind to the EGF receptor family of receptor tyrosine kinases. The first evidence that EGF receptor ligands may be involved in cumulus cell function was reported by Dekel and Sherizly (2) and by Downs (3). More recently, considerable attention has been focused on members of the growth-factor families in controlling oocyte maturation and ovulation (1, 4, 5), because the restricted expression of LH receptors on cumulus cell and the oocyte itself raised the possibility that many LH effects were indirect and may be transmitted via these growth factors in a paracrine manner (6). Some studies have demonstrated that EGF and TGFα may also regulate the growth of follicles (7, 8). It was recently demonstrated that
LH (or hCG) stimulation induced the rapid and transient expression of the messenger RNA encoding EGF family members AR, epiregulin, and betacellulin in rodent ovaries (4, 9) and human ovaries (10). It has been shown clearly in several species that EGF receptor ligands mediate expansion of the cumulus–oocyte complex (4, 11–13). Finally, incubation of follicles with these growth factors recapitulates the morphological and biochemical events triggered by LH, including cumulus expansion and oocyte maturation (4). Likewise, addition of AR resulted in complete stimulation of the resumption of meiosis in rat follicle–enclosed oocytes (14). Thus, these studies strongly suggest that these EGF-related growth factors are synthesized in granulosa cells upon LH surge and can act in a paracrine manner to mediate LH signaling throughout the follicle. However, AR itself has been shown to increase the expression of AR mRNA and AR protein production in murine cumulus–oocyte complex, depending on the cyclooxygenase–prostaglandin cascade (15), which suggests the presence of autocrine pathways by AR. Amphiregulin also was reported to have been induced by FSH stimulation in primary rat granulosa cell culture and to be involved in granulosa cell differentiation (16). These in vitro data were collected mainly from studies using rodent ovaries. In contrast, few studies have examined the role of EGF receptor ligands in human ovary (10, 17–19). To the best of our knowledge, only one report elsewhere has demonstrated the presence of EGF receptor ligand in human follicular fluid (FF) (20). To clarify which EGF receptor ligand is most important in human follicular maturation, we measured three distinct EGF receptor ligands, that is, EGF, TGFα, and AR, all of which share a common EGF receptor (ErbB1) (21), in FF that was obtained from patients who underwent IVF-ET in our institute, and we also explored the relationship between IVF-ET outcome and the level of the EGF receptor ligand that is most abundant in human FF. In a study elsewhere, we reported that the diffusion of exogenous hCG into the FF may be an important predictor of IVF outcome and that the level of hCG in FF may reflect the degree of hCG signals to follicles (22). Thus, we explored the association between the hCG level and EGF receptor ligand level in FF.

MATERIALS AND METHODS

Collection of Human FF

Human FF was obtained from women aged 22 to 44 years (median, 33 years) who were undergoing IVF at Fukuoka University Hospital (Fukuoka, Japan), mainly as a result of either male-factor infertility or tubal-factor infertility. The Ethics Committee of Fukuoka University approved this study, and written informed consent was obtained from all subjects. Ovarian stimulation was performed by using GnRH analog (Suprecur; Aventis Pharma, Tokyo, Japan), beginning either on day 1 of the cycle (short protocol: 85 cycles) or in the midluteal phase of the previous cycle (long protocol: 77 cycles) and followed by the daily administration of pure FSH (Fertinorm P; Serono Japan Co., Ltd., Tokyo, Japan) and hMG (Humegon; Organon Japan Co., Ltd., Osaka, Japan), starting from day 3 of the cycle. In both cases, stimulation was continued until at least two leading follicles achieved an optimum size of 18 mm, as confirmed by transvaginal ultrasound (Aloka; SSD-3500, with a curvilinear transvaginal transducer of 7.5 MHz, Tokyo, Japan), after which hCG (Mochida Pharmaceuticals Co., Ltd., Tokyo, Japan) was administered. Oocyte retrieval was performed transvaginally, 34 to 37 hours after hCG administration. All follicles with a diameter of >10 mm were punctured. The mean (± SD) number of retrieved oocytes was 3.6 ± 2.9. Venous blood samples also were obtained from all patients just before oocyte retrieval. Aspirated FF at oocyte retrieval was collected separately from each ovary. Overall, we obtained FF samples from 301 ovaries of 97 patients. Ovum pickups were undertaken twice in 16 patients, 3 times in 12 patients, and 4 times in 4 patients. Among these patients who underwent repeated ovum pickups, 3 patients conceived. We could not obtain FF from each follicle because this may have prolonged the amount of time required for follicular aspiration, and because we could obtain only a small amount of FF from a follicle with a small diameter. Therefore, each FF sample represented the pooled contents of multiple follicles from each ovary. The mean number of follicles evaluated by transvaginal ultrasound was 7 ± 5. Cell structures and debris were removed from venous blood and FF by centrifugation at 1,861 × g for 10 minutes. After centrifugation, serum samples and FF were stored at −20°C until assayed for EGF receptor ligands and hCG.

Immunoassay for Human EGF, AR, TGFα, and hCG in Human FF

Concentrations of EGF, AR, and TGFα in human FF were determined by using a commercially available sandwich ELISA kit (DuoSet Kit; R&D Systems Inc., Minneapolis, MN) in accordance with the manufacturer’s instructions, using tetramethyl benzidine as a substrate. We chose these three EGF receptor ligands from among several ligands because EGF, TGFα, and AR all share a common EGF receptor (ErbB1) (21) and also because a sandwich ELISA kit was commercially available for these three ligands only during the study period. Samples were assayed in triplicate. The absorbance at 455 nm was read with a reference wavelength of 650 nm by using a model 680 series microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA). Levels of EGF, AR, and TGFα were calculated from the linear areas of respective standard curves obtained by using Microplate Manager, version 5.2 (Bio-Rad Laboratories). The mean value was used as the representative value. The EGF, AR, and TGFα concentrations for each sample were calculated after a standard curve was generated by using a dilution series of human recombinant proteins. The lower limits for the detection of EGF, AR, and TGFα were 3.9, 15.6, and 7.8 pg/mL, respectively. Thus, when the amount was less than the limit of detection, the EGF, AR, and TGFα values were recorded as 3.9, 15.6, and 7.8 pg/mL, respectively. We measured the concentration of exogenous hCG in some of the FF samples to explore the correlation between AR and exogenous hCG in FF.
Concentrations of hCG in human FF were determined by using a commercially available ELISA kit (DSL-10-8300 Active, Intact hCG ELISA kit; Cosmo Bio Co., Ltd, Webster, TX) in accordance with the manufacturer’s instructions. The intraassay and interassay coefficients of variation were 9.6% and 4.6%, respectively.

Evaluation of Embryo Quality and Fertilization Rate

After ovum pickup, the obtained oocytes were placed in insemination medium 1–4 hours before insemination with 50,000–500,000 motile spermatozoa per milliliter of medium, as reported elsewhere (22). The presence of two pronuclei was identified 12–16 hours after insemination, and the embryos were placed in growth medium. Oocytes were judged to be fertilized on the basis of the presence of two pronuclei, and the fertilization rate (FR) was calculated as the number of oocytes fertilized, divided by the number of total oocytes obtained in each ovary. All samples were divided into three groups according to FR: FR of <40% (low FR), FR between 40% and 80% (middle FR), and FR of >80% (high FR). Embryo quality was classified at the two- to four-cell stage according to the criteria of Veeck (23). A maximum of three embryos at this stage were transferred to the uterus on day 2 after ovum pickup. Pregnancy was considered to be established after identification of a gestational sac.

Statistical Analysis

Results are reported as mean ± SEM. The levels of the three EGF receptor ligands in FF or serum and the AR levels in the groups with different embryo qualities or FRs were compared by Fisher’s protected least significant difference test. Spearman’s test was used to determine whether the coefficient of correlation (r) between the hCG level and AR level in FF was significantly different from zero. All statistical analyses were performed by using Microsoft Excel, version 11.3.5 (Microsoft, Redmond, WA), and the SAS software package (version 9.1; SAS Institute Inc, Cary, NC) at Fukuoka University. A P value of <.05 was considered statistically significant.

Table 1: Concentrations of EGF receptor ligands in human follicular fluid and serum.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Location</th>
<th>EGF (pg/mL) Mean ± SD (range)</th>
<th>n</th>
<th>TGFα (pg/mL) Mean ± SD (range)</th>
<th>n</th>
<th>AR Mean ± SD (range)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Follicular fluid</td>
<td>4.6 ± 0.4 (3.9–16.0)</td>
<td>52</td>
<td>16.1 ± 1.0 (9.8–37.7)</td>
<td>52</td>
<td>108.4 ± 6.3 ng/mL a (0.8–1,189.4)</td>
<td>301</td>
</tr>
<tr>
<td>Serum</td>
<td>241.5 ± 29.1 b (82.0–422.3)</td>
<td>16</td>
<td>29.4 ± 3.7 (10.8–58.3)</td>
<td>16</td>
<td>32.5 ± 5.9 pg/mL (15.6–446.4)</td>
<td>132</td>
<td></td>
</tr>
</tbody>
</table>

aP<.01 vs. EGF and TGFα in follicular fluid.
bP<.05 vs. TGFα and AR in serum.


RESULTS

We measured three kinds of EGF receptor ligands, EGF, TGFα, and AR, which share the same EGF receptor (ErbB1), in human serum and FF (Table 1). The level of EGF in human FF was very low (4.6 ± 0.4 pg/mL, n = 52). Indeed, EGF was less than the cutoff value in 92.3% (48/52) of the FF samples examined. For these samples, because the concentrations were considered to be 3.9 pg/mL, as mentioned in Materials and Methods, the true value of EGF in FF may be lower. This is consistent with reports elsewhere (1, 19). However, EGF was the most abundant of these three EGF receptor ligands in serum (241.5 ± 29.1 pg/mL, n = 16), and there was significantly more EGF than TGFα or AR (P<.05). The level of EGF in serum was ≥52 times as high as that in FF. The levels of TGFα in FF and serum were 16.1 ± 1.0 (n = 52) and 29.4 ± 3.7 pg/mL (n = 16), respectively, and thus the concentration of TGFα in FF was less than that in serum. This suggests that TGFα may diffuse from serum to FF rather than being specifically produced in follicles. The level of AR in serum was 32.5 ± 5.9 pg/mL, which is almost the same as that of TGFα in serum. However, the level of AR in FF was remarkably high (108.4 ± 6.3 ng/mL, n = 301). The AR concentration in FF was almost 3,000 times as high as that in serum, which strongly suggests that AR may be specifically produced in follicles. The AR concentration in FF was at least 6,100 times and 57,000 times as high as those of TGFα and EGF, respectively. These results suggest that AR may act as a second messenger in the hCG signal pathway in human follicle, and thus we performed further analysis, especially with regard to AR.

Figure 1 shows the association between embryo quality and the concentration of AR in FF. The concentration of AR in cases with good embryo quality (i.e., cases in which >60% of embryos obtained were classified as grade 1 or 2 according to Veeck’s criteria; n = 15; mean age, 33 ± 3 years) was 73.4 ± 17.7 ng/mL, and that in cases with poor embryo quality (i.e., cases in which <60% of embryos obtained were classified as grade 1 or 2; n = 80; mean age, 34 ± 4 years) was 123.0 ± 21.3 ng/mL. Although there was no significant difference between these two groups both in AR level and
mean age, the concentration of AR in cases with good embryos tended to be lower than that in cases with poor embryos.

Next, we explored the relationship between the AR concentration in human FF and FR (see Materials and Methods). The mean ages in low-, middle-, and high-FR groups were 34 ± 5, 32 ± 4, and 34 ± 4 years, respectively. There were no significant differences among these three groups. The mean numbers of retrieved oocyte in low-, middle-, and high-FR groups were 3.2 ± 3.3, 4.6 ± 2.9, and 3.0 ± 2.0, respectively, and the number of oocytes in middle-FR groups was significantly higher than that in low- and high-FR groups (P < .05). The AR concentrations in the low-, middle-, and high-FR groups were 126.2 ± 21.9 (n = 69), 89.6 ± 14.4 (n = 47), and 73.0 ± 7.8 ng/mL (n = 45), respectively (Fig. 2). There was a significant difference in AR concentration between the low-FR group and high-FR group (P < .05), and AR was negatively associated with FR.

Figure 3 shows the relationship between the concentration of AR in FF and the dose of hMG used for controlled ovarian hyperstimulation in the IVF cycle. When we defined two groups (a high-AR group [group H, n = 15], in which the concentrations of AR were within the uppermost 5% [≥312.9 ng/mL], and a poor-responder group [group P, n = 16], in which it was necessary to use hMG more than in the uppermost 5% [≥3,900 IU] to obtain mature follicles), neither group included cases of women who became pregnant (pregnancy group, group G; n = 20), as seen in Figure 3. Therefore, we examined the correlation between the concentration of AR and exogenous hCG in FF, as shown in Figure 4 (r = 0.50, P = .024, slope factor = −0.57).

**DISCUSSION**

Amphiregulin first was isolated from serum-free conditioned media of MCF-7 human breast carcinoma cells and is a potent

![Figure 1](image1.png)

**FIGURE 1**
Concentration of AR in human FF in relation to embryo quality that was evaluated according to Veeck’s criteria (mean ± SEM). NS = not statistically significant.

![Figure 2](image2.png)

**FIGURE 2**
Concentration of AR in human FF in relation to FR (mean ± SEM). There was a significant difference between the low-FR group and high-FR group, but comparisons between the other two pairs indicated were not significant (NS).

![Figure 3](image3.png)

**FIGURE 3**
Relationship between the concentration of AR (Y axis) and the dose of hMG used for controlled ovarian hyperstimulation in IVF (X axis). Open circles, samples obtained from patients who became pregnant. Closed circles, samples obtained from patients who did not achieve pregnancy. No instances of pregnancy were found in either the high-AR group or the poor-responder group.
mitogen for fibroblasts and keratinocytes (24, 25). Initially, AR was demonstrated in human ovarian epithelial cells, but not in granulosa cells (18). However, more recent studies have demonstrated up-regulation of the AR gene in granulosa cells. For example, Fru et al. (26) reported that the expression of AR mRNA in granulosa cells of rhesus macaques was undetectable before hCG administration but was induced 3 hours after hCG and remained elevated at 24 hours. The same result was obtained in rats (9). Furthermore, some studies have demonstrated that AR itself mimics the action of LH on the resumption of oocyte maturation (4, 14). Some studies have suggested that AR is involved in follicular maturation in the human ovary. Rimon et al. (27) reported, based on results with a DNA microarray, that the gene coding AR was dramatically up-regulated by LH (286-fold) and by the direct adenylate cyclase activator forskolin (859-fold) in primary human granulosa cells that were obtained from IVF patients. Furthermore, in human granulosa cells, it has been demonstrated that LH, forskolin (10), FSH (19), and PGE2 (28) induced the expression of AR mRNA or protein. We have no previous studies that have demonstrated that LH (or hCG) increased the expression of AR mRNA or protein. In the present study, we demonstrated for the first time that AR is the most abundant EGF receptor ligand among three members of the EGF receptor ligand family (EGF, TGFα, and AR), as measured in human FF obtained at ovum pickup for infertile patients undergoing IVF-ET. The abundance of AR in FF is consistent with findings from studies elsewhere that have suggested that AR is involved in oocyte maturation. However, to our surprise, we found that AR is inversely related to oocyte quality, FR, and pregnancy outcome. Furthermore, the concentration of AR also was inversely related to that of exogenous hCG, which we used as an index of hCG signal to follicle (22). Thus, the elevation of AR does not imply the competence of oocyte maturation, and we can speculate that hCG injection may decrease the AR concentration in FF to trigger oocyte maturation. This result is in sharp contrast to results of previous studies that have demonstrated that LH (or hCG) increased the expression of AR mRNA or protein. We have no data regarding the longitudinal time-dependent changes in EGF receptor ligands in human FF because it is difficult to collect FF from immature oocytes before hCG administration in a clinical setting. However, in murine granulosa cells, the increase in AR protein by hCG reached a peak at about 8 hours after hCG injection and then decreased gradually and returned to the baseline level at 16 hours. If the same time-dependent changes take place in human beings, it is possible that hCG increased AR early after injection. Amphiregulin also decreases relatively quickly, and a delayed decrease in AR may have a negative effect on oocyte maturation (15).

It is well recognized that the binding of LH or hCG to its receptor stimulates adenylate cyclase and hence cyclic adenosine monophosphate production, leading to cyclic adenosine monophosphate–mediated events, predominantly via the protein kinase A–signaling pathway. In addition, other kinase-signal transduction pathways have been implicated in an LH-induced cascade of events, including protein kinase C. Furthermore, granulosa cell differentiation by FSH also uses multiple signal transduction pathways (16, 30), and AR plays a substantial role in these processes. On the basis of the results of the present study, it is not possible to clarify what kind of signal pathway is involved in the cascade of events in periovulatory human follicles induced by LH or hCG and what role AR plays in this cascade. In rodent, Jo and Curry (31) demonstrated that hCG increased AR and that AR increased the expression of mRNA for the transcription factor Runx1 in cultured granulosa cells, which suggests that AR acts by inducing gene expression.

It has not yet been established whether AR acts directly on the oocyte itself or indirectly via cumulus cells, or both. Park et al. (4) reported that hCG acts on mural granulosa cells and stimulates the expression of AR, epiregulin, and betacellulin. These EGF receptor ligands act on cumulus cells and promote germinal vesicle breakdown and cumulus cell expansion. Shimada et al. (15) demonstrated that hCG induced AR mRNA in both mural granulosa cells and cumulus cells. These results indicated that AR acts on cumulus cells rather than directly on oocytes.

In this study, the level of EGF in human FF was very low and was less than that in serum. In fact, it was below the cutoff level in about 92.3% of the samples. Thus, EGF may not play a major role in hCG signal transduction, at least in human beings. Gómez et al. (32) reported that EGF, when added to culture media before insemination, had no positive effects on either the FR or blastomere development of human oocyte. This is consistent with our finding of scant EGF in FF. However, similar results have not been seen in other species. For example, it has been revealed that EGF promotes germinal vesicle breakdown and meiosis progression in mouse (33).
Furthermore, the same positive effect of EGF added to culture medium on the germinal vesicle breakdown rate also was reported in the in vitro maturation of cumulus-denuded human immature oocytes by Goud et al. (34). This means that EGF may be important at an early stage of oocyte maturation, but not at a late, periovulatory stage of maturation. However, using immunohistochemical techniques, Maruo et al. (17) and Reeka et al. (20) reported that the expression of EGF in human granulosa cells became apparent in the antral follicles during the midfollicular phase and increased as the size of the follicle increased. Thus, granulosa cells at a periovulatory stage expressed a considerable amount of EGF, which suggests that EGF may be physiologically important at a late stage of follicular development. These results are in conflict with our data. However, Reeka et al. (20) also reported that EGF was not detected in FF. The cause of the discrepancy between the expression of EGF in granulosa cells and its paucity in FF was not resolved. Westergaard and Anderson (35) reported that EGF was detected in preovulatory follicles in women undergoing ovarian hyperstimulation for ovulation induction, as with our subjects, but the level of EGF in FF was 50% of the level in serum, indicating that EGF in FF of preovulatory follicles may be passively diffused from the circulation. In our study, the level of EGF in FF was very much lower than that in serum, and thus the same speculation is pertinent.

The level of TGFα in human FF was distinctly less than that of AR. However, TGFα was detectable in all FF samples. Tamura et al. (18) found that TGFα was present predominantly in theca cells of antral follicle. Qu et al. (8), using an immunohistochemical technique, reported that in human follicles, TGFα was expressed in various cells, including oocytes, granulosa cells, theca cells, and stroma cells, at several developmental stages. As mentioned before, Reeka et al. (20) also reported that the expression of TGFα was detected in granulosa cells and theca cells at various developmental stages, including atretic follicles. Thus, we cannot exclude the possibility that TGFα is involved in the cascade of events in human follicular maturation.

This study has some limitations. First, because of technical limitations, we did not obtain FF from individual separate follicles but rather from all follicles in each ovary. Thus, FF was obtained from both mature and immature follicles. We could not exclude the possibility that this may affect the results in our comparison of the levels of three EGF receptor ligands. Second, all of the study participants were Japanese, so the results may not be applicable to other races and ethnicities. Third, FF was obtained from infertile patients who were stimulated with hMG, not from normal fertile females, even though the causes of infertility were mainly male factors and tubal factors. It is possible that there may have been some differences in physiological follicular maturation.

In conclusion, we have demonstrated that AR is very abundant in human FF and may play an important role in the hCG signal pathway in human ovary. Levels of AR were inversely related to FR and hCG levels in FF, and the elaborate interaction of AR with hCG or LH may be required for a better outcome in IVF-ET.

REFERENCES


