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INCREASING LEVELS OF AMPHIREGULIN IN FOLLICULAR FLUIDS ARE ASSOCIATED WITH HUMAN OOCYTE MATURATION

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Abstract

Luteinizing hormone stimulates ovarian somatic cells to induce hormone release for oocyte maturation. Accumulating evidences in rodents indicate importance of epidermal growth factor (EGF)-like growth factors [amphiregulin (*AREG*), epiregulin (*EREG*) and betacellulin (*BTC*)] from the somatic cells in oocyte maturation. Here, we investigated an involvement of ovarian EGF-like growth factors in human oocyte maturation. Using isolated human ovarian cells, we found the expression of *AREG*, *EREG* and *BTC* mRNAs in granulosa and cumulus cells by RT-PCR, and AREG protein in cumulus cells, suggesting their production in ovarian somatic cells. In human follicular fluids aspirated from individual follicles undergoing *in vitro* fertilization-embryo transfer and granulosa cells obtained by centrifugation of the fluid, the association between AREG, EREG, and BTC levels and oocyte maturation was examined by using enzyme-linked immunosorbent assay or quantitative real-time RT-PCR. **AREG protein level was significantly higher in the follicular fluids that yielded mature oocytes.** However, *EREG* mRNA level in granulosa cells was insignificant between mature and immature oocytes, and BTC protein was absent from all follicular fluids examined. Our findings suggest an important role for AREG, but not EREG and BTC, in optimal maturation of human oocytes.

Key words : amphiregulin, EGF-like growth factors, follicular fluid, oocyte maturation

Introduction

In vertebrates, ovulation and final maturation of oocytes occur in response to stimulation by pituitary-derived luteinizing hormones (LH) that act on the somatic granulosa and theca cells surrounding the oocyte. LH stimulates the somatic cells to induce hormone release for oocyte maturation, as oocytes lack LH receptors. Shortly after stimulation by the preovulatory

surge of LH, oocytes arrested in late prophase resume meiosis, characterized by germinal vesicle breakdown (GVBD), chromosome condensation, and extrusion of the first polar body in preparation for fertilization and early embryonic development. Recent studies have demonstrated that the endocrine hormone LH stimulates ovarian production of insulin-like 3 from theca cells[1] and follicular fluid meiosis-activating sterol (FF-MAS) from cumulus cells[2,3] to promote GVBD. Furthermore, hormonal regulation of first polar body extrusion has been shown to be induced by brain-derived neurotrophic factor (BDNF)[4,5], FF-MAS and its related compounds[6,7], and leptin[8].

Although epidermal growth factor (EGF) induces oocyte maturation[9,10], physiological EGF production in ova-

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ries is not regulated by LH[11,12]. Recently, EGF-like growth factors were found to be potent inducers for nuclear maturation of oocytes in rodents, and their production was upregulated by LH stimulation[11,13]. EGF-like growth factors comprise closely related proteins that include transforming growth factor alpha, heparin-binding EGF-like growth factor, amphiregulin (AREG) and epiregulin (EREG), betacellulin (BTC), epigen, and neuregulins[14]. All the EGF-like growth factors bind with different specificity and activate four EGF receptor (EGFR) subfamily (ErbB1-4)[14]. Among the EGF-like growth factors, *AREG*, *EREG* and *BTC* transcripts are expressed in rodent granulosa cells and their levels were increased by treatment with human chorionic gonadotropin (hCG)[11,15-17]. The upregulation of AREG expression after hCG treatment has also been confirmed at the protein level by immunoassays in ovaries[17]. AREG and EREG exhibit strong activities for induction of GVBD as well as cumulus expansion[11,16].

Despite increasing evidence of ovarian EGF-like growth factors that induce nuclear maturation of oocytes in animals, no substantial findings of induction of the nuclear maturation by those factors have been reported in humans. The expression of *AREG* and *EREG* mRNAs[18-20], and AREG protein[18,20] has been demonstrated in isolated granulosa cells. Furthermore, the levels of *AREG* and *EREG* transcripts in cultured granulosa cells were increased by treatment with LH[18]. However, little is known about their contribution on nuclear maturation of oocytes in human. To explore whether the nuclear maturation of oocyte in women undergoing *in vitro* fertilization and embryo transfer (IVF-ET) is related to the levels of EGF-like growth factors in follicles, we investigated (i) the expression of AREG, EREG, and BTC in human ovarian cells, (ii) their presence in the follicular fluid, and (iii) the association of either the levels of AREG protein in follicular fluid or the levels of EREG transcripts in granulosa cells with nuclear maturity of the oocyte.

Materials and methods

Sample collection from patients undergoing IVF-ET

The study population included 16 patients (20 cycles) 34.7 ± 1.1 years old (mean±SE). All patients were from couples undergoing IVF with intracytoplasmic sperm injection (ICSI) treatment for male-factor infertility. Fourteen cycles constituted a standard institutional stimulation protocol of ovarian downregulation with gonadotropin releasing hormone agonist (Nasanyl : Pfizer, Tokyo, Japan) followed by controlled ovarian stimulation with human menopausal gonadotropin (HMG) (HMG Nikken : Kowa pharmaceutical, Tokyo, Japan, or HMG injection Teizo : ASUKA pharmaceutical, Tokyo, Japan). Six cycles were treated with clomiphene citrate (Clomid : Shionogi, Osaka, Japan) followed by HMG administration, and oocyte maturation was induced using hCG (Gonatropin : ASUKA pharmaceutical, Tokyo, Japan) injection. We collected individual follicular fluid and matched cumulus-oocyte complex from total 67 follicles. In addition, serum samples were also collected in each cycle at the time of oocyte retrieval.

During ovarian stimulation, follicular monitoring was performed via ovarian ultrasonography, and oocyte maturation was triggered with 5,000 or 10,000 IU of hCG when the largest follicle was 19 mm or greater in size. At the time of oocyte retrieval, follicular fluid was aspirated separately from each follicle under ultrasound-guided transvaginal retrieval[21], and the aspiration tube was flushed between aspirates. Individual follicular fluid was centrifuged at 1,000 rpm for 10 min to remove the cellular component. The clear supernatant fraction was stored at $-80°C$ for enzyme-linked immunosorbent assay (ELISA).

Following oocyte retrieval, cumulus-oocyte complexes (COCs) were transferred to human tubal fluids (HTF) medium, and some cumulus cells were stripped from the COC with a 27G needle under a stereomicroscope (Olympus, Tokyo, Japan) subjected to RNA extraction and immunocytochemistry. After removal of remaining cumulus cells, the oocytes were classified according to nuclear maturity : mature [metaphase II (MII) stage], or immature [germinal vesicle (GV) or metaphase I (MI)

stage] oocyte.　ICSI was then performed on MII stage oocytes only.

All patients gave written informed consent to participate in the study, and the experimental design and involvement of human subjects in this study were approved by the ethics committees of Akita University Hospital.

RT-PCR

For RT-PCR, unfertilized oocytes after ICSI or conventional IVF were collected, mural granulosa cells were collected from the follicular aspirates as individual clumps or sheets of cells under the stereomicroscope, and normal ovaries were obtained from patients undergoing surgery for uterine carcinoma presenting no metastasis or invasion into the ovaries.　Total RNA was extracted using the RNeasy Micro kit or RNeasy Mini kit (Qiagen K.K, Tokyo, Japan).　RT-PCR was performed as described previously[22].　Primers were based on GenBank accession numbers ; *AREG* (NM_001657), *EREG* (NM_001432), *BTC* (NM_001729), and β-*actin* (NM_001101).　The primers were as follows : *AREG,* sense 5′-CTGCGAAGGACCAATGAGAG-3′, antisense 5′-ATTGAGGTCCAATCCAGCAG-3′ ; *BTC,* sense 5′-TTCACTGTGTGGTGGCAGAT-3′, antisense 5′-TGTG-GTGGTAGCTGCACAGT-3′.　The primers for *EREG* and β-*actin* were the same as those for real-time RT-PCR described below.　The program of PCR cycles was as follows : denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min.　For the positive control, human ovary cDNA was amplified simultaneously.　For the negative control, a specimen in which water was substituted for mRNA was amplified.

Quantitative real-time RT-PCR of transcript levels in granulosa cells collected from the follicular aspirates as described above was performed using a Smart Cycler II (Takara, Ootsu, Japan) as described previously[5,23].　The primers and probes for *EREG* and β-*actin* were as follows : *EREG*, sense 5′-CCATGGCTGACCTCTG-GA-3′, antisense 5′-CAGTCGTGTGATTGCACCAG-3′ ; and probe 5′-6-carboxy-fluorescein (FAM)-CTT-GTCTCCATGTGTATCCATGCATT-6-carboxy-tetra-methyl-rhodamine (TAMRA)-3′ ; β-*actin* ; sense 5′-TCTGTGTGGATTGGTGGCTCTA-3′, antisense 5′-CTGCTTGCTGATCCACATCTG-3′, and probe 5′-FAM-

CTTGCCCACAGCCTTGGCAGC-TAMRA-3′.

The PCR program was as follows : denaturation at 95°C for 10 sec, followed by 60 cycles of amplification.　Cycling conditions were as follows : denaturation at 95°C for 5 sec, and annealing/elongation at 60°C for 20 sec.　To correct for differences in RNA quality and quantity between samples, data were normalized based on β-*actin* transcript levels.

Immunocytochemistry

Because AREG protein has been shown to express in human granulosa cells[18-20], we tried to determine the expression of AREG protein in human cumulus cells.　Cumulus cells were incubated with hyaluronidase (80 IU/ml) (Medi Cult, Jyllinge, Denmark) for 1-2 min, followed by 2 washes with HTF.　For cell culture, cumulus cells from 13 COCs of 3 patients were pooled and then plated onto Lab-Tek 16 Chamber Slide culture chambers (Nunc, Naperville, IN).　The cumulus cells were adjusted to 20×10^3 cells/well in Ham's F-10 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, and were cultured at 37°C under 5% CO_2 in air for 48 h.

At the end of culture, immunocytochemistry was performed as described[24].　The cumulus cells were fixed with 1.5% paraformaldehyde for 45 min at room temperature, and then permeabilized with 0.2% Triton X-100 (Sigma, St. Louis, MO) for 5 min.　Then, fixed cells were treated with blocking buffer including 10% normal goat serum for 30 min, and were incubated with goat anti-human amphiregulin antibody (R&D systems, Minneapolis, MN) diluted at 15 μg/ml for 1 h at room temperature.　After three washes, the cells were incubated with secondary antibodies [ENVISION kit /HRP (DAB) (Dako cytomation, Tokyo, Japan)] for 1 h at room temperature.　Bound antibodies were visualized with diaminobenzidine tetrahydrochloride.　Counter staining was performed with hematoxylin.　For the negative controls, the cells were treated as described previously except that the primary antibody was omitted.

ELISA and radioimmunoassay (RIA)

ELISAs for AREG and BTC were performed as described previously[5] using ELISA kits (R&D sys-

tems). The follicular fluids were diluted 1 : 160 in AREG, and 1 : 3 in BTC with PBS before use with the ELISA kits. The minimum detectable levels were 7.8 pg/ml, and intra- and inter-assay coefficients of variation were 4.5% and 6.3%, respectively. The concentrations of estradiol in the follicular fluids were also determined by using RIA (Mitsubishi Chemical Medicine, Tokyo, Japan). The minimum detectable levels were 10 pg/ml.

Statistical analysis

The Wilcoxon-Mann-Whitney test (Kaleida Graph 4.0, Hulinks, Tokyo, Japan) was performed for the comparison of AREG concentration in follicular fluids and the expression levels of *EREG* mRNA in granulosa cells. Statistical significance was established at the $P < 0.05$ level.

Results

Expression of EGF-like growth factors mRNAs in human isolated ovarian cells

Using isolated ovarian cells, the localization of EGF-like growth factors in ovary was determined by RT-PCR (Fig. 1). Transcripts of *AREG*, *EREG* and *BTC* were detected in granulosa cells. Although cumulus cells expressed *AREG* and *BTC* mRNAs, the expression of *EREG* mRNA was not detected. In oocytes, *EREG* mRNA was expressed, whereas expression of *AREG* and *BTC* mRNAs was not detected.

Expression of AREG protein in cultured human cumulus cells

We confirmed the expression of AREG protein in cultured cumulus cells (Figs 2, A and B). Strong AREG signals were detected in the cytoplasm of cultured cumulus cells (Fig. 2A), whereas no specific expression was observed in the negative controls (Fig. 2B).

The levels of AREG protein in follicular fluids with mature and immature oocytes

To assess the association of the levels of EGF-like growth factors in follicular fluid with nuclear maturity of oocyte, we quantified the levels of AREG and BTC protein in follicular fluids (Fig. 3). Of 67 follicle aspirates, the mature group represented follicular fluids yielding
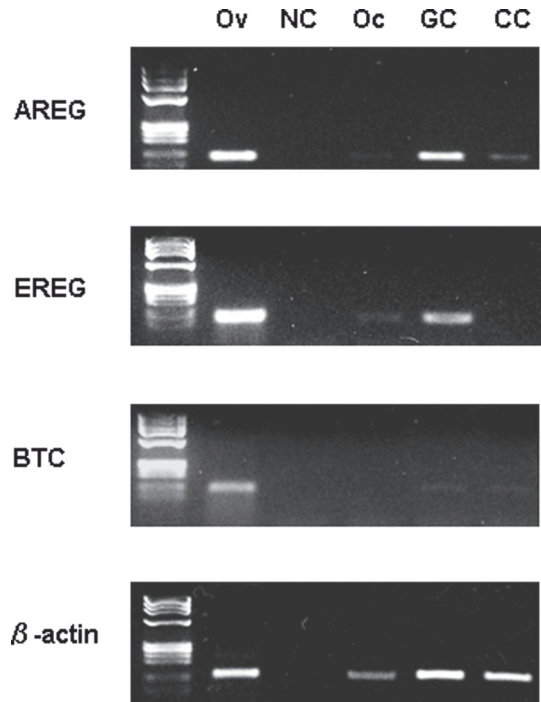

Fig. 1. RT-PCR detection of amphiregulin (*AREG*), epiregulin *(EREG)*, and betacellulin (*BTC*) mRNAs in isolated human ovarian cells. For internal controls, β-actin was amplified simultaneously in each PCR reaction. Total ovarian cDNA was used in the positive controls, whereas no template DNA was included in the negative controls. The molecular marker, ΦX 174-Hae III digest ; Ov, ovary ; NC, negative control ; Oc, oocyte ; GC, granulosa cell ; CC, cumulus cell.

MII stage oocytes ($n=43$), while the immature group represented follicular fluids yielding GV and MI stages oocytes ($n=18$). Because the occurrence of degenerative changes likely affects on gene expressions in mural and cumulus granulosa cells, six follicular aspirates yielding degenerated oocytes were excluded from the study. The level of AREG protein in the mature group was significantly higher than that of the immature group. However, BTC protein was not detected in follicular fluids (data not shown). In addition, both AREG and BTC proteins were undetectable in the serum samples (data not shown).
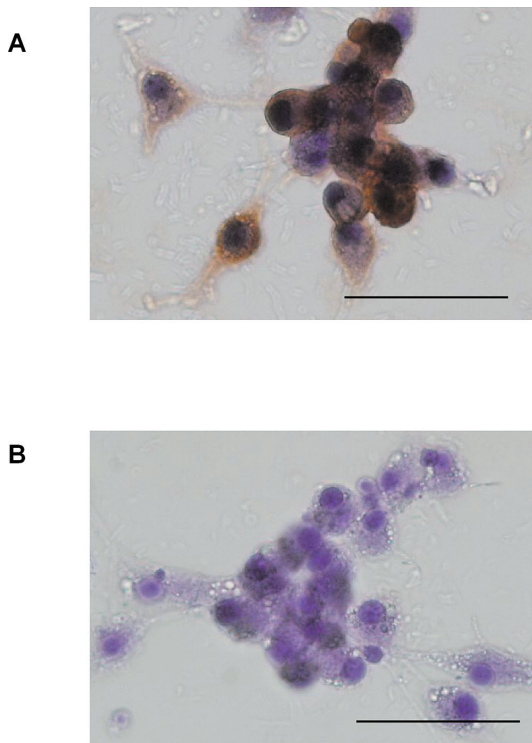
A

B

Fig. 2. Immunohistochemical detection of AREG protein in human cumulus cells. (A) AREG protein was detected as brown spots in the cytoplasm of cultured cumulus cells. (B) For the negative controls, the cells were subjected to the same method but in the absence of primary antibody. (Scale bars, 50 μm)

The levels of *EREG* mRNA in granulosa cells with mature and immature oocytes

Due to the lack of a suitable antibody to evaluate expression levels of EREG proteins, we quantified the levels of *EREG* mRNA in granulosa cells instead of measuring of EREG protein in follicular fluid (Fig. 4). The mature group represented granulosa cells obtained from follicular fluids yielding MII stage oocytes ($n=30$), while the immature group represented granulosa cells obtained from follicular fluids yielding germinal vesicle and MI stages oocytes ($n=13$). The numbers of samples in each group were lower than those of follicular fluid, because samples with suboptimal RNA extraction were excluded from the results. In contrast to the upregulation of AREG expression in the mature group, there was no significant difference in *EREG* mRNA between the ma-
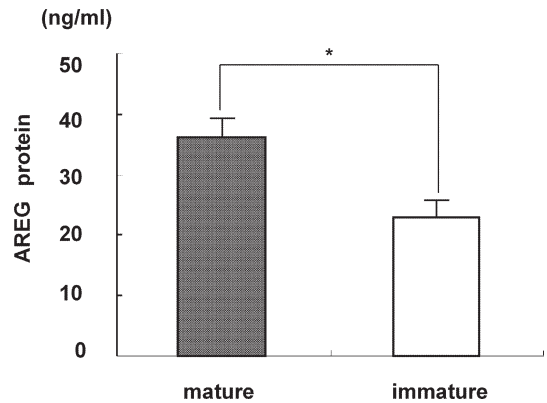

Fig. 3. Increased levels of AREG protein in the follicular fluid yielding MII stage oocytes. The levels of AREG protein were measured using ELISA. Mature : follicular fluids yielding MII stage oocytes ($n=43$) ; immature : follicular fluids yielding germinal vesicle and MI stages oocytes ($n=18$). Data are expressed as the mean$\pm$ SE. *P<0.01
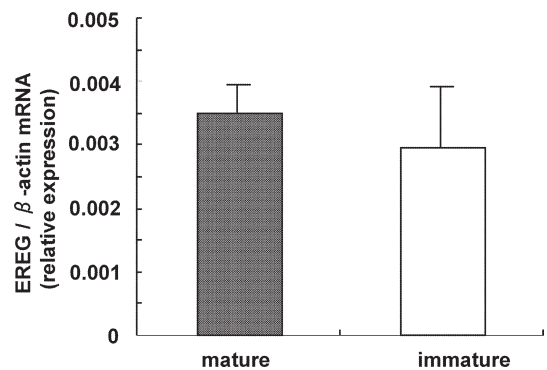

Fig. 4 The levels of *EREG* mRNA in granulosa cells obtained from follicles yielding mature or immature oocytes. Transcript levels for EREG in granulosa cells were determined using real-time RT-PCR. Levels of *EREG* mRNA were normalized based on those for *β-actin* in the same sample. Mature : follicular fluids yielding MII stage oocytes ($n=30$) ; immature : the follicular fluids yielding GV and MI stages oocytes ($n=13$). Data are expressed as the mean$\pm$SE.

ture and immature groups.

Discussion

Although the expression of *AREG* and *EREG* mRNAs

and AREG protein has been shown in human granulosa cells[18-20], there is no systemic data regarding the expression of AREG, EREG, and BTC in ovarian cells. In the present study, we detected *AREG* mRNA in granulosa and cumulus cells, and AREG protein in cumulus cells. We further demonstrated the presence of AREG protein in human follicular fluid and that its levels in follicular fluids yielding the oocytes with completion of meiosis I were higher than those yielding immature oocytes. Because AREG protein was not detected in serum at the time of oocyte retrieval, high follicular fluid concentrations of AREG are likely due to local production by granulosa and cumulus cells. Although EREG protein could not be quantified due to the lack of a suitable antibody, no marked difference in *EREG* mRNA was observed between the granulosa cells with mature and immature oocytes. In addition, transcript of *EREG* was not detected in cumulus cells. In consistent with low expressions of *BTC* transcripts in granulosa and cumulus cells, BTC protein was not detected in any of the follicular fluids. These findings suggest that, of the three EGF-like growth factors (AREG, EREG and BTC), positive association of the levels of AREG protein in follicular fluid with nuclear maturation of human oocytes. Although the amounts of the granulosa and cumulus cell component may affect on the levels of AREG protein in follicular fluid, the concentrations of estradiol in follicular fluids that reflect on the amount of those cells were similar between the mature and immature groups (mature, 369 ± 38.3 ng/ml ; immature, 337 ± 29.3 ng/ml).

Previous study demonstrated that the expressions of *AREG* mRNA and protein are increased in human granulosa cells after LH treatment[18,20]. These results further support the hypothesis of the contribution of AREG on LH-induced nuclear maturation of human oocytes. We also found that *AREG* mRNA and protein were expressed in cumulus cells. Because LH receptor expression is restricted in cumulus cell[25], LH stimulation may not directly induce AREG production in cumulus cells. Recently, similar to LH stimulation, PGE2 stimulation has been shown to increase the expression of AREG protein in human granulosa and cumulus cells[20]. Because LH-induces PGE2 production in mammalian granulosa cells[26-28], AREG expression in cumulus cells may be regulated by PGE2 produced in granulosa cells in response to LH.

In rodents, studies have demonstrated that the expression of EGF-like growth factors is upregulated by treatment with hCG in rat (AREG and EREG)[29] and mouse granulosa cells (AREG, EREG and BTC)[11,16,17]. Using DNA microarray analyses in mouse ovaries, the expressions of *AREG* and *EREG*, or *BTC* mRNA have been shown to reach their highest levels at 2 or 5 h after hCG treatment, respectively[11]. After treatment with hCG, the strong signals for AREG, EREG and BTC are detected in mural granulosa cells by *in situ* hybridization[16]. At the protein level, AREG protein was also detected in mouse cumulus cells by immunofluorescence staining of COCs[17]. Although BTC protein was not detectable in human follicular fluid, all AREG, EREG, and BTC induce GVBD in follicle-enclosed oocytes in rodents[15,16]. The discrepancy between the studies in rodents and human may be caused by species differences or experimental conditions.

In the present study, we demonstrated the positive association of AREG, but not EREG and BTC, with nuclear maturation of human oocytes. To further clarify the contributions of EGF-like growth factors on nuclear maturation of human oocytes, induction of *in vitro* oocyte maturation by treatment with EGF-like growth factors would be required. However, such experiments are difficult to perform due to ethical considerations. In addition to our findings, several ovarian factors, such as insulin-like growth factor (IGF)-I, IGF-binding protein-3[30], FF-MAS[2,3], and angiogenin[31] are detected in human follicular fluid and have been shown to be related to oocyte maturation. Future studies would clarify whether these factors play redundant roles or complement each other.

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