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Evidence for the Presence of Keratinocyte Growth Factor (KGF) in Human Ovarian Follicles

YUTAKA OSUGA*, KAORI KOGA*, OSAMU TSUTSUMI*,**, TETSU YANO*, KOJI KUGU*, MIKIO MOMOEDA*, RYUGO OKAGAKI*, AKIHIKO SUENAGA*, TOSHIHIRO FUJIWARA*, AKIHISA FUJIMOTO*, HIROTAKA MATSUMI*, HISAHIKO HIROI* AND YUJI TAKETANI*

** Department of Obstetrics and Gynecology, University of Tokyo, Hongo 7–3–1, Bunkyo-ku, Tokyo 113–8655, Japan*
*** CREST, Japan Science and Technology, Kawaguchi Center Building 1–8, Honcho 4-chome, Kawaguchi City, Saitama 332–0012, Japan*

Abstract. The presence of keratinocyte growth factor (KGF) in human follicular fluid (FF) was investigated in a total of 145 FFs obtained during oocyte retrieval for in vitro fertilization (IVF) from 29 patients with no apparent endocrine disorders. The concentrations of KGF, estradiol, progesterone, testosterone and human chorionic gonadotropin (hCG) in FF were measured by enzyme-linked immunosorbent assay. FF samples contained relatively higher amounts of KGF (2194±87 pg/ml), whereas its concentrations in serum were below assay limit (<31.2 pg/ml). Concentrations of KGF in FF were positively correlated with both progesterone ($r=0.311$, $p<0.0005$) and testosterone ($r=0.230$, $p<0.01$) concentrations in FF. However, KGF concentrations were not significantly correlated with estradiol and hCG concentrations. KGF in FF was detected as a broad band (26~29 kD) by immunoblotting, the size being reduced by 7kD after N-glycosidase treatment. In an *in vitro* experiment, KGF suppressed the basal and hCG-stimulated progesterone production by cultured human luteinized granulosa cells. In summary, we demonstrated the presence of KGF in human ovarian follicles, suggesting its possible role as a local factor in regulating human ovarian functions.

Key words: Ovary, Keratinocyte growth factor, Follicular fluid, IVF-ET

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Keratinocyte growth factor (KGF), also known as fibroblast growth factor 7 (FGF 7), is one of the ten known members of the fibroblast growth factor family [1]. KGF is a stromal cell-derived mitogen for epithelial cells. The receptor for KGF primarily localized to epithelial cells [2] is a transmembrane tyrosine kinase receptor, which is an alternatively spliced variant of FGF receptor-2.

In ovaries, KGF mRNA is detected in bovine thecal cells [3] and in bovine small luteal cells [4], with KGF proteins being secreted from these cells. In addition, the expression of KGF receptor mRNA has been demonstrated in bovine granulosa cells [5].

The biologic effects of KGF within the ovary appear to be multifold. For instance, KGF induces proliferation of bovine granulosa cells [3], while it reduces basal and FSH-stimulated aromatase activity in bovine and rat granulosa cells [5].

Given these observations, it is intriguing to speculate that KGF may play a role during ovarian follicular development and ovulation by modulating gonadotropin actions.

However, thus far, no information regarding KGF in human ovaries is available. As a first step in exploring a physiological role of KGF in human ovaries, we asked whether KGF is present in follicular fluid (FF). Here we demonstrated the presence of considerable amounts of KGF in FF, highlighting a

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Correspondence to: Dr. Yuji TAKETANI, Department of Obstetrics and Gynecology, University of Tokyo, Hongo 7–3–1, Bunkyo-ku, Tokyo 113–8655, Japan

possible role of KGF in the human ovary.

MATERIALS AND METHODS

Subjects

After obtaining informed consent, 29 infertile women undergoing in vitro fertilization and embryo transfer (IVF-ET) for tubal factor or male factor were recruited in this study. Their age ranged from 26 to 40 years (mean±SEM, 34.9±0.7 years). The women were pretreated with gonadotropin releasing hormone analogue (buserelin acetate, Suprecur; Aventis Pharma, Tokyo, Japan), starting in the midluteal phase of the preceding cycle. After verifying ovarian suppression by observing the ovaries and endometrium by ultrasonography, daily intramuscular injections of 150–300 IU human menopausal gonadotropin (HMG; Nikken, Tokyo, Japan) were given depending on individual responses. When the diameter of the leading follicle reached 17 mm or greater, human chorionic gonadotropin (hCG) at a dose of 10,000 IU (hCG; Mochida, Tokyo, Japan) was administered. Transvaginal ultrasound-guided oocyte retrieval was performed 35 hours later. During oocyte collection, each follicle was aspirated separately and the FF was collected into a sterile plastic tube by electricity-driven suction. The oocyte was isolated from the aspirate and its degree of maturity was assessed according to the classification of Hoshi *et al.* [6]. After isolation of the oocyte, aspirated FF was immediately centrifuged (1500 xg), and the supernatant was stored at −80°C until use. Only follicles in which an oocyte was clearly identified were analyzed, with a total of 145 FFs being determined for concentrations of KGF and hormones stated below. Serum was also sampled from each woman just before the oocyte collection.

Isolation and culture of luteinized granulosa cells

Isolation and culture of human luteinized granulosa cells (GCs) were described in our previous study [7]. To obtain GCs for culture, FF was aspirated with repeated flushes. All the follicular aspirates and flush medium from each woman were mixed and centrifuged at 200 xg for 5 min to obtain a cell pellet, which was resuspended in phosphate-buffered saline (PBS) with 0.1% hyaluronidase and incubated at 37°C in a shaking water bath for 30 min. Suspension was layered onto Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 150 xg for 30 min. GCs recovered from the interface were washed with PBS. Isolated GCs were resuspended in DMEM medium containing 10% FBS, 100 units/mL penicillin, 0.1 mg/mL streptomycin and 0.25 μg/mL amphotericin B. The cells were plated at a density of 25,000 cells/well in 24-well plate and kept at 37°C in a humidified 5% CO_2/95% air environment. After 48 h, media were replaced with the medium containing 5% FBS, and the cells were cultured without (control) or with hCG (150 ng/mL; Mochida, Tokyo, Japan) and/or rhKGF (100 ng/mL; Genzyme Techne, Minneapolis, MN) for 24 h. Conditioned medium was collected, centrifuged and stored at −80°C for subsequent analysis.

Hormone Measurement

Concentrations of estradiol, progesterone, testosterone and hCG in FFs were measured in duplicate with the use of the Serono SR1 analyzer (Serono, Allentown, PA, USA). The standard preparation of hCG was WHO 1st IRP (75/537).

KGF concentrations in FFs and sera were measured in duplicate, using a specific enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). The limit of sensitivity of this ELISA was 31.2 pg/mL. The intra-assay coefficients of variation were <10%. All assays were conducted by investigators who had been blinded to the clinical data.

For the measurement of progesterone concentrations in culture media, progesterone enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) was used.

Partial purification of KGF

The KGF in FF were partially purified by heparin-Sepharose affinity chromatography. The slurry of 1.0 g Sepharose CL-6B (Pharmacia Inc., Piscataway, NJ) was incubated with approximately 100 mL FF with gentle stirring for 3 hours at 4°C. The mixture was poured into an open column. The packed column was washed with PBS and eluted with 20 mL

NaCl in step wise concentrations of 200 mM, 400 mM, 600 mM, 800 mM and 1 M. Because more than 95% of KGF determined by ELISA was in the eluted fraction of 600 mM NaCl, further procedure was done using this fraction. The eluted fraction was further concentrated to ~100 μL by Bio-Max 10 (Millipore, Bedford, MA) and Centricon 10 (Millipore).

Deglycosylation experiments

Fifteen μL of partially purified KGF was incubated with 5 μL (5 units) of N-Glycosidase F (Boehringer-Mannheim, Mannheim, Germany) in PBS at 37°C for 48 hours.

Western blotting

Glycosylated and nonglycosylated partially purified KGF was resolved by gradient (10–20%) SDS-PAGE under reduced conditions in the parallel lane with *E.coli*-derived recombinant human KGF (R&D Systems). The proteins were transferred to nitrocellulose membranes and incubated with anti-human KGF goat polyclonal antibody (R & D Systems) using the ECL Western blotting system (Amersham, Buckinghamshire, England).

Statistic analysis

Data are expressed as means±SEM. Correlation analysis was performed with linear regression analysis. Data were evaluated using ANOVA with post hoc analysis (Fisher's protected least significance) for multiple comparisons. A $p<0.05$ was accepted as statistically significant.

RESULTS

In all the FFs examined, concentrations of KGF were above the limit of sensitivity of ELISA. Concentrations of KGF in FF were 2194±87 pg/ml. On the contrary, all serum samples measured less than the limit of sensitivity, 31.2 pg/ml. The volumes of FFs and the concentrations of estradiol, progesterone, testosterone and hCG in FFs were shown in Table 1.

As shown in Fig. 1, simple regression analysis showed that the concentrations of KGF in FFs were positively correlated with those of progesterone ($r=0.311$, $p=0.0002$) and testosterone ($r=0.230$, $p=0.006$). In contrast, there was no significant correlation between the concentrations of KGF and those of estradiol or hCG.

KGF concentrations in FFs were not related to the maturation stage of corresponding oocytes. No significant correlation was detected between KGF concentrations and the volume of FF. In 7 women who became pregnant following IVF-ET, their mean KGF concentrations (1754±308 pg/ml) in FFs appeared to be lower than those in the remaining non-pregnant women (2428±195 pg/ml), but not significantly.

Western blotting of KGF is shown in Fig. 2. A broad band of approximately 26–29 kD was detected in the lane of partially purified KGF obtained from FF. Treatment with N-glycosidase F decreased the size of KGF by 7 kD, thus producing 20–22 kD sized KGF. The band of N-glycosidase treated KGF appeared to contain two distinct bands which differed in

Table 1. Concentrations of keratinocyte growth factor (KGF), estradiol, progesterone, testosterone and human chorionic gonadotropin (hCG) in follicular fluid and the volume of follicular fluid aspirated.

	Mean±SE	Median	Range
KGF (pg/ml)	2194.0±87.1	1915.4	257.1-5551.0
Estradiol (ng/ml)	2757.0±128.9	2391.5	369.7-8567.1
Progesterone (μg/ml)	10.0±0.5	9.9	0.7-39.0
Testosterone (ng/ml)	11.2±1.0	7.7	1.7-68.0
hCG (mIU/ml)	153.3±8.7	117.0	16.9-441.6
volume (ml)	4.0±0.2	3.7	1.4-10.4

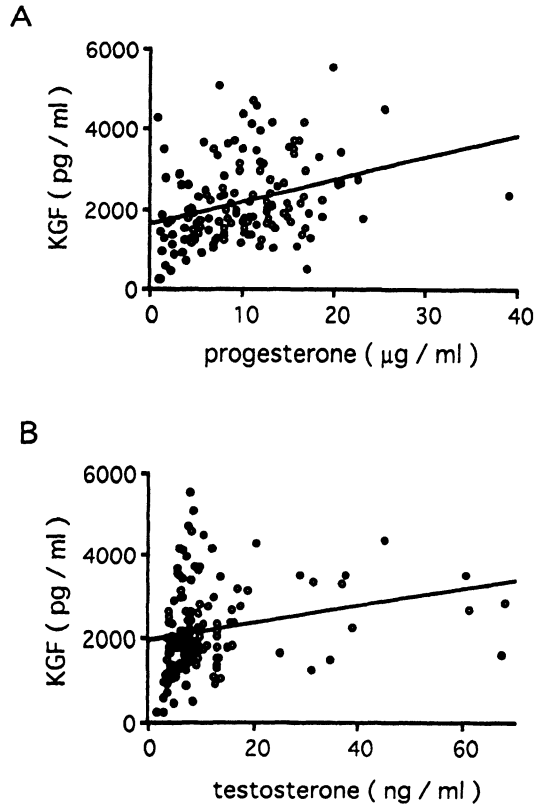

Fig. 1. A, Correlation between concentrations of keratinocyte growth factor (KGF) and progesterone in follicular fluid ($y=54.3x+1650.6$; $r=0.311$; $p<0.0005$). B, Correlation between concentrations of KGF and testosterone in follicular fluids ($y=20.3x+1966.3$; $r=0.230$; $p<0.01$).

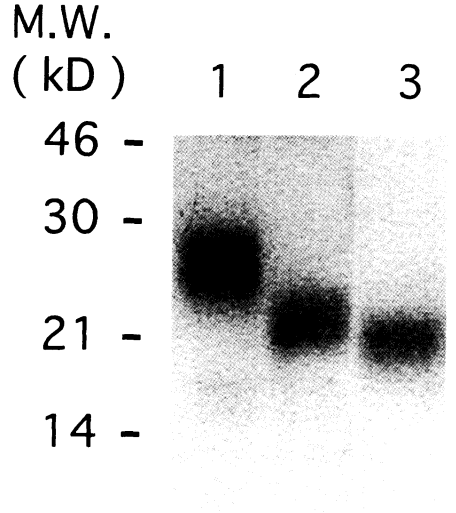

Fig. 2. Western blotting analysis of keratinocyte growth factor (KGF) with an anti-human KGF goat polyclonal antibody. Partially purified KGF in follicular fluids with heparin-Sepharose with or without N-glycosidase treatment alongside *E. coli*-derived recombinant human KGF were separated on gradient SDS-PAGE (10–20% gel) under reducing conditions. *lane 1*, KGF in FFs; *lane 2*, KGF in FFs with N-glycosidase treatment; *lane 3*, recombinant human KGF.

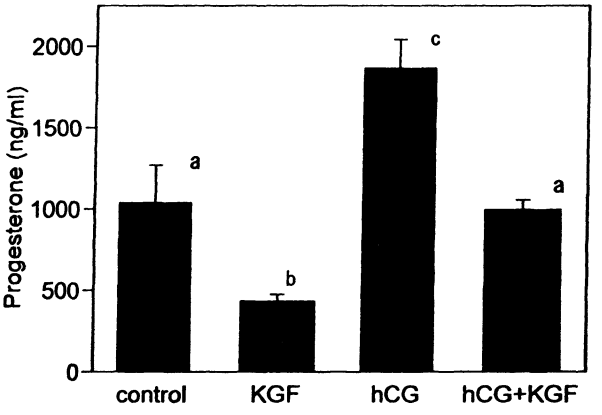

Fig. 3. Progesterone production by cultured granulosa cells. Human luteinized granulosa cells were cultured without (control) or with recombinant human KGF (100 ng/ml) and/or hCG (150 ng/ml) for 24 hours. Progesterone concentration in the culture media was determined and presented as mean±SEM of pentaplicate cultures. Bars with different superscript letters differ from each other ($p<0.05$).

molecular weight by 1~2 kD. The lower band is the same in molecular weight as *E. coli*-derived recombinant human KGF.

Cultured human GCs treated with hCG produced an approximately 80% increase in the amount of progesterone compared with that in the absence of hCG. Interestingly, the addition of KGF resulted in an approximately 50% reduction in progesterone production regardless of the presence of hCG (Fig. 3).

Discussion

The present study is the first to demonstrate that FF samples but not sera contain considerable amounts of KGF, suggesting that KGF is locally produced in human ovarian follicles.

Molecular size of KGF derived from bovine luteal tissues and bovine theca cells is reported to be 28 kD

[3, 4]. In cultured endometrial stromal cells, 20, 40 and 50 kD species of KGF have been shown [8]. In the present study, partially purified KGF obtained from human FF was shown to be of 26–29 kD size. The N-glycosidase treatment reduced the size of KGF from FF, yielding KGF of 20–22 kD, which is essentially the same as the size of *E. coli*-derived recombinant human KGF. In addition to heterogeneous N-glycosylation, several posttranslational modifications, such as partial oxidation, partial sulfation, O-glycosylation were known in KGF recombinantly expressed in Chinese hamster ovary cells [9]. The broad bands shown in this study may reflect such posttranslational modifications.

Sex steroid hormones are known to regulate KGF expression. In human prostatic stromal cells, KGF expression is increased by androgen [10]. In addition, progesterone stimulates KGF expression in rhesus endometrial stromal cells [11]. The current data demonstrated that the concentrations of KGF in FF are correlated with those of progesterone and testosterone. We therefore considered the possibility that sex steroid hormones might stimulate KGF expression in human ovarian follicles as observed in rhesus endometrium and in human prostate.

Various cytokines and growth factors, such as interleukin-1 (IL-1), IL-6, tumor necrosis factor-α, platelet-derived growth factor (PDGF), all of which are present in human ovarian follicles, are shown to induce KGF expression in human skin fibroblasts [12, 13]. These findings leave open another possibility of the involvement of cytokines/growth factors in regulating KGF expression in human ovaries.

The physiological role of KGF in follicles remains to be established. In the present study, KGF inhibited the basal and hCG-stimulated progesterone production by GCs. Inhibitory effect of KGF on progesterone production was also reported in cultured bovine granulosa cells [5], suggesting a similar effect of KGF both in the human ovary and the bovine ovary. In vitro studies using bovine or rat follicles suggested that KGF augmented granulosa cell proliferation [3]. KGF has further been shown to promote the development of rat preantral follicles [14]. Recently, Parrot et al. postulated an intriguing concept of a positive feedback loop between thecal cells and granulosa cells by demonstrating that both KGF and hepatocyte growth factor (HGF) stimulate the expression of Kit ligand (KL) in bovine granulosa cells on the one hand, and that KL stimulates the expression of KGF and HGF in thecal cells on the other hand [15]. HGF has been demonstrated to be present in considerable amounts in human ovarian follicles, suggesting a role in human ovaries [16]. Coupled with these findings, the presence of KGF in the ovarian follicle supports the contention that KGF has a possible physiological role in human folliculogenesis as well.

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