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Establishment and Characterization of a Steroidogenic Human Granulosa-Like Tumor Cell Line, KGN, That Expresses Functional Follicle-Stimulating Hormone Receptor

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ABSTRACT

We established a steroidogenic human ovarian granulosa-like tumor cell line, designated KGN, from a patient with invasive ovarian granulosa cell carcinoma. KGN had a relatively long population doubling time of about 46.4 h and had an abnormal karyotype of 45,XX, 7q-, -22. A steroid analysis of the cultured medium by RIA performed 5 yr after the initiation of culture showed that KGN was able to secrete pregnenolone and progesterone, and both dramatically increased after stimulation with $(Bu)_2$cAMP. However, little or no secretion of 17α-hydroxylated steroids, dehydroepiandrosterone, androstenedione, or estradiol was observed. The aromatase activity of KGN was relatively high and was further stimulated by $(Bu)_2$cAMP or FSH. These findings showed a pattern similar to that of steroidogenesis in human granulosa cells, thus allowing analysis of naturally occurring steroidogenesis in human granulosa cells. Fas-mediated apoptosis of KGN was also observed, which mimicked the physiological regulation of apoptosis in normal human granulosa cells. Based on these findings, this cell line is considered to be a very useful model for understanding the regulation of steroidogenesis, cell growth, and apoptosis in human granulosa cells. (*Endocrinology* **142:** 437–445, 2001)

STUDIES OF THE physiological functions of the human ovaries have recently made significant progress, and important findings on the regulation mechanisms of oogenesis, folliculogenesis, ovarian atresia, and steroidogenesis continue to increase (1). Primary culture systems of human granulosa cells isolated from ovarian follicles have often been used for the studies of granulosa cells (2–11). Human granulosa cells are obtainable mainly from *in vitro* fertilization programs. However, they are only obtainable in small numbers, and they do not survive in culture for extended cell generations. Such difficulties in obtaining and maintaining primary culture systems and also in preparing uniform cell populations in sizable amounts have often prevented the performance of the detailed analyses on molecular and cell biological levels.

During the last 2 decades, although several animal ovarian granulosa cell lines, mostly immortalized by oncogenes and oncoviruses have been reported (12–25), only five human granulosa cell lines have been established (26–30). These human granulosa cell lines include 1) lines established by the long-term culture of human granulosa cell tumor cell (26, 27), 2) human granulosa-lutein cells immortalized with SV40 large T antigen (28), 3) a line established by a forced introduction of the human papillomavirus gene to the primary human granulosa cells (29), and 4) a line most recently established by triplet transfection of primary human granulosa cells with the SV-40 DNA, Ha-*ras* oncogene, and a temperature-sensitive (ts) mutant of the p53 tumor suppresser gene (30). Although these human cell lines are useful and offer some promise of further discoveries in this field, none was reported to express functional FSH receptor.

In the present study we established a new line of human granulosa-like cells from a tumor specimen enucleated from a patient who showed a local recurrence of a granulosa cell tumor after menopause. We consider this cell line, KGN, to be very unique and useful, because it maintains most physiological activities, including the expression of functional FSH receptor, as well as the same pattern of steroidogenesis and Fas-mediated apoptosis as those observed in normal granulosa cells.

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Materials and Methods

Case history

A 63-yr-old woman with a tumor in her pelvic space was admitted to the gynecology division of Kyushu-Rosai Hospital in April 1984. After a series of clinical and laboratory examinations, a diagnosis of ovarian

cancer stage III was made, and a surgical operation (total abdominal hysterectomy, bilateral salpingo-oophorectomy, and omentectomy) was performed in May 1984. The histopathological diagnosis indicated granulosa cell carcinoma (Fig. 1, A and B). In December 1993, the tumor recurred in the pelvic region. A portion of the granulosa tumor tissue obtained at the time of reoperation in January 1994 was used as the source of the cell culture.

Establishment of the KGN cell line

A specimen of enucleated granulosa tumor tissue was finely minced and dispersed to cells by treatment with 0.25% collagenase at 37 C for 1 h. Thereafter, the cells were cultured for several passages in a 1:1 mixture of DMEM and Ham's F-12 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Sera Laboratory Ltd., Sussex, UK), penicillin (100 U/ml), streptomycin (100 μg/ml), and HITES (hydrocortisone, insulin, transferrin, estradiol, and sodium selenite) (31). After the 10th passage, the amounts of HITES in the culture medium were gradually decreased. After the 15th passage, the cells were maintained in a DMEM/Ham's F-12 medium supplemented with 10% FCS alone. After that, the cells were passaged every 1–2 weeks with 0.25% trypsin-1 mM EDTA, and the morphologically homologous cell population was thus obtained.

In vitro cell morphology and growth characteristics

The morphological appearance of the KGN cells in a monolayer culture was observed by phase difference microscopy (Nikon, Tokyo, Japan). The rate of cellular proliferation was measured for monolayer cultures of KGN in a logarithmic growth phase at a starting concentration of 2×10^5 cells/dish in 60-mm petri dishes (Falcon 3002, Becton Dickinson and Co., Rutherford, NJ). The population doubling time was determined by cell counting at 24-h intervals for 5 days while changing the medium (DMEM/Ham's F-12 with 10% FCS) every other day. The determinations were carried out with three dishes for each experiment.

Immunohistochemical staining of cytochrome P450arom

The KGN cells were plated on cover glass and fixed with 4% paraformaldehyde at 4 C for 1 h. After treatment with 0.2% Triton X-100 for 2 min, the cells were incubated with 0.3% H_2O_2 in methanol for 30 min at room temperature to avoid nonspecific endogenous peroxidase reaction. Next, the cells were preincubated with 2% skim milk in PBS (pH7.5) for 1 h, and then incubated with diluted rabbit antiserum raised against human cytochrome P450arom (32) (1:500 in PBS) at 4 C for overnight. Control KGN cells were also incubated with normal (preimmune) rabbit serum (1:500 in PBS). After the cells were washed with PBS three times, the antibody-antigen complexes were detected by the streptavidin-biothin-peroxidase method using a Histofine kit (Nichirei, Tokyo, Japan). The specific staining was identified by the presence of brown reaction procedures.

Chromosome analysis

The chromosomes were examined in exponentially growing KGN cells (passage 20) in an *in vitro* culture. The karyotype was analyzed by standard trypsin G-banding and was described according to the ISCN (33).

Measurement of the steroid content in medium secreted from KGN

KGN cells were inoculated on culture dishes (Falcon 3002) and cultured for 3 days in a medium containing 10% FCS. After 3 days, when the cells reached confluence, they were transferred to medium containing 10% dextran-treated charcoal-treated FCS (DCS) and then further cultured for 6–72 h in the presence or absence of 10^{-5}–10^{-2} M $(Bu)_2$cAMP (Wako Pure Chemical Industries Ltd., Tokyo, Japan) or 10^{-10}–10^{-6} M phorbol 12-myristate 13-acetate (TPA; Wako Pure Chemical Industries Ltd.). The steroid contents of progesterone, 17α-hydroxyprogesterone, dehydroepiandrosterone, and androstenedione secreted into the culture medium were assayed using the respective commercial RIA kits (Diagnostic Products, Los Angeles, CA). Pregnenolone, 17α-hydroxypregnenolone, estrone (E_1), and estradiol (E_2) were measured by SRL Co. Ltd. (Tokyo, Japan) using the respective RIA systems (34). The antibody against 17α-hydroxypregnenolone cross-reacts 1.0% with pregnenolone, 0.5% with 17α-hydroxyprogesterone, 0.2% with 16α-pregnenolone, and 0.4% with 20α-progesterone and 20β-progesterone. The antibody against the 17α-hydroxyprogesterone cross-reacts 0.6% with progesterone, 2.1% with 11-deoxycortisol, 3.2% with 17α-hydroxypregnenolone, and 3.8% with 17α-hydroxypregnenolone sulfate.

Measurement of cAMP content in KGN cells

We measured the content of cAMP in KGN cells under the stimulation of human FSH (hFSH; Sigma, St. Louis, MO) by RIA using a commercially available kit (Yamasa Syoyu Co. Ltd., Chiba, Japan) (35). The biological potency of hFSH was about 7000 IU/mg. Cultured KGN cells at confluent state in petri dishes (Falcon 3002, Becton Dickinson and Co.,

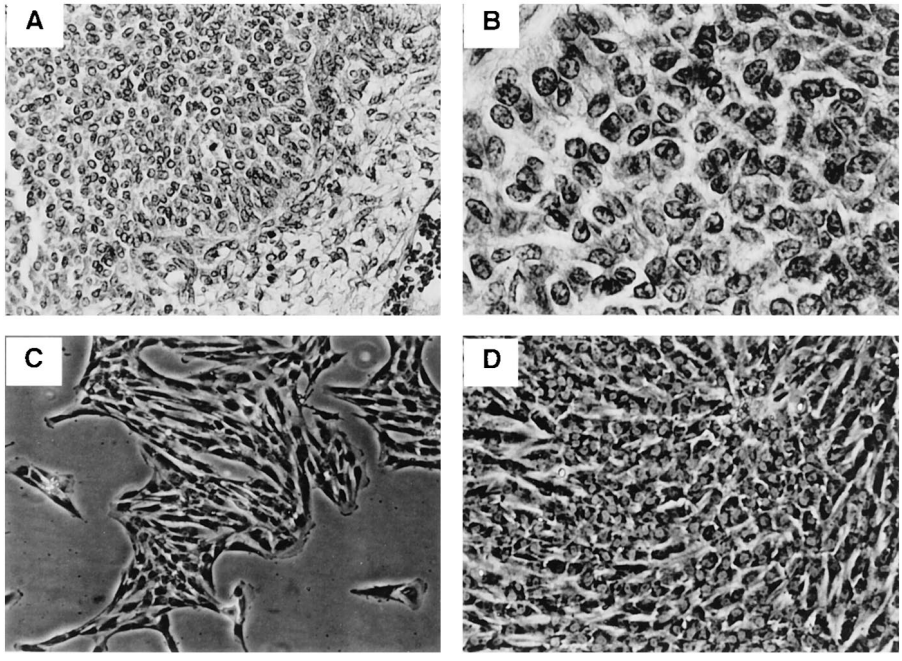

FIG. 1. Microscopic findings of the granulosa cell tumor (the origin of KGN cell line; A and B) and cultured KGN cells (C and D). Sections of the original tumor tissue stained with hematoxylin and eosin (HE) exhibits a coffee-bean like nuclear appearance, which is typically observed in specimens of granulosa cell origin (A, ×100 magnification of the original; B, ×400). Cultured KGN cells examined by phase difference microscopy showed different shapes depending on the density of the cells in culture (C and D).

Inc., Franklin Lakes, NJ) were transferred to the medium containing 1.0% (wt/vol) BSA (Sigma) and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma). The cells were further cultured for 3 h in the presence or absence of 0.5–500 ng/ml hFSH or 10^{-6} M forskolin (Sigma). For the measurement of intracellular cAMP, cells were washed twice with PBS, lysed with 3 ml 0.1 M HCl, scrapped into Eppendorf tubes using a rubber policeman, and sonicated three times for 20 sec each time on ice with sonicater (Cell disruptor-185, SmithKline Co.). The cell debris was precipitated by centrifugation for 10 min at 4 C, and cAMP in the supernatant was measured.

Aromatase assay

The aromatase activity of KGN was determined by measuring the amount of $[^3H]H_2O$ released upon the conversion of [1β-^{3}H]androstenedione to estrone by a modification of the method of Ackerman *et al.* (36). KGN cells were plated on a petri dish (Falcon 3001) in culture medium with 10% FCS. At confluence, the culture medium was replaced with DMEM/Ham's F-12 containing 10% DCS and incubated for another 12 h in the presence or absence of 10^{-5}–10^{-2} M $(Bu)_2$cAMP (Wako Pure Chemical Industries Ltd.), 10^{-10}–10^{-6} M TPA(Wako Pure Chemical Industries Ltd.), 0.5–500 ng/ml hFSH (Sigma), 5–5000 mIU/ml human menopausal gonadotropin (hMG; Teikoku Zouki Co. Ltd., Tokyo, Japan), 0.1–100 IU/ml hCG (Teikoku Zouki Co. Ltd.), or 10^{-8}–10^{-5} M dexamethasone (Sigma). After treatment, the cells were further incubated with 12.5 nM [1β-^{3}H]androstenedione (NEN Life Science Products, Boston, MA; SA, 27.5 Ci/mmol) for 12 h. After incubation, the medium (2.0 ml) was transferred to tubes containing 1.0 ml ice-cold 30% (wt/vol) trichloroacetic acid and then centrifuged to remove precipitated protein. The cells were harvested using 0.25% trypsin-1 mM EDTA to determine the protein concentration. The following protocol for the extraction of the medium to measure the amount of $[^3H]H_2O$ was performed exactly as previously described (37). Finally, the amount of radioactivity in the $[^3H]H_2O$ was corrected by subtracting the blank values from each sample. The cell protein content was determined using a micro bicinichoninic acid kit (Pierce Chemical Co., Rockford, IL) after the cells were dissolved in 1.0 N NaOH. The aromatase activity was expressed as picomoles per mg cell protein. As a control, the aromatase activities of human granulosa cells (obtained from *in vitro* fertilization programs), human fibroblast, and HOS cell (human osteoblast-like cell line) (37) were measured in the same manner.

To ensure that KGN cells can produce estrogens, they were incubated with 10^{-5} M androstenedione for 72 h in the presence or absence of 10^{-3} M $(Bu)_2$cAMP, and the contents of E_1 and E_2 secreted into the culture medium were measured by RIAs as described above.

FSH binding assay

To determine whether KGN cells have functional FSH receptor, an [^{125}I]FSH binding study was performed in the manner described by Li *et al.* (38). In brief, KGN cells were inoculated on 12-well culture plates (Falcon) and cultured for 2 days in a medium containing 10% FCS. After 2 days, when the cells reached confluence, they were washed twice with binding buffer [DMEM/Ham's F-12 medium containing 0.5% (wt/vol) BSA] and then incubated with [^{125}I]hFSH (SA, 155 TBq/mmol; concentration, 787 kBq/ml; NEN Life Sciences Products) in the presence of different concentrations of cold hFSH (Sigma) at 37 C for 1 h in the binding buffer and lysed with 1 M NaOH. The amount of bound radioactivity was counted using a γ-counter. Specific binding was defined as that remaining after subtraction of nonspecific binding from the total amount of [^{125}I]FSH binding. Protein concentrations of the cell lysate were determined using a commercial protein assay kit (micro bicinchoninic acid kit, Pierce Chemical Co.).

Effects of interferon-γ and anti-Fas antibody on cultured KGN cells

To determine the utility of the KGN cell line for an experiment on apoptosis, Fas-mediated apoptosis was tested as an example. KGN cells were inoculated on culture dishes (Falcon 3001) at a concentration of 1×10^5 cells/dish. After incubating for 24 h, the cells were transferred to the medium containing 10% DCS. Then, the cells were preincubated for 24 h in the presence of 100 IU/ml interferon-γ (Shionogi Co. Ltd., Osaka, Japan) (39, 40). Next, monoclonal antihuman Fas antibody, CH-11 (Medical and Biological Laboratories Co. Ltd., Nagoya, Japan), the active-form antihuman Fas antibody (IgM-type antibody derived from mouse) that stimulates post-Fas signaling by binding to Fas (41), was added to the cell culture at 1 μg/ml. At the same time, 1 μg/ml mouse IgM (Medical and Biological Laboratories Co. Ltd.) was added to the control culture. All cells on the culture dishes were collected 24 and 48 h after the addition of CH-11. Dead cells were counted by the trypan blue dye exclusion method, and the ratio of dead cells was compared with the control value. DNA fragmentation was examined by 1.8% agarose-TBE gel electrophoresis of the DNA samples extracted from the collected cells following a previously described protocol (42).

Statistics

All experiments were carried out more than three times with triplicate plates per point. All values represent the mean ± SD. A one-factor ANOVA was used for statistical evaluation. $P < 0.05$ was considered to indicate statistical significance.

Results

Morphology and growth characteristics of KGN cells in vitro

KGN cells grew as an adherent monolayer, and stable proliferation of the cells took place during more than 100 passages over almost 5 yr. The cultured cells were proliferated in multilayers after they reached confluence, and no contact inhibition was noted. They demonstrate a spindle shape in the low cell density state (Fig. 1C) and changed to an epithelial cell-like shape in the high cell density state (Fig. 1D). They had an estimated doubling time of about 46.4 h *in vitro*.

Karyotype analysis

The chromosome counting of 50 metaphase KGN cells revealed the modal peak to be 45. G-Banded karyotype analyses of 10 KGN cells exhibiting 45 chromosomes revealed all to be an abnormal karyotype of 45,XX, 7q-, -22. Namely, the 7q deletion and monosomy 22 were observed (data not shown).

Immunohistochemical staining of KGN cells with antibody against cytochrome P450arom

To show the clonality of this cell line as a steroidogenic cell, we immunohistochemically stained the cells with normal rabbit serum or immune rabbit serum against human cytochrome P450arom. As shown in Fig. 2, compared with the cells stained with normal rabbit serum as a control (Fig. 2A), most of the KGN cells were specifically stained with antiserum against human cytochrome P450arom, especially in the perinuclear region, although the intensities were somewhat different among the cells (Fig. 2, B and C). These results indicate that a large percentage of KGN cells are steroidogenic cells, which really expresses cytochrome P450arom.

Steroidogenic activities of KGN cells

The steroidogenic activities of KGN cells were evaluated by the steroid concentration in the cultured medium using RIA. Figure 3 indicates the levels of the various steroids in the medium secreted from 10^6 KGN cells for 24 h in the

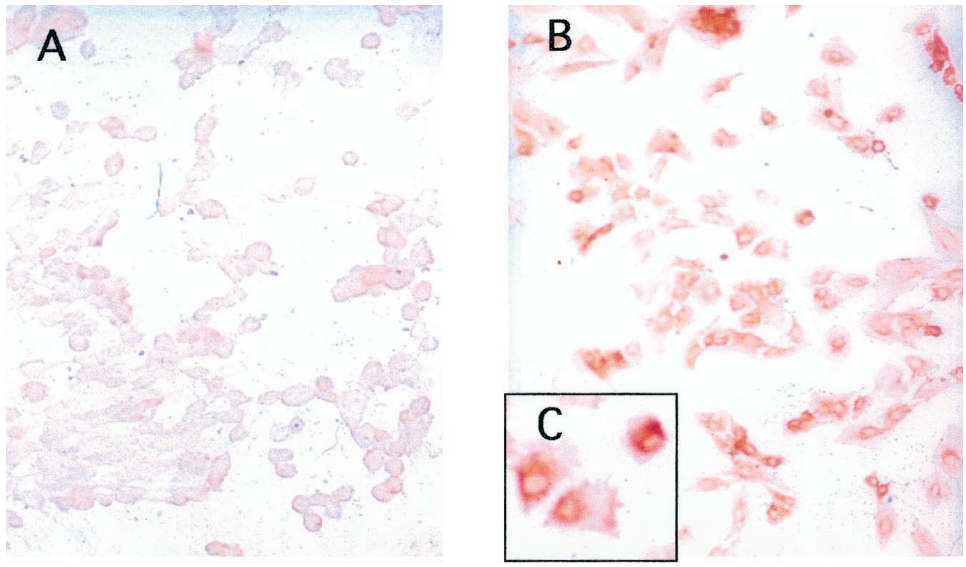

FIG. 2. Immunohistochemical staining of cytochrome P450arom in KGN cells. The KGN cells were stained with normal (preimmune) rabbit serum (A) or rabbit antiserum against human cytochrome P450arom (B and C). The immunoreactivity of cytochrome P450arom was observed in the perinuclear region of most KGN cells (C), whereas no specific staining was observed with preimmune serum (A). Magnification: A and B, ×100; C, ×400.

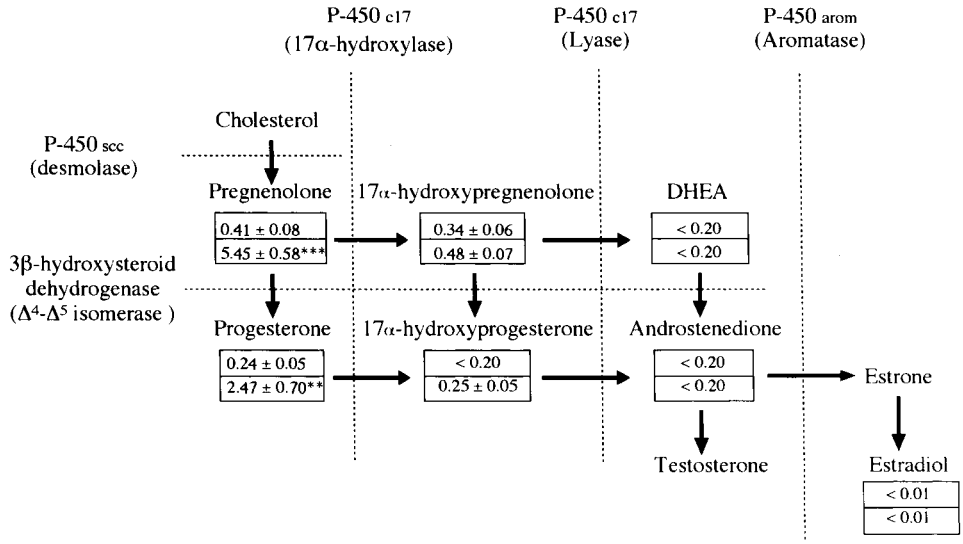

FIG. 3. Steroid secretion by KGN cells cultured in steroid-free FCS supplemented with DMEM/Ham's F-12 medium, as determined by RIA. The steroidogenic pathways in the granulosa cells and the actual steroid hormone values are indicated. The values in the *upper* and *lower boxes* indicate the basal (nonstimulated) and 10^{-4} M $(Bu)_2$cAMP (db-cAMP)-stimulated steroid hormone concentrations (nanograms per ml) in the medium secreted from KGN cells (10^6 cells) for 24 h, respectively. Each value indicates the mean ± SD of three experiments, with triplicate plates per point. **, $P < 0.01$; ***, $P < 0.001$ (*vs.* the nonstimulating condition).

presence or absence of 10^{-4} M $(Bu)_2$cAMP. KGN cells showed detectable levels of basal secretion of pregnenolone and progesterone in the medium, both of which significantly increased after stimulation with 10^{-4} M $(Bu)_2$cAMP. On the other hand, the basal concentrations of 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, androstenedione, dehydroepiandrosterone, and estradiol in the medium were either very low or undetectable, and none of the above steroids showed any significant change after the stimulation with 10^{-4} M $(Bu)_2$cAMP for 24 h.

Regulation of progesterone production in KGN cells

Regarding the regulation of progesterone secretion from KGN cells, the effect of $(Bu)_2$cAMP was tested (Figs. 4 and 5). A significant and time-dependent increase in the secretion of progesterone compared with basal secretion was noted during stimulation with 10^{-3} M $(Bu)_2$cAMP for 24–72 h (Fig. 4). Further, a dose-dependent increase in the secretion of progesterone was observed during stimulation with 10^{-5}–10^{-2} M $(Bu)_2$cAMP for 24 and 48 h (Fig. 5). The effect of the C kinase stimulator, TPA, was also tested (Fig. 6). Although 10^{-3} M $(Bu)_2$cAMP caused a dramatic increase in the secretion of progesterone compared with the basal secretion (0.26 ± 0.08 *vs.* 8.50 ± 1.83 ng/10^6 cells·48 h; mean ± SD; n = 3; $P < 0.01$), the coincubation with TPA (10^{-8}–10^{-6} M) significantly suppressed the $(Bu)_2$cAMP-stimulated secretion of progesterone (coincubation with 10^{-8} M TPA, 3.24 ± 1.03; 10^{-7} M TPA, 0.45 ± 0.21; 10^{-6} M TPA, 0.77 ± 0.34 ng/10^6 cells·48 h).

Aromatase activities in KGN

To determine whether the aromatase activity of KGN cells was regulated in a manner similar to that in primary cultured human granulosa cells, it was determined by measuring the $[^3H]H_2O$ released upon the conversion of [1β-^{3}H]androstenedione to estrone. The KGN cells maintained in control medium (10% DCS containing culture medium) exhibited a relatively high aromatase activity (0.84 ± 0.23 pmol/mg protein; mean ± SD; n = 3), which was about 300 times higher than that of HOS cells (3.24 ± 2.64 fmol/mg protein; n = 3) and about 50 times higher than that of primary cultured

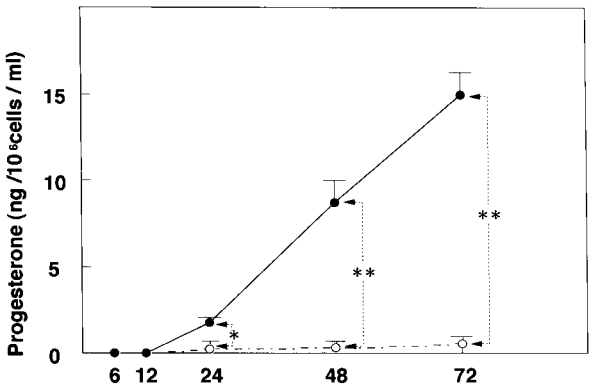

FIG. 4. Kinetics of progesterone production in cultured KGN cells. KGN cells were incubated with or without 1×10^{-3} M $(Bu)_2$cAMP. Progesterone content in the culture medium (DMEM/Ham's F-12 with 10% DCS) was measured by RIA as described in *Materials and Methods*. Cell number was counted by the trypan blue dye exclusion method. Each value indicates the mean ± SD of three experiments, with triplicate plates per point. Progesterone contents secreted from $(Bu)_2$cAMP-stimulated cells are significantly higher than those from nonstimulated cells at 24–72 h. *, $P < 0.05$; **, $P < 0.001$ (*vs.* corresponding values).

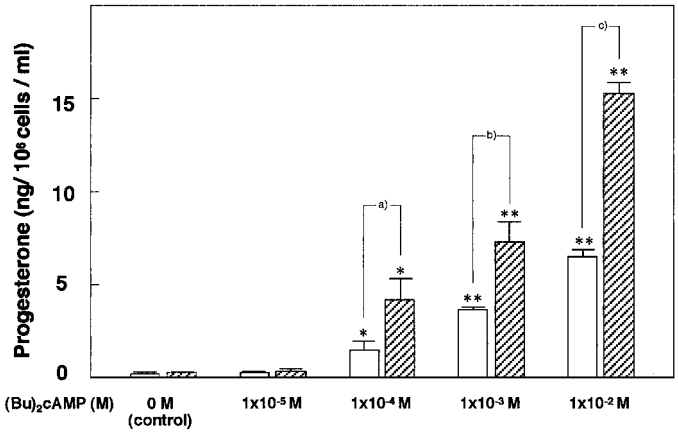

FIG. 5. Stimulation of progesterone production by $(Bu)_2$cAMP in KGN cells. KGN cells were incubated with or without (control) $(Bu)_2$cAMP (1×10^{-5} to 1×10^{-2} M) for 24 h (□) and 48 h (▨). Progesterone content was measured by RIA. Each value indicates the mean ± SD of three experiments, with triplicate plates per point. *, $P < 0.01$; **, $P < 0.001$ [*vs.* control (absence of $(Bu)_2$cAMP)]. a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$ (*vs.* corresponding values).

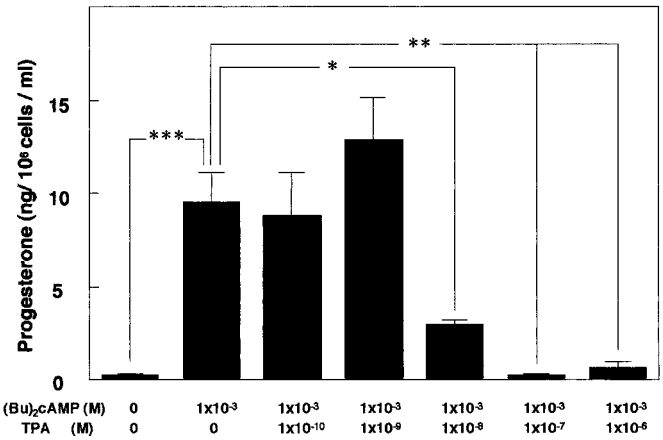

FIG. 6. Effect of TPA on progesterone release from $(Bu)_2$cAMP-treated KGN cells. KGN cells were cultured with or without $(Bu)_2$cAMP (1×10^{-3} M) and/or TPA (1×10^{-10} to 1×10^{-6} M) for 48 h. After 48-h incubation, each conditioned medium was collected, and concentrations of progesterone in the medium were measured by RIA. Each value indicates the mean ± SD of three experiments, with triplicate plates per point. *, $P < 0.05$; **, $P < 0.01$ (compared with corresponding values).

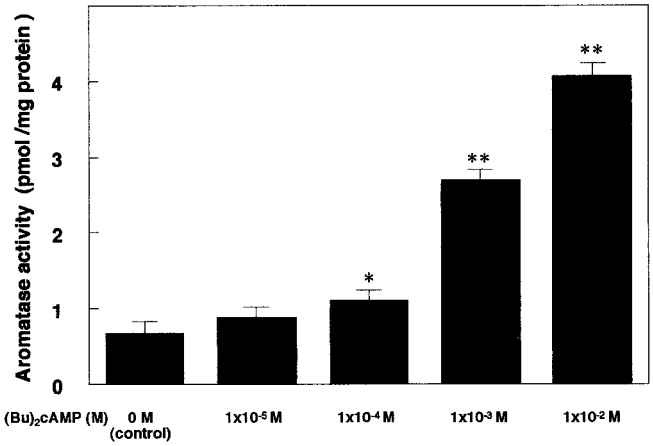

FIG. 7. Concentration effect of $(Bu)_2$cAMP on aromatase activity in KGN cells. KGN cells were preincubated with (1×10^{-5} to 1×10^{-2} M) or without (control) $(Bu)_2$cAMP for 12 h, and aromatase activity was assayed as described in *Materials and Methods*. Each value indicates the mean ± SD of three experiments, with triplicate plates per point. *, $P < 0.05$; **, $P < 0.01$ (*vs.* control).

human skin fibroblasts (19.4 ±0.7 fmol/mg protein; n = 3), but was about 50 times lower than that of primary cultured human granulosa cells (41.5 ± 8.3 pmol/mg protein; n = 3). When KGN cells were treated with 10^{-5}–10^{-2} M $(Bu)_2$cAMP, a dose-dependent and significant increase in the aromatase activity was observed (a minimum effective dose, 1×10^{-4} M; Fig. 7). Whereas, the treatment of KGN with 10^{-10}–10^{-6} M TPA in the presence or absence of 10^{-3} M $(Bu)_2$cAMP caused no significant change in the aromatase activity (data not shown). Treatment of KGN cells with 5–5000 mIU/ml hMG (Fig. 8A) and 0.5–500 ng/ml hFSH (Fig. 9) caused a dose-dependent and significant increase in aromatase activity. The minimum effective doses for the stimulation of aromatase activity were 50 mIU/ml hMG (1.83 ± 0.35 pmol/mg protein; control, 0.86 ± 0.14 pmol/mg protein; mean ± SD; n = 3; $P < 0.05$) and 50 ng/ml hFSH (2.35 ± 0.47 pmol/mg protein; control, 0.96 ± 0.15 pmol/mg protein; n = 3; $P < 0.01$). On the other hand, no significant changes in aromatase activity were noted during stimulation with 0.1–100 IU/ml hCG (Fig. 8B). A slight, but significant, increase in aromatase activity was observed during treatment with 10^{-8}–10^{-5} M dexamethasone for 12 h in a dose-dependent manner (data not shown). The maximum stimulation was observed with 10^{-6} M dexamethasone (1.71 ± 0.19 pmol/mg protein; control, 0.92 ± 0.16 pmol/mg protein; n = 3; $P < 0.01$). These results suggest that aromatase activity in KGN cells is responsive to FSH partly through the protein kinase A-mediated activation and is also under the regulation of glucocorticoid.

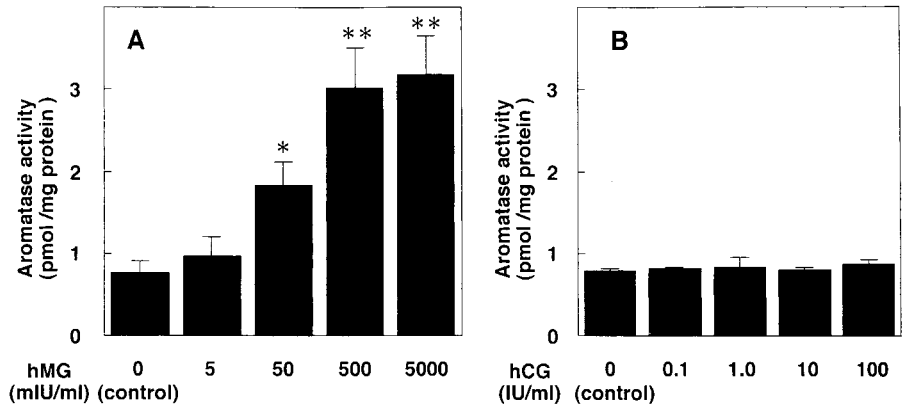

FIG. 8. Concentration effect of hMG or hCG on aromatase activity in KGN cells. KGN cells were preincubated with or without hMG (5–5000 mIU/ml; A) or hCG (0.1–100 IU/ml; B) for 12 h, and aromatase activity was assayed as described in *Materials and Methods*. Each value indicates the mean ± SD of three experiments, with triplicate plates per point. *, $P < 0.05$; **, $P < 0.01$ (*vs.* control).

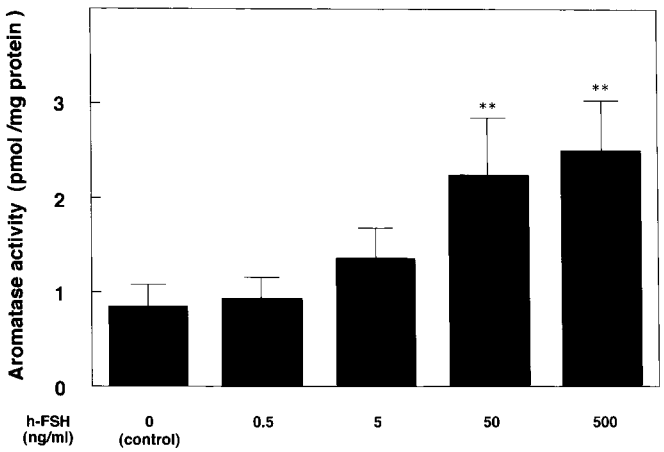

FIG. 9. Concentration effect of FSH on aromatase activity in KGN cells. KGN cells were preincubated with (0.5–500 ng/ml) or without hFSH for 12 h, and aromatase activity was assayed as described in *Materials and Methods*. Each value indicates the mean ± SD of three experiments, with triplicate plates per point. **, $P < 0.01$ *vs.* control.

TABLE 1. Estrone and estradiol formation from androstenedione by KGN cells

	Dose of $(Bu)_2$cAMP (M)	
	0	1×10^{-3}
Estrone (pg/10^6 cells·72 h)	103.7 ± 6.5	1165 ± 2.0[a]
Estradiol (pg/10^6 cells·72 h)	170.7 ± 3.5	4060 ± 170[a]

KGN cells were incubated with 1×10^{-5} M androstenedione for 72 h with or without $(Bu)_2$cAMP (1×10^{-3} M). Each medium was collected, and concentrations of estrone and estradiol were measured by RIA. Each value indicates the mean ± SD of three experiments with triplicate plates per point.

[a] $P < 0.0001$ *vs.* control [$(Bu)_2$cAMP, 0 M].

Next, to ensure the real capability of estrogen production by KGN cells, they were incubated with 10^{-5} M androstenedione (substrate for estrogens) for 72 h in the presence or absence of 10^{-3} M $(Bu)_2$cAMP, and the contents of E_1 and E_2 secreted into the culture medium were measured by RIAs. As shown in Table 1, significant amounts of E_1 (103.7 ± 6.5 pg/10^6 cells) and E_2 (170.7 ± 3.5 pmol/10^6 cells) were produced and secreted in the culture medium, and significant increases in the levels of E_1 (11.2-fold) and E_2 (23.8-fold) were noted during stimulation with 10^{-3} M $(Bu)_2$cAMP.

Intracellular cAMP accumulation by FSH stimulation

After 3-h stimulation with 5–500 ng/ml hFSH, the levels of intracellular cAMP increased dose dependently (Table 2). The minimal effective dose of hFSH was 50 ng/ml ($P < 0.05$ *vs.* control). However, the magnitude of the stimulation of intracellular cAMP production by hFSH was much smaller than that by forskolin (1 μM forskolin; 683 ± 36 pmol/10^6 cells).

FSH binding to KGN cells

An appreciable level of specific FSH binding to KGN cells was demonstrated by the competitive binding assay of [^{125}I]hFSH. Namely, Scatchard analysis of the data demonstrated a linear plot (data not shown), and the estimated FSH binding capacity to KGN cells (Bmax) and the K_d value for FSH binding were 1.79 fmol/mg protein and 4.65×10^{-10} M, respectively.

Effect of interferon-γ and active-form anti-Fas antibody (CH-11) on cultured KGN cells

To determine whether KGN cells exemplify the Fas-mediated apoptotic phenomenon observed in human granulosa cells, KGN cells were exposed to active-form antihuman Fas antibody (CH-11) (40) for 24 or 48 h after pretreatment with 100 IU/ml interferon-γ. Interferon-γ pretreatment was used to facilitate the Fas-induced apoptotic mechanism partly by the induction of Fas antigen (39, 40). Although the pretreatment with only interferon-γ did not cause any significant change in the ratio of the number of dead cells to that of the total cells, the addition of CH-11 to the interferon-γ-pretreated cells caused a dramatic increase in the ratio (Fig. 10A). DNA fragmentation was recognized by electrophoresis in the DNA specimens extracted from the KGN cells cultured in the presence of CH-11 in a time-dependent manner (Fig. 10B).

Discussion

The incidence of granulosa cell carcinoma ranges from 1.6–3.0% of all cases of ovarian tumor and comprises about 10% of all cases of ovarian cancer (43). The most common age of patients with granulosa cell carcinoma is fifties to sixties, corresponding to the peri- to postmenopausal age, thus lead-

TABLE 2. Concentration effect of FSH on intracellular cAMP production in KGN cells

	Dose of h-FSH (ng/ml)			
	0	5	50	500
Intracellular cAMP (pmol/10^6 cells)	12.0 ± 1.5	14.5 ± 2.0	22.6 ± 3.7[a]	37.7 ± 2.5[b]

KGN cells were incubated with (5–500 ng/ml) or without human FSH (h-FSH) for 12 h. Intracellular cAMP contents were measured as described in *Materials and Methods.* Each value indicates the mean ± SD of three experiments, with triplicate plates per point.

[a] $P < 0.01$ *vs.* control.

[b] $P < 0.001$ *vs.* control.

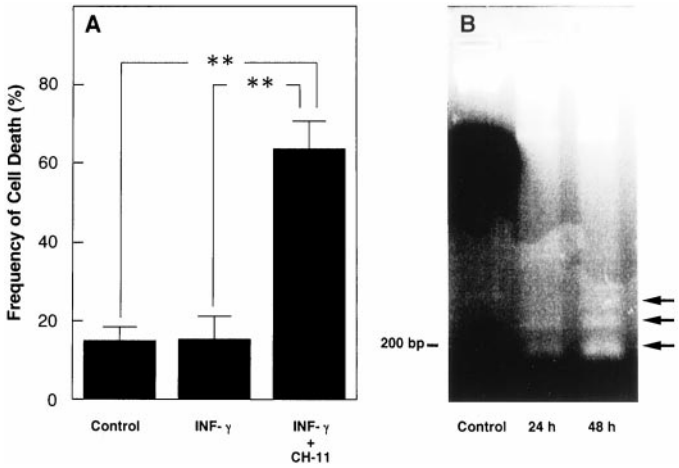

FIG. 10. Induction of apoptosis of KGN cells by the addition of interferon-γ and the active-form anti-Fas antibody. The incubation of KGN cells with active form anti-Fas antibody (CH-11) for 48 h, after treatment with 100 IU/ml interferon-γ (IFN-γ) for 24 h significantly increased the ratio of dead cells. Interferon-γ was pretreated for the induction of Fas antigen. A, Determination of the ratio of dead cells by the trypan blue dye exclusion method. The values represent the mean ± SD of triplicate samples. **, $P < 0.01$ *vs.* control values. B, DNA extracted from the cells treated with both interferon-γ and anti-Fas antibody (CH-11) showed a ladder in the electrophoretic pattern due to the fragmentation of DNA associated with apoptosis. The *arrows* indicate the DNA ladder.

ing to the hypothesis that ovarian hyperstimulation may cause carcinogenesis in granulosa cells (44). We established a granulosa-like tumor cell line, KGN, from this particular patient's tumor. The long-term and stable proliferation over almost 5 yr and the abnormal karyotype, 7q-, -22 (7q deletion and monosomy 22), indicate this cell line to be truly derived from carcinoma cells, not from normal granulosa cells. The karyotype is probably related to the malignant character of this granulosa cell, as frequent abnormalities of chromosome 7 or 22, such as allelic loss of 7q31.1 and monosomy 22, have been reported in ovarian tumors, including ovarian granulosa cell tumors (45–48). In particular, the finding of a highly frequent allelic loss at 7q31.1 in primary ovarian carcinomas strongly suggested the existence of a tumor suppressor gene on this locus (47).

As for the mechanism of steroidogenesis in ovarian tissues, a two-cell, two-gonadotropin theory has been proposed, which assumes that ovarian estrogen synthesis may require both thecal and granulosa cells. It is assumed that minor androgens mainly produced in thecal cells under the control of LH are transported to granulosa cells and then are converted to estrogen under the control of FSH (49, 50). Most of the enzymatic or immunohistochemical findings of animal or human granulosa cells correlate with this theory, as the activity and expression of P450arom were present in normal granulosa cells, whereas those of P450C17, a single enzyme mediating both 17α-hydroxylase and 17,20-lyase activities (51) were either very low or undetectable in the cells (6, 52). However, several investigators have reported the presence of both 17α-hydroxylase activity and estrogen production in human granulosa cells (2–4, 26). The inconsistency of such studies may be due to the difference in the culture condition or to the stage of follicular development at which the experiments were performed, as a development-related difference in steroidogenesis of human granulosa cell has been suggested (10, 11).

In our KGN cells, based on the steroid concentrations secreted for 24 h, basal and cAMP-stimulated production of pregnenolone and progesterone was observed, whereas very low or undetectable levels of 17α-hydroxylated steroids and androgens were produced either before or after treatment with $(Bu)_2$cAMP. A slight detection of 17α-hydroxypregnenolone or 17α-hydroxyprogesterone in the medium is probably due to the slight cross-reactivities of respective antibodies with other steroids in the RIAs, as no significant increase in these steroids was observed after stimulation with cAMP for 24 h. In addition, P450C17 transcript roughly assessed by RT-PCR was undetectable before and after treatment with cAMP for 24 h (data not shown). However, it is not completely denied that even such an undetectable level of P450c17 expression may still contribute to some detection of 17α-hydroxylated steroids. Because of the absence or low level formation of androstenedione, no detectable formation of estradiol was observed in the KGN cells incubated in 10% DCS medium despite the presence of aromatase activity. However, when the cells were exogenously provided with enough androstenedione, detectable amounts of E_1 and E_2 were produced and secreted by KGN, indicating the real capacity of KGN cells to produce estrogens. All of these findings together indicate the mechanism of steroidogenesis in KGN to be quite similar to that in normal granulosa cells (6, 50, 52), regarding the concept of two-cell, two-gonadotropin theory. Furthermore, the suppressive effect of TPA (relatively high dose) on cAMP-induced progesterone production in KGN mimicked the pattern observed in normal granulosa cells (53, 54).

In the previously established granulosa cell lines, little has been reported on the aromatase activity and its regulation, except a report by Rainey *et al.* (29) demonstrating the enhanced expression of P450arom messenger RNA by forskolin in a transformed human granulosa cell line (HGL5). This is probably because aromatase activity has proven difficult to maintain in long-term cultures of granulosa cells. In our

study the basal aromatase activity of KGN cells was easily measurable and was relatively high compared with those of other aromatase-expressing cells such as fibroblast and osteoblast (37). Although the aromatase activity of KGN cells was about 50–100 times lower than that of primary cultured human granulosa cells measured by us or reported by Steinkampf *et al.* (8), it was highly inducible by treatment with $(Bu)_2$cAMP or FSH, but was only slightly inducible by treatment with glucocorticoid. These patterns of regulation regarding the aromatase activity in KGN cells were quite similar to those observed in normal human granulosa cells (5, 8), suggesting granulosa cell-specific promoter usage of the human P450arom gene (55, 56). Actually, the usage of granulosa cell-specific promoters, consisting of either exon 1c or 1d (or called promoter II) of the human aromatase gene, in KGN cells was confirmed by RT-PCR analysis (data not shown). Thus, the continued expression of P450arom in this cell line is also very useful for the study of regulation of P450arom in human granulosa cells.

The marked increase in aromatase activity caused by treatment with hFSH or hMG suggests that this cell line expresses functional FSH receptor. The concomitant increase in the levels of intracellular cAMP by hFSH treatment further supports this finding. Indeed, specific [^{125}I]FSH binding to KGN cells was observed by binding assays, and the estimated binding capacity and K_d were almost similar to those noted in the ovarian cancer of sex cord-stromal origin (57). The expression of the FSH receptor on KGN cells and its responsiveness to FSH are worthy of note, as no clear response to FSH has been demonstrated in any of the previously established human granulosa cell lines (26–30). As most of the previous human cell lines have been established from normal granulosa cells by the forced introduction of a part of oncogenes or viral genes (26–30), it has been speculated that the native FSH receptor might be lost upon cell transformation (1). In contrast, our cell line, KGN was established by long-term culture of cells from an originally transformed human granulosa cell tumor, probably resulting in the preservation of physiologically more normal conditions. In a rat granulosa cell line, to overcome the absence of native FSH receptor, a stable transformant cell line expressing FSH receptor has been obtained by the forced introduction of FSH receptor gene (21). In contrast, treatment with hCG caused no change in the aromatase activity of KGN cells. The responsive pattern of aromatase activity to gonadotropin in human granulosa cells has been reported to be different depending on the developmental stage of the follicle; in granulosa cells from immature follicles, treatment with FSH, but not LH, increased aromatase activity, whereas in mature granulosa cells, both treatments markedly stimulated aromatase activity (9). These findings suggest that the developmental stage of KGN cells may be close to that of immature granulosa cells.

In recent years the involvement of apoptosis in granulosa cells regarding the process of follicular atresia has been established, and several mechanisms of the apoptosis have been proposed (1). As one such mechanism, the Fas-Fas ligand system has been reported to be important for the apoptosis of granulosa cells using a primary culture system (39, 40). However, a molecular biological analysis using the primary culture system is usually limited, because relatively large populations of uniform cells are required for such analyses. In this respect it is also very meaningful that the Fas-mediated mechanism of apoptosis could be reproduced in our KGN cells by exposure to interferon-γ and the active form of Fas antibody. This fact enables us to perform a detailed analysis of the mechanism using our newly established cell line and may also provide insight for a new therapeutic approach to the granulosa cell tumor.

In conclusion, we established a new ovarian granulosa-like tumor cell line, KGN, from a granulosa cell tumor. The KGN cells had steroidogenic activities similar to those of normal granulosa cells and expressed functional FSH receptor. The relatively high level of aromatase activity of KGN cells will greatly help in the study of P450arom gene regulation as well as in the study of the aromatase inhibitor as an anticancer drug. In addition, it is highly probable that the Fas-mediated apoptotic mechanism in normal granulosa cells is maintained in KGN cells. This cell line is therefore expected to allow us to study various aspects of the physiological regulation of human granulosa cells in the future.

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