Roles of luteinizing hormone/chorionic gonadotropin receptor in anchorage-dependent and -independent growth in human ovarian surface epithelial cell lines

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(Received May 20, 2003/Revised September 18, 2003/Accepted September 22, 2003)

Epithelial ovarian carcinomas are thought to arise from cells of ovarian surface epithelium (OSE) covering the free surface of the human ovary. Two immortalized human cell lines, OSE2a (non-tumorigenic) and OSE2b-2 (tumorigenic), were previously established from normal OSE cells of a reproductive-age patient. In the present study, we found that expression of luteinizing hormone (LH)/chorionic gonadotropin (CG) receptor (LH/CGR) is present in OSE2a cells and absent in OSE2b-2 cells. In OSE2a cells, a low concentration (10^3 mIU/ml) of CG enhanced anchorage-dependent growth via up-regulation of insulin-like growth factor-1 (IGF1), whereas a high concentration (10^5 mIU/ml) of CG induced anchorage-independent growth and down-regulation of *IGF1* expression. To investigate involvement of other genes in LH/CGR-related tumorigenicity, we compared cDNA expression arrays of OSE2a and OSE2b-2 cells, and found that the following genes had lower expression in OSE2b-2 than in OSE2a: *integrin β1*, *intercellular adhesion molecule-1 (ICAM1)*, and *Waf1/Cip1*. Subsequent semiquantitative reverse transcription polymerase chain reaction using OSE2a cells showed that expression of *integrin β1* was down-regulated by a high concentration (10^5 mIU/ml) of CG. These results suggest that LH/CGR affects anchorage-dependent and -independent growth by mediating up- and down-regulation of IGF1 and integrin β1. Repetitive and excessive activation of LH/CGR may cause genetic alteration of its signal transduction pathway, resulting in stimulation of growth of OSE cells, initiation of ovarian carcinogenesis, and cancer progression. (Cancer sci 2003; 94: 953–959)

Epithelial ovarian carcinoma constitutes approximately 90% of ovarian malignancies, and is generally thought to derive from ovarian surface epithelium (OSE),[1] which descends from embryonic coelomic mesothelium and gives rise to the müllerian ducts.[2] A number of gene alterations related to ovarian carcinogenesis have been found in studies using ovarian carcinoma specimens and cell lines. There have been reports of inactivation of tumor suppressor genes (*p53*, *BRCA1*, *BRCA2* and *PTEN*) and activation of oncogenes (*Ki-ras*, c-*erbB-2*, c-*myc*, *AKT2* and *PIK3CA*) in cases of epithelial ovarian carcinoma.[3] The early molecular changes and sequence of genetic events in ovarian carcinogenesis are not fully understood, because of a lack of adequate experimental systems using human cells. Recently, *in vitro* models using normal and immortalized human OSE cells have been developed to study the characteristics of OSE cells.[4,5] More recently, Ong *et al*. produced an ovarian adenocarcinoma by transforming human OSE cells using simian virus 40 (SV40)/E-cadherin.[6] Nitta *et al*. established 6 human OSE cell lines by using SV40 large tumor (LT) antigen to immortalize normal cells from 5 patients with gynecologic diseases.[7] Two of those lines, OSE2a and OSE2b, were derived from OSE cells of a reproductive-age patient. OSE2a was non-tumorigenic in athymic mice. An OSE2b-2 cell line was selected from colonies of OSE2b in soft agar. OSE2b-2 produced disseminated tumors on the peritoneal surface and induced ascites after being injected intraperitoneally. These tumors were histologically diagnosed as undifferentiated carcinoma.[7] OSE2a and OSE2b-2 can be used as *in vitro* models for analyzing initiation and development of epithelial ovarian carcinogenesis.

Results of epidemiological studies suggest that gonadotropins are involved in initiation of ovarian carcinogenesis.[8,9] Two gonadotropins, luteinizing hormone (LH) and chorionic gonadotropin (CG), bind to a common transmembrane glycoprotein receptor, LH/CG receptor (LH/CGR),[10] which is involved in activation of adenylyl cyclase (AC) and phospholipase C (PLC) $β_2$.[11–13] LH/CGR is expressed in gonads and various non-gonadal tissues.[14] Recent studies have shown that this receptor is expressed in normal human OSE cells,[15–18] and also that CG can stimulate anchorage-dependent growth of normal human OSE cells via up-regulation of insulin-like growth factor-1 (IGF1).[18]

Our goal is to clarify the regulatory mechanisms of gene expression involved in the LH/CG-LH/CGR signal pathway of ovarian carcinogenesis. In the present study, we evaluated the effects of CG and IGF1 on anchorage-dependent and -independent growth using OSE2a and OSE2b-2 cells, and investigated the involvement of CG, IGF1 and tumor-related genes in LH/CGR expression in OSE2a cells.

Materials and Methods

Cell culture. OSE2a and OSE2b-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 containing 10% fetal bovine serum (FBS), as described elsewhere.[7] After cell passage, both lines were cultured in medium with 10% FBS (serum-containing) or without FBS (serum-free) until the cells had grown to 80% confluence, with the following concentrations of CG or IGF1: human CG (hCG; Mochida, Tokyo), 0, 10^2, 10^3, 10^4 or 10^5 mIU/ml (0, 2.22×10, 2.22×10^2, 2.22×10^3 or 2.22×10^4 ng/ml); IGF1 (Sigma, St. Louis, MO), 0, 10 or 10^2 ng/ml. The medium was changed every day until the cells were harvested. The cells were harvested 24 h after the last change of medium, followed by RNA extraction.

Reverse transcription (RT)-polymerase chain reaction (PCR) analysis. Using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD), total RNA was extracted from OSE2a and OSE2b-2 cells. RT-PCR was then performed to synthesize cDNA from 5 μg of the total RNA using the "SuperScript" First-strand Synthesis System (Life Technologies). Simultaneously, negative control procedures were performed without reverse transcriptase. The primer sequences for RT-PCR were as follows: *LH/CGR*, 5′-

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ATG CTT TTC AAG GGA TGA ATA ATG A-3′ (sense) and 5′-CAC ATC GGG GTG TCT TGG GTA A-3′ (antisense); *IGF1*, 5′-TCT TGA AGG TGA AGA TGC ACA CCA-3′ (sense) and 5′-AGC GAG CTG ACT TGG CAG GCT TGA-3′ (antisense); *IGF-R*, 5′-ACC CGG AGT ACT TCA GCG CT-3′ (sense) and 5′-CAC AGA AGC TTC GTT GAG AA-3′ (antisense); *AC*, 5′-CAC CGC AAA ATA CTT AGA TGA CG-3′ (sense) and 5′-CCT TCT CCT GCA AGA TCT CAC AC-3′ (antisense); *PLC* β_2, 5′-CCA GGG GCT ATA AGA GCA AC-3′ (sense) and 5′-CAC CAC CGT GAG TGT CTT CAG C-3′ (antisense); *integrin β1*, 5′-GTT ACA CGG CTG CTG GTG TT-3′ (sense) and 5′-CTA CTG CTG ACT TAG GGA TC-3′ (antisense); *intercellular adhesion molecule-1 (ICAM1)*, 5′-TGA CCA GCC CAA GTT GTT GG-3′ (sense) and 5′-ATC TCT CCT CAC CAG CAC CG-3′ (antisense); *Waf1/Cip1*, 5′-GTC ACA GGC GGT TAT GAA AT-3′ (sense) and 5′-CTA GGC TGT GCT CAC TTC AG-3′ (antisense); *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* (internal control), 5′-GGT CAT CCC TGA GCT GAA CG-3′ (sense) and 5′-TTC GTT GTC ATA CCA GGA AAT-3′ (antisense). For each message, semiquantitative RT-PCR was performed using the optimal number of PCR cycles (20–45) to yield product levels in the linear portion of the serial dilution curve. The PCR products were fractionated on a 2% agarose gel and visualized by ethidium bromide staining. All PCR conditions were optimized for quantification of message content relative to *GAPDH* product levels. Amplification of the correct sequence was verified by direct sequencing of each PCR product. For evaluation of *IGF1* and *integrin β1* expressions under CG treatment, the intensity of each band was quantified with NIH Image Version 1.61 Software. Message levels were expressed as the ratio of the signal intensity of the PCR products of *IGF1* and *integrin β1* to that of *GAPDH*. The reproducibility of the quantitative measurements was evaluated by three independent experiments. The means and standard deviations of the triplicate measurements were calculated and are shown in the plots.

Immunocytochemistry. For immunocytochemical evaluation, immunoperoxidase staining was performed using the DAKO Envision System (Dakopatts, Glostrup, Denmark). For the primary antibody, we used rabbit polyclonal antisera raised against a synthetic peptide consisting of an N-terminal amino acid sequence (positions 15 to 38) of human LH/CGR, which was kindly provided by Dr. C. V. Rao (University of Louisville Health Sciences Center, Louisville, KY). Nuclear staining was performed with 1% methyl green in water. Chorionic villi were used as a positive control. Negative control staining was performed by omitting the primary antibody and replacing it with non-immune rabbit serum.

Assay for cell proliferation (anchorage-dependent growth). OSE2a and OSE2b-2 cells were plated in separate 96-well microtiter plates at a density of 2×10^3 cells/well and allowed to grow for 24 h in DMEM with or without 10% FBS and with or without CG (0, 10^3 or 10^5 mIU/ml) or IGF1 (0, 10 or 10^2 ng/ml). The Biotrak Cell Proliferation ELISA System Version 2 (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for the cell proliferation assay. Briefly, cells were incubated in 5-bromo-2′-deoxyuridine (BrdU) labeling solution. Following cell fixation and DNA denaturation, specimens were incubated in peroxidase-labeled anti-BrdU. Subsequently, (3,3′,5,5′-tetramethylbenzidine) (TMB) was added as a substrate to detect immune complexes. Absorbance was calculated from the absorbance at 450 nm, which was measured using a microtiter plate reader.

Assay for colony formation (anchorage-independent growth). The ability of OSE2a and OSE2b-2 cells to form colonies in a semi-solid medium was estimated. Then, 2×10^3 or 2×10^4 cells were suspended in 1 ml of top agar consisting of 0.3% agarose (Sigma, St. Louis, MD) and DMEM/F-12 supplemented with 10% FBS, with or without CG or IGF1 at the concentrations used in the assay for cell proliferation. The top agar was plated onto 1 ml of solidified bottom agar consisting of 0.5% agarose in the media used for the top agar. In each dish, the number of colonies was counted. Colony efficiency was calculated as the ratio of the number of colonies counted to the number of cells seeded.

Human cDNA expression array. Total RNA was extracted from OSE2a and OSE2b-2 cells cultured in serum-containing medium, and mRNA was isolated from the total RNA using "Dynabeads" oligo (dT) 25 (Dynal A.S, Oslo, Norway), then treated with DNase (Amersham Pharmacia Biotech). cDNA probes labeled with [α-^{32}P] dATP (Amersham Pharmacia Biotech) were synthesized from mRNA from OSE2a and OSE2b-2 cells using Moloney murine leukemia virus reverse transcriptase (Clontech, Heidelberg, Germany). The labeled cDNA probes were hybridized side-by-side to 2 identical "Atlas" human cDNA expression arrays (Clontech). After a high-stringency wash, the hybridization pattern was analyzed by autoradiography. To evaluate the array results, spot densitometry was performed using the NIH Image Version 1.61 Software. The densitometric value of each spot was normalized to the sum of values for the 3 housekeeping genes (*β-actin, 23 kDa highly basic protein, ribosomal protein S9*). Relative ratios were calculated by comparing normalized spot densitometric values of gene expression between OSE2b-2 and OSE2a.

Statistical analysis. Data from the proliferation assay and colony formation assay were analyzed using the ANOVA test for significant differences. Values are presented as mean±SD. Differences with a P value of <0.05 were considered significant.

Results

Expression of LH/CGR, IGF1 and IGFR in human OSE cell lines. RT-PCR and immunocytochemical analysis showed that LH/CGR was expressed in OSE2a cells, but not in OSE2b-2 cells when both lines were cultured in serum-containing medium (Fig. 1, A and B). The OSE2a PCR product had a single 531-bp band corresponding to exons 5 to 11 of *LH/CGR* mRNA. In RT-PCR, expression of *IGF1* and *IGFR* was detected in OSE2a and OSE2b-2 cells in serum-containing medium. The 2 cell lines had similar levels of *IGFR* expression, independent of the presence or absence of 10% FBS (Fig. 1C). In OSE2a cells, expression of *IGF1* was much lower in serum-free medium than in serum-containing medium (Fig. 1C). In OSE2b-2, there was little difference in *IGF1* expression between cells cultured with or without serum, and the *IGF1* expression level was lower than that of OSE2a cells in serum-containing medium.

LH/CGR-dependent expression of *AC*, *PLC*, *IGF1* and *IGFR* in OSE2a cells. Expression of *AC* was up-regulated by treatment with 10^3 mIU/ml of CG, but was not up-regulated by treatment with 10^5 mIU/ml. There was no difference in *AC* expression between 0 and 10^5 mIU/ml of CG (Fig. 2). Expression of *PLC* β_2 was dependent on the concentration of CG, with expression highest in cells treated with 10^5 mIU/ml of CG (Fig. 2). Expression of *IGF1* was slightly up-regulated by treatment with 10^3 mIU/ml CG, and was suppressed by treatment with 10^5 mIU/ml to a level lower than that of the untreated control (Fig. 2). Expression of *IGFR* was not altered by treatment with CG (Fig. 2).

Cell proliferation of adherent human OSE cell lines (anchorage-dependent growth). For OSE2a cells cultured in medium containing 10% FBS and 0, 10^3 or 10^5 mIU/ml of CG, cell proliferation was significantly enhanced by 10^3 mIU/ml of CG, to 127±4% of the level of proliferation in cultures without CG (Fig. 3A; P<0.005). In the absence of FBS, CG did not stimulate cell proliferation of OSE2a (data not shown). In addition, IGF1 treatment caused enhanced proliferation of OSE2a cells in the absence of FBS (Fig. 3B; P<0.05). Cell proliferation of

OSE2b-2 was not altered by any tested concentration of CG or IGF1 in the culture medium (data not shown).

Colony formation of human OSE cell lines in soft agar (anchorage-independent growth). Without CG treatment, the colony formation efficiencies of OSE2a and OSE2b-2 cells were 0.44±0.12% and 3.71±0.42%, respectively. Colony formation efficiencies of OSE2a cells with 10^3 and 10^5 mIU/ml CG were 0.46±0.22% and 1.29±0.18%, respectively. Colony formation of OSE2a cells was enhanced by 10^5 mIU/ml of CG, to 2.9 times the value of cells without CG (Fig. 4, A and B; $P<0.005$). IGF1 did not affect colony formation of OSE2a cells (data not shown). In OSE2b-2 cells, neither CG nor IGF1 affected colony formation (data not shown).

Human cDNA expression array and semiquantitative RT-PCR in human OSE cell lines. Of the 588 genes represented on the expression arrays, approximately 60 genes (10%) had detectable levels of gene expression in both cell lines. Genes with a relative ratio (OSE2b-2/OSE2a) >1.5 were considered to exhibit increased expression, and those with a ratio <0.67 were considered to exhibit decreased expression. Nine of the 588 genes (1.5%) were found to have decreased expression, and no genes were found to have increased expression (Table 1, Fig. 5, A and B). Expression of the genes *Waf1/Cip1*, *ICAM1* and *integrin β1* was decreased to less than half in OSE2b-2 cells (Table 1, Fig. 5B). Because the focus of the present study was on genes downstream from *LH/CGR*, OSE2a cells expressing *LH/CGR* were screened by semiquantitative RT-PCR for *Waf1/Cip1*, *ICAM1* and *integrin β1* in culture medium containing different concentrations of CG. The analysis showed that *integrin β1* expression was slightly up-regulated by 10^3 mIU/ml CG and greatly down-regulated by 10^5 mIU/ml CG, irrespective of the presence or absence of serum (Fig. 6, A and B). The expression pattern correlated positively to that of IGF1 cultured in medium containing CG. When OSE2a cells were treated with IGF1, *integrin β1* was expressed in a dose-dependent manner in serum-free medium (Fig. 6C). In contrast, expression of *Waf1/Cip1* and *ICAM1* was not altered by CG treatment (data not shown). In OSE2b-2 cells, these expressions of *IGF1* and *integrin β1* were not affected by addition of CG (data not shown).

Discussion

Epidemiological findings have suggested possible risk factors for epithelial ovarian cancer. Nulligravidity and history of infertility have been found to be fairly consistent predictors.[19] An early age at menarche and a late age at menopause have also been cited as ovarian cancer risk factors.[20] The accepted risk factors are associated with increased stimulation of ovaries by pituitary gonadotropins. Recent findings suggest an association between drugs administered to induce ovulation and development of ovarian carcinoma.[8, 9] These findings strongly support the hypothesis that frequent and incessant ovulation plays an

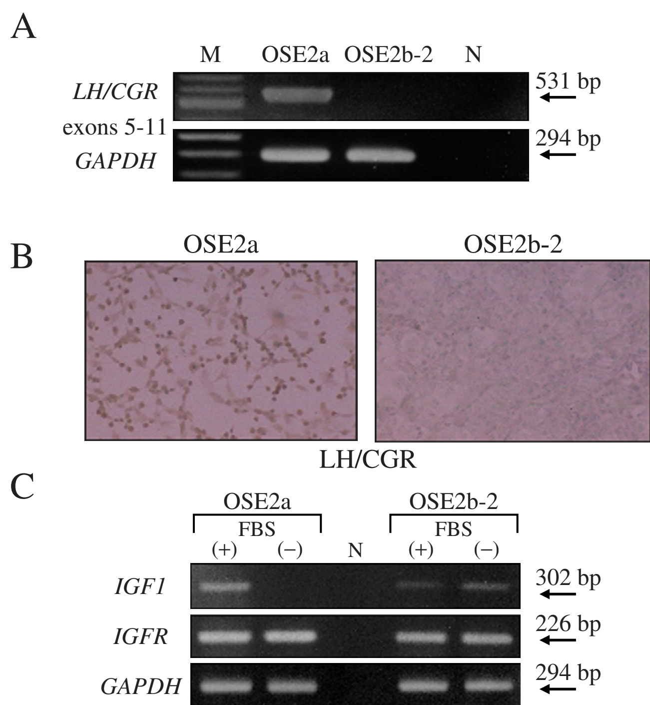

Fig. 1. RT-PCR and immunocytochemistry of *LH/CGR* and RT-PCR of *IGF1* and *IGFR* in OSE2a and OSE2b-2 cells. A. The ethidium pattern of RT-PCR shows that OSE2a expresses *LH/CGR*, but OSE2b-2 does not. The PCR product is a single 531-bp band (exons 5–11 of *LH/CGR*). The lower panel shows that *GAPDH* is expressed at equal levels in the 2 OSE cell lines. B. In immunocytochemistry using rabbit polyclonal antibody against human LH/CGR, OSE2a cells are stained positively, but OSE2b-2 cells are not (methyl green staining). Original magnification: ×40. C. In OSE2a, moderate expression of *IGF1* was detected in the presence of 10% FBS, whereas *IGF1* expression was extremely low in the absence of FBS. Expression of *IGF1* was lower in OSE2b-2 than in OSE2a in the presence of 10% FBS; in contrast, it was higher in OSE2b-2 than in OSE2a in the absence of FBS. In both cell lines, there was little difference in *IGFR* expression between the presence and absence of FBS.

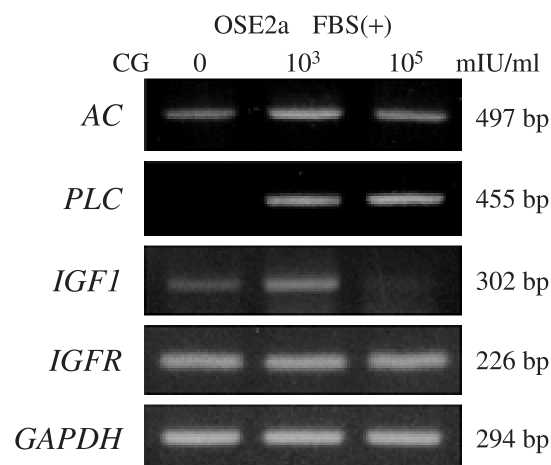

Fig. 2. RT-PCR of *AC, PLC, IGF1* and *IGFR* in OSE2a cells. The expression level of *AC* was highest at 10^3 mIU/ml of CG, whereas the expression level of *PLC* was dose-dependent and highest at 10^5 mIU/ml of CG. The level of expression of *IGF1* was highest at 10^3 mIU/ml of CG, and lowest at 10^5 mIU/ml of CG. The expression levels of *IGFR* and *GAPDH* as an internal control were not affected by the concentration of CG.

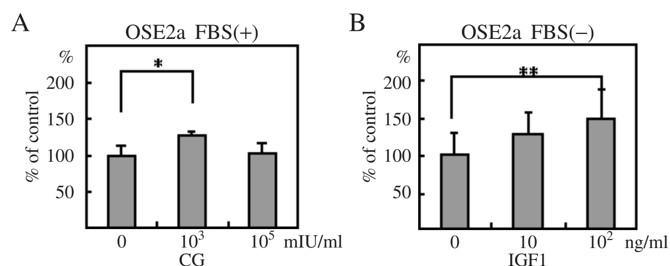

Fig. 3. Assay of cell proliferation (anchorage-dependent growth) using OSE2a cells. A. In the presence of 10% FBS, cell proliferation of OSE2a was significantly enhanced by 10^3 mIU/ml of CG, but was not enhanced by 10^5 mIU/ml. * $P<0.005$. B. Cell proliferation of OSE2a was significantly enhanced by 10^2 ng/ml of IGF1. ** $P<0.05$.

important role in ovarian carcinogenesis.[21] Ovulation is induced by a surge of LH from the pituitary gland or by CG injection for infertility therapy. LH and CG both bind to LH/CGR, which is expressed in normal OSE cells[14–17] and in 50% to 60% of epithelial ovarian carcinomas.[16] Recently, Syed *et al*. reported that LH significantly enhanced cell growth of OSE cells, and that normal OSE cells in primary cultures exhibited the best responses, followed by immortalized normal OSE cell lines and ovarian carcinoma cell lines.[17] Kuroda *et al*. showed that CG can enhance proliferation of normal OSE cells via up-regulation of IGF1.[18] It has been shown that *IGFR* is expressed in normal OSE cells and ovarian carcinoma specimens.[18, 22–24] Also, *in vitro* studies have shown that IGF1 and IGFR mediate adherent proliferation of the human ovarian carcinoma cell lines OVCAR-3 and CaOV-3.[22] These findings suggest that the LH/CG-LH/CGR and IGF1-IGFR systems are involved in growth of normal and neoplastic OSE cells. However, the interaction between these 2 systems in ovarian tumorigenicity is not fully understood.

In the present study, *LH/CGR* was expressed in OSE2a cells, which expressed *IGF1* and *IGFR* in serum-containing medium. Unlike *IGFR*, which was expressed at similar levels in serum-free and serum-containing medium, *IGF1* expression was dependent on the presence of serum. In the presence of serum, cell proliferation and *IGF1* expression of OSE2a cells were significantly enhanced at a low concentration (10^3 mIU/ml) of CG, but were not enhanced at a high concentration (10^5 mIU/ml). In the absence of serum, CG treatment did not promote cell proliferation, and expression of *IGF1* was greatly decreased by all concentrations of CG, whereas IGF1 treatment caused dose-dependent enhancement of cell proliferation. This suggests that the low concentration of CG enhanced anchorage-dependent growth via up-regulation of IGF1 in the presence of serum. In contrast, colony formation in soft agar was not enhanced by treatment with the low concentration of CG or any concentration of IGF1. However, it was obviously enhanced by treatment with the high concentration of CG. When OSE2a cells were treated with CG, the expression level of *IGF1* was inversely related to efficiency of colony formation.

The present results suggest that there are 2 different signaling pathways that involve LH/CGR in anchorage-dependent and -independent growth of OSE cell lines that express LH/CGR. One pathway is activated by a low concentration of CG, which stimulates anchorage-dependent growth via up-regulation of *IGF1*. The other pathway is activated by a high concentration of CG, which stimulates anchorage-independent growth via down-regulation of *IGF1*. It is generally accepted that LH/CGR directly activates 2 different signals, AC/cAMP and PLC/inositol phosphate, via Gi and Gs proteins coupled to LH/CGR.[10–13] Gudermann *et al*. reported that CG stimulated AC and PLC in mouse L cells expressing LH/CGR, and that a 20-to100-fold greater concentration of CG was needed to activate PLC than to activate AC.[11] Similarly, in the present study, *AC* and *PLC* were expressed in OSE2a cells and their expression level was altered independently of treatment with CG. These findings provide a possible mechanism for 2 different signaling pathways via LH/CGR for anchorage-dependent and -independent growth of OSE cells.

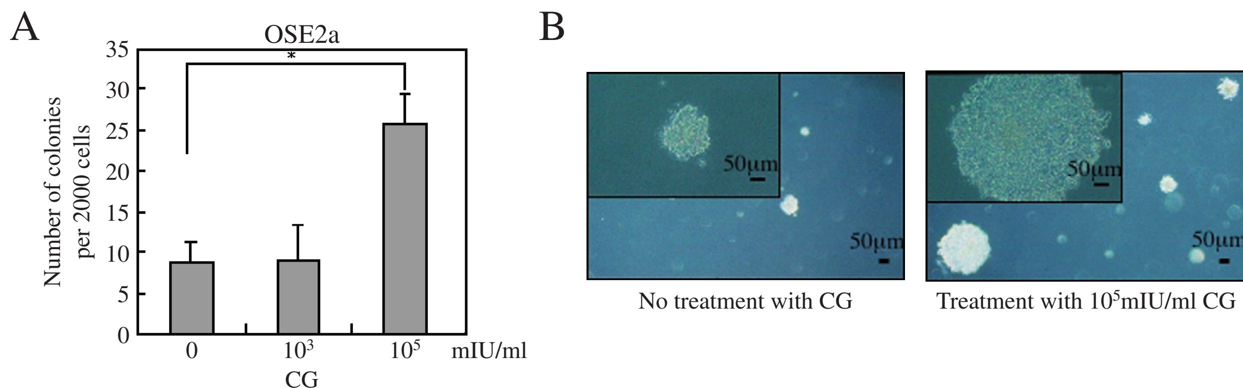

Fig. 4. Assay of colony formation in soft agar (anchorage-dependent growth) using OSE2a cells. A. In OSE2a, 10^5 mIU/ml of CG significantly stimulated colony formation in soft agar. The numbers of colonies in 2×10^3 OSE2a cells were 8.9±2.46 (CG, 0 mIU/ml), 9.1±4.38 (CG, 10^3 mIU/ml) and 25.8±3.62 (CG, 10^5 mIU/ml). * $P<0.005$. B. Phase-contrast microscopic photographs show 2×10^4 OSE2a cells suspended in soft agar with and without treatment with 10^5 mIU/ml of CG. Left: there is a single colony, approximately 130 μm in size, without CG treatment. Right: there are 4 colonies ranging from 100 to 500 μm in size after treatment with 10^5 mIU/ml of CG. Original magnification: ×4, ×10 (inset).

Table 1. Nine genes with decreased expression in OSE2b-2 cells

Gene name	Position	Genebank #	OSE2b-2/OSE2a
ICAM-1	1	J03132	0.31
Waf 1/Cip1	2	U09579	0.44
Integrin β1	3	X07979	0.47
c-AMP-dependent transcription factor ATF-4	4	D90209	0.51
Transcription elongation factor S-II	5	M81601	0.56
Zinc finger X-chromosomal protein	6	X59738	0.58
CIP 2	not shown	L25876	0.60
N-Cadherin	7	M34064	0.63
Cyclin A	not shown	X51688	0.64

Position: location in Fig. 5B.
Genebank #: Genebank accession number.
OSE2b-2/OSE2a: densitometric value of OSE2b-2/densitometric value of OSE2a.

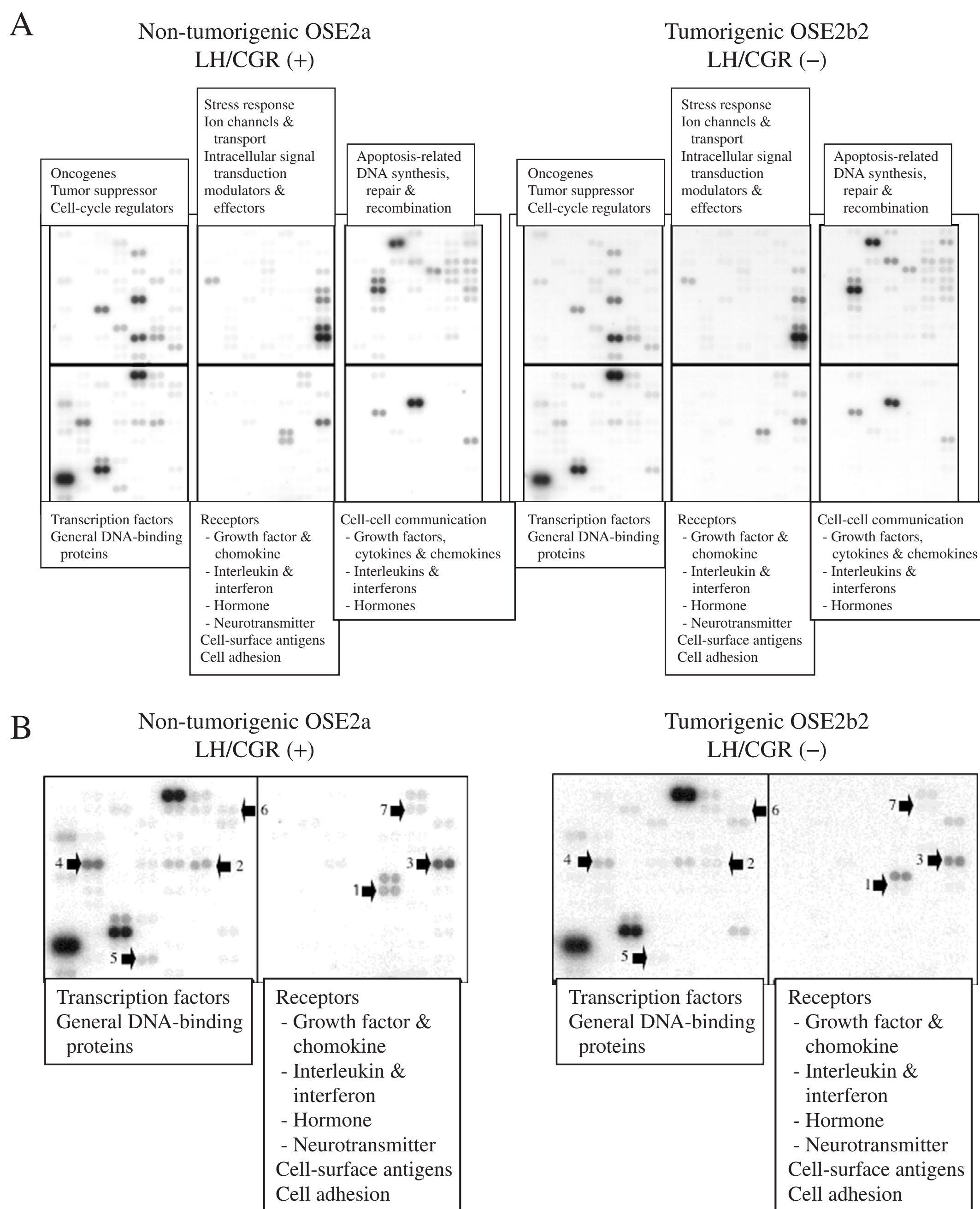

Fig. 5. Atlas of human cDNA expression array for OSE2a and OSE2b-2 cells. A. Whole profiles of expression arrays of OSE2a (left) and OSE2b-2 (right) are shown. Of the 588 genes, approximately 60 genes (10%) had detectable levels of gene expression in OSE2a and OSE2b-2. Nine of the 588 genes (1.5%) had decreased expression, and no genes had increased expression. B. Part of the expression profile of OSE2a is shown at the left, and part of the expression profile of OSE2b-2 is shown at the right. The arrows and numbers indicate genes with relative ratios (OSE2b-2/OSE2a) <0.67 (Table 1).

Unlike OSE2a cells, OSE2b-2 cells, which have high capacity of anchorage-independent growth and tumorigenicity, did not express LH/CGR. The expression level of *IGF1* was lower in OSE2b-2 cells than in OSE2a cells in the serum-containing medium, and was not dependent on the presence of serum. Moreover, treatment with CG did not affect anchorage-dependent or -independent growth. We hypothesized that expression of some genes could be altered by mutation or amplification of a downstream modulator gene of signaling pathways involving LH/CGR in OSE2b-2 cells. Consequently, we assessed cDNA expression arrays of OSE2a and OSE2b-2 cells, to identify alterations in expression of genes other than *IGF1*. In OSE2b-2 cells, there was markedly decreased expression of the genes *ICAM1*, *integrin β1* and *Waf1/Cip1*, which code for adhesion molecules and a cell cycle regulator, respectively. Although no specific gene alteration was identified, we speculate that OSE2b-2 cells have a gene alteration that results in decreased expression of these 3 genes. Down-regulation of these 3 genes may be related to tumorigenicity and capacity for anchorage-independent growth.

Semiquantitative RT-PCR showed that *integrin β1* expression was affected by CG treatment in OSE2a cells expressing

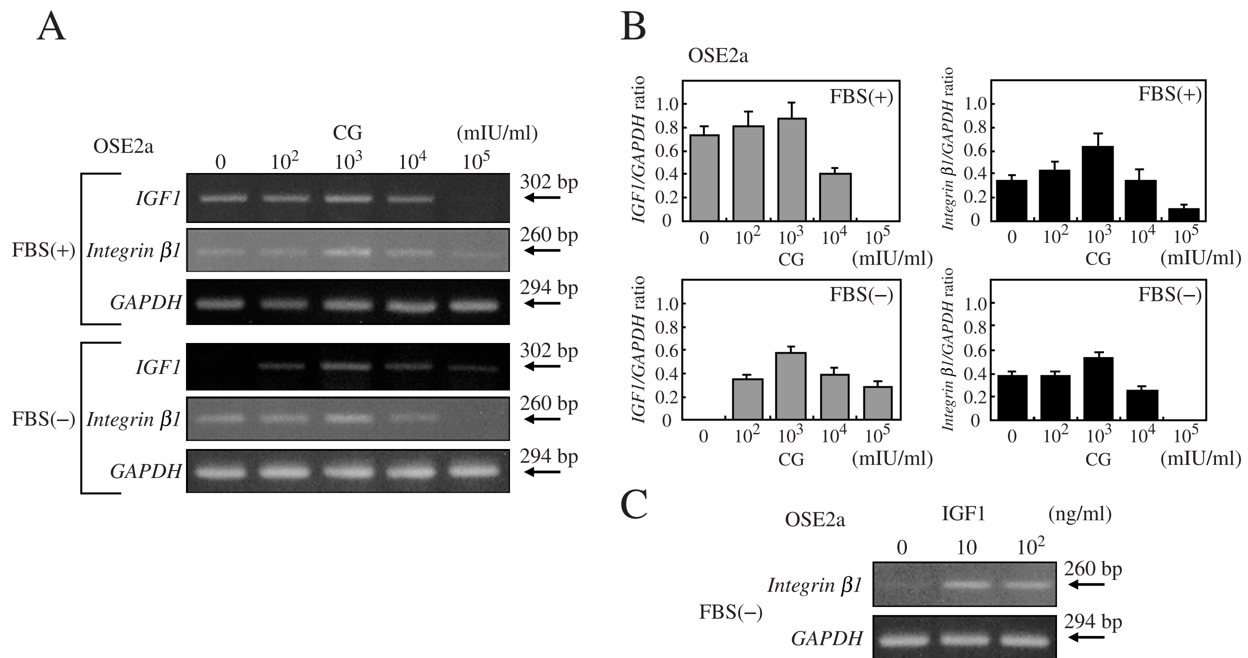

Fig. 6. RT-PCR of *IGF1* and *integrin β1* under CG treatment and *integrin β1* under IGF1 treatment in OSE2a cells. A. Ethidium patterns of *IGF1* and *integrin β1* under CG treatment are shown. B. Ratios of *IGF1* and *integrin β1* to *GAPDH* were calculated using the NIH Image Version 1.61 Software in three independent experiments. *Integrin β1* expression was up-regulated by 10^3 mIU/ml of CG and down-regulated by 10^5 mIU/ml of CG, with or without FBS. *Integrin β1* and *IGF1* had similar expression patterns under CG treatment. C. An ethidium pattern of *integrin β1* under IGF1 treatment is shown. IGF1 treatment in the absence of FBS induced *integrin β1* expression in OSE2a cells.

LH/CGR, whereas expression of *ICAM1* and *Waf1/Cip1* was not affected. *Integrin β1* expression was greatly suppressed by a high concentration of CG, which promotes anchorage-independent growth. Furthermore, the expression pattern of *integrin β1* positively correlated with that of *IGF1* in the presence of CG. When OSE2a cells were treated with IGF1, *integrin β1* was expressed in a dose-dependent manner in serum-free medium. This suggests that integrin β1 is a downstream modulator of a signaling pathway that involves IGF1 and LH/CGR. It has been shown that *integrin β1* expression is lower in hepatocellular carcinoma cells than in normal hepatocytes, and that overexpression of *integrin β1* suppresses colony formation and tumor formation in nude mice.[25] In the present study, expression of *integrin β1* was lower in OSE2b-2 cells than in OSE2a cells. Thus, down-regulation of *integrin β1* is induced by CG treatment in OSE cell lines expressing LH/CGR, and it may be related to their anchorage-independent growth. Intriguingly, loss of *integrin β1* expression is shown in 85% of epithelial ovarian carcinoma specimens.[26] Clarification of the mechanism of down-regulation of integrin β1 via LH/CGR may help to elucidate ovarian carcinogenesis.

Ovulation is induced by a physiological LH surge and therapeutic CG treatment. OSE cells covering the wall of a mature follicle are destroyed during ovulation, and others then proliferate to heal the resulting wound.[2, 27] The present findings suggest that LH/CGR is involved in regeneration of OSE cells after ovulation *in loco*. Repetitive and excessive activation of LH/CGR may cause alteration of LH/CGR-dependent transcriptional genes in OSE cells. As a result, OSE cells may autonomously grow independently of LH/CG stimulation when gene alteration results in signal transduction downstream from LH/CGR.

In conclusion, in OSE cells, expression of LH/CGR plays an essential role in regulating anchorage-dependent and -independent growth, via putative multiple signaling pathways including AC/PLC, IGF1/IGFR and integrin β1.

We wish to thank Professor C. V. Rao (University of Louisville Health Sciences Center, Louisville, KY) for providing rabbit polyclonal antibody against human LH/CG-R. This work was supported by a Grant-in-Aid for Scientific Research (12671616) from the Japan Society for the Promotion of Science, and a Grant-in-Aid for Encouragement of Young Scientists (13770926) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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