Abstract. The tumor-suppressive effects of human chorionic gonadotropin (hCG) against human breast cancer cells were examined. In cell viability assays, hCG inhibited the growth of three human breast cancer cell lines (estrogen receptor (ER)-positive KPL-1 and MCF-7, and ER-negative MKL-F cells), and the growth inhibition activity of hCG was most pronounced against KPL-1 cells (luteinizing hormone/chorionic gonadotropin receptor (LHCGR)-positive and luminal-A subtype). In hCG-treated KPL-1 cells, immunoblotting analysis revealed the expression of tumor suppressor protein p53 peaking at 12 h following treatment, followed by cleavage of caspase-9 and caspase-3 at 24 h and 48 h, respectively. KPL-1-transplanted athymic mice were divided into 3 groups: a sham-treated group that received an inoculation of KPL-1 cells at 6 weeks of age followed by daily intraperitoneal (i.p.) injection of saline; an in vitro hCG-treated KPL-1 group that received an inoculation of KPL-1 cells pre-treated with 100 IU/ml hCG in vitro for 48 h at 6 weeks of age, followed by daily i.p. injection of saline; and an in vivo hCG-treated group that received an inoculation of KPL-1 cell inoculation at 6 weeks of age, followed by daily i.p. injection of 100 IU hCG. The daily injections of saline or hCG continued until the end of the experiment when mice reached 11 weeks of age. KPL-1 tumor growth was retarded in in vitro and in vivo hCG-treated mice compared to sham-treated controls, and the final tumor volume and tumor weight tended to be suppressed in the in vitro hCG-treated group and were significantly suppressed in the in vivo hCG-treated group. In vivo 100-IU hCG injections for 5 weeks elevated serum estradiol levels (35.7 vs. 23.5 pg/ml); thus, the mechanisms of hCG action may be directly coordinated via the p53-mediated mitochondrial apoptotic pathway and indirectly through ovarian steroid secretion that elevates estrogen levels. It is thus concluded that hCG may be an attractive agent for treating human breast cancer expressing both LHCGR and ER.

Human breast cancer is influenced by female reproductive history. Early menarche and late menopause, both of which increase the duration of ovarian steroid exposure, are associated with increased risk, while bilateral oophorectomy is associated with reduced risk (1). Paradoxically, young age at full-term pregnancy, which results in high physiological levels of ovarian hormone exposure during the gestational period, substantially lower the risk (2-5). Parity-induced protection against breast cancer is a universal phenomenon, common in women of all ethnicities, and is the only normal physiological condition that consistently protects against breast cancer without known side-effects. However, the phenomenon of women not wanting to have children has recently increased. Therefore, mimicking the pregnancy condition by treating nulliparous women with pregnancy hormones may be an attractive strategy for reducing human breast cancer risk. The protective effects of pregnancy have also been observed in rats (6), and parity-induced protection can be reproduced in animals by short-term treatment (approximately equivalent to the gestational period of rodents, 21 days) with estrogen and progesterone (7).

Human chorionic gonadotropin (hCG) is a pregnancy hormone produced by the placental syncytiotrophoblastic cells that is essential for maintaining pregnancy. hCG interacts with the luteinizing hormone/chorionic gonadotropin receptor (LHCGR) and promotes the maintenance of the corpora lutea during pregnancy. Female transgenic mice that overexpress hCG have elevated serum levels of estrogen (8) and progesterone from the corpora lutea (9). LHCGR, which mediates the effects of hCG, is present in normal, benign, and malignant human breast epithelial cells in vivo and in cultured human breast cell lines (10, 11). The effects of hCG can be directly mediated through LHCGR in breast cancer cells or indirectly mediated through accelerated steroid secretion from the ovary. Young virgin rats treated with hCG for a short duration suppress 7,12-
dimethylbenz[e]anthracene-induced (12, 13) and N-methyl-N-nitrosourea (MNU)-induced rat mammary carcinogenesis (14). The hCG-mediated suppression of LHCGR-negative and estrogen receptor (ER)- and/or progesterone receptor (PgR)-positive MNU-induced mammary cancer was due to accelerated ovarian steroid secretion that elevates estrogen and progesterone levels same as the levels in pregnancy (14).

To date, the precise molecular mechanism of anti-cancer properties of hCG against LHCGR-positive breast cancer cells has not been identified, and the systemic application of hCG on human breast cancer cells transplanted into athymic mice has not been studied. Therefore, the present study was designed to elucidate the hCG effects on human breast cancer cell growth in vitro to determine the molecular mechanisms of action. Moreover, human breast cancer cells were transplanted into female athymic BALB/c mice to examine the in vivo effects of hCG.

Materials and Methods

Breast cancer cell lines and reagents. For cell proliferation assays, we used three human breast cancer cell lines. KPL-1 is a human breast cancer cell line established from the pleural effusion of a breast cancer patient, and it is ER-positive, PgR-negative, human epidermal growth factor receptor (HER)2-negative, which is the luminal-A subtype (15, 16). The MCF-7 cell line was derived from pleural effusion and expresses both ER and PgR (17). The MCF7 cell line is an ER-independent transfectant cell line of MCF-7 (18). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St. Louis, MO, USA) with 10% fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA) in 5% CO2/95% humidified air at 37°C. hCG was purchased from Mochida Pharmaceutical (Tokyo, Japan). Primary antibodies and antisera used in western blotting and immunohistochemistry were as follows: anti-LHCGR antisera (Acris, Herford, Germany), anti-α-LHCGR antibody (clone:DO-7, DAKO, Glostrup, Denmark), anti-caspase-9 antisera (Abcam, Cambridge, UK), anti-caspase 3 antibody (clone: 19, BD Biosciences), anti-ER antibody (clone: 6F-11, Novoceastra, Newcastle Upon Tyne, UK), anti-PgR antibody (clone: 10A9, Biodesign, Saco, ME, USA), anti-HER2 antisera (Dako), and anti-actin antibody (clone: AC-15, Sigma).

Cell proliferation assays. The growth inhibitory effects of hCG in cell cultures were determined by using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay (19). KPL-1, MCF-7, or MFK-L cells were seeded at 5x10^3 cells per well in 96-well plates. The cells were treated with experimental medium containing 0 (control), 10, 50, 100, 150, and 200 IU/ml hCG for 48 h. After incubation with experimental medium, MTT was added, and the resulting reactions were analyzed in a plate reader (iMark, BioRad, Hercules, CA, USA). Cell viabilities were expressed as a percentage of the controls.

SDS-PAGE and western blotting. KPL-1 cells were treated with 100 IU hCG for 12, 24, and 48 h. After washing with PBS (−), cell pellets were homogenized with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40, and 0.1% sodium dodecyl sulfate [SDS]), and the cell lysates were centrifuged at 13,000 g for 20 min at 4°C. Protein concentrations were measured by the DC protein assay kit (BioRad). Proteins (50 μg) from each sample were electrophoresed on SDS-PAGE gels and electroblotted onto transfer membranes (Hybond-P PVDF membranes; Amersham Biosciences, Buckinghamshire, UK). The transfer membranes were blocked with 5% non-fat powdered milk in Tris-buffered saline-Tween (TBST) and incubated with each primary antibody overnight followed by incubation with secondary antibodies. The secondary antibodies were HRP-conjugated anti-mouse IgG (Cell Signaling Technology, Danvers, MA, USA) or HRP-conjugated anti-sheep IgG (Vector Laboratories, Burlingame, CA, USA). Protein bands were visualized by using ECL plus reagent (Amersham Biosciences) and analyzed with a luminol-image analyzer (LAS-4000 mini; Fuji Film, Tokyo, Japan). The intensity of each protein band was compared against the intensity of the loading control (actin) and expressed as the relative intensity. Values of relative band intensities were calculated from three independent samples.

Athymic mouse cell-transplantation assay. The protocol for the in vivo study is shown in Figure 1. Four-week-old female athymic BALB/c mice were purchased from Charles River Japan (Atsugi, Japan). All animals were housed in an environmentally-controlled animal room (22±2°C, 60±10% humidity, and 12-h light/dark cycle) with woodchip bedding and free access to a commercial pellet diet (CMF 30 Gy; Oriental Yeast, Chiba, Japan). Athymic mice were randomly separated into three groups: a sham-treated group (n=8) that received an inoculation of 1x10^7 viable KPL-1 cells in 100 μM DMEM supplemented with 10% FBS into the right thoracic mammary fat pad at 6 weeks of age followed by daily intraperitoneal injection (ip) of saline; an hCG in vitro-treated group (n=6) that received an inoculation of 1x10^7 viable KPL-1 cells preincubated with 100 IU hCG/ml medium for 48 h followed by daily injection of saline; and an hCG in vivo-treated group (n=9) that received an inoculation of viable KPL-1 cells similar to the sham-treated group followed by daily i.p. injection of 100 IU hCG in 0.1 ml saline. All mice received either saline or hCG until the end of the experiment when the mice were 11 weeks of age (5 weeks after KPL-1 cell inoculation). The tumor volume was calculated as length × width × height × 0.5. At the time of sacrifice, circulating blood was sampled by cardiac puncture for the measurement of serum hCG and estradiol values by radioimmunoassay (SRL Inc., Hachioji, Japan), and non-tumorous mammary glands and reproductive organs from host animals as well as transplanted KPL-1 tumors were collected for histological and immunohistochemical evaluation. All animal procedures were approved by the Animal Experimentation Committee of Kansai Medical University.

Histological examination, immunohistochemistry, and TUNEL staining. Mouse non-tumor mammary glands and reproductive organs as well as KPL-1 tumors were harvested at the time of sacrifice. KPL-1 tumors and mouse organs were weighed and fixed in 10% neutral-buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (HE) for routine histological examination. The expression of ER, PgR, HER2, and LHCGR in the KPL-1 tumors of athymic BALB/c mice was evaluated by immunohistochemistry with the labeled streptavidin-biotin (LSAB) method (LSAB staining kit; Dako), according to the manufacturer’s instructions, and the reactions were visualized by using 3,3'-diaminobenzidine (DAB) as a chromogen. Apoptosis in KPL-1 tumors was detected by using TdT-mediated dUTP-digoxigenin nick end-labeling (TUNEL) performed with the TACS 2 TdT-Replinisher.
kit (Trevigen Inc, Gethersburg, MD, USA). TUNEL-positive cells in the tumor sections were counted from over 1,000 cells, and the percentage of positive cells was calculated.

**Statistics.** All results are expressed as means±standard error (SE). Data were analyzed with the unpaired t-test or Mann-Whitney U-test after assuring the homogeneity of variances.

**Results**

**Growth inhibitory effects of hCG against KPL-1, MCF-7, and MKL-F cells in vitro.** All three human breast cancer cell lines expressed LHCGR, as illustrated by immunoblotting (Figure 2A). To determine the effects of hCG in culture, both ER-positive (KPL-1 and MCF-7) and ER-negative (MKL-F) human breast cancer cell lines were treated with five different concentrations of hCG. hCG inhibited the growth of human breast cancer cells, and the growth inhibition was most prominent in KPL-1 cells (Figure 2B). The growth of KPL-1 cells was maximally inhibited at concentrations of 50 IU/ml hCG or greater. The concentration of 100 IU/ml hCG, which suppressed all three of the human breast cancer cell lines, was selected for the following KPL-1 study.

**Changes in the expression of apoptosis-related proteins.** To assess the anti-proliferative mechanisms of hCG against human breast cancer cells, changes in the expression of apoptosis-related proteins in KPL-1 cells were examined by SDS-PAGE and western blotting. Representative results are shown in Figure 3A. A concentration of 100 IU/ml hCG activated p53 expression, and the difference in relative p53 expression between control and 100 IU/ml hCG at 12 hours was significant (Figure 3B). Cleavage of caspase-9 and -3 was enhanced in cells co-cultured with 100 IU/ml hCG, reaching peak levels at 24 h for caspase-9 and 48 h for caspase-3. Therefore, the apoptotic pathway consisted of the time-dependent activation of p53 followed by the cleavage of caspase-9 and then caspase-3.

**Effects of hCG on the growth of KPL-1 cells inoculated into athymic mice.** In the athymic mouse KPL-1 transplantation assay, the average body weights of mice were similar among the three groups, and daily hCG injections did not influence body weight gain throughout the experiment (Figure 4A). However, growth retardation of KPL-1 cells at the inoculation sites occurred in the in vitro and in vivo hCG-treated groups compared to sham-treated controls. The final tumor volume and tumor weights in the in vitro hCG group tended to be lower and in the in vivo hCG group were significantly lower when compared to the sham-treated group (sham-treated, 1463.0±277.4 mm³ and 1185.1±143.1 mg; hCG in vivo, 774.2±151.9 mm³ and 699.1±169.4 mg; hCG in vitro, 861.2±323.3 mm³ and 850.9±304.9 mg) (Figure 4B and 4C). Apoptotic cells labeled with TUNEL staining were significantly increased in tumor sections from in vivo hCG-treated mice as compared to sham-treated mice (sham-treated, 0.42±0.05; hCG in vivo, 1.35±0.33) (Figure 4D), while TUNEL-positive cells in the in vitro hCG-treated tumors were compatible to sham-treated controls. In an immunoblipping study with protein lysates from inoculated tumors, p53 in the in vivo hCG treatment group was up-
regulated as compared to the sham-treated group (sham-treated, 0.21±0.05; in vivo hCG-treated, 0.78±0.13) (Figure 4E). Again, the cleavage of caspase-9 and -3 was enhanced in KPL-1 tumors of the in vivo hCG-treated group. The growth of KPL-1 cells in the in vitro hCG-treated group was relatively suppressed as compared to the sham-treated group, but the difference did not reach statistical significance; other than direct hCG action to KPL-1 cells may exist to achieve significant growth suppression of KPL-1 cells in in vivo hCG-treated mice.

Morphology of KPL-1 tumors, mammary glands, and reproductive organs of host mice. Histologically, the locally growing KPL-1 tumors were arranged in a trabecular and/or glandular pattern and composed of polygonal cells possessing round or oval nuclei, and conspicuous nucleoli were accompanied by mitotic figures (Figure 5A). By light microscopy it was shown that hCG treatment did not influence the morphology of KPL-1 tumors; the amounts of mitotic and apoptotic cells were almost equivalent among groups. Immunohistochemically, the majority of KPL-1 tumor cells showed ER-positive nuclear staining, whereas staining for PgR (data not shown) and HER2 was completely negative. In addition, LHCGR clearly labeled the cytoplasm of KPL-1 cells. Thus, the intrinsic subtype of KPL-1 cells is luminal-A and LHCGR-positive. Just as hCG mediated direct effects via LHCGR in vitro, hCG may directly suppress KPL-1 tumor growth in vivo. The ovaries of sham-treated mice contained corpora lutea and follicles at different maturational stages. In contrast, the ovaries of 100 IU hCG–treated mice developed increased corpora lutea, some of which were hemorrhagic, accompanied by hyperplasia of theca cells and interstitial cells and inconspicuous follicles (Figure 5B). The mammary glands of sham-treated mice were mainly composed of mammary ducts, but lobulo-alveolar development appeared in hCG-treated mice. These changes in the hCG-treated mouse reproductive organs and mammary glands were morphologically suggestive of hyperestrogenic status.

Changes in serum hormone levels after hCG injection in athymic mice. Serum hCG and estradiol levels of mice treated daily with or without 100 IU hCG for 5 weeks from 6 weeks of age until sacrifice at 11 weeks of age are shown in Table I. The hCG levels in the sham-treated group were below the detectable levels, but hCG treatment resulted in a significant increase in the serum hCG level (0 vs. 2.0 ng/ml). Although the value did not reach statistical significance, serum estradiol
at a level of <0.2 ng/ml was undetectable. Estradiol (E2) at a level of p<0.01 vs. sham-treated mice.

Table I. Serum hormone levels in female athymic BALB/c mice treated daily with 100 IU hCG or saline for 5 weeks from 6 weeks of age until sacrifice at 11 weeks of age.

<table>
<thead>
<tr>
<th>Treatment¹</th>
<th>hCG (ng/ml)²</th>
<th>Estradiol (pg/ml)³</th>
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<tbody>
<tr>
<td>Sham-treated</td>
<td>0</td>
<td>23.5±8.2</td>
</tr>
<tr>
<td>100 IU hCG</td>
<td>2.0±0.3*</td>
<td>35.7±10.5</td>
</tr>
</tbody>
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¹Each group consists of 5 rats.
²Human chorionic gonadotropin (hCG) at a level of <0.2 ng/ml was undetectable.
³Estradiol (E2) at a level of <20.0 pg/ml was undetectable. *p<0.01 vs. sham-treated mice.

levels in the in vivo hCG-treated group tended to increase to almost pregnancy levels (23.5 vs. 35.7 pg/ml).

Discussion

hCG is a potent anticancer agent against several types of neoplastic cells. Treatment with hCG inhibits human immunodeficiency syndrome-related Kaposi’s sarcoma cells through the enhancement of hCG receptor-dependent cell apoptosis (20, 21). hCG suppresses SKBR3, MCF-7, MDA-MB-231, MDA-MB-468, and T47D human breast cancer cells in culture; hCG shows a significant direct effect on both ER-positive and ER-negative human breast cancer cells (22). Indeed, our results from the cell proliferation assay (Figure 2) show that 100 IU/ml hCG inhibited the growth of both ER-positive (KPL-1 and MCF-7) and ER-negative (MKL-F) human breast cancer cell lines. An immunoblotting assay revealed that all three human breast cancer cell lines used in the present study possess LHCGR. These results indicate that the effects of hCG on human breast cancer cell growth seem to be mediated through LHCGR.

hCG exhibits anti-neoplastic potential through various cell kinetic pathways. Several Authors have demonstrated that the anti-proliferative properties of hCG are accompanied by the activation of programmed cell death in previous studies (13, 20-23). 100-IU daily hCG i.p. inhibits chemically-induced rat mammary carcinogenesis by activation of apoptosis-related genes (13). In fact, our in vivo results with TUNEL staining showed an increase of apoptotic cells in the KPL-1 tumors of hCG-treated female athymic mice. When mouse mammary carcinoma cells that stably express hCG are transplanted into BALB/c athymic mice, the tumor cell growth is inhibited due to up-regulation of apoptosis-related proteins including p53 (24).

In our current results from the immunoblotting assay, treatment with 100 IU/ml hCG induced apoptosis in KPL-1 cells by up-regulation of tumor suppressor protein p53, followed by cleavage of caspase-9 and then caspase-3, respectively. Caspase is one of the essential factors in programmed cell death. Caspase-9 is a key mediator in the mitochondria-dependent apoptotic pathway that stimulates caspase-3 down-stream (25). Our immunoblotting results showed that p53 activation peaked 12 h after hCG stimuli, followed by the cleavage of caspase-9 and caspase-3, which peaked at 48 and 72 h, respectively (Figure 3). These results indicate that hCG may induce apoptosis via a p53-mediated mitochondrial apoptotic pathway. A transcriptome assay revealed that the p53-regulatory element of CCAAT/enhancer binding protein beta (Cebpβ) was upregulated in hCG-treated rat mammary epithelial cells (26). These findings are comparable with our hypothesis that the anti-proliferative properties of hCG in LHCGR-positive human breast cancer cells may depend on the regulation of p53.

Topical (intratumoral) application of hCG suppresses SKBR3 human breast cancer cells transplanted into female athymic mice (22). The present study is the first report showing that systemic application of hCG suppressed the growth of tumors in a human breast cancer xenograft model. hCG suppressed the growth of KPL-1 cells transplanted into female BALB/c athymic mice without major toxicity (Figure 4). The direct action of hCG on breast cancer implies that the tumor cells were LHCGR-positive (Figure 5); and that hCG acted directly on breast cancer cells. In the present study, in vivo hCG treatment significantly suppressed tumor growth in athymic mice. LHCGR-positive KPL-1 cells pre-treated in culture with 100 IU/ml hCG for 48 h tended to have suppressed growth, but the level of suppression was not statistically significant.

The main functions of hCG are to support gestation, and one of its major functions is to promote progesterone production (27). In addition to progesterone production, female transgenic mice that overexpress hCG have elevated serum levels of estradiol from the corpora lutea (8). In agreement with a previous study, daily hCG injections caused the development of increased hemorrhagic corpora lutea accompanied with theca cells and interstitial cell hyperplasia in mice (8). Moreover, lobulo-alveolar development of the mammary gland appeared in hCG-treated mice (Figure 5). These changes in the ovaries and mammary glands may be explained by high estrogen levels. LHCGR expressed in the corpora lutea stimulated the production of ovarian steroids, and increased serum estrogen and progesterone levels, mimicking pregnancy for a short duration (not more than 20 weeks), suppressed MNU-induced mammary carcinoma in rats (7). In our previous investigation, mechanisms of the growth inhibitory action of hCG against LHCGR-negative MNU-induced mammary carcinoma were mainly due to accelerated ovarian steroid secretion that elevated estrogen and progesterone levels compared to those of pregnancy (14). In the present study, the serum estradiol levels were relatively elevated in hCG-treated mice (Table I). Taken together, these results indicate that in addition to direct hCG effects via LHCGR in KPL-1 cells, the indirect action of hCG due to the hypersecretion of estradiol from ovaries that mimics pregnancy levels may mitigate the growth of transplanted KPL-1 human breast cancer cells.
Transgenic hCG-β associated with mouse α-subunit and formed high levels of biologically-active dimeric hCG that mediated substantial lobulo-alveolar development, followed by the development of mammary carcinomas (9). Importantly, despite the persistent elevation of hCG levels, ovariectomy abolishes mammary carcinoma in transgenic hCGβ mice. hCG caused long-term aberration of ovarian function (production of estrogen and progesterone) that seems to be related to mammary tumorigenesis. Estrogen and progesterone administration for a long duration (>20 weeks) abolishes mammary cancer–suppressing effects in rats (28). In the present study, the secretion of estrogen from ovaries was limited to a short duration (5 weeks). In contrast to short-term administration of hCG shown to suppress breast
cancer, opposite results also exist (29). When hCG was given to virgin female transgenic mice carrying activated rat-ERBB-2 oncogene (HER2) for a short duration, mammary carcinoma development was accelerated with short latency. Also, hCG enhanced the in vitro proliferation and in vivo metastasis of breast cancer cells expressing ERBB-2 receptor (HER2) and LHCGR (29). Therefore, the intrinsic subtype of breast cancer should be considered prior to the use of hCG treatment.

In conclusion, hCG effectively suppressed LHCGR- and ER-positive KPL-1 human breast cancer cell growth both in vitro and in vivo. The mechanisms of action may be direct effects through the activation of programmed cell death via the p53-mediated mitochondrial apoptotic pathway and indirect effects through ovarian steroid secretion that elevates endogenous estrogen levels for a short duration. Therefore, short-term systemic hCG treatment may be an attractive strategy for LHCGR- and ER-positive human breast cancer control. However, the intrinsic subtype should be considered before hCG application because cancer promotion may occur in some types of breast cancer. It is, therefore, suggested that hCG exposure in relation to the intrinsic subtype should be further investigated.

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References


Figure 5. A. Morphology and immunohistochemical profiles of KPL-1 tumors growing in female athymic BALB/c mice. Immunohistochemical staining showed that KPL-1 tumor was estrogen receptor (ER)- and luteinizing hormone/chorionic gonadotropin receptor (LHCGR)-positive, while ERBB-2 (HER2) was negative. B. Changes in morphology of mouse ovaries and mammary glands with or without hCG treatment in vivo. Intraperitoneal injection of 100 IU hCG for 5 consecutive weeks caused multiple hemorrhagic corpora lutea in the ovary and lobulated ductal branching in the mammary gland.