Human chorionic gonadotropin (hCG), a hormone produced by the placenta, is essential for maintaining pregnancy. The tumor-suppressive property of hCG against palpable N-methyl-N-nitrosourea (MNU)-induced rat mammary carcinomas was examined. A single intraperitoneal injection of 60 mg/kg MNU was administered to female Lewis rats. When the MNU-induced mammary tumors reached a palpable size (≥1 cm in diameter), 0, 100, or 300 IU hCG were intraperitoneally injected 5 times per week for 4 weeks (a total of 20 injections). At the end of the treatment period, the 300 IU hCG treatment had significantly suppressed the growth of MNU-induced mammary carcinomas as compared to the sham treatment. The lower dose of hCG (100 IU) did not exert a significant effect. Final tumor volume and tumor wet weight, respectively, were as follows: sham-treated, 8151.3±1367.1 mm³ and 6011.3±1042.2 mg; 100 IU hCG, 7480.6±2011.2 mm³ and 5613.5±1142.0 mg; and 300 IU hCG, 3925.0±875.3 mm³ and 3482.4±817.3 mg. Serum estrogen and progesterone levels were markedly elevated to pregnancy levels in 300 IU hCG-treated animals, while serum hCG levels remained low, resulting in significantly increased ovarian and uterine weights with multiple corpora lutea in the ovary and cystically dilated endometrial glands. Immuno-histochemical studies revealed that almost all hCG-treated mammary carcinoma cells expressed estrogen and progesterone receptors, whereas luteinizing hormone/chorionic gonadotropin receptor was barely detectable. In conclusion, 300 IU hCG treatment for a short duration (4 weeks) suppressed the growth of overt and palpable MNU-induced mammary carcinomas. The mechanism of action may be through accelerated ovarian steroid secretion with the elevation of estrogen and progesterone levels to those in pregnancy.

Breast cancer is the most common neoplasm among women in both developing and developed nations and is the principal cause of death from cancer among women globally (1). Epidemiologically, reproductive history, such as early age at menarche, nulliparity, late age at first birth, late age at any birth, low parity, and late menopause, are related to breast cancer risk. In particular, the incidence of breast cancer is lower in parous women than in nulliparous women, and women who experience a full-term pregnancy before 20 years of age have a 50% reduction in breast cancer risk as compared to women who do not (2, 3). The protective effects of pregnancy on mammary cancer risk are also supported by data from experimental animal models (4). Parity protection seems to be related to pregnancy-related hormones; mammary carcinogenesis is suppressed in animals which receive short-term estrogen and progesterone treatment that mimics the hormonal milieu of pregnancy at a young age (5).

Human chorionic gonadotropin (hCG) is a pregnancy hormone produced by the placenta that is essential for maintaining pregnancy. The levels of hCG increase exponentially during the first trimester of pregnancy and then rapidly decrease to low steady-state levels. hCG interacts with the luteinizing hormone/choriogonadotropin receptor (LHCGFR) and promotes the maintenance of corpora lutea during pregnancy. Women with high serum hCG levels tend to be at a lower risk of breast cancer than women with low hCG levels (6). Maternal breast cancer risk decreases with increasing levels of hCG in pregnancy, especially for pregnancies before the age of 25 (7). Women who had received daily injections of hCG for weight reduction at a young age have a considerably reduced risk of breast cancer, and this association is particularly evident among nulliparous women (8). Women who have had choriocarcinoma, which is associated with exposure to a high level of hCG, have a significant reduction in breast cancer risk as compared to the general population, regardless of age and race (9). These findings are consistent with a possible long-term protective effect of elevated levels of circulating hCG on subsequent breast cancer risk.
In rodent mammary carcinogenesis, Russo and Russo’s group have demonstrated the chemopreventive property of hCG (10-12). Young virgin female Sprague-Dawley rats treated with 100 IU hCG for 21 days prior to exposure to the mammary carcinogen 7,12-dimethylbenz[a]anthracene (DMBA) exhibited a suppressed mammary carcinoma yield (11). The progression of mammary carcinomas in virgin Sprague-Dawley rats was inhibited by injections of 100 IU hCG for 40 consecutive days starting 21 days after DMBA exposure, when intraductal proliferation (ductal hyperplasia, DH) occurs in the mammary gland (12). hCG treatment inhibited the progression of mammary carcinomas by blocking the development of DH and ductal carcinoma in situ (DCIS). These results indicate that hCG may have a chemopreventive and/or chemotherapeutic potency against breast cancer. However, the possible chemotherapeutic effect of hCG on overt and palpable mammary carcinoma (at the stage of invasive ductal carcinoma; IDC) has not been tested in rodents. Moreover, hCG treatment up-regulates apoptosis-related and differentiation-associated molecules in the mammary gland (10), but the precise mechanism of the effect of hCG on the suppression of breast cancer remains unclear. Therefore, the aims of the present study were to determine if intraperitoneal injection of hCG can suppress the growth of overt N-methyl-N-nitrosourea (MNU)-induced mammary cancer in female Lewis rats and to determine the mechanisms of hCG action.

Materials and Methods

Animals and hCG treatment. Sixty female Lewis rats that were 2 weeks of age were purchased from Charles River Japan (Atsugi, Japan). All pups were housed 5 per cage with one nursing mother in an environmentally controlled animal room (22±2°C, 60±10% humidity, and 12-h light/dark cycle) with wood-chip bedding. After an acclimatization period of 1 week, all rats were intraperitoneally injected with 60 mg/kg MNU (Sigma, St. Louis, MO, USA) dissolved in physiologic saline containing 0.05% acetic acid immediately prior to injection. After this, they had free access to a commercial pellet diet (CMF 30 Gy; Oriental Yeast, Chiba, Japan). All pups were housed 5 per cage with one nursing mother in an environmentally controlled animal room (22±2˚C, 60±10% humidity, and 12-h light/dark cycle) with wood-chip bedding. After an acclimatization period of 1 week, all rats were intraperitoneally injected with 60 mg/kg MNU (Sigma, St. Louis, MO, USA) dissolved in physiologic saline containing 0.05% acetic acid immediately prior to injection. After this, they had free access to a commercial pellet diet (CMF 30 Gy; Oriental Yeast, Chiba, Japan) and fresh water throughout the experiment. The MNU-induced rat mammary tumorigenesis model allowed us to easily induce carcinomas and monitor the tumor growth. After the initiation of mammary carcinogenesis when the maximum diameter of mammary tumors reached a palpable size (32 rats developed mammary tumor ≥1 cm in diameter between 11 to 17 weeks of age), rats with mammary tumor ≥1 cm in diameter were divided into three groups; 10 rats in a group treated with saline only, 12 rats treated with 100 IU hCG and 10 rats in treated with 300 IU hCG. Physiological saline or hCG (Mochida Pharmaceutical, Tokyo, Japan) dissolved in physiological saline was injected intraperitoneally once per day, 5 times per week, for 4 weeks (20 total injections). Throughout the experiment, tumor volume was calculated with the standard formula; volume=length×width×height×0.5. At the time of sacrifice, circulating blood was sampled by cardiac puncture for the measurements of serum estrogen, progesterone, and hCG values by radioimmunoassay (SRL Inc., Tachikawa, Japan) and mammary tumors and reproductive organs were collected for histological and immunohistochemical evaluation. All animal procedures were approved by the Animal Experimentation Committee of Kansai Medical University.

Histological examination of mammary tumors and reproductive organs. Mammary tumors, non-tumoral mammary glands, and reproductive organs were harvested at the time of sacrifice. Tissues were weighed and fixed in 10% neutral-buffered formalin, embedded in paraffin, cut into 4-μm sections and stained with hematoxylin and eosin (H&E) for routine histological examination. Immunohistochemistry and TUNEL staining. The expression levels of proliferating cell nuclear cell antigen (PCNA), estrogen receptor (ER), progesterone receptor (PgR), and luteinizing hormone/chorionic gonadotropin receptor (LHCGR) were evaluated by immunohistochemistry with the labeled streptavidin-biotin (LSAB) method (LSAB staining kit; Dako, Carpinteria, CA, USA) according to the manufacturer’s instructions, and the reactions were visualized using 3,3′-diaminobenzidine (DAB). Anti-PCNA antibody (clone PC-10; Novocastra, Newcastle upon Tyne, UK), anti-ER antibody (clone 6F11; Novocastra), anti-PgR antibody (clone 10A9; Biodesign, Saco, ME, USA), and anti-LHCGR antibody (clone polyclonal; Acris, Herford, Germany) were used. Positivity in the mammary carcinomas was evaluated quantitatively. For the PCNA positive rate, at least 500 cancer cells were counted and the percentage of positively stained nuclei was calculated. For immunohistochemical scoring of ER, PgR, and LHCGR, at least 300 cancer cells were counted in three different areas of each tumor, and the percentage of the stained cells was converted to a grade as follows: grade 0 (negative), <15%; grade 1 (low), ≥15%-24%; grade 2 (medium), ≥25%-64%; and grade 3 (high), ≥65%. The staining intensity was assessed and subdivided into the following four grades: grade 0 (negative); grade 1 (low); grade 2 (medium); and grade 3 (strong). The final immunohistochemical score was calculated by multiplying the percentage grade by the intensity grade (13). Apoptosis was evaluated by TdT-mediated dUTP-digoxigenin nick end-labeling (TUNEL) performed with an in situ apoptosis detection kit (Apop-Tag; Millipore, Billerica MA, USA). To calculate the TUNEL-positive cells, more than 1,000 cancer cells were counted, and the percentage of the positively stained cells was calculated.

Statistical analysis. All results are expressed as the mean±standard error (SEM). Data were analyzed with the unpaired t-test or Mann-Whitney U-test after assuring the homogeneity of variances.

Results

Effect of hCG on the growth of MNU-induced rat mammary carcinoma. We started the hormone treatment when the MNU-induced mammary tumors reached a palpable size (≥1 cm in diameter). Changes in the growth curves of mammary tumor as expressed by tumor volume showed that the 300 IU hCG treatment suppressed mammary tumor growth, whereas the 100 IU hCG treatment did not yield a marked change as compared to sham-treated tumors (Figure 1A and B). Final tumor volume and tumor wet weight were as follows: sham-treated, 8151.3±1367.1 mm³ and 6011.3±1042.2 mg; 100 IU hCG, 7480.6±2011.2 mm³
and 5613.5±1142.0 mg; 300 IU hCG, 3925.0±875.3 mm³ and 3482.4±817.3 mg, respectively. All MNU-induced rat mammary tumors were histologically categorized as invasive papillary, tubular or cribriform adenocarcinomas. hCG caused a dose-dependent suppression of the growth of MNU-induced mammary carcinoma. The tumor volume and the tumor wet weight were significantly different between the sham-treated group and the 300 IU hCG-treated group at the termination of the experiment (p<0.05, respectively).

Histological detection of proliferative/apoptotic cells in rat mammary carcinomas after hCG treatment. In mammary carcinomas from 100 and 300 IU hCG-treated rats, no remarkable morphological differences were seen as compared to tumors from sham-treated rats (Figure 2A to C). Immunohistochemically, the percentage of PCNA-positive mammary cancer cells was significantly reduced in adenocarcinomas treated with 300 IU hCG as compared with the other two groups (Figure 2D and E vs. F), whereas all three groups had similar frequencies of TUNEL-labeled cells (Figure 2G to I). Therefore, the growth-suppressive mechanism of hCG against MNU-induced mammary carcinomas was a significant decrease in cell proliferation with no significant increase in apoptotic cell death.

Table I. Serum hormone levels and weights of reproductive organs of female Lewis rats treated with or without hCG for 4 consecutive weeks.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Estradiol (pg/ml)</th>
<th>Progesterone (ng/ml)</th>
<th>Uterine wet weight (mg)*</th>
<th>Ovarian wet weight (mg)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham-treated</td>
<td>45.58±9.60</td>
<td>16.02±5.19</td>
<td>256.32±12.58</td>
<td>67.62±5.15</td>
</tr>
<tr>
<td>100 IU hCG</td>
<td>71.24±10.27</td>
<td>105.98±32.44*</td>
<td>244.97±7.44</td>
<td>88.65±6.70*</td>
</tr>
<tr>
<td>300 IU hCG</td>
<td>98.30±25.37*</td>
<td>103.60±44.67*</td>
<td>308.16±24.10*</td>
<td>111.27±10.31*</td>
</tr>
</tbody>
</table>

*Wet weights of reproductive organs are expressed as mg per 100g body weight. *p<0.05 compared with sham-treated group.

Hormone receptor status of mammary carcinomas. Immunohistochemical staining and the quantitative scores of ER, PgR, and LHCGR in MNU-induced mammary carcinomas are shown in Figure 3. Regardless of hCG treatment, ER and PgR were intensely stained in almost all of the mammary carcinoma cells (Figure 3 A to F), while immunoreactions with LHCGR were hardly detectable in the groups (Figure 3G to I). Immunohistochemical scores of ER, PgR, and LHCGR among the three groups were similar.

Changes in reproductive organ structure and function and serum hormone levels after hCG treatment. Repeated intraperitoneal injections of hCG with the present dose regimens were tolerable in all rats. At the time of autopsy, hCG-treated animals had no remarkable toxicity as evaluated by body weight gain, and the major organs including liver, lung, and digestive tracts, were all normal. Representative ovarian and uterine structure at the termination of the experiment is shown in Figure 4. The uterine and ovarian wet weight and the levels of serum estrogen and progesterone at the termination of the experiment are listed in Table I. hCG treatment, especially at a dose of 300 IU, yielded a significant increase in both the uterine and ovarian weights, as compared with sham-treated rats. Increased ovarian weight was due to...
an increase in the ovarian area occupied by corpora lutea (Figure 4B vs. A); LHCGR expression was seen in the cytoplasm of the corpora lutea (data not shown). Uterine enlargement was due to cystically enlarged endometrial glands (Figure 4D vs. C). Moreover, serum estrogen and progesterone values were significantly elevated in 300 IU hCG-treated animals. However, serum hCGβ levels were not altered among the groups (<0.1 ng/ml in most of the rats from all groups; data not shown). Consequently, these results indicate that repeated hCG treatments led to the hypersecretion of endogenous estrogen and progesterone from the ovary.

Discussion

hCG, which is structurally similar to chorionic gonadotropin produced by the placenta of rats and other rodents, caused a dose-dependent protective effect on rat mammary cancer growth. Although a previous study showed that DMBA-induced mammary carcinoma was suppressed when hCG was given at the DH stage (12), in the present study, growth suppression was seen in MNU-induced mammary carcinoma when hCG was given at the IDC stage (when tumors were palpable). Growth of the 4T1 mouse mammary carcinoma cell line with stable expression of hCGβ (4T1-hCGβ) transplanted into BALB/c mouse mammary glands was significantly reduced and tumor vessel formation was attenuated when compared with a mammary cancer cell line without hCGβ expression (4T1); significant apoptosis was detected in hCGβ-expressing mammary cancer cells, as well as enhancement of Bax protein expression (14). hCG injection, directly into SKBR3 human breast cancer cells, transplanted into athymic mice, significantly increased apoptosis, while the cell proliferation rate was unchanged (15). In an in vitro study, hCG treatment reduced cell viability in SKBR3, MCF-7, MDA-MB-231, MDA-MB-468, and T47D human breast cancer cell lines (15). Although the hCG action was through LHCGR in MCF-
Figure 3. Immunoexpression of estrogen receptor (ER), progesterone receptor (PgR) and luteinizing hormone/chorionic gonadotropin receptor (LHCGR) in N-methyl-N-nitrosourea (MNU)-induced mammary carcinomas in female Lewis rats. A, B, and C: ER expression; D, E, and F: PgR expression; G, H, and I: LHCGR expression. Immunohistochemical scores for ER, PgR, and LHCGR in all three groups were similar. All images shown at ×400 magnification.

Figure 4. Histology of ovaries and uterus shown by H&E staining. A: Sham-treated rat ovary showing corpora lutea and follicles. B: 300 IU hCG-treated rat ovary showing multiple corpora lutea throughout the whole ovary. C: Sham-treated rat uterus showing normal structure. D: 300 IU hCG-treated rat uterus showing cystically dilated endometrial glands. All images shown at ×400 magnification.
7 cells (16), a direct correlation between the response to hCG and the expression levels of LHCGR is lacking (15). Tumor growth is a balance between cell proliferation and cell death. hCG accelerates apoptosis of cells of DMBA-induced mammary carcinomas (17), while in the present study, although the number of TUNEL-labeled cells was not altered, the proliferating cell rate was significantly decreased after 300 IU hCG treatment.

LHCGR mediates the effects of hCG. hCG effects may take place directly through LHCGR in breast cancer cells. MCF-7 cells contain functional LHCGR, and hCG treatment results in a steady-state decrease in ER mRNA and protein levels and inhibits cell growth (18). Although the cause or consequence is not known, LHCGR is up-regulated in invasive breast cancer as compared to pre-invasive breast cancer (19). However, LHCGR expression is detected in only some human breast carcinomas (13). An examination of 1551 human breast carcinomas and 42 human breast cancer cell lines found that LHCGR was undetectable in 62% (955/1551) of breast cancer samples and 98% (41/42) of human breast cancer cell lines (20). The direct action of hCG on breast cancer implies the existence of corresponding receptors in these tissues. Taken together, the unaltered ER and PgR status and the hardly detectable LHCGR from mammary carcinomas in the present study indicate that hCG may not act directly on mammary cancer cells. Rather, the effects of hCG may take place indirectly through the LHCGR in ovaries, thereby influencing steroid hormone production.

Female transgenic mice that overexpress hCG have elevated serum levels of estradiol (21) and progesterone from the corpora lutea (22). Histologically, hCG treatment causes enlarged ovaries with multiple corpora lutea accompanied by an enlarged uterine horn. In hCGβ transgenic mice, prolonged hCG exposure promotes lobuloalveolar development, followed by the occurrence of mammary carcinomas (22, 23). Importantly, despite the persistent elevation of hCG levels, ovariectomy abolishes mammary carcinomas in mice (22). hCG-induced aberration of ovarian function (production of steroid hormones) for a long duration seems to be responsible for mammary tumorigenesis. LHCGR was expressed in the corpora lutea of the rat ovary, which stimulated the production of estrogen and progesterone that reached pregnancy levels. The ranges of circulating 17β-estradiol and progesterone levels in rats during pregnancy are 55-630 pg/ml for 17β-estradiol and 45-130 ng/ml for progesterone (5).

Pregnancy levels of estrogen and progesterone administered to young rats for a short duration effectively suppress the initiation and promotion stages of MNU-induced rat mammary carcinogenesis (5, 23, 24). Even estrogen alone can exert this effect and block the progression from DH to DCIS (25). However, the cancer-suppressing effects are lost with long-term estrogen and progesterone treatments (>20 weeks) (26).

In conclusion, 300 IU hCG treatment for a short duration (4 weeks) suppressed the growth of overt and palpable MNU-induced mammary carcinomas, and the mechanism of action may be through accelerated ovarian steroid secretion that elevates estrogen and progesterone levels to those in pregnancy. Our results showed that short-term hCG lacked major toxicity in animals. Long-term exposure of hCG in the MNU-induced model of mammary carcinoma should be further investigated.

Acknowledgements

We thank Ms. T Akamatsu for her technical assistance and Ms A Shudo for preparing the manuscript. This work was supported by a Grant-in-Aid for Young Scientific Research (B) (23791506) from the Japan Society for the Promotion of Science.

References