Direct Modulation of Tumor Suppressor Connexin 26 Gene by Human Chorionic Gonadotropin in Rat Mammary Glands

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ABSTRACT

Human chorionic gonadotropin (hCG) has been shown to reduce the incidence of carcinogen-induced rat mammary tumors. Because connexin 26 (Cx26), a tumor suppressor gene candidate, can be up-regulated in mammary epithelial cells during lactation, we examined the in vivo and ex vivo effects of hCG on Cx26 expression in rat mammary tissues and used its effect on the expressions of β-casein and Cx43 as controls. The Cx26 mRNA and protein expressions were up-regulated by daily administration of 100 units of hCG, starting on day 5 and reaching a 14-fold maximum increment on days 16 through 21. It remained elevated above the basal level even 20 days after hCG withdrawal. The changes in β-casein expression ran parallel to that of Cx26, whereas the expression of Cx43 was down-regulated. There was no correlation between steroidal hormone levels and Cx26 expression, except for the first 5 days of hCG treatment. In the ex vivo organ culture system, expression of mammary glands to 10 units/ml hCG for 5 days up-regulated Cx26 but had no effect on β-casein expression. These results imply a direct induction of the tumor suppressor Cx26 gene by hCG in mammary epithelial cells, a mechanism unrelated to lactation.

INTRODUCTION

Gap junctional communication plays a critical role in tissue development and differentiation (1–3). The gap junctions are formed by members of a protein family called Cxs (review in Ref. 4). Six Cx molecules form a hexamer called connexon, and the gap junction channel is formed by two connexons, one from each of the two adjacent cells. The channel allows the passage of regulatory factors with a molecular weight less than 1000 (5).

The mammary luminal epithelial cells express mainly Cx26, whereas the myoepithelial cells and surrounding stromal fibroblasts express Cx43 (6). During lactation, there is a marked up-regulation of Cx26, presumably to facilitate and coordinate all mammary epithelial cells to pursue their destined physiological function in milk production (6, 7). This up-regulation of Cx26 in mammary epithelium is reminiscent of another physiological phenomenon occurring in uterine myometrium, where Cx43 expression increases 5–9-fold at term to synchronize contractile activity during labor (8, 9).

Whereas Cx26 is up-regulated in mammary epithelial cells during lactation, its expression is markedly reduced or undetectable in most breast cancers (10). When HeLa cells were transfected with various types of Cx, only the Cx26 gene exerts a strong negative growth effect (11, 12). Similarly, the Cx26 gene, when introduced into MCF-7 breast cancer cells by transfection, confers not only a reduction of tumor growth potential but also a restoration of cellular differentiation (13). These results strongly imply that Cx26 is a tumor suppressor gene. Agents capable of up-regulating Cx26 mRNA expression could, therefore, be exploited for their potential use in breast cancer prevention.

There is compelling evidence to indicate that lactation at early ages play a protective role against breast cancer (14, 15). Similarly, hCG-induced pseudopregnancy could also reduce the tumor incidence in rat mammary tumor models (16). Because mammary Cx26 is up-regulated in the late stage of pregnancy and during lactation and Cx26 is a tumor suppressor gene candidate, we decided to study the effect of hCG on Cx26 expression in rat mammary glands. Furthermore, receptors for luteinizing hormone/hCG are present in normal and malignant mammary epithelial cells (17), it is therefore conceivable for hCG to exert its direct action upon mammary epithelial cells rather than mediating through steroidal and lactogenic hormones. Our ex vivo study may further delineate whether hCG has such a direct effect on the up-regulation of Cx26 gene expression.

MATERIALS AND METHODS

Animals. Fifty-day-old female Sprague Dawley rats (Harlan, Indianapolis, IN) were divided into control (vehicle) and experimental (hCG) groups. The rats were housed at a constant temperature (22°C) and in an imposed diurnal cycle with the light period from 6 a.m. to 6 p.m. Food (rodent pellets; Harlan Tek-Lad, Madison, WI) and water were available ad libitum. The experimental rats received hCG, 100 units/day i.p. for 21 days, followed by hCG withdrawal for an additional 20 days. Four rats were sacrificed at each of the following time points: on days 1, 3, 5, 8, 16, and 21 of hCG treatment; and on days —¿3, —¿5, —¿10, —¿15, and —¿20 of hCG withdrawal. The control rats were sacrificed on the days corresponding to days 1, 8, and 21 of hCG treatment and day —¿20 of hCG withdrawal. Mammary tissues were dissected out and frozen immediately in liquid nitrogen. They were stored in liquid nitrogen until used for immunocytochemistry staining and Northern blot analysis. The hCG preparation, Profasi, was purchased from Serono Laboratory (Norwell, MA). The dosage chosen (100 units/day) was based on previous experiences in the literature (16) as well as ours in the prevention of carcinogen-induced rat mammary tumors.

Blood specimens for 17β-estradiol and progesterone assays were collected at the time of sacrifice. The 17β-estradiol RIA assay was based on the manufacturer’s procedure (Pantex, Santa Monica, CA) with coefficients of variation ranging from 7 to 11%. The chemiluminescent immunoassay for progesterone was performed also according to the manufacturer’s procedure with coefficients of variation ranging from 5.6 to 13.6% (Sanofi Diagnostics Pasteur, Inc., Chaska, MN). Attempts to measure the plasma prolactin levels were unsuccessful, because of erratic results seen in the control rats secondary to the lack of a reliable method for standardizing the animal stress levels at sacrifice.

Organ Culture of Rat Mammary Glands. Three 90-day-old Sprague Dawley female virgin rats were ovariecctomized and then sacrificed 1 week later. Mammary glands from thoracic and abdominal regions of each rat were dissected out, cut into small pieces, pooled, and equally divided into four culture flasks. The flasks were allocated into the control and hCG (10 units/ml) groups and incubated for 3 and 5 days in 10 ml of DMEM medium (Life Technologies, Inc.) containing 5% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in 100% oxygen. The experiment was duplicated in an additional four rats. The 5-day results from these two experimental sets were combined and compared between the controls and the hCG-treated group using the paired one-tailed Student’s t test.

We found that cell-cell communication (dyadic coupling) in MCF-10A human mammary epithelial cells could be markedly enhanced by hCG at all three

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4 The abbreviations used are: Cx, connexin; DMBA, 7,12-dimethylbenz(a)anthracene; hCG, human chorionic gonadotropin; GAD, glycericdehyde-3-phosphate dehydrogenase.
concentration levels ranging from 1, 10, to 100 units/ml. A similar concentration range was also used by other investigators (18). The middle level (10 units/ml) was chosen for our ex vivo experiments to ensure an adequate exposure of epithelial cells to hCG.

**Immunocytochemistry.** The frozen tissues were sectioned (5-μm thickness) on a cryostat and fixed in absolute ethanol. After PBS washing and blocking with 1.5% horse serum (Vector Laboratory, Burlingame, CA), monoclonal antibody against Cx26 (Zymed, San Francisco, CA) at 1:500 dilution or monoclonal antibody against Cx43 (Chemicon, Temecula, CA) at 1:50 dilution was added to the sections and incubated at 4°C overnight. Then biotinylated horse anti-mouse IgG antibody, diluted at 1:200 in 3% horse serum/PBS, was added and incubated at room temperature for 30 min. After washing with PBS, streptavidin-fluorescein (1:1000 in PBS; Amersham, Arlington Heights, IL) was added. The degree of fluorescence was examined under a Zeiss fluorescent microscope.

**RNA Isolation and Northern Blot Analysis.** Total RNA was isolated from rat mammary glands by the method of Chomczynski and Sacchi (19) using phenol and guanidine isothiocyanate TRIzol reagent (Life Technologies, Inc.). RNA (20 μg/lane) was loaded on a 1% agarose gel, separated by electrophoresis, and transferred to a positive charged nylon membrane (ICN Biomedicals, Costa Mesa, CA). For hybridization, antisense RNA 32P-labeled probes were generated using rat Cx26 cDNA, rat Cx43 cDNA (both provided by David Paul, Harvard University), rat β-casein cDNA (provided by Jeffrey Rosen, Baylor University), and mouse GAD cDNA (provided in the Ambion NorthernMaxi kit). The hybridization was performed at 42°C overnight, and washings were according to the manufacturer’s procedure (ICN Biomedicals).

**RESULTS**

**In Vivo Up-Regulation of Mammary Gland Cx26 mRNA Expression by hCG.** Injection of hCG at a dose of 100 units daily into 50-day-old Sprague Dawley female rats clearly up-regulated the mammary gland Cx26 mRNA expression. The results of Northern blot analysis on the pooled mammary gland RNA from four rats are shown in Fig. 1A. Such up-regulation started on day 5 of hCG injection and reached the maximum levels by days 16–21. When compared with the control basal level, there was a sustained Cx26 up-regulation, even 20 days after hCG withdrawal.

The changes in β-casein mRNA expression ran in parallel with those of Cx26 (Fig. 1). It reached the maximum on day 16 and remained elevated on day −20. On the other hand, Cx43 mRNA expression that represents the major connexin in myoepithelial and stromal cells steadily declined from days 8–21 of hCG treatments, then fully recovered 5 days after hCG withdrawal.

When Northern blot analysis was run on RNA extracts from each rat, the composite data from four separate experimental sets are shown in Fig. 1B. The degree of Cx mRNA expression was adjusted by the amount of GAD loading controls and presented as a Cx:GAD ratio. The ratio of Cx26:GAD expression was around 0.31 in the controls that increased to 4.1 on day 16 and 4.4 on day 21 of hCG treatment, an ~14-fold increment by hCG. On the other hand, the Cx43:GAD expression ratio declined from 1.8 of the control to 0.5 of hCG treatment on days 8–21, an over 3-fold decrease. However, there was also a steady decline of Cx43 with age in the controls.

**In Vivo Regulation of Cx26 and Cx43 Proteins in Mammary Glands by hCG.** Immunocytochemistry stains for Cx26 and Cx43 were carried out on frozen sections of the mammary glands. In the control group, Cx26 expression was rarely detectable in luminal epithelial cells (Fig. 2d), whereas Cx43 expression was abundant in the myoepithelial cells and surrounding fibroblasts (Fig. 2g). The Cx26 expression became detectable by 5-day hCG treatments (Fig. 2e) and was abundant by day 21 (Fig. 2f). Conversely, the Cx43 expression dwindled, especially from day 5 to day 21 of hCG treatments (Fig. 2, g, h, and i).

Changes in Circulatory Estradiol and Progesterone Levels by hCG Treatment. Following administration of hCG, there was a progressive increment of blood 17β-estradiol and progesterone levels, starting on day 5 and reaching the maximum by day 16 (Fig. 3). Interestingly, by day 21, both hormones abruptly returned to their basal levels despite of the continuing daily injections of hCG. The changes in progesterone levels were particularly impressive; it mimics those changes observed in a full-term pregnancy.

**Ex Vivo Regulation of Cx26 mRNA Expression by hCG in Organ Culture.** The direct effect of hCG on mammary gland Cx26 was assessed by organ culture with mammary glands collected from three separate rats (experiment 1). After culture for 3 and 5 days with or without 10 units/ml hCG in the medium, the tissues were subjected to Northern blot analysis. In comparison with the controls, hCG...
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Fig. 2. Immunocytochemistry stains for Cx26 and Cx43 on the frozen section of rat mammary glands after 5 days and 21 days of hCG (100 units/day) injections, i.p. The morphological alterations of mammary glands from hCG treatment are shown in H&E staining (a–c). Punctuate fluorescent spots demonstrated by monoclonal antibody against Cx26 were rarely visible in control mammary glands (d) but became abundant on the membranes of the luminal epithelial cells (LE) after hCG treatment (e and f). Conversely, the intensity of Cx43 fluorescent spots surrounding the myoepithelial (ME) cells and fibroblasts dwindled from the control (g) to 21-day hCG mammary glands (i). Mouse IgG (Sigma Chemical Co.) was used as control primary antibody (j–l). X500.
clearly caused an increment of Cx26 mRNA expression in the mammary glands from rats 2 and 3, especially after a 5-day exposure (Fig. 4A). The results derived from rat 1, however, was not as conclusive.

In support of the results from the in vivo study, hCG appears also to down-regulate the Cx43 expression of mammary glands from rats 2 and 3 in the organ culture system. Clearly, hCG had no effect on β-casein expression of the mammary glands in this ex vivo culture system.

The 5-day hCG ex vivo experiment was duplicated in another set of four rats (experiment II). The results were combined with that of experiment I and expressed as relative mRNA expressions of Cx26, Cx43, and β-casein normalized by GAD (Fig. 4B). The statistical analysis was based on a paired one-tail Student’s t test. The up-regulation of Cx26 by 5-day hCG exposure was highly significant ($P = 0.004$). The down-regulation of Cx43, however, was only marginally significant ($P = 0.054$). On the other hand, the relative β-casein expressions were barely detectable in both the controls and the explants following a 5-day hCG exposure.

**DISCUSSION**

Since the early 1970s, epidemiological studies have shown that breast cancer risk could be reduced by as large as 50–60% in women whose first childbirth occurred before age 20 (14, 15). A recent report has linked the reduction of breast cancer risk to lactation (20). There is a strong correlation between the degree of risk reduction and age of first lactation, whereas the correlation with accumulative duration of lactation during life, although present, is much weaker. Similar protective effects could also be achieved in rat mammary tumor models. Pseudopregnancy induced by hCG could drastically reduce the incidence of DMBA-induced rat mammary tumors by 50–70% (16). The mechanism for such protective effects from lactation and hCG is still largely unknown, although two popular theories, cellular maturation and apoptosis, have been postulated (21–24).

This protective effect is rather puzzling, especially because it is well known that prolactin strongly promotes mammary carcinogenesis in these rodent mammary tumor models (25, 26). We started questioning whether the breast cancer risk reduction may be attributed to factors associated with lactation rather than prolactin-induced lactation per se.

Our present study on Cxs provides at least four interesting clues on the effects of hCG and implies that breast cancer risk reduction may be mediated through mechanism(s) totally unrelated to lactation:

(a) There is a late dissociation between Cx26 expression and the circulatory hormone levels. In our experiments, the increase of Cx26 expression coincides only briefly with the surge of estrogen and progesterone levels during the first 5 days of hCG treatment. After 16 days of hCG treatment, however, such association disappears; the Cx26 up-regulation is sustained even when steroid hormones have returned to their basal levels. Although up-regulation of Cx26 by estrogens has been reported (27), the sustained up-regulation has to be controlled by a mechanism other than estrogens. It is quite surprising to see a sharp decline of blood steroidal hormone levels on day 21, even when rats were still receiving hCG, a phenomenon reminiscent of a true full-term pregnancy in preparation for parturition on day 21.

(b) Our second interesting observation is a down-regulation of Cx43 expression by hCG in the mammary glands. However, these results should be interpreted with caution. After hCG treatment, there is a disproportional increment of luminal epithelial cells versus myoepithelial and stromal cells. The trend of Cx43 decline in controls may have reflected the expansion of luminal epithelial cells with advance of age. However, such a bias would be negligible in the ex vivo system with a short-term organ culture, where we still observed a decrease in Cx43 mRNA expression. Most importantly, the results of immunocytochemistry staining for Cx43 protein have supported the contention that hCG down-regulated Cx43 not only in stromal tissue in general but also specifically in the location where myoepithelial cells reside. Interestingly, down-regulation of Cx43 mRNA is also seen in uterine myometrium during pregnancy before reaching parturition (9) and is presumably responsible for maintaining uterine quiescence during pregnancy (28). Conceivably, a down-regulation of Cx43 in mammary myoepithelial cells by hCG may serve a similar physiological function to prevent a premature milk excretion before parturition.

(c) The up-regulation of Cx26 appears to be a direct effect of hCG. In mammary organ cultures, hCG exposure up-regulates the Cx26...
gene but has no effect on β-casein mRNA expression. These results suggest that hCG may activate these two genes through different mechanisms, a direct effect on Cx26 and an indirect modulation on β-casein through prolactin. Therefore, it is highly possible that the protective effect of hCG against rat mammary tumor development may be mediated through the direct activation of tumor suppressor gene(s), a mechanism distinctly separated from that of lactation. (d) Our final observation pertains to a prolonged action of hCG on Cx26. Although the rats received only a short-term hCG treatment, i.e., a 21-day duration mimicking a full-term pregnancy, the effect on Cx26 mRNA up-regulation appears to linger for a much longer period of time, lasting at least 20 days after hCG withdrawal in our study. Whether the observed degree and duration of modulation of this suppressor gene is sufficient to contribute to the reduction of breast cancer risk is the subject of continuing investigation. Using recombinant hCG or subunits of hCG may further define the active component(s) that are responsible for the modulation of Cx26 and potentially of other suppressor genes. We have recently cloned the upstream genomic sequence of the human Cx26 gene (29). That will certainly facilitate our understanding on the molecular mechanism of up-regulation of this tumor suppressor gene by hCG.

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