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Report

Enhancement of radiosensitivity of the MCF-7 breast cancer cell line with human chorionic gonadotropin

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Summary

Secretion of human chorionic gonadotropin (hCG) during pregnancy induces differentiation of the mammary gland, thereby making breast tissue less susceptible to carcinogenesis. HCG binds to specific hCG receptors on mammary epithelial cells inducing changes in gene expression that can inhibit cell proliferation and, therefore, interfere with tumorigenesis. Since breast cancer cells also contain a relatively high level of the hCG receptor, hCG has potential as a therapeutic agent. We postulated that hCG might also enhance the radiosensitivity of breast cancer cells and, therefore, be useful as an adjunctive therapy. In the present study, MCF-7 breast cancer cells grown in cell culture were treated with hCG (0.2–5 IU/ml) for 24 h prior to exposing the cells to 0 Gy, 3 Gy, 4 Gy, or 5 Gy of radiation. Following irradiation, the MCF-7 cells were incubated either in the presence or absence of hCG. Cell survival was monitored with an MTT assay 1 day, 4 days, and 7 days after irradiation. All of the concentrations of hCG tested enhanced radiosensitivity of MCF-7 cells. The maximum enhancement occurred with MCF-7 cells that had been exposed to 2 IU/ml of hCG for at least 24 h prior to irradiation with 4 Gy. The use of higher concentrations of hCG or a higher dose of radiation did not increase the enhancement effect. Treatment of MCF-7 cells with hCG for only 24 h was sufficient to achieve the maximum effect. However, maintaining the cells in hCG beyond 24 h increased the effectiveness of the lowest hCG concentration. Using a linear-quadratic equation to analyze the data, we determined that the use of hCG would result in an 8–10% reduction in MCF-7 cell survival at a dose of 2 Gy, a typical dose used in conventional cancer therapy.

Introduction

Human chorionic gonadotropin (hCG), a glycoprotein hormone secreted by the human placenta during pregnancy, is an important mediator of fetal development. In recent years, hCG has also emerged as a mediator of carcinogenesis. The initial indication of a connection between hCG and cancer occurred in 1983 when it was reported that patients with urogenital and non-urogenital cancers frequently have elevated serum hCG levels [1]. Acevedo et al. [2] subsequently reported that hCG is expressed on the cytoplasmic membranes of 74 established cancer cell lines of various types and origin. Furthermore, a study of hCG *in vivo* revealed a possible correlation between hCG expression and metastatic potential [3]. Other investigators discovered that free hCG can interact with cells by binding to a specific transmembrane glycoprotein receptor [4]. The hCG receptor has been detected in gonadal tissues as well as male and female non-gonadal tissues [5].

The possibility that purified hCG can produce a beneficial, receptor-mediated effect with certain types of cancer has been investigated. Several studies [6–8] have shown that hCG can block tumorigenesis and metastasis of Kaposi's sarcoma. Investigators found that biopsy specimens of AIDS-related Kaposi's sarcoma contained hCG receptors whereas biopsies of normal skin did not. The dose-dependent anti-

Kaposi's activity of hCG was attributed primarily to the induction of apoptosis.

In a study focusing on prostate cancer, Chiao et al. [9] were able to demonstrate a dose-dependent inhibitory effect on the proliferation of LNCaP cells, an androgen-dependent prostate cancer cell line known to express relatively high levels of hCG receptor protein [10]. As with Kaposi's sarcoma, the hCG-induced effect was primarily attributed to an increase in apoptosis. When non-androgen-dependent prostate cancer cell lines were tested, hCG was able to modulate cell growth apparently by blocking G_1 cells from entering the replicating phases of the cell cycle.

In the case of breast cancer, studies have shown that hCG inhibits mammary tumorigenesis through induction of mammary gland differentiation *in vivo* and inhibition of cell proliferation *in vitro* and *in vivo* [11–14]. Furthermore, studies have shown that hCG induces an acceleration in the expression of apoptotic genes [15, 16]. Functional hCG receptor protein and hCG receptor mRNA have been detected in normal breast epithelial cells, cells from breast cancer tissues, and breast cancer cell lines [11, 17]. One particular breast cancer cell line, MCF-7, which contains a relatively high level of the hCG receptor, has been used extensively to study hCG activity *in vitro* [11,13, 17–19].

The inhibitory effects of hCG on cell proliferation encouraged us to look for additional beneficial effects of hCG. In the present study, we demonstrate that hCG can enhance the radiosensitivity of MCF-7 cancer cells.

Materials and methods

Materials

The MCF-7 cell line (ATCC HTB-22), a human breast cancer cell line of epithelial origin, was obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in Minimal Essential Medium (MEM) with Earle's salts (GibcoBRL, Life Technologies, Rockville, MD) supplemented with 10% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD), and antibiotics (penicillin, gentamycin, and fungizone). The cells were cultured in sterile, flat-bottom 96-well microtiter plates (Costar, Cambridge, MA). Purified hCG was obtained from American Pharmaceutical Partners, Inc. (Los Angeles, CA). Recombinant hCG was obtained from Sigma (St. Louis, MO).

Cell culture

Each well of a microtiter plate was seeded with 5,000 MCF-7 cells (200 µl). The cells were incubated at 37°C in 5% CO_2 for 48 h prior to use. At 48 h of growth, the cells were typically 25% confluent.

Treatment with hCG and radiation

The growing cultures of MCF-7 cells were treated with various concentrations of hCG before irradiation. Specifically, cell culture medium was removed from each cell culture well and replaced with fresh cell culture medium containing hCG (0.2, 1, 2, 5 IU/ml) or without hCG (controls). The cells were then incubated for 24 h prior to being irradiated. In one set of experiments, the cell culture medium containing the hCG was removed just before irradiation and replaced with fresh medium without hCG. In a second set of experiments, the hCG containing media was not removed. Cells were exposed to either 0 Gy, 3 Gy, 4 Gy, or 5 Gy of radiation using a 250 kVp X-ray machine (Phillips) operated at 250 kV and 15 mA with a 1 mm copper filter. Exposure doses were calculated using an ionization chamber, and absorbed doses were calculated using appropriate temperature, pressure, and Roentgen to Gy conversion factors. The absorbed dose rate was approximately 1 Gy/min. Following irradiation, the cells were incubated for 1, 4, or 7 days at 37°C in 5% CO_2 and then assayed to determine cell survival.

MTT assay

Cell survival was evaluated using a modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously [20]. Briefly, 30 µl of MTT (5 mg/ml, Sigma, St. Louis, MO) were added to each well of the MCF-7 cell culture microtiter plate. After 4 h of incubation at 37°C, all fluid was removed and 100 µl of dimethyl sulfoxide (DMSO) were added to each well. Plates were shaken vigorously for 5 min on a rotary shaker to solubilize the MTT-formazan product. Absorbance at 530 nm was measured with a Dynex MRX Revelation microtiter plate reader (Dynex Technologies, Inc., Chantilly, VA).

Experimental plan and statistical calculations

Because of the large number of variables to be tested and logistical considerations, it was necessary to repeat experiments a number of times. For example,

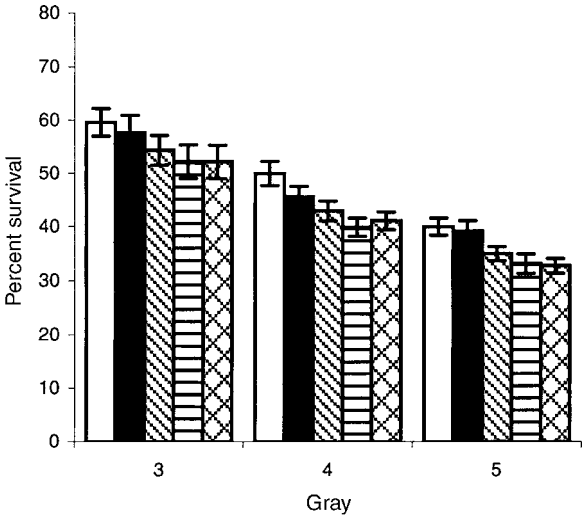

Figure 1. Survival of MCF-7 cells treated with 0 IU/ml □, 0.2 IU/ml ■, 1.0 IU/ml ▨, 2.0 IU/ml ▤, and 5.0 IU/ml ⊠ of purified hCG for 24 h prior to exposure to 3 Gy, 4 Gy, and 5 Gy of radiation compared to control cells (non-irradiated MCF-7 cells). MCF-7 cell survival was determined by MTT assay 1, 4 and 7 days after irradiation. The day 7 data are presented. Error bars represent the mean of the variance of four experiments.

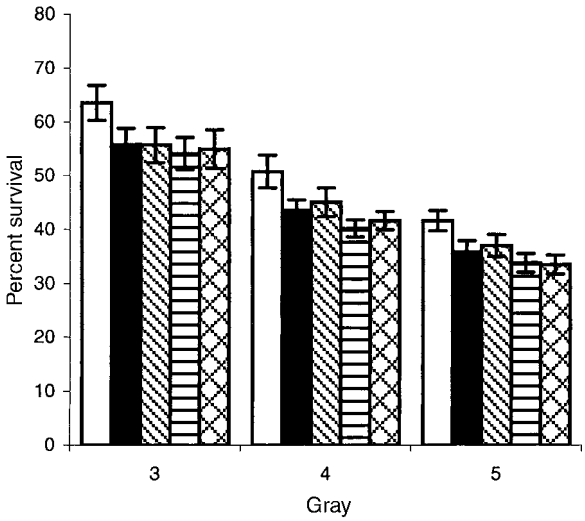

Figure 2. Survival of MCF-7 cells treated with 0 IU/ml □, 0.2 IU/ml, ■ 1.0 IU/ml ▨, 2.0 IU/ml ▤, and 5.0 IU/ml ⊠ of purified hCG both before and after exposure to 3 Gy, 4 Gy, and 5 Gy of radiation compared to control cells (non-irradiated MCF-7 cells). MCF-7 cell survival was determined by MTT assay 1, 4, and 7 days after irradiation. The day 7 data are presented. Error bars represent the mean of the variance of six experiments.

experiments in which MCF-7 cells were exposed continuously to purified hCG were repeated 6 times. Experiments in which cells were only exposed to purified hCG prior to irradiation were repeated 4 times. Finally, there was a single experiment in which cells were exposed to recombinant hCG both before and after irradiation. Each experiment with purified hCG included six replicates for each concentration of hCG and each dose of radiation. The single experiment with recombinant hCG included 12 replicates. Since each repeat experiment resulted in slightly different survival endpoints despite the use of identical test conditions, the cumulative data from the various experiments (see Figures 1–3) are presented as the mean of the means of individual repeat experiments and the mean of the corresponding standard deviations. In addition, the statistical significance of differences between means of various experiments was determined using the combined data at the 95% confidence level. The calculations were made using the F-test for variance and student t-test for means.

Analysis of cellular response

The basic data generated in these experiments were the MTT responses at days 1, 4, and 7 post-irradiation. To help understand the results, the data on day 7 post-

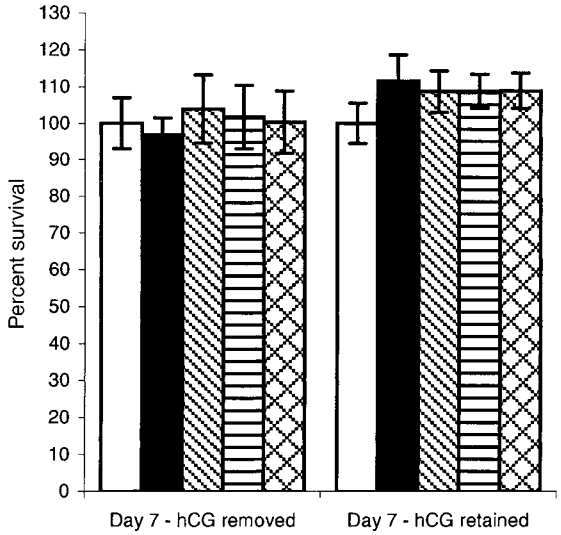

Figure 3. Survival of non-irradiated MCF-7 cells treated with 0 IU/ml □, 0.2 IU/ml ■, 1.0 IU/ml ▨, 2.0 IU/ml ▤, and 5.0 IU/ml ⊠ of purified hCG compared to control cells (non-irradiated, non-hCG-treated MCF-7 cells). Data are presented from post-irradiation day 7 for MCF-7 cells exposed to hCG for only 24 h (hCG removed) and for cells maintained in hCG for a total of 8 days (hCG retained). Error bars represent the mean of the variance of six experiments.

irradiation were analyzed using the linear-quadratic (LQ) equation in which two parameters of effect can be described. In the equation, fractional survival ($F \times S$) is related to dose (D, Gy) by the expression $F \times S = \exp\ -(\alpha D + \beta D^2)$ where α (Gy^{-1}) and β (Gy^{-2}) represent inactivation constants [21, 22]. Operationally, the fractional survival data were fitted using a transform of the above equation (i.e., $-F \times S/D = \alpha + \beta D$) [23]. The α and β values were calculated from a linear regression analysis of the transformed data using standard statistical procedures [24]. The LQ parameters derived from the 7-day MTT were then used to obtain estimates of the surviving fractions of MCF-7 cells at a dose of 2 Gy, a typical dose used in conventional radiation therapy.

Results

The effect of hCG on cell survival following the exposure of MCF-7 cells to 3 Gy, 4 Gy, and 5 Gy doses of radiation is shown in Figure 1. The hCG was removed from MCF-7 cells just prior to irradiation. Therefore, the total exposure time of cells to hCG was only 24 h. On the day following irradiation, cell survival remained unchanged. However, on the 4th and 7th days after irradiation, differences were noted in survival based on both hCG concentration and radiation dose. As expected, an increase in the radiation dose corresponded to a decrease in cell survival independent of an exposure to hCG. However, for each dose of radiation tested, hCG-treated cells exhibited decreased survival when compared to control cells (no hCG). The hCG-related effect on cell survival was concentration-dependent. The greatest effect was observed 7 days after irradiation with MCF-7 cells exposed to 4 Gy of radiation and 2 IU/ml of hCG. In this case, the difference in cell survival between the hCG-treated cells and the control cells (no hCG) was 10.1% (39.8% v.s. 49.9% survival, p-value < 0.001). The use of a higher concentration of hCG (5 IU/ml) did not enhance the effect further. Cell survival with 5 IU/ml of hCG was not statistically different than cell survival with 2 IU/ml of hCG at radiation doses of 3 Gy, 4 Gy, or 5 Gy. However, the lowest concentration of hCG tested (0.2 IU/ml) was statistically less effective (p-value > 0.05) than 2 IU/ml at enhancing radiosensitivity.

The effect of hCG on survival of MCF-7 cells when hCG is present in the culture media both before and after irradiation is shown in Figure 2. On the day following irradiation, there was no apparent decrease in cell viability. However, by the 4th and 7th days after irradiation, an effect was clearly evident. Again, as expected an increase in the dose of radiation resulted in a decrease in cell survival independent of exposure to hCG. However, the hCG-treated MCF-7 cells exhibited a further decrease in survival when compared to control cells (no hCG). The hCG effect was less concentration-dependent than the effect seen in Figure 1 where the exposure time to hCG was only 24 h. The greatest effect was observed 7 days after exposure of MCF-7 cells to 4 Gy of radiation in the presence of 2 IU/ml of hCG. Under these conditions, the difference in survival between the hCG-treated cells and the control cells (no hCG) was 10.6% (40.1% v.s. 50.7%, p-value < 0.001). Again, cell survival with a higher concentration of hCG (5 IU/ml) was not statistically different than the survival with 2 IU/ml. However, with the continuous exposure of MCF-7 cells to hCG, the survival at the lowest concentration (0.2 UI/ml) was similar to the cell survival at the higher hCG concentrations on the 7th day following irradiation with 3 Gy, 4 Gy, and 5 Gy.

Survival at 2 Gy was determined using the linear-quadratic equation described in the Materials and methods. The α and β inactivation constants obtained from our analysis of the MTT data at day 7 are listed in Table 1. We calculated that hCG causes an 8–10% reduction in cell survival at 2 Gy.

It is important to note that the decrease in MCF-7 cell viability that occurred with hCG and radiation together was not detected when MCF-7 cells were exposed to the same concentrations of hCG in the ab-

Table 1. Linear-quadratic inactivation constants and survival at a dose of 2 Gy for irradiation of MCF-7 breast cancer cells

hCG (IU/ml)	$\alpha(Gy^{-1})$	$\beta(Gy^{-2})$	Survival at 2 Gy
hCG before irradiation			
0.0	0.155	0.005	0.72
0.2	0.182	0.002	0.69
1.0	0.180	0.008	0.68
2.0	0.216	0.002	0.64
5.0	0.208	0.003	0.65
hCG before and after irradiation			
0.0	0.116	0.012	0.76
0.2	0.181	0.005	0.68
1.0	0.190	0.002	0.68
2.0	0.192	0.006	0.66
5.0	0.173	0.001	0.68

sence of radiation (Figure 3). The data were derived from non-irradiated control cells tested by MTT assay on day 7. The viability of non- irradiated MCF-7 cells exposed to hCG (0.2–5 IU/ml) was statistically equal to the viability of non-irradiated cells grown without hCG (p-value > 0.05), except in one case. The exception occurred with 0.2 IU/ml of hCG when hCG was maintained in the culture media (p-value $= 0.042$). Note that the total exposure time of MCF-7 cells to hCG was 24 h when hCG was removed from the cell cultures and 8 days when the hCG was retained in the cell culture media throughout the experiment.

Finally, MCF-7 cells exposed to recombinant hCG before and after irradiation showed an enhancement in radiosensitivity similar to that obtained with purified hCG (data will be presented in a subsequent report).

Discussion

Of all the tissues that have been investigated, breast tissue has provided the most dramatic evidence of hCG-modulation of carcinogenesis. Several studies have demonstrated that endogenous hCG released during pregnancy causes breast glandular epithelium to differentiate, which in-turn results in an inhibition of cell proliferation, an increase in DNA repair capabilities of the mammary epithelium, and a decrease in the binding of carcinogen to mammary cell DNA [12, 14]. Other studies have shown that purified hCG can interfere with mammary tumorigenesis either by activating genes involved in apoptosis or by up-regulating the synthesis of inhibin [12, 13, 15, 16]. Inhibin, a member of the transforming growth factor (TGF)-β group of morphogenesis- and differentiation-related proteins, is known to have tumor suppressive activity.

Many of the *in vitro* studies involving hCG and breast tissue have utilized the MCF-7 breast cancer cell line. MCF-7 cells express a relatively high level of hCG receptor m-RNA and protein and are inhibited by hCG [11, 17]. hCG can regulate gene expression and stimulate the production of inhibin in MCF-7 cells [13, 18]. Based on the extensive use of MCF-7 cells, we decided to use this cell line to study enhancement of radiosensitivity by hCG.

Our results show that hCG does enhance the radiosensitivity of MCF-7 cells. The maximum response of MCF-7 cells to hCG was virtually identical for cells exposed to hCG for only 24 h prior to irradiation and cells exposed to hCG both before and after irradiation. Therefore, it appears that the hCG-induced cellular events responsible for the increase in radiosensitivity occur relatively rapidly and that continuous exposure of cells to hCG is not required to sustain the effect. However, according to our data, prolonged exposure of MCF-7 cells to hCG tends to increase the effectiveness of lower concentrations of hCG. The use of 2 IU/ml of hCG together with 4 Gy of radiation consistently yielded the greatest response. The use of a higher concentration of hCG did not provide any additional benefit.

The primary result of the linear quadratic (LQ) analysis of the hCG pre-treatment (24 h) and hCG pre- and post-treated MCF-7 cultures was that all concentrations of hCG (0.2–5.0 IU/ml) reduced cell survival as compared to the sham-irradiated controls (Table 1). This reduction in survival was by approximately 8% (pre-treatment) and 10% (pre- and post-treatment) using the surviving fractions at a dose of 2 Gy as determined from the LQ parameters as a measure of effect. The main effect appears to be exerted by an increased α inactivation coefficient in the hCG-treated cultures. Such an effect would be useful for radiation therapy as it suggests that the hCG effects would be most pronounced at lower radiation doses.

While we note that these are relatively small changes in cellular response that we have documented, the decrease in survival is consistently found for all eight of the hCG experimental treatments as compared to their respective control treatments. In this regard, we therefore suggest that the effects of hCG in cell culture on intrinsic radiation sensitization are real, albeit small. At the moment, we are not sure if the hCG treatment can affect repair of radiation lesions. Although the pre- and post-treatment data show a 10% inhibition in cellular growth as compared to the 8% seen for pretreatment alone suggests this may be true, the differences are small and require further data on other cell lines. Other investigators [25] have shown that MCF- 7 cells are relatively sensitive to x-irradiation with a high intrinsic α inactivation coefficient and, therefore, a relatively low repair capacity after irradiation. Therefore, in retrospect it might have been better to use a cell line with a smaller α coefficient for these studies.

In our study, hCG alone did not interfere with the growth of MCF-7 cells. This finding appears to contradict previous reports of direct hCG-induced inhibition of MCF-7 cells [11, 13]. A careful examination of the data from the previous studies provides two possible explanations. Alvarado et al. [13] reported that

hCG-induced inhibition of MCF-7 cells decreases in a dose-dependent manner at hCG concentrations below 10 IU/ml. Therefore, the concentrations of purified hCG used in our experiments (0.2 to 5 IU/ml) may be too low to inhibit cell growth. In another study, Lojun et al. [11] reported that 0.15. IU/ml of hCG inhibited MCF-7 cell growth gradually over a period of 6 days. However, the inhibition only occurred when cells were grown in MEM with minimal (0.05%) fetal bovine serum (FBS). The hCG did not inhibit MCF-7 cells when the cells were grown in MEM with 10% FBS, the same concentration of FBS used in our cell culture medium. The authors state that the minimal amount of FBS was necessary for the cells to survive. Unfortunately, there is no evidence presented that their cells were actually proliferating. We utilized conditions appropriate for cell growth not just cell survival. It is important to note that 100 IU/ml of hCG has been the standard dose used to study inhibition of cell growth and induction of apoptosis in breast cells both *in vivo* and *in vitro* [6, 16]. Furthermore, the *in vitro* studies have utilized cell culture media containing 10% FBS.

In the present study, we did not attempt to characterize the mechanism of action responsible for the hCG-induced enhancement of radiosensitivity in MCF-7 cells. However, our data strongly suggest that hCG may have value as an adjunctive therapy when combined with traditional anti-cancer therapies, particularly as there are no apparent side effects at the concentrations used in this study and others [26].

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