

Modulating Activity of Human Chorionic Gonadotropin on Growth and Tumorigenesis of Prostate Cancer Cells

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ABSTRACT

The hypothesis that human chorionic gonadotropin (hCG) is a nonandrogenic hormone regulating the proliferation and development of prostate cancer cells was investigated. The supplementation of human androgen-dependent LNCaP cancer cells with hCG caused growth reduction, apoptosis, and cell death. The hormone showed an identical dose- and time-dependent effect on cells grown in medium with or without steroids, suggesting an hCG mechanism independent of androgen. In androgen-independent prostatic cancer cell lines, hCG showed primarily a growth-modulating effect. It mediated a blocking of G₁ cells from entering into S phase. The analyses of hCG's effect on clonogenesis with androgen-independent JCA-1 cells revealed that hCG caused a drastic reduction in the number of tumor cell colonies and tumorigenesis. The demonstration of hCG receptors in the prostate complements these findings, indicating a direct functional role of hCG in this organ.

INTRODUCTION

HUMAN CHORIONIC GONADOTROPIN (hCG) is an oncofetal tumor marker that has been found at unusually high concentrations in the sera of greater than 30% of patients having testicular interstitial-cell tumors, choriocarcinoma, pancreatic carcinoma, and embryonal carcinoma. Elevated serum hCG also appears, but less frequently, in patients with ovarian adenocarcinoma, teratoma, and carcinoma of the breast, urinary bladder, stomach, colon, and lungs, as well as adenoma and multiple myeloma.¹

Acevedo et al^{2,3} identified an hCG-like antigen in bacteria isolated from the urine of cancer patients but not in the same bacteria obtained from healthy subjects. Later, those investigators showed that cultured human cancer cells express hCG on the cell membrane and suggested that there exists a direct correlation between the concentration of hCG or hCG-like molecules and the invasiveness of the cells.⁴

McMichael^{5,6} demonstrated that a relatively low concentration of hCG can be used to treat a variety of malignancies in

man and animals successfully and that chorionic gonadotropin from other species could be used with equal efficacy. Likewise, Lunardi-Iskanar et al⁷ showed that hCG inhibits metastasis and tumorigenesis of Kaposi's sarcoma cells in mice. Gill and associates⁸ recently reported the successful treatment of patients with Kaposi's sarcoma by intralesional administration of hCG.

We have investigated the effect of hCG on the growth and development of human prostate cancer cells. Prostatic carcinoma is the most commonly diagnosed cancer among men in the United States. The growth of some prostatic cells is hormonally dependent, while in others, it is not. Androgen is known as the principal regulating factor for those hormone-sensitive cells. Reported herein are data derived from exposing several androgen-independent or -dependent cell lines to hCG *in vitro*. The results suggest that hCG has a dose-dependent regulatory effect on the proliferation and development of prostatic cancer cells. Recently, the expression of hCG receptor gene transcript and the receptor protein were demonstrated in rat and human prostates.^{9,10} The hCG receptors detected in the human gland were present with normal, hyperplastic, and

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carcinoma tissues and also some cell lines.⁹ The treatment of rat prostate with hCG caused an increase in cAMP formation, suggesting that the receptors are functional in mediating the hCG action.¹¹

MATERIALS AND METHODS

Growth Assays

Human prostatic cancer cell cultures were exposed to hCG at various concentrations at initiation. The hCG was prepared in saline (Schein Pharmaceutical, Baxter, NJ), and cultures supplemented with saline in place of hCG were used as controls. The assays were performed in triplicate. The androgen-depen-

dent prostatic cancer line LNCaP was seeded at 2×10^5 cells/mL of RPMI-1640 medium with either 5% charcoal-stripped (CS) fetal bovine serum (FBS) (HyClone) or heat-inactivated regular FBS (GIBCO). The prostate cancer cell lines PC-3 and JCA-1¹² were seeded at 1×10^5 cells/mL of RPMI-1640 medium and 5% heat-inactivated regular FBS. For cell analyses, attached cells were removed by trypsinization, with cells floating in the medium also being included. Cell counts and viability were determined by the trypan blue method. Cell-cycle phases and apoptotic cell determination were performed with a Coulter Profile II cytometer (Coulter, Miami, FL) according to described procedures.¹⁵⁻¹⁷ Cells were fixed with 80% ethanol at 4°C and incubated on ice before they were stained with propidium iodide at 50 $\mu\text{g/mL}$ and analyzed within 24 h. Peripheral blood lymphocytes stained identically were used as an internal reference standard for DNA content.

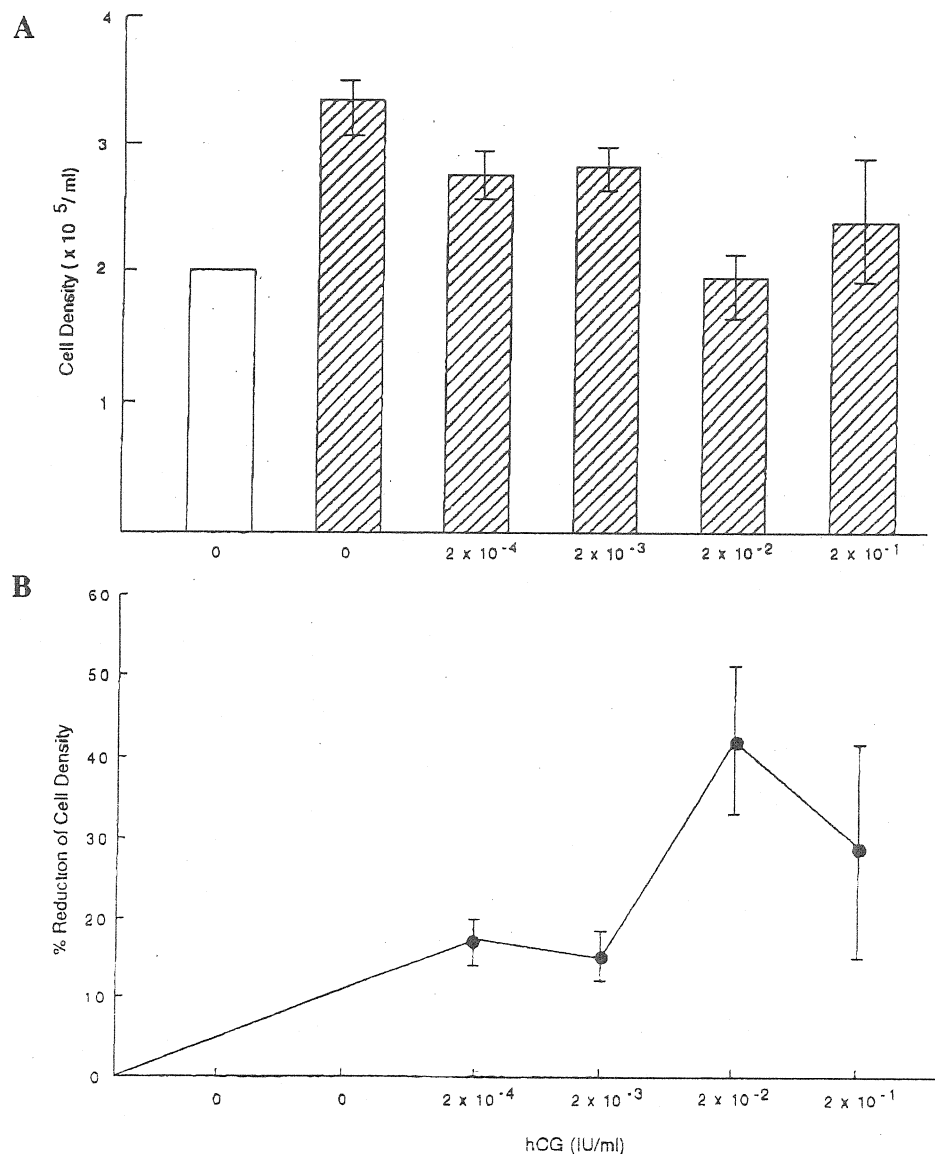


FIG. 1. Androgen-dependent LNCaP cells cultured in RPMI-1640 medium and 5% steroid-depleted FBS were supplemented with hCG at various concentrations. (A) Initial cell density (open bar) and cell density after 1 day of incubation (hatched bars). (B) Percent reduction of cell density in hCG-treated cultures. Vertical bars indicate ranges of four separate experiments.

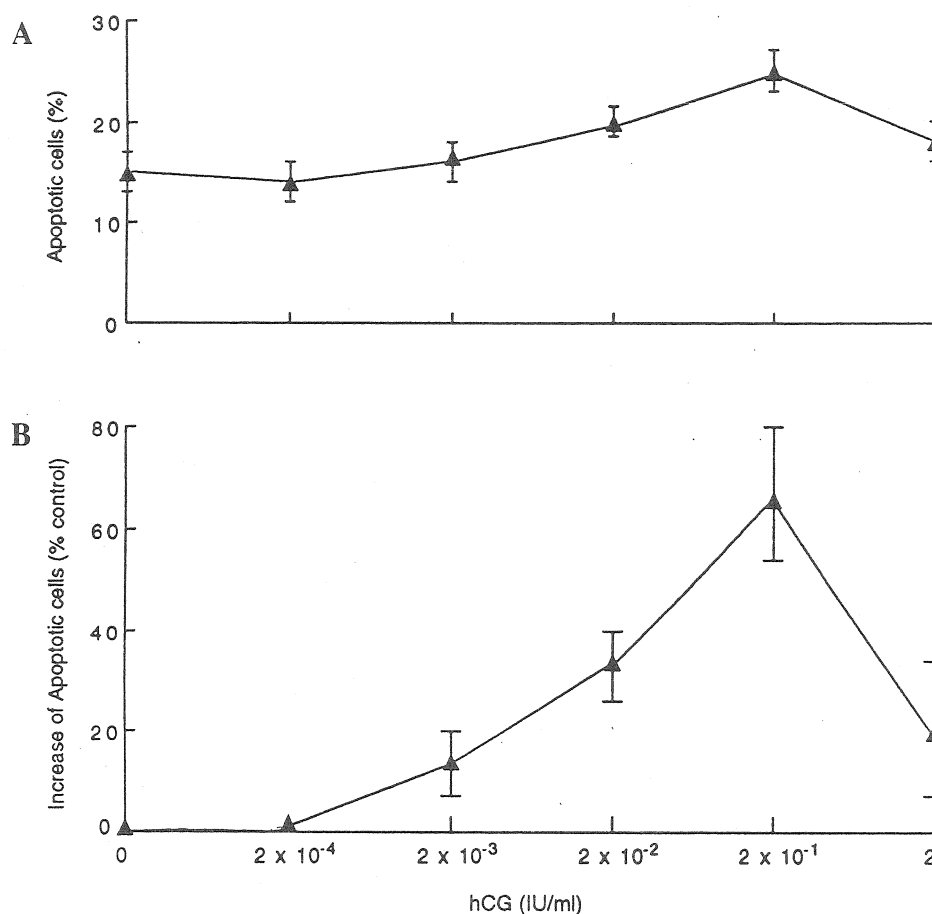


FIG. 2. Androgen-dependent LNCaP cells were grown in steroid-depleted FBS medium without or supplemented with hCG at various concentrations. (A) Increase of apoptotic cell proportions in cultures with hCG supplementation. Each point represents mean of two separate experiments with triplicate cultures for each condition. (B) Percent increase of apoptotic cells in hCG-treated cultures compared with saline-supplemented control cultures.

Clonogenic Assay

The influence of hCG on androgen-independent prostatic cancer cells was analyzed with an *in vitro* clonogenic assay.¹² Cells of the JCA-1 line were plated in the upper agar layer of a double-layer soft-agar culture at 5×10^2 cells/plate with RPMI-1640 medium and 10% heat-inactivated regular FBS. Cells in some of the upper agar layers were exposed to hCG at various concentrations or to saline (control). Colonies (>50 cells) were counted after a 12- to 14-day incubation period at 37°C. The number of colonies represents the mean of triplicate cultures.

DNA Gel Electrophoresis and Immunostaining

The DNA was extracted from LNCaP cells treated with or without hCG for 48 h, as well as from LNCaP or human leukemic HL-60 cells incubated with 0.3 μ M camptothecin as described elsewhere.¹³ The genomic DNA was extracted by the phenol-chloroform method using RNase A and proteinase K. The DNA was then precipitated by isopropanol and dissolved in TE buffer.¹³ Electrophoresis was performed on a 1% agarose gel at 20 mA for 5 h. The DNA fragments from a phage λ HindIII digest were used as molecular weight markers.

Intracellular bcl-2 antigen was detected by an indirect im-

munofluorescent antibody technique using a mouse IgG anti-human bcl-2 antibody (type 124; Dako, Carpinterie, CA). The cells were fixed first with 1% formaldehyde on ice and then 70% ethanol overnight. Antibody staining was performed in the presence of 1% BSA, and a fluorescein-labeled goat IgG-(Fab')₂ antibody was used as the second antibody. Cells stained with a nonspecific mouse IgG as the first antibody were used as a background. The proportion of the positively stained cells and the mean fluorescence intensity were analyzed with the intensity channels on a Coulter Profile flow cytometer.

RESULTS

Androgen-dependent LNCaP cells cultured with FBS depleted of free steroids were exposed to hCG at various concentrations. Compared with control cultures, hCG showed a modulating effect on cell density, which could be observed after 1 to 2 days of incubation. Figure 1A shows that a decrease of cell density and growth rate was observed, with the magnitude being dependent on the hCG concentration. Approximately 12% to 25% reduction of cell density was observed with hCG at 2×10^{-4} IU/mL and that increased to approximately 27%

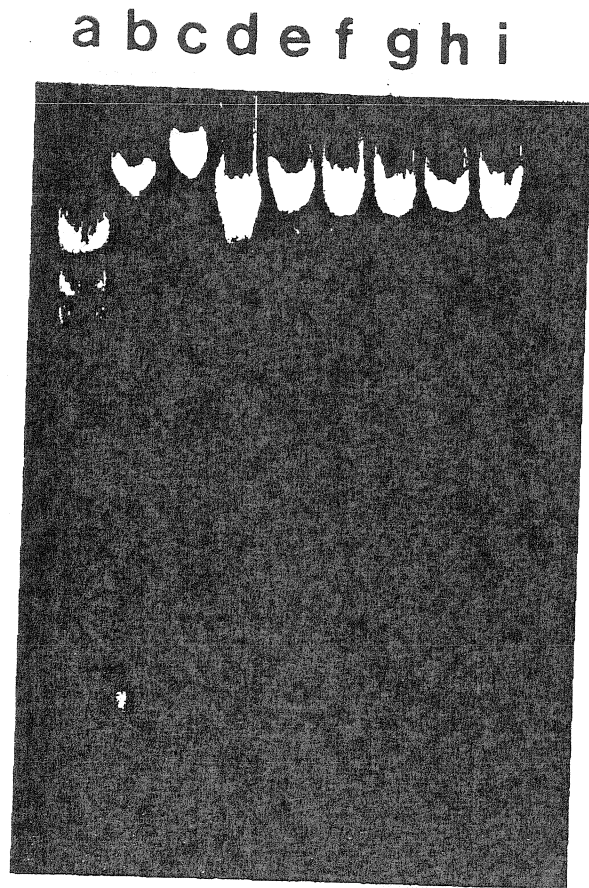


FIG. 3. Genomic DNA from hCG-treated LNCaP cells. Lane a, molecular weight marker; Lane b, genomic DNA extracted from camptothecin-treated HL-60 cells; Lane c, control with genomic DNA from untreated LNCaP cells; Lane d, LNCaP cells treated by camptothecin. Lanes e-i are LNCaP cells treated with hCG at 2, 2×10^{-1} , 2×10^{-2} , 2×10^{-3} , or 2×10^{-4} IU/mL, respectively.

to 54% with 2×10^{-2} IU/mL (Fig. 1B). The cell density in some cultures treated for 1 day with hCG at a concentration such as 2×10^{-2} IU/mL was lower than the initial seeding density. The viability of these cultures was also reduced, and dead cells were detectable.

Figure 2A depicts an increase in the apoptotic cell populations in the cultures that had lower cell density and viability. A significant increase of the apoptotic cell population could be observed in cultures treated with hCG at a concentration as low as 2×10^{-3} IU/mL (Fig. 2B). The results suggest that hCG can mediate apoptotic death of LNCaP cells. Electrophoresis was performed to determine the characteristics of the degraded chromosomal DNA in LNCaP cells. Figure 3 reveals that degraded DNA, appearing primarily as large fragments, was present in LNCaP cells treated with various hCG concentrations, but not in the untreated cells. Control LNCaP cells treated with the known apoptotic agent camptothecin showed a pattern similar to that in the hCG-treated LNCaP cells (Fig. 3). This DNA fragmentation pattern is similar to that described for other apoptotic human prostate cancer cells.¹⁴ These results showed that generation of larger DNA fragments rather than the internucleosomal DNA cleavage represents the essential apoptotic signal.

The expression of *bcl-2* antigen in LNCaP cells with or without exposure to hCG was determined by indirect fluorescent antibody technique. The mean fluorescence intensity representing *bcl-2* in LNCaP cells showed a decrease after exposure to hCG for 1 to 2 days. The reduction was observed at all hCG dosages used, from 2×10^{-5} to 2×10^{-1} IU/mL. Compared with untreated cells, an average of 20.9% (range 6.1%–50.2%) reduction was observed in the treated cells. These results indicate that diminished *bcl-2* oncogene expression, which overrides programmed cell death mechanisms, may be associated with an increase in apoptotic cells.

Figure 4 depicts the finding that hCG had a similar effect on LNCaP cells grown in medium supplemented with either heat-inactivated or steroid-depleted FBS. The magnitude of reduction in cell density and viability was similar in these two types

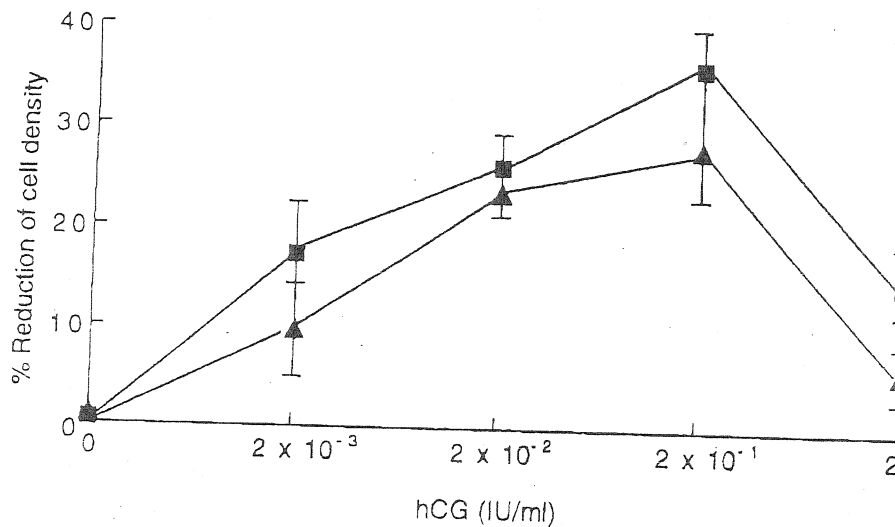


FIG. 4. Percent reduction of density compared with controls of androgen-dependent LNCaP cells cultured in RPMI-1640 medium with either 5% cs FBS (■—■) or 5% heat-inactivated regular FBS (△—△). Some cultures were supplemented with various concentrations of hCG, whereas control cultures received saline. Values are means of three separate experiments. The hCG had similar effects on cells grown with either regular or steroid-depleted FBS.

of cultures. These results suggest that hCG interacts directly with cells, independent of the serum steroid effect.

Human chorionic gonadotropin was also introduced to androgen-independent prostatic cancer cells PC-3 and JCA-1, and its effect was analyzed. Depending on the dose, the hCG-treated cultures displayed a pattern of alternating reduction or increase in cell density. A significant cell density decrease was observed in the hCG ranges of 2×10^{-2} to 30 IU/mL. The magnitude of the decrease was greater after a 3-day exposure to hCG than

after 1 or 2 days (Fig. 5A). Maximum growth modulation was seen after the cells had been treated with hCG 20 IU/mL: approximately a 14% to 35% reduction was observed.

Figure 5B depicts the reduction of the replicating cell populations in these cultures as quantified from their cell-cycle phase distribution. Approximately 30.2% S and G₂M phases of the untreated JCA-1 cells decreased to approximately 22.8% after treatment with hCG 20 IU/mL, representing approximately a 24.5% reduction of the replicating cell population. This de-

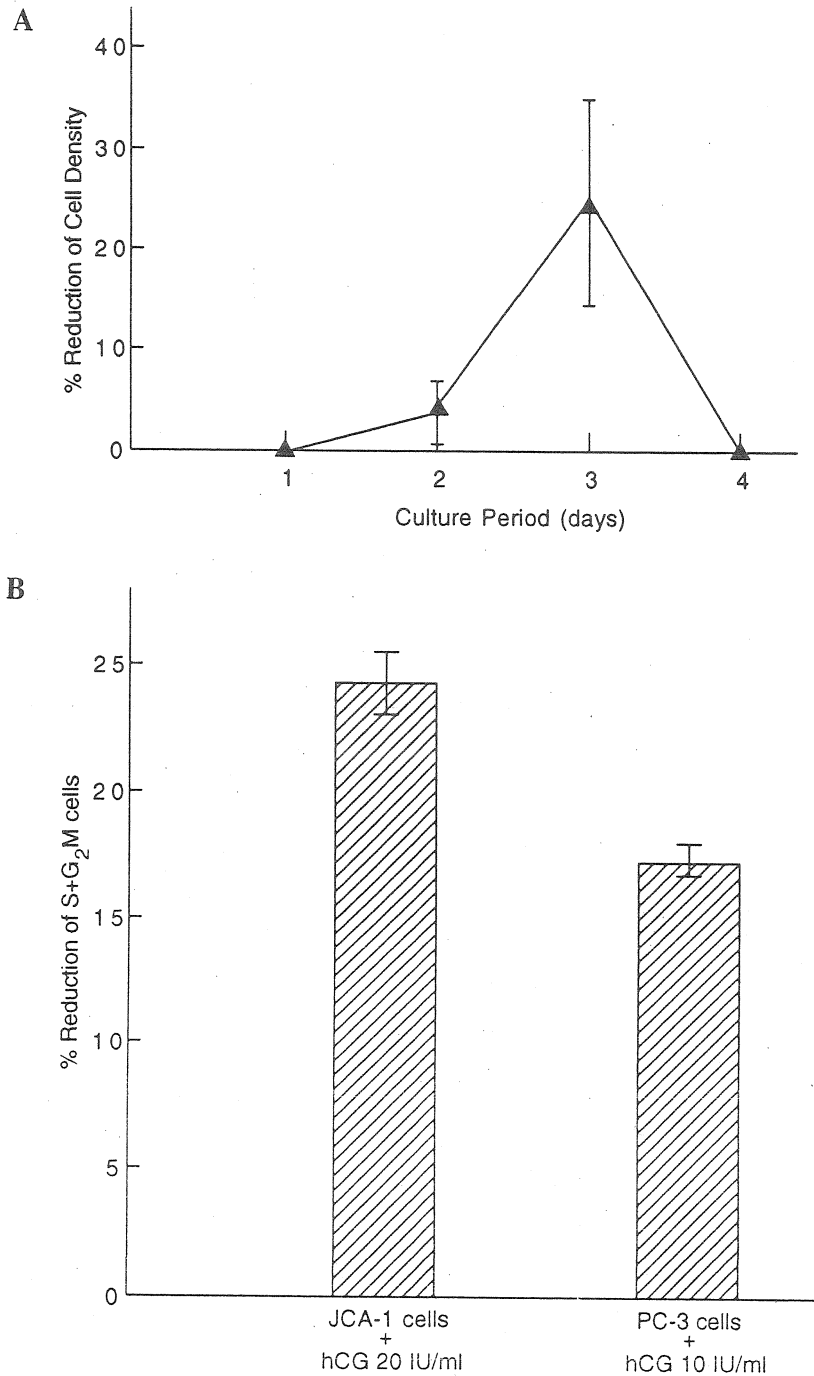


FIG. 5. Effect of hCG on androgen-independent prostate cells. (A) Percent reduction of density of JCA-1 cells supplemented with hCG 20 IU/mL compared with control cells receiving saline. (B) Percent reduction of replicating population of JCA-1 cells supplemented with hCG 20 IU/mL for 3 days and PC-3 cells given 10 IU/mL for 4 days.

TABLE 1. hCG MEDIATES INHIBITION OF TUMORIGENESIS OF ANDROGEN-INDEPENDENT PROSTATIC CANCER JCA-1 CELLS IN CLONOGENIC *IN VITRO* ASSAY

	hCG Concentration (IU/mL)						
	0	2×10^{-1}	2×10^{-2}	2×10^{-3}	2×10^{-4}	2×10^{-5}	2×10^{-6}
No. of colonies							
Mean	25 ± 5	1	1	1	1	1	4
Range	22-28	—	—	—	—	—	3-5

crease of replicating cells was accompanied by an increase in phase G₁ cells. In addition, the growth rate of treated cultures was lower than that of control cultures.

A similar growth-modulating effect of hCG was seen with PC-3 cells. Figure 5B shows that approximately a 17.9% reduction of the replicating cells was present after treatment with hCG at 10 IU/mL for 4 days. These results indicate that the mechanism of action for the growth modulation involves a blocking of G₁ cells from entering the replicating phases of the cell cycle. The effect of hCG on cell growth lasted for 3 to 4 days after initial exposure. Cell proliferation later recovered to that of untreated control cells, indicating that the hCG effect was reversible. Androgen-independent cells treated with hCG showed only minor changes in viability and apoptotic proportion, suggesting that hCG did not cause significant apoptosis compared with that found in LNCaP cells.

The effect of hCG on clonal growth was analyzed with androgen-independent JCA-1 cells. A dose-dependent effect of hCG in reducing the number of colonies was observed. The number of regular colonies was significantly reduced at the hCG dosages that caused growth reduction. Small clusters or single cells remained viable in these cultures. Table 1 shows that compared with control cells, which produced approximately 25 colonies/ 5×10^{-2} cells, JCA-1 cells incubated with hCG from 2×10^{-3} to 20 IU/mL had approximately 1 colony per 5×10^{-2} cells, a reduction of approximately 95%.

DISCUSSION

This research demonstrated for the first time that hCG at certain concentration ranges induces apoptosis and modulates the growth and tumorigenesis of prostate cancer cells. The demonstration of hCG receptors on normal and pathological prostate epithelial cells^{9,10} has supported the observation that hCG can directly affect the development of prostate cancer cells.

Human chorionic gonadotropin exerts a dose-related effect of growth reduction and apoptosis on the androgen-dependent LNCaP cells. An increase of the apoptotic cell population and decrease of *bcl-2* oncogene expression could be observed within 24 to 48 hours of hCG exposure. The apoptotic effect was the same in culture media with and without steroids, suggesting a direct hCG effect on the cells independent of androgen mediation. In contrast, hCG exerted primarily a growth-modulating activity on androgen-independent prostatic cancer cells, as confirmed by cell counting, cell-cycle phase distribution, and clonal

growth. The slower growing cancer cells had a decreasing percentage of cells in S phase coupled with an increase in the percentage of cells in G₁. The characteristics of the cell-cycle phase distribution indicated that the blocking of the entry of G₁ cells into the S and G₂M phases may be one of the mechanisms by which hCG contributes to slower growth. The different hCG response in apoptosis among cell lines may reflect variations in cell types. The androgen-dependent LNCaP cells was reported to express higher levels of hCG receptor mRNA transcripts and protein than the androgen-independent cell lines,⁹ which may be an underlying cause for the different quantitative response. The different hCG responses may also be related to the production of hCG-induced specific cytokines, which in turn influence cellular development. These cell lines have been found to release numerous cytokines, including transforming growth factors.¹⁸

Our finding of an hCG effect on proliferation and apoptosis supports the interpretation that the hCG receptors in the prostate are functional.^{9,11} Chorionic gonadotropin and luteinizing hormone (LH) bind to a common membrane receptor. This fact has raised the question of whether LH is similar to hCG in that it has a dose-related effect in mediating prostatic cell proliferation and function. The activity of LH in this regard has not been clearly described and is currently under investigation. Nevertheless, the presence of functional receptors for hCG/LH in the prostate strongly indicates that these molecules are nonandrogenic hormones regulating the development of the prostate gland.

Compared with the growth modulation in liquid cultures, the magnitude of the hCG effect on the anchorage-independent clonal growth was significantly greater. Consistent with this observation was a similar experiment in which hCG overwhelmingly inhibited the clonogenic growth of a human Kaposi's sarcoma cell line *in vitro*.⁷ Those authors also described the killing of Kaposi's tumor cells in nude mice by hCG and suggested that tumorigenesis was blocked. Whether hCG possesses anti-tumor activity other than interference with cell proliferation remains to be investigated.

The scope of hCG activity and its mechanism in the prostate have not been fully elucidated. Questions regarding how to control the hCG effect and whether hCG requires certain cofactors for its function need to be addressed. It is interesting that hCG has now been documented to modulate the growth of two different types of cancer cells; i.e., Kaposi's sarcoma⁸ and prostate cancer. One could speculate that hCG must affect a common characteristics of these two different cancers. Understanding these hCG mechanisms becomes important, as they could be applied to cancer control.

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