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Hormonal Control of Breast Cancer Cell Growth

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INTRODUCTION

Breast cancer is the neoplasm most frequently diagnosed in American women. It is also responsible of 17 percent of female cancer deaths, and the number one cause of cancer-related death in non-smoking women (1,2). These dismal statistics are aggravated by the facts that the incidence of this disease is steadily rising in most western societies and in oriental countries traditionally known to have a low incidence of breast cancer (3-5), and by the lack of a clear understanding of the cause of this worldwide increase. Breast cancer has long been recognized to be a hormone dependent malignancy (6,7). Combined clinical and experimental studies have determined that both hormone deprivation and hormone supplementation have a preventive and therapeutic value (7). Other important endocrinological influences have been found to modify the risk of developing breast cancer. Increased risk is associated with early menarche, late menopause, nulliparity or late first full term pregnancy, while opposite conditions, such as late menarche, early pregnancy and early menopause exert a protective effect (8-11). Although the cause and the time of initiation of the carcinogenic process are not known, epidemiological, clinical, and experimental data have identified the period between menarche and the first full term pregnancy as a "window" of critical importance in the lifetime risk of developing breast cancer. The understanding of the complex interactions involved in the initiation and progression of breast cancer requires the design of studies in which all possible variables are carefully controlled. *In vivo* and *in vitro* experimental models provide the ideal conditions for exploring the mechanisms through which these interactions influence the risk of mammary cancer development (12-16).

The widely utilized animal model of induction of rat mammary tumors by administration of the chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) to Sprague-Dawley rats has allowed the PI to determine that the differentiation of the mammary gland is the single most important factor in determining the susceptibility or resistance of the mammary gland to develop malignancies. Full differentiation of the mammary gland occurs after a full term pregnancy, although treatment of young virgin rats with the placental hormone human chorionic gonadotropin (hCG) exerts an effect on the mammary gland's lobular development, inhibition of cell proliferation, increase in the DNA repair capabilities of the mammary epithelium and activation of genes controlling programmed cell death similar to that induced by pregnancy. Both processes inhibit the initiation of chemically induced mammary carcinogenesis (17-26). HCG also inhibits the progression of rat mammary carcinomas *in vivo*, and *in vitro* it inhibits the proliferation of human breast epithelial cells (27-29).

An understanding of the mechanisms through which hCG inhibits the proliferation of both immortalized and neoplastic human breast epithelial cells would provide researchers indispensable clues on the role of this important pregnancy hormone in the prevention of breast cancer. The observed direct *in vitro* effect led us to postulate that hCG acts via a receptor for either inducing the activation of genes controlling programmed cell death (PCD), or arresting the cells in specific phases of the cell cycle (30). The maintenance of the homeostatic balance between cell proliferation,

differentiation, and cell death in human cells requires intrinsic regulatory mechanisms that counterbalances growth stimulatory effects. It is known that checkpoint controls are modulated by the synthesis and degradation of cyclins and phosphorylation by cyclin-dependent kinases (cdks) (31-34). Negative regulatory forces are provided by tumor suppressor proteins such as the p53 and the retinoblastoma (pRb) proteins (35) and by cyclin-dependent kinase inhibitors, such as roscovitine, that inhibits the kinase activity of cdc2/cyclin B, cdk2/cyclin A, and cdk2/cyclin E complexes (31, 36-38). Since it has been reported that roscovitine prevents the cell cycle progression of mammalian cells at the G1/S and the G2/M checkpoints and blocks the transactivation of cyclin A by myc (38-41), we felt it necessary to compare the effects of this purine-derived cdk inhibitor with that of hCG on the proliferative activity of malignant and non-malignant human breast epithelial cells. This information would be correlated with our studies on the effect of hCG treatment on cell cycle progression, determined by flow cytometry, and on the expression of genes that modulate PCD.

Subject

The fact that hCG has a direct inhibitory effect on the proliferation of human breast epithelial cells *in vitro* (27-29) led us to postulate that one of the mechanisms responsible of this inhibition is the activation of genes controlling programmed cell death (PCD), leading to apoptosis, as it has been shown in other systems (24-26,42,43). PCD is a physiological form of active cell death that has been associated with specific phases of development that control cell proliferation and differentiation. It is regulated through a phylogenetically conserved mechanism, such as the ced-3, ced-4 and ced-9 genes in *C. elegans* (44-51). The gene product of ced-3 has homology to a cysteine protease, the mammalian interleukin-1- β converting enzyme (ICE) and several ICE-related genes such as CPP32/Yama (52-61). Progression of apoptosis in mammalian cells has also been associated with increased expression of testosterone repressed prostate message-2 (TRPM2) gene (62-64). Enhanced expression of TRPM2 and of transforming growth factor (TGF)- β has been demonstrated in human breast cancer cells following estrogen ablation (65,66). The product of the proto-oncogene bcl2, on the other hand, is known to play a role in promoting cell survival and inhibiting apoptosis (67-70), although bax, a bcl-2 gene product that homodimerizes and forms heterodimers with bcl-2; has been shown to accelerate PCD (71,72). It has been suggested that the ratio of bcl2 to bax determines survival or death following an apoptotic stimulus (73). C-myc has been demonstrated to play a crucial role in the induction of apoptosis in several cell types, either through p53 dependent or p53-independent pathways (74,75). An important downstream target for p53 is the WAF1/CIP1 gene, which is transcriptionally regulated by wild type p53 (76). P21, the gene product of WAF1/CIP1, acts as a potent inhibitor of cyclin dependent kinases (77,78) and thus may mediate the p53 induced cell cycle arrest and apoptosis following DNA damage (79). Since the orderly progression of cells through the various phases of the cell cycle is governed by sequential activation and deactivation of specific complexes of cyclins and cdks, while uncontrolled cell growth, the hallmark of neoplastic cells, is due in part to deregulation of cell cycle checkpoint functions, we tested the effect of roscovitine, an olomoucine-related purine that is a potent and selective inhibitor of cdc2 and cdk2 (39,40) for comparison with the effects of hCG on the *in vitro* growth of

human breast epithelial cells. The transit of treated cell through the various phases of the cell cycle is under study utilizing flow cytometry, that provides a rapid and accurate way of determining the phase of the cell cycle through determination of the relative DNA content of cell populations.

Purpose

Our work has been designed with the purpose of determining whether the inhibition of cell proliferation induced by hCG in human breast epithelial cells is mediated by modulating the expression of specific genes that control PCD or by blocking the cells in a specific phase of the cell cycle. If this is found to be the mechanism, then the level of expression of specific cyclins and cdks would allow us to determine the specific pathways through which hCG operates for inhibiting tumor cell growth. For these purposes, gene expression was studied in hCG-treated normal immortalized human breast epithelial cells (HBEC) MCF10F; the malignant breast cell line MCF-7, BP1-E cells, a benzo(a)pyrene (BP)-transformed cell line derived from MCF-10F cells, and the urothelial cancer cell line T24 (80-83). In order to clarify the mechanisms through which hCG inhibits the proliferation of HBEC we compared the *in vitro* effects of this hormone with those of roscovitine, an inhibitor of cdc2 and cdk2, and their influence on the cell cycle was determined by flow cytometry.

Scope of Research

Our studies, that were initiated with the purpose of clarifying why pregnancy in humans and in experimental animal models reduces the incidence of mammary cancer (2-12), led us to discover that a single placental hormone, human chorionic gonadotropin (hCG) administered to virgin rats inhibits both the initiation and progression of chemically induced mammary cancer (18-20,84,85). This protective effect was the result of the induction of mammary gland differentiation, manifested as profuse lobular formation with elimination of undifferentiated structures, mainly terminal end buds (TEBs), reduction of the proliferative activity of the mammary epithelium and increased synthesis of inhibin, a growth factor with tumor suppressor activity. HCG treatment inhibits tumor initiation when given before the carcinogen, and tumor progression when administered even 20 days after the carcinogen. This phenomenon is mediated by the induction of an increase in the expression of TRPM2, ICE, bcl-XS, c-myc and p53, and elevation in the apoptotic index in the non-tumoral mammary glands. HCG treated animals develop considerably fewer tumors than controls, and their mammary adenocarcinomas exhibit an increased expression of p53, c-myc and ICE genes in comparison with the levels detected in the adenocarcinomas developed by the animals treated with DMBA alone. These observations indicate that hCG induces programmed cell death in the mammary gland initiated in the carcinogenic process, that this process is p53 dependent, and is modulated by c-myc expression. Our data also indicate the possibility that a cell death program dependent of the bcl2 family exists, because of the potential involvement of p53, bcl-XS and Bax in apoptosis (24-26). The objective of this proposal is to validate observations made in the experimental animal model for their extrapolation to the human disease. This approach is supported by our observations that hCG inhibits the proliferation of human breast epithelial cells *in vitro*, data that were already discussed in the previous Annual Report. The inhibition

of *in vitro* cell growth of both the spontaneously immortalized human breast epithelial cell line MCF-10F and of the breast adenocarcinoma metastatic cell line MCF-7 indicate that this hormonal treatment is of great potential value for the treatment of already established breast cancers, in addition to representing a promissory model for the design of protocols of breast cancer prevention.

Background of Previous Work

Chorionic gonadotropin is a glycoprotein hormone secreted early in pregnancy by the developing embryo (86). Its main known function, which is virtually identical to that of the pituitary luteinizing hormone (LH), is the stimulation of the production of gonadal steroid hormones through its interaction with the lutropin-choriogonadotropin-receptor (LH-CG-R) (87). This receptor has been traditionally known to be present in the granulosa cells of the ovary in the female and in the testicular Leydig cells in the male. In the ovary, upon interaction with its receptor, hCG increases adenyl cyclase activity, an effect mediated by intracellular membrane associated G proteins; this, in turn, results in cAMP increases, leading to steroid and polypeptide hormone synthesis (86-88). Research performed in our laboratory has uncovered novel functions for this hormone, such as an inhibitory effect on the initiation and progression of chemically-induced rat mammary carcinogenesis (84,85), and the inhibition of the *in vitro* proliferative activity of human breast epithelial cells (HBEC) (27-29).

We have determined that under *in vivo* conditions hCG exerts a protective effect on the mammary gland from chemically induced carcinogenesis, mainly through the induction of differentiation, inhibition of cell proliferation, increase in the DNA repair capabilities of the mammary epithelium and activation of genes controlling programmed cell death (17-26). The fact that hCG has a direct inhibitory effect on the proliferation of human breast epithelial cells *in vitro* (27-29) led us to postulate that one of the mechanisms responsible of this inhibition is the activation of genes controlling programmed cell death (PCD), as it has been shown in other systems (24-26,42,43). Programmed cell death (PCD) is a physiological and phylogenetically conserved form of active cell death that has been associated with specific phases of development that control cell proliferation and differentiation (44-49). In *C. elegans* the *ced-3*, *ced-4* and *ced-9* genes regulate PCD; among these, the gene product of *ced-3* has homology to a cysteine protease, the mammalian interleukin-1- β converting enzyme (ICE) (50,51). Subsequent studies have revealed the presence of several ICE-related genes, such as CPP32/Yama, also called apopain (52-61). *In vitro* studies have suggested that a protease cascade involving the ICE protease family acts to transduce apoptotic signals, such as TX/ICH2/ICE rel-II (89,90), which can cleave the precursor of CPP32/Yama (55). The inhibition or mutation of these genes inhibits apoptosis induced by various stimuli, observations that confirm the importance of their role in driving apoptosis (52,59-61). Progression of apoptosis in mammalian cells has also been associated with increased expression of testosterone repressed prostate message-2 (TRPM2) gene (62-64). Enhanced expression of TRPM2 and of TGF- β has been demonstrated in human breast cancer cells following estrogen ablation (65,66). The product of a proto-oncogene, *bcl2*, promotes cell survival and inhibits apoptosis (67-70). The existence of

bcl-2 related gene products that accelerate programmed cell death has been reported (71,72). One of these gene products, termed bax, has been shown to homodimerize, as well as to form heterodimers with bcl-2; it has been suggested that the ratio of bcl2 to bax determines survival or death following an apoptotic stimulus (73). C-myc has been demonstrated to play a crucial role in the induction of apoptosis in several cell types, either through p53 dependent or p53-independent pathways (74,75). An important downstream target for p53 is the WAF1/CIP1 gene, which is transcriptionally regulated by wild type p53 (76). P21, the gene product of WAF1/CIP1, acts as a potent inhibitor of cyclin dependent kinases (77,78) and thus may mediate the p53 induced cell cycle arrest and apoptosis following DNA damage (79).

The orderly progression of cells through the various phases of the cell cycle is governed by sequential activation and deactivation of specific complexes of cyclins and cyclin-dependent kinases (cdks) which regulate a series of checkpoints that determine cell cycle progression (91-93). Uncontrolled cell growth, the hallmark of neoplastic cells is due in part, to deregulation of cell cycle checkpoint functions. Checkpoint controls are modulated by the synthesis and degradation of cyclins and phosphorylation by cyclin-dependent kinases (31). Several human cdks have been identified and defined (32,33). These include cdc2 (cdk1), cdk2, cdk3, cdk4, cdk5, cdk6, and cdk7. In human cells, cdc2 associates with cyclin A and cyclins D1-D3; cdk2 forms complexes with cyclin A, cyclins D1-D3, and cyclin E; cdk4, cdk5, and cdk6 bind to cyclins D1-D3 while cdk7 associates with cyclin H (33). With the exception of cdk3 whose regulatory cyclin subunit has not been identified, each step in the cell cycle is known to be regulated by activated cyclin/cdk complexes (33,34). To maintain homeostatic balance between cell proliferation, differentiation and cell death, human cells possess intrinsic regulatory mechanisms that counterbalance the growth stimulatory effects of cdks. Negative regulatory forces are provided by tumor suppressor proteins such as the p53 and the retinoblastoma (pRb) proteins (35) and by cyclin-dependent kinase inhibitors (43). These cdk inhibitors which appear to block cell cycle progression in G1 include p21^{Cip1}, p27^{kip1}, p57^{kip2}, p15^{INK4B}, p16^{INK4A}, and p18^{INK4C} and p19^{INK4D} (36,37).

The discovery that cyclin-dependent kinases were deregulated in human tumors and in human carcinoma cell lines (31,94), coupled with reports of the therapeutic potential of natural cdk inhibitors in tumor suppression (31) encouraged a vigorous search for pharmacological inhibitors of cyclin-dependent kinases. The studies led to identification of chemical cdk inhibitors such as flavopiridol, butyrolactone, and the purine olomoucine (95). Recently, roscovitine, an olomoucine-related purine was found to be a potent and selective inhibitor of cdc2 and cdk2 (39,40). Concentrations of roscovitine at micromolar levels were observed to exert strong inhibitory effect on the kinase activity of cdc2/cyclin B, cdk2/cyclin A, and cdk2/cyclin E complexes (38). It was also reported that roscovitine prevented the cell cycle progression of mammalian cells at the G1/S and the G2/M checkpoints and blocked the transactivation of cyclin A by Myc (39-41). In view of the demonstrated antimitotic and potential antitumor effect of roscovitine, we felt it necessary to study the effects of this purine-derived cdk inhibitor on the proliferative activity of malignant and non-malignant human breast epithelial cells. Thus, the main purpose of the present study was to evaluate the effect of

roscovitine on the growth kinetics and viability of immortal and neoplastic human breast epithelial cells.

BODY

Experimental Methods and Procedures

Study of the Effect of hCG on Cell Proliferation and gene Expression: The spontaneously immortalized human breast epithelial cells MCF-10F, which originated from the mortal cells MCF-10M, and clone BP1-E, derived from benzo(a)pyrene (BP) transformed MCF-10F cells, and MCF-7, a human breast carcinoma cell line, and T24, a human bladder carcinoma cell line were used in these experiments. MCF-10F and the BP-transformed cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F-12 (1:1), supplemented with equine serum, insulin, hydrocortisone, epidermal growth factor, cholera toxin, and antibiotics. MCF-7 cells were grown in DMEM/F-12 medium supplemented with 5% fetal calf serum, antibiotics and insulin, and T24 cells in the same medium, but containing 10% fetal calf serum. The cells were cultured at 37°C in a humidified atmosphere under 5% CO₂. The cell proliferation assay was performed as described above.

Each one of the cell lines listed above were plated in six T150 flasks (Falcon, Lincoln Park, NJ) concentration of 1×10^4 cells per cm². When the cells reached 80% confluence they were treated with 100 IU hCG (Profasi [Serono Laboratories, Inc., Randolph, MA]). The cells were harvested at the end of the 24 and 120 hour-treatment, and immediately frozen in liquid nitrogen for RNA extraction. Polyadenylated RNA was isolated from all the treated and control cells described above using a Fastrack mRNA isolation kit (Invitrogen, Inc. CA). Five to 10 µg of RNA were electrophoresed through 1.2% formaldehyde agarose gels and capillary transferred to nylon membranes, and fixed by U.V. cross linking. Rat TRPM2, ICE and human bcl2 cDNA probes were subcloned into p-bluescript vector in the orientation of T3 promoter and were used for preparing riboprobe. The probes were labeled with (α -³²P) CTP by random primer labeling kit. Gene specific oligonucleotides were synthesized for bcl-XL and bcl-XS as described by Li et al. (96), end labeled and used as probes. Riboprobes were prepared for the detection of bcl2, TGF- α and TGF- β by *in vitro* transcribing the linearized template DNA with (α -³²P) UTP and then purified by the Biospin column. The nylon membrane blot was prehybridized, hybridized in hybridization solution containing 50% formamide, 5xDenhardt's reagent, 2xSSC, 0.5%SDS and 100 µg of salmon sperm DNA at 42°C overnight. Then membranes were washed twice in 2XSSC, 0.1%SDS at room temperature for 15 min, 1XSSC, 0.1% SDS at 55°C, followed by 0.1XSSC, 0.1% SDS at 60°C and exposed to a Kodak X-omat film with an intensifying screen at -70°C. Relative mRNA contents of each experimental group were determined by scanning laser densitometry. Northern blots were stripped and then reprobed sequentially with additional probes. The human β -actin probe was employed as a control for verifying load consistency and RNA integrity. Densitometric results were normalized by expressing the units obtained for a specific transcript relative to the units obtained for actin transcript. The

expression of β -actin mRNA was not affected by any of the treatments applied to the cells in this study.

Effect of hCG Treatment on MCF-10 F Cell Cycle: Flow Cytometric Analysis

MCF-10F cells were grown in DMEM/F12 (1:1) with additives under standard culture conditions. Cells in their 98 passage were plated in T75 flasks at a concentration of 10×10^4 cells/cm². When the cells reached 30% confluence, they were treated with 100 UI/ml hCG (Profasi [Serono Laboratories, Inc., Randolph, MA]) for 24-, 48-, or 72-hour periods; the hormone and culture medium were changed daily. Control cells were treated with the same volume of vehicle in which hCG had been dissolved. The cells were harvested by trypsinization at the above indicated time periods. After trypsinization, control and treated cells were fixed in ethanol, treated with ribonuclease and finally stained with propidium iodide (PI). Analysis was carried out using excitation wavelength of 488 nm and a detection wavelength of 510-550 nm. PI is an orange-red fluorescent dye that binds to double-stranded nucleic acids.

Effect of Roscovitine on the Proliferative Activity of Human Breast Epithelial Cells

MCF-7, MCF-10F, and MDA-MB-123, an estrogen receptor-negative human breast carcinoma cell line (98), were routinely cultured in T75 flasks. Fully confluent cells were harvested, counted with hemocytometer and seeded into 96-well plates. After 24 hours in culture, the cells were treated daily with 5, 10, 20, and 40 μ g/ml roscovitine (Calbiochem, La Jolla, CA) dissolved in DMSO and incubated for a length of time ranging from 24 to 144 hours. Control cells were incubated under identical conditions with the same volume of DMSO-containing medium.

Results

Effect of hCG treatment on the proliferative activity of human epithelial cells: The quantification of the number of viable cells revealed that by 24 hr. of hormonal treatment there were no significant differences between control and hCG-treated MCF-10F, BP1-E and T24 cells. MCF-7 cells, on the other hand, exhibited a significant inhibition (Fig. 1). By 120 hours in culture, control cells had shown a 20-60 fold increase in the number of viable cells, while hCG-treated MCF-10F, MCF-7, and BP1E-Tp cells did not differ in the number of viable cells from the values observed at 24 hr. T24 cells showed the same rate of cell growth in the control and in the hCG-treated cells (Fig.1).

Activation of programmed cell death genes by hCG treatment of MCF-10F cells: Northern blot analysis indicated that the 24 hr hCG treatment induced an elevation in the expression of TRPM2 by 2 fold, ICE by four fold, TGF- β by 2.5 fold, c-myc by 2 fold, p53 by 3 fold, and p21^{WAF1/CIP1} by 3 fold. The treatment did not modify the expression of bcl-2 in comparison with its respective control cells (Fig 2). By 120 hr of hormonal treatment the expression of all the apoptotic genes remained at the same levels observed at 24 hr. There was a slight increase in bcl2, and a decrease in the expression of c-myc, but the differences were practically negligible. Of interest was the observation that in the control cells an increment was observed with respect to the levels detected in the 24 hr control cells.

Activation of programmed cell death genes by hCG treatment of MCF-7 cells: The 24 hr hCG treatment of MCF-7 cells significantly induced an increase in the expression of TRPM2 (2.5 fold), TGF- β (3 fold), c-myc (3.5 fold), p-53 (4 fold) and p21^{WAF1/CIP1} (3 fold) transcripts as compared to the control cells (Fig. 3). The ICE transcript could not be detected in MCF-7 control cells, and hCG treatment did not modify its expression. The levels of bcl2 significantly declined after the hormonal treatment in comparison with control cells (Fig. 3). By 120 hr of hormonal treatment the levels of TRPM2, TGF- β , p53, and p-21 remained at the same levels observed after 24 hr treatment. The differences with their respective controls, however, had disappeared because of the elevation in the expression of these genes in control cells. The expression of bcl2 was more intense in the 120 hr than in the 24 hr control, and hCG treatment almost completely inhibited its expression.

Activation of programmed cell death genes by hCG treatment of chemically transformed human breast epithelial cells: The 24 hr hCG treatment of the transformed human breast epithelial cells BP1-E produced a significant increase in the expression of ICE by greater than two fold, but increased only slightly the expression of TRPM2, TGF- β , p53, and p21^{WAF1/CIP1} genes, whereas the expression of bcl2 and c-myc was down-regulated by the treatment (Fig. 4). Very little change in gene expression was observed in the 120 hr with respect to the 24 hr hCG-treated cells, but a notable increase in the expression of TRPM2, ICE, and p21^{WAF1/CIP1} was observed in control cells, in which the level of expression of these genes was similar to those of hCG-treated cells.

The hormonal treatment of BP1-E-Tp cells for 24 hr increased 2 fold the expression of TRPM2 (Fig. 5). This increase was less pronounced in BP1-E cells. The expression of bcl2 slightly decreased, and that of c-myc, TGF- β , and p-53 was not significantly modified in any of the cell lines in comparison to their respective controls. P21^{WAF1/CIP1}, on the other hand, was significantly increased (2.2 fold) in BP1E-Tp cells (Fig. 5). The 120 hr treatment induced changes similar to those observed in BP1E cells, namely increased expression of p53 and p21^{WAF1/CIP1} in treated cells, and increased expression of TRPM2, and ICE in both control and treated cells.

Effect of hCG treatment on the expression of programmed cell death genes in T24 cells: The 24 hr treatment of T24 cells with hCG did not alter significantly the expression of TRPM2, bcl2, p53 and c-myc, but it induced a slight increase in the expression of TGF- β and p21^{WAF1/CIP1} (Fig. 6). By 120 hr of treatment the expression of TRPM2 had increased in both control and hCG treated cells, and the same phenomenon was observed in the expression of bcl2 and TGF- β . The expression of c-myc, on the other hand, was lower in treated than in control cells, and that of p53 and p21^{WAF1/CIP1} was almost indistinguishable. The postulated model of the effect of hCG on cell cycle arrest and apoptosis is shown in Figure 7.

Analysis Effect of hCG (Profasi) Treatment on the MCF-10 F Cell Cycle

Flow cytometric analysis of hCG (Profasi) treated MCF-10F after trypsinization, ethanol fixation, ribonuclease treatment and propidium iodide (PI) staining was carried out using excitation wavelength of 488 nm and a detection wavelength of 510-550 nm. At 48 hours in culture 53.1% of control cells were in G1, 26.4% in S and 20.4% in the G2-M phases of the cell cycle. Cells treated with Profasi for the same period of time had 52.8% of the cells in G1, 25.4% in S and 21.7% in G2-M. By 72 hours the percentages in control cells were: 61.8% in G1, 20.1% in S and 18.0 in G2-M, while Profasi treated cells had a higher percentage of cells in G1, 63.7, fewer cells in S, 17.6%, and fewer cells in G2-M, 17.9. Control cells showed an increase in the percentage of cells in G1 at 72 hours with respect to 48 hours, an indication that as the cells approach confluence they become arrested in this phase of the cycle. A greater percentage of Profasi treated cells were in G1 at 72 hours, an indication that there was a trend to a greater accumulation of cells in this phase of the cycle related to the hormonal treatment, although the differences with controls were not statistically significant. Studies on the effect of hCG on MCF-10F and MCF-7 cells at 24, 48, 72, 96, 120 and 168 hours, with correlations with cell proliferation assay are ongoing in our laboratory.

Effect of Roscovitine on the Proliferative Activity of Human Breast Epithelial Cells: Exposure of MCF-7 cells to different concentrations of roscovitine ranging from 5 to 40 $\mu\text{g/ml}$ reduced the growth rate of the cells in culture. It was evident from the growth curve in Figure 8 that cells exposed to 40 $\mu\text{g/ml}$ roscovitine failed to proliferate in all time points examined. Compared to the controls, cells treated with 5 $\mu\text{g/ml}$ grew exponentially, but at a reduced rate, while cells exposed to 10 and 20 $\mu\text{g/ml}$ were unable to attain exponential growth. Data of roscovitine effect on the number of viable MCF-7 cells illustrated in Figure 9, demonstrates that the cdk inhibitor has a dose- and time-dependent effect on cell viability. There was no increase in cell numbers in the group of wells treated with 20 and 40 $\mu\text{g/ml}$ roscovitine after 24 hours of exposure. Treatment of MCF-10F cells to 5, 10, 20, and 40 $\mu\text{g/ml}$ roscovitine for 24, 48, 72, 96, and 120 hours showed that the proliferative activity of the cells was inhibited in a dose- and time-dependent fashion. In comparison to the controls, exposure of the cells to 5 $\mu\text{g/ml}$ resulted in a considerable decrease in the proliferative rate of MCF-10F cells after 48 hours of treatment (Fig. 10). Treatments with 10, 20, and 40 $\mu\text{g/ml}$ resulted in abrogation of exponential cell growth. It is evident from Figure 11 that exposure to 5, 10, 20 and 40 $\mu\text{g/ml}$ for 24 hours had little or no effect on the number of viable MCF-10F cells. However, all concentrations of roscovitine markedly decreased the number of viable cells after a 72 hour treatment. These results suggest that the proliferative activity of this breast epithelial cell line was suppressed by roscovitine.

MDA-MB-231 control cells grew exponentially after a 48-hour incubation and attained confluence after 120 hours in culture. The growth kinetics of cells exposed to 5 $\mu\text{g/ml}$ roscovitine was similar to that of control cells, but suppression of cell proliferation occurred after a 96-hour incubation (Fig. 12). Cells treated with 10 $\mu\text{g/ml}$ attained exponential growth but with a reduced rate of proliferation, while 20 and 40 $\mu\text{g/ml}$ treatments prevented the exponential growth of the cells. Moreover, reduction in number of viable cells was evident in the group of cells exposed to 40 $\mu\text{g/ml}$ roscovitine

after 24 hours, and this trend persisted throughout the entire duration of the study. It was also observed that the number of viable cells was severely decreased in the group that received 10, 20, and 40 $\mu\text{g/ml}$ after 72 hours in culture (Fig. 13). Reduction in cell numbers in cells exposed to 5 $\mu\text{g/ml}$ occurred only after 96 hours of roscovitine exposure.

Discussion

The results of the present investigation demonstrate that treatment of human breast epithelial cells with the human placental hormone chorionic gonadotropin obtained from the urine of pregnant women, which is clinically used for induction of ovulation, among other applications (98), inhibits the proliferative activity of the cells and induces activation of genes controlling programmed cell death. This effect was observed only in HBEC, either the normal immortalized MCF-10F, or the chemically transformed pre- and tumorigenic cells BP1E and BP1E-Tp, respectively, and the malignant metastatic MCF-7 cells, the urothelial cells T24 were not affected by this treatment. The difference in responsiveness observed between HBEC and urothelial cells at the level of activation of the genes that control programmed cell death coincides with the selective inhibitory effect of hCG on *in vitro* HBEC proliferation, which is not observed in T24 cells (29). This specificity of action might be attributed to the presence of a receptor to hCG that we have detected in HBEC (unpublished observations), and that has been recently reported in rat mammary epithelial cells (99). Although all HBEC are growth inhibited and express activation of programmed cell death under hCG treatment, the response of the cells to the hormonal treatment varies depending upon the basic biological conditions of the cells. For example, most of the genes controlling programmed cell death were activated in the immortalized, but otherwise normal HBEC MCF10-F cells, whereas the chemically transformed cells BP1E and BP1E-Tp and the metastatic human breast carcinoma cell line MCF-7 exhibited differences which might be related to variations in the pathway of activation of programmed cell death (Fig. 7).

In our experimental model, activation of TRPM2 and TGF- β genes by hCG treatment occurred in MCF-10F and MCF-7 cells, but not in the chemically transformed HBEC and the T24 cell lines, an observation that supports the concept that activation of these two genes might be dependent on specific cell characteristics. Induction of TRPM2 transcript has been shown during chemotherapeutic regression of a mouse bladder tumor (100). Although enhanced expression of TRPM2 and TGF- β genes has been reported in regressing human breast cancer cells following estrogen ablation and in prostatic tumors after hormone withdrawal (65,101), we have observed that in the rat mammary carcinoma model, activation of TRPM2 occurs under maximal hormonal stimulation (109). TRPM2 has been reported to be stimulated in MCF-7 cells by 1,25-dihydroxyvitamin D3, which also inhibits cell proliferation (102).

Another gene that has been shown to be relevant in the induction of apoptosis is the ICE gene. ICE belongs to a protease family, which includes ICE and CPP32/Yama, or apopain (55-57,103). The role of ICE in driving the cells to apoptosis has been confirmed by the observations that its inhibition and/or mutation inhibits apoptosis induced by various

stimuli (50,61,104). In our experimental system, the expression and behavior of the ICE gene under hCG treatment differs among the different cell lines tested. It is significantly increased in MCF-10F and in the transformed BP1-E and BP1E-Tp cells, but it is barely expressed and does not respond to the hormonal treatment in MCF-7 cells. In T24 cells, on the other hand, the gene is present, but its expression is not modified by hCG treatment.

Several lines of evidence indicate that induction of apoptosis can be mediated by both p53 and c-myc (74), which are the major players in the context of growth arrest and apoptosis. It has also been demonstrated that c-myc mediated apoptosis requires functional p53 (105,106). Furthermore, recent evidence indicates that induction of apoptosis can be mediated by the tumor suppressor p53 through its downstream target gene p21^{WAF1/CIP1} (74,76). We have found that under hCG treatment the expression of p53 and c-myc is significantly increased in both MCF-10F and MCF-7 cells, but it is not modified in either BP1E, BP1E-Tp or T24 cells. These observations are relevant to the light that deregulated expression of c-myc is often associated with an increased incidence of cell death, usually by apoptosis. It has been suggested that cell proliferation and cell death are tightly coupled or overlapping processes that are both driven by c-myc, although once they are established, cell growth and cell death are independently modulated by other genes and other external factors (45,75,96). C-myc mediated apoptosis requires functional p53 (105-107), as demonstrated by the induction of apoptosis by activation of c-myc in p53 +/+ fibroblasts, which is preceded by stabilization of p53, whereas in quiescent p53-null fibroblasts c-myc activation did not result in apoptosis (105). Whereas in mammary and other epithelial cells in culture, both p53 dependent and p53 independent apoptosis pathways have been identified (62,106-110), our observations that p53 was significantly activated by hCG indicated that this gene is involved in the process of programmed cell death, in association with the inhibition of *in vitro* cell proliferation. Using northern blot analysis, we have further shown profound induction of p21^{WAF1/CIP1} mRNA in all the cell lines treated with hCG. Based upon these observations it can be concluded that hCG first arrests the progression of cell cycle by inducing p53 and its target gene p21^{WAF1/CIP1} and then proceeds towards apoptosis (Fig.7). In the case of chemically transformed cell lines, we did not find any change in the levels of p53, but there was a profound induction of WAF1/CIP1 mRNA. The possibility exists that WAF1 was induced by hCG independently of p53, as it has been shown in other systems (111).

The molecular details of p53-induced cell death are not fully understood, but conserved features include activation of proteases of ICE class (112). This mechanism is supported by our findings that hCG treatment induces an increase in the expression of both p53 and ICE in MCF-10F cells. This postulate, however, is not supported in MCF-7 cells, in which ICE is barely expressed, and it is not modified by the hCG treatment, supporting the concept that each cell type may respond differently to hormonal stimuli in the activation of these genes. Another possible involvement of p53 in apoptosis is the regulation by members of the bcl2 multiprotein family (78,79,113,114). Some of the members of the bcl2 family which are overproduced in a variety of human cancers (i.e., bcl2, bcl-XL) are blockers of cell death, while others,

such as Bax and bcl-XS, are promoters of apoptosis, and their levels might be reduced in some types of cancers (78,79,113,114). In the present study we found that hCG treatment did not modify the expression of bcl2 in MCF-10F cells, but it induces its down-regulation in MCF-7 cells. The fact that p53 and bcl2 expression is differently modulated by the hormonal treatment is a strong indication that alternative pathways might be operational in the activation of programmed cell death genes by hCG. In the performance of these studies we observed that control cells, after 120 hours in culture exhibited an elevation in the level of expression of apoptotic genes which was similar to the levels observed in the 24 hr hCG-treated cells, indicating that hCG accelerates the process of gene activation that has been reported to be associated with confluence (115). Our observations led us to conclude that a 24 hour treatment of immortalized, chemically transformed and malignant human breast epithelial cells with hCG activates programmed cell death genes even before an arrest of cell growth has become evident. Of relevance is the fact that hCG that in vivo acts as a preventive and tumoristatic agent (19,84,85), and an inhibitor of in vitro cell proliferation (29,81,82,85), may utilize different pathways for activating programmed cell death genes, depending upon the degree of expression of neoplastic phenotypes.

Our study of the proliferative responses of different human breast epithelial cell lines to roscovitine, a potent chemical inhibitor of cdc2 and cdk2 utilizing MCF-7, an estrogen receptor-positive human breast carcinoma cell line (80,81), MCF-10F, an estrogen receptor-negative immortal human breast epithelial cell line (82) and MDA-MB-231, a highly malignant estrogen receptor-negative human breast epithelial cell line (98) demonstrated that roscovitine suppressed the proliferation of the three breast epithelial cell lines in a dose- and time-dependent fashion. Cell proliferation measurements with the WST-1 colorimetric assay revealed that roscovitine treatment reduced the number of viable cells and prevented the exponential growth of all the cell lines examined.

The cellular and molecular mechanisms responsible for the antiproliferative effect of roscovitine on human breast epithelial cells is not yet defined, however, its ability to inhibit cdk kinase activity by competition for ATP (39) may prevent the phosphorylation of pRb and thus inhibit cell growth. The possibility that roscovitine effects are mediated by estrogen receptors is unlikely because data from the present study indicate that similar growth responses were elicited from both estrogen receptor-positive and estrogen receptor-negative cell lines by roscovitine. Evidence from classical enzymological studies have elegantly demonstrated that roscovitine selectively inhibits the kinase activity of cdc2/cyclin B, cdk2/cyclinE and cdk2/cyclin A complexes (38,40). Since these activated cdk complexes are required for progression of cells through different phases of the cell cycle, it is conceivable that the antiproliferative of roscovitine may be due to its ability to block the cell cycle-propelling activity of the activated kinases and induce growth arrest.

Suppression of cdk2 kinase activity is one of the mechanisms utilized by proliferative signals to regulate cell cycle progression during G1. Previous studies had implicated p21^{Cip1} and p27^{Kip1} in mediating the effects of negative proliferative signals such as contact inhibition, TGF- β , and DNA damage (116,117). Moreover, overexpression of p21^{Cip1}, a universal inhibitor of cyclin-dependent kinases has been shown to inhibit the proliferation of several mammalian cells (40). Our results indicate

that roscovitine mimics the action of natural cdk inhibitors by inducing cell growth arrest in our experimental model system. The substantial reduction in the number of viable human breast epithelial cells observed in our experiments might also be due to the ability of this potent cdk inhibitor to induce physiological cell death. Evidence from recent studies have shown that olomoucine, and butyrolactone-1 also known to inhibit cdk activity, enhanced drug-induced apoptosis in human leukemia cells and murine mammary cell lines (118,119). Like roscovitine, olomoucine and butyrolactone-1 are thought to inhibit cdk1 and cdk2 protein kinase activity by competition with ATP (38,39). Thus, it is very likely that roscovitine treatment triggers apoptotic cell death in human breast epithelial cells. Data obtained from this study suggest that roscovitine is a potential antineoplastic agent. To facilitate the translation of roscovitine's efficacy to the clinical setting, a more detailed study to elucidate the mechanisms responsible for the antiproliferative effect of this potent cdk inhibitor would be necessary. Such experiments are now underway in our laboratory. The relevance of our findings lies in the potential use of hCG as a chemopreventive and chemotherapeutic agent in breast cancer, utilizing the detection of activation of programmed cell death genes as an early end point in the action of this hormone on the target tissues.

Recommendations in Relation to the Statement of Work

Numerous discoveries on the role of hCG in malignancies have occurred since this grant application was submitted. A protective effect of hCG from breast cancer risk in women has been confirmed by epidemiological studies of Bernstein et al. (120), who identified a group of women that were administered hCG as part of a weight loss regimen or infertility treatment. In nulliparous women with a maximum body mass index lower than 27.5 kg/m^2 breast cancer risk was substantially reduced (multivariate odds ratio 0.30, 95 % confidence interval = 0.10-0.96) in (120). Two different groups, besides our own, have identified the presence of LH/CG receptor in human breast epithelial cells and in breast cancers (121,122). Furthermore, hCG treatment of nude mice injected with Kaposi's sarcoma cells inhibits tumor growth, and both local and systemic treatment of AIDS patients with commercially available forms hCG causes remission of Kaposi's sarcoma lesions (123-125). A controversy on the purity of commercially available forms of hCG and what specific fraction of this hormone is responsible for the effects observed arose among the groups working on the effect of hCG on tumor cells led us to compare the effect of different preparations of hCG on human breast and bladder carcinoma cell lines. The development of a recombinant hCG by a pharmaceutical company (Serono-Benelux, Geneva, Switzerland) added a new dimension to our studies on the effect of hCG on cell proliferation and gene expression. We have carried out preliminary studies of the effect of recombinant hCG on the ovary and the mammary gland of young virgin rats. An excellent response of the ovaries and a profuse lobular development after a 15 day treatment in materials collected up to now (unpublished results) indicate that this highly purified compound will offer excellent opportunities for testing the specificity of this hormone on its multiple target sites.

CONCLUSIONS

Our observations led us to conclude that treatment of immortalized, chemically transformed and malignant human breast epithelial cells with hCG activates programmed cell death genes even before an arrest of cell growth has become evident. Of relevance is the fact that hCG *in vivo* acts as a preventive and tumoristatic agent. *In vitro* it acts as an inhibitor of cell proliferation, an indication that this hormone might utilize different pathways for either activating programmed cell death genes or inhibiting specific cell cycle dependent kinases, depending upon the degree of expression of neoplastic phenotypes. The relevance of these findings lies in the potential use of hCG as a chemopreventive and chemotherapeutic agent in breast cancer, utilizing the detection of activation of programmed cell death genes as an early end point in the action of this hormone on the target tissues.

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APPENDIX

Legend of Figures

Figures 1-13

Legends of Figures

Figure 1. Effect of hCG treatment on cell growth. MCF-10F, MCF-7, BP1-E-Tp, and T24 cells were treated daily with 100 IU/ml hCG and harvested at 24 and 120 hours cell growth determination by WST-colorimetric assay. Control cells were treated with vehicle only. Values represent the mean number of viable cells ($\times 1000$) $\pm$ SD of three wells from two experiments.

Figure 2. Histogram showing the expression of TRPM2, ICE, bcl-2, TGF- β , c-myc, p53 and p21 mRNA relative to their respective controls in MCF-10F cells treated with hCG for 24 hours. Relative mRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β -actin.

Figure 3. Histogram showing the expression of TRPM2, bcl-2, TGF- β , c-myc, p53 and p21 mRNA relative to their respective controls in MCF-7 cells treated with hCG for 24 hours. Relative mRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β -actin.

Figure 4. Histogram showing the expression of TRPM2, ICE, bcl-2, TGF- β , c-myc, p53 and p21 mRNA relative to their respective controls in BP1-E cells treated with hCG for 24 hours. Relative mRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β -actin.

Figure 5. Histogram showing the expression of TRPM2, ICE, bcl-2, TGF- β , c-myc, p53 and p21 mRNA relative to their respective controls in BP1-E-Tp cells treated with hCG for 24 hours. Relative mRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β -actin.

Figure 6. Histogram showing the expression of TRPM2, bcl-2, TGF- β , c-myc, p53 and p21 mRNA relative to their respective controls in T24 cells treated with hCG for 24 hours. Relative mRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β -actin.

Figure 7. Postulated model of hCG-induced cell cycle arrest and apoptosis in human breast epithelial cells. In the presence of hCG for 24 hours breast epithelial cells the binding of the hormone to a newly discovered, putative membrane receptor, that triggers a cascade of programmed cell death gene activation through the cAMP-PKA pathway, as well as through activation of TGF- β . In the chemically transformed BP1-E and BP1-E-Tp cells in which TGF- β , c-myc and p53 are not activated, it is postulated that p21 activation might proceed through an alternative pathway, i.e., TF/DF (wavy arrow).

Figure 8. Effect of roscovitine on growth kinetics of MCF-7 cells. Cells were grown in medium supplemented with 5% fetal bovine serum, exposed to different concentrations of roscovitine dissolved in DMSO and harvested at 24, 48, 72, 96, 120, and 144 hours.

Cell proliferation was determined by WST-1 microplate assay. Values represent the mean $\pm$ SD of six wells from two experiments.

Figure 9. Effect of roscovitine on number of viable MCF-7 cells. Cells grown in medium supplemented with 5% fetal bovine serum were treated with different concentrations of roscovitine and harvested at 24, 48, 72, 96, 120, and 144 hours for WST-1 colorimetric assay. Values represent the mean $\pm$ SD of six wells from two experiments.

Figure 10. Effect of roscovitine on growth kinetics of MCF-10F cells. Cells were grown in medium supplemented with 5% horse serum and exposed to 5, 10, 20, and 40 μ g/ml roscovitine and harvested at 24, 48, 72, 96, and 120 hours for WST-1 cell proliferation assay. Values represent the mean $\pm$ SD of six wells from two experiments.

Figure 11. Effect of roscovitine on number of viable MCF-10F cells. Cells grown in medium supplemented with 5% horse serum were treated with different concentrations of roscovitine and harvested at 24, 48, 72, 96, and 120 hours for WST-1 colorimetric assay. Values represent the mean $\pm$ SD of six wells from two experiments.

Figure 12. Effect of roscovitine on growth kinetics of MDA-MB-231 cells. Cells were grown in medium supplemented with 10% fetal calf serum, treated with different concentrations of roscovitine and harvested at 24, 48, 72, 96, and 120 hours for WST-1 cell proliferation assay. Values represent the mean $\pm$ SD of six wells from two experiments.

Figure 13. Effect of roscovitine on number of viable MDA-MB-231 breast carcinoma cells. The cells were cultured in medium supplemented with 10% fetal calf serum; exposed to different concentrations of roscovitine and harvested at 24, 48, 72, 96, and 120 hours for WST-1 colorimetric assay. Values represent the mean $\pm$ SD of six wells from two experiments.

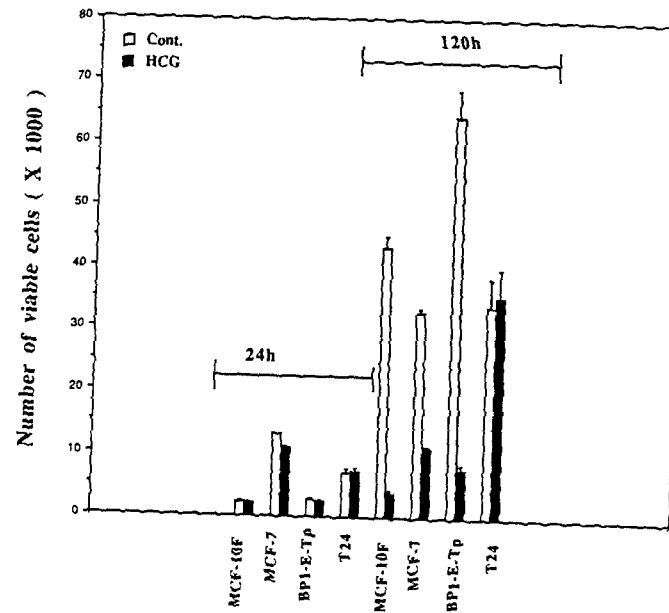


Figure 1

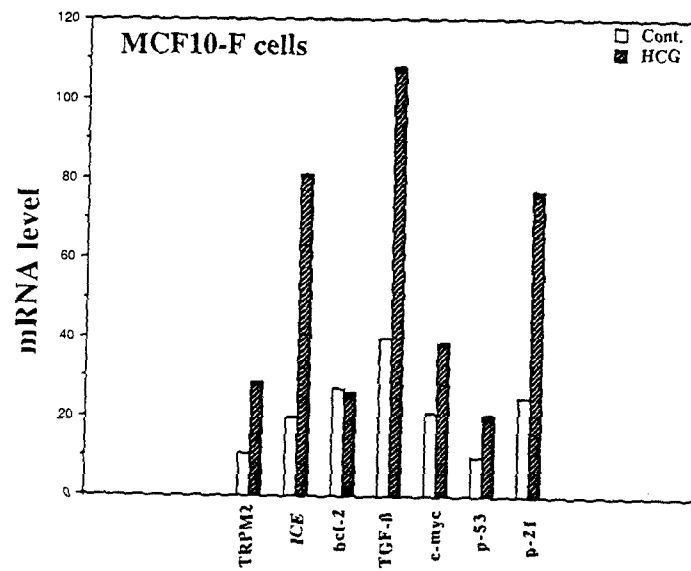


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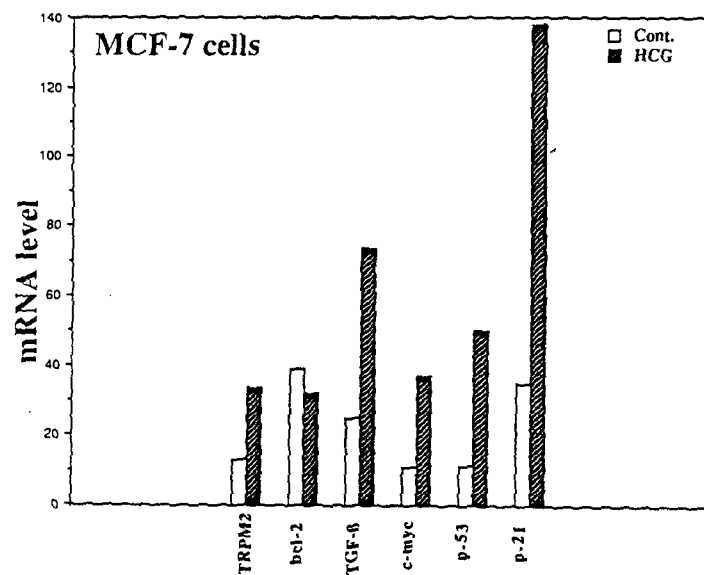


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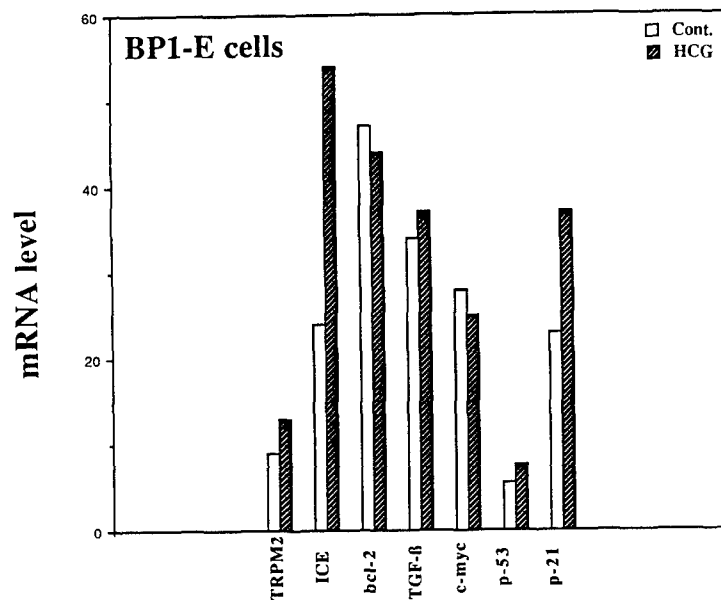


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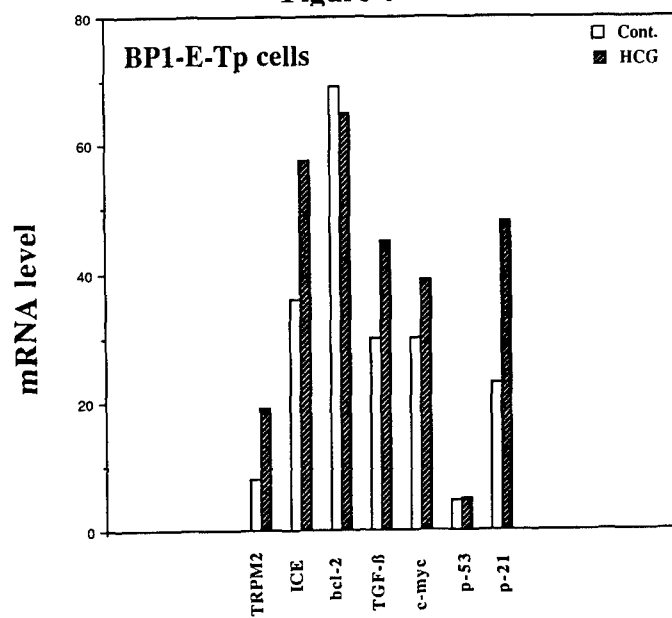


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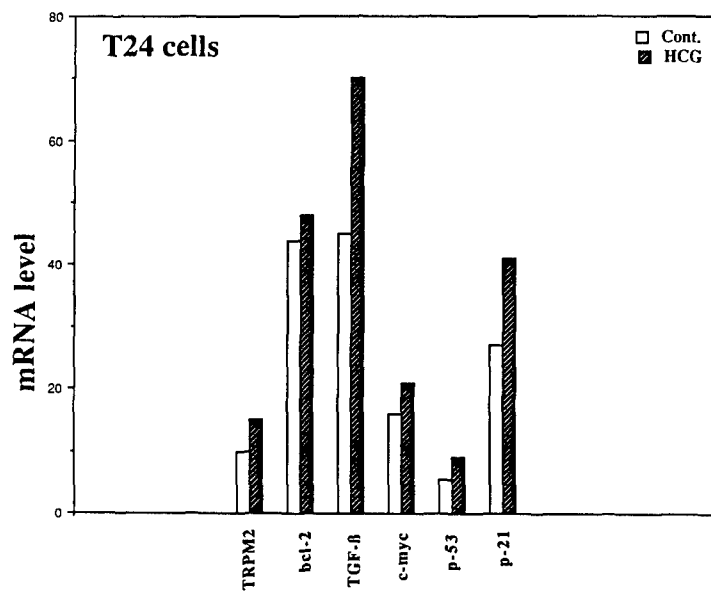


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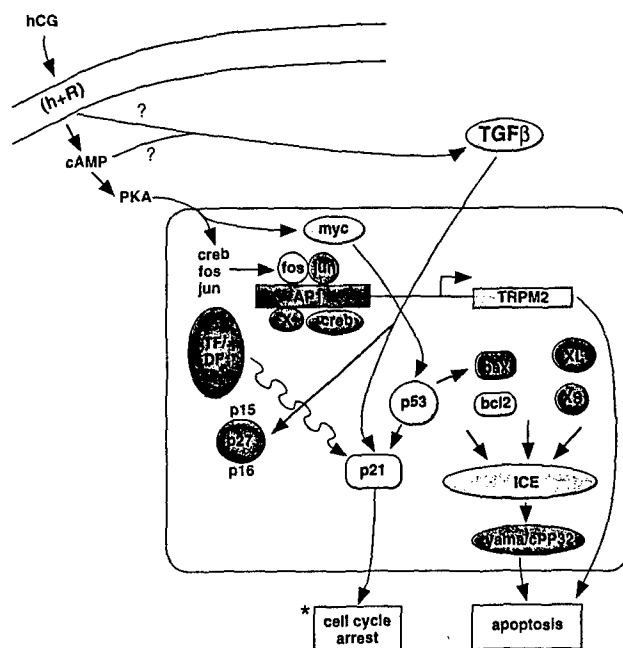


Figure 7

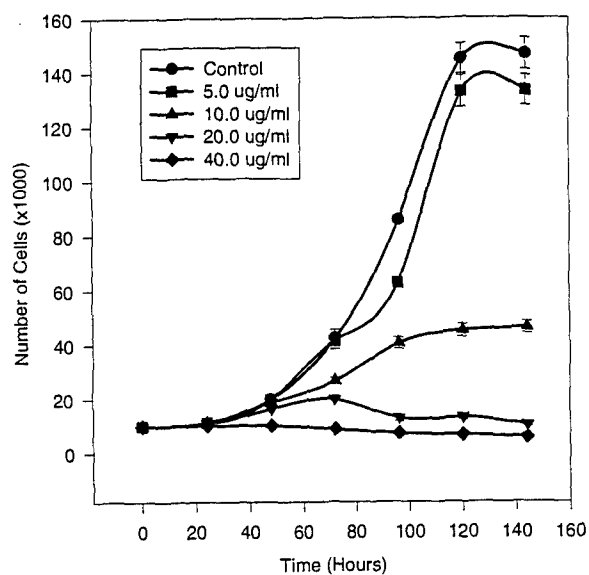


Figure 8

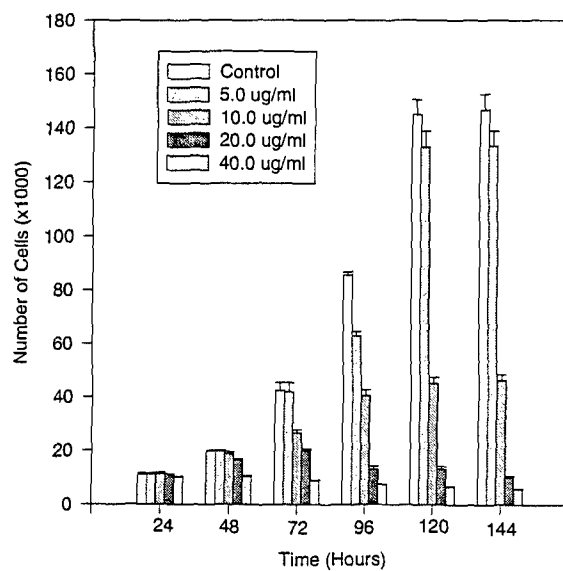


Figure 9

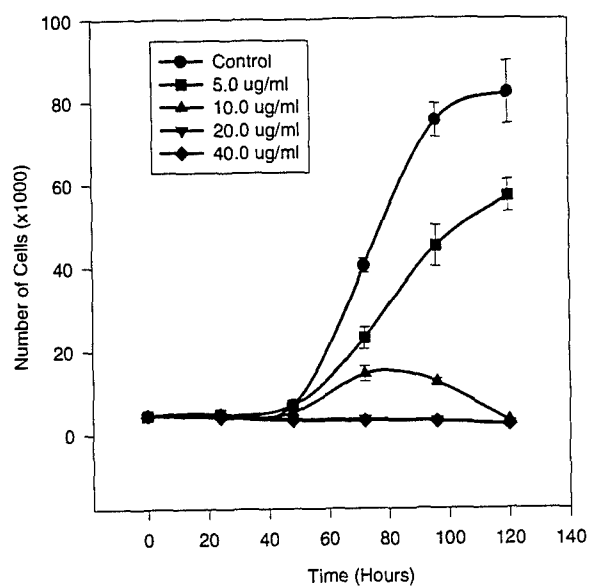


Figure 10

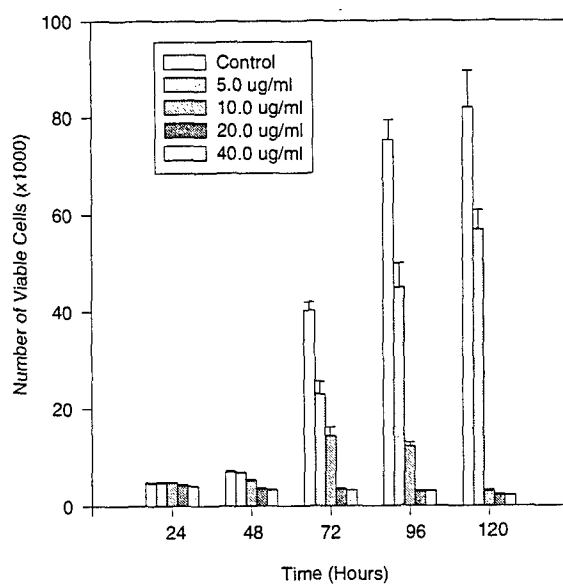


Figure 11

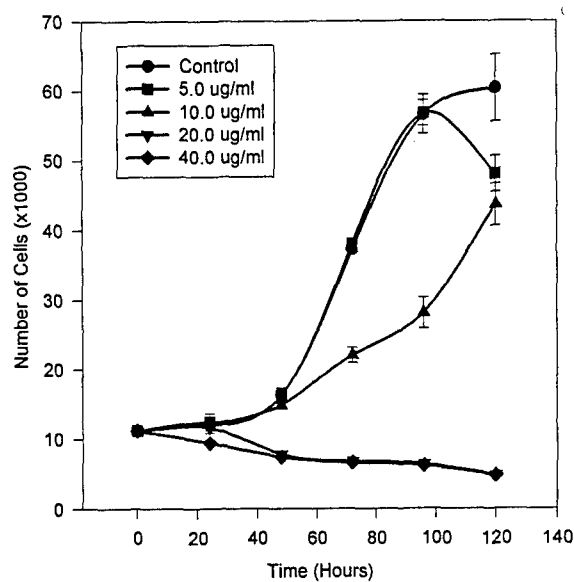


Figure 12

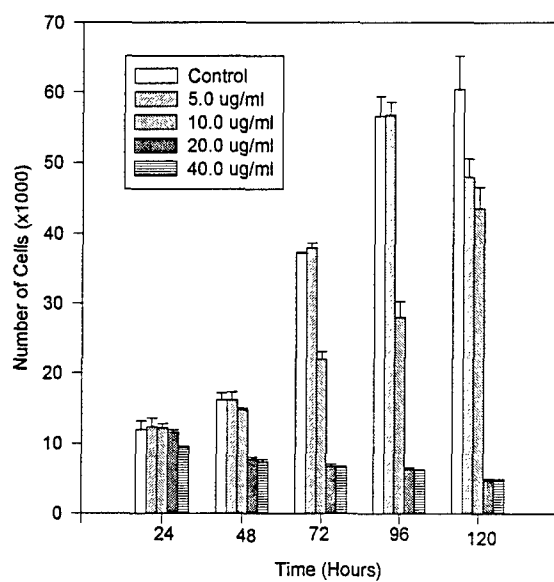


Figure 13