

Abstract

Breast cancer is a fatal disease whose incidence is gradually increasing in most industrialized countries and in all ethnic groups. Primary prevention is the ultimate goal for the control of this disease. The knowledge that breast cancer risk is reduced by early full-term pregnancy and that additional pregnancies increase the rate of protection has provided novel tools for designing cancer prevention strategies. The protective effect of pregnancy has been experimentally reproduced in virgin rats by treatment with the placental hormone human chorionic gonadotropin (hCG). HCG prevents the initiation and inhibits the progression of chemically induced mammary carcinomas by inducing differentiation of the mammary gland, inhibiting cell proliferation, and increasing apoptosis. It also induces the synthesis of inhibin, a tumor suppressor factor, downregulates the level of expression of the estrogen receptor alpha (ER- α) by methylation of CpG islands, imprinting a permanent genomic signature that characterizes the refractory condition of the mammary gland to undergo malignant transformation. The genomic signature induced by hCG is identical to that induced by pregnancy and is specific for this hormone. Comparison of the mammary gland's genomic profile of virgin Sprague-Dawley rats treated daily with hCG for 21 days with that of rats receiving 17 β -estradiol (E₂) and progesterone (Pg) (E₂+ Pg) revealed that in hCG-treated rats 194 genes were significantly up-modulated (>2.5 log₂-folds) (p <0.01) and commonly expressed, whereas these genes were not expressed in the E₂+ Pg group. The

genomic signature induced by hCG and pregnancy included activators or repressors of transcription genes, apoptosis, growth factors, cell division control, DNA repair, tumor suppressor, and cell-surface antigen genes. Our data indicate that hCG, like pregnancy, induces permanent genomic changes that are not reproduced by steroid hormones and in addition regulates gene expression through epigenetic mechanisms that are differentiation-dependent processes, leading us to conclude that hormonally induced differentiation offers enormous promise for the primary prevention of breast cancer.

Introduction

Breast cancer, the fatal disease most frequently diagnosed in American women, is increasing in incidence at a rate of approximately 6% per year in women from all ethnic groups (Greenlee et al. 2001; Jemal et al. 2002). Similar trends are observed worldwide, even in countries characterized by their low breast cancer incidence (De Waard and Thijssen 2005; Forbes 1997; Howe et al. 2001). Improved detection methods, the identification of women at higher risk by family history or detection of germline mutations in the BRCA1 and BRCA2 genes (Warmuth et al. 1997), and diagnosis at an early stage have resulted in a decline in breast cancer mortality in the United States (Greenlee et al. 2001; Jemal et al. 2002; Forbes 1997). Although genetic predisposition accounts for fewer than one in ten cases of breast cancer, women that are carriers of BRCA1/BRCA2 germline mutations are at an

85% lifetime risk of developing breast cancer, with a significantly earlier age of onset of the disease (Chang and Elledge 2001). Current strategies to prevent breast cancer in women at high risk are prophylactic mastectomy, oophorectomy, or antiestrogen therapy (Armstrong et al. 2005), whereas for the general population, trials of dietary changes with reduced fat intake designed to mimic the diets of countries with low breast cancer incidence are advocated. Opponents of this approach argue that only a lifetime dietary change can decrease the risk of breast cancer, and therefore major dietary changes undertaken now may not alter breast cancer incidence for another generation (Tymchuk et al. 2000). Another strategy capitalizes in a unique feature of breast cancer, its estrogen dependence, which can be manipulated to control growth or prevent tumor development utilizing either selective estrogen receptor modulators (SERMs), such as tamoxifen (King et al. 2001; Narod et al. 2000; Baum 2002; Mouridsen et al. 2001; Robertson et al. 2001), or aromatase inhibitors (AIs), such as Arimidex, Letrozole, and Exemestane (Janov et al. 2001; Tymchuk et al. 2000). However, the inability to predict precisely who will develop breast cancer has required the implementation of broad, population-based strategies utilizing preventative measures that have significant side effects and require a protracted treatment. These drawbacks have made these strategies not widely acceptable to a majority of treated women who would not have developed breast cancer even if untreated (Gail et al. 1989).

In light of the fact that more than 50% of breast cancer cases remain unexplained by personal characteristics and other traditionally accepted risk factors, that once the disease becomes metastatic it becomes incurable (Mirza et al. 2002), and the observed worldwide increased incidence of the disease, effective interventions for its primary prevention are urgently required (De Waard and Thijssen 2005; Dunn et al. 2000; Hoffmann et al. 2001; Matsumoto and Yamane 2000; Mirza et al. 2002).

Up to now the possibilities of successfully preventing breast cancer have been hindered by the lack of identification of a definitive causal agent or a mechanism responsible of its initiation. Currently, only inheritance of cancer-predisposing

genes (Mirza et al. 2002; King et al. 2001; Lynch and Casey 2001; Narod et al. 2000; Khurana et al. 2000; Narod 2001; Riggs 2001; Stoutjesdijk and Barentsz 2001; Hartmann et al. 2001), and radiation exposure at a young age (McGregor et al. 1977; Boice et al. 1991; Clemons et al. 2000; Cutuli et al. 2001; Janov et al. 2001) have been identified as a mechanism or causal agent associated with cancer initiation. Multidisciplinary studies based on epidemiological, endocrinological, experimental, and statistical findings have found a direct association of breast cancer risk with nulliparity, confirming conclusions reached by the Italian physician Bernardino Ramazzini, who, almost 300 years ago, considered breast cancer an occupational disease because of its association with celibacy and nulliparity in nuns (Ramazzini 1961). A large body of evidence has confirmed that nulliparity increases breast cancer risk by 30% when compared with parous women (Blair et al. 1999; Ewertz et al. 1990; Fraumeni et al. 1969). A reduction in lifetime breast cancer risk has been found to be conferred by an early first full-term pregnancy (Trapido 1983; MacMahon et al. 1970; Chie et al. 2000; Holmberg et al. 2001; Vessey et al. 1985; Kelsey and Horn-Ross 1993; Lambe et al. 1996). In addition, a greater number of full-term pregnancies increases the protection. Each additional birth after the first reduces the breast cancer risk by 7% in the absence of breastfeeding (Collaborative Group on Hormonal Factors in Breast Cancer 2002). Breastfeeding confers an additional protection of 4.3% reduction in risk for each year a woman breastfeeds (Collaborative Group on Hormonal Factors in Breast Cancer 2002), even if they are carriers of BRCA1 germline mutations (Jernstrom et al. 2004).

Physiological Basis of Breast Cancer Prevention

The above-described epidemiological observations indicating that pregnancy significantly reduces the lifetime risk of developing breast cancer provide a window of opportunity for learning how and why this physiological condition exerts such a protective effect. Due to the complexity of the carcinogenic process, this event does

not explain all the aspects of this complex disease; nevertheless, data obtained in experimental models have served as a blue print for developing a new paradigm in breast cancer prevention (I.H. Russo and J. Russo 1993, 1994, 1996; I.H. Russo et al. 1990a, b; J. Russo et al. 1977; Srivastava et al. 1998; Mgbonyebi et al. 1996; Tahin et al. 1996; J. Russo and I.H. Russo 2000; Alvarado et al. 1993, 1994). Our studies have unraveled the biological principle underlying the protection conferred by an early first full-term pregnancy, demonstrating experimentally that it induces in the breast the expression of a specific signature in response to the differentiation of this organ driven by the reproductive process. This signature serves, in turn, as a biomarker associated with lifetime decreased breast cancer risk. More importantly, we have harnessed this biological principle by demonstrating in an experimental model that a short treatment with human chorionic gonadotropin (hCG), a placental hormone secreted during pregnancy, induces the same genomic signature as pregnancy, inhibiting not only the initiation but also the progression of mammary carcinomas, stopping the development of early lesions, such as intraductal proliferations, and carcinomas in situ (CIS) (Srivastava et al. 1998). These observations indicate that hCG administered for a very short period of time has significant potential as a chemopreventive agent, exerting a long-lasting inhibition of the transforming potential of the normal cell without altering the organ's physiology. This new biological concept implies that when the genomic signature of protection or refractoriness to carcinogenesis is acquired, the hormonal treatment with hCG is no longer required, contrasting with the need of continuous administration of currently used chemopreventive agents for suppressing a metabolic pathway or abrogating the function of an organ (King et al. 2001; Narod et al. 2000).

Epidemiological and Clinical Basis for the New Paradigm

Epidemiological and clinical evidence indicates that endocrinological and reproductive influences play major roles in breast cancer. It has long been known that the incidence of breast cancer

is greater in nulliparous than in parous women (Nix 1964; Kelsey and Horn-Ross 1993; Lambe et al. 1996; J. Russo et al. 1982). Changes in lifestyle, that in turn influence the endocrinology of women, have been observed during the last decades in American women, namely a progressive decrease in the age of menarche (Tanner 1973; Kelsey and Horn-Ross 1993) and a progressive increase in the age at which a woman bears her first child (Lambe et al. 1996). The significance of these changes is highlighted by the reduction in breast cancer risk associated with late menarche and the completion of a full-term pregnancy before age 24, with further reduction in the lifetime breast cancer risk as the number of pregnancies increases (Kelsey and Horn-Ross 1993; Lambe et al. 1996; J. Russo et al. 1982). Women who undergo their first full-term pregnancy after age 30, on the other hand, appear to be at higher risk of breast cancer development than nulliparous women, suggesting that parity-induced protection against breast cancer is related to the timing of a first full-term pregnancy. Pregnancy is also associated with a transient increase relative to nulliparous women, lasting 10–15 years, followed thereafter by a decreased risk; the protection conferred lasts a lifetime (Lambe et al. 1996). Of interest is the fact that women from different countries and ethnic groups exhibit a similar degree of parity-induced protection from breast cancer, regardless of the endogenous incidence of this malignancy (Rao et al. 1994; Coe 1998). This observation suggests that the reduction in breast cancer risk associated with early first full-term pregnancy does not result from factors specific to a particular environmental, genetic, or socioeconomic setting, but rather from an intrinsic effect of parity on the biology of the breast (Apter 1996; Coe 1998; Gaudette et al. 1996; Kelsey and Horn-Ross 1993; Lambe et al. 1996; Rao et al. 1994; J. Russo et al. 1982; Shivers and Miller 1997). Environmental, genetic, and socioeconomic factors, among others, affect the endocrine milieu, indirectly influencing the breast's susceptibility to developing cancer. These observations indicate that an early first full-term pregnancy modifies, through mechanisms still poorly understood, specific biological characteristics of the breast that result in a lifetime decreased risk of cancer development

Experimental Animal Studies: Role of Pregnancy and Chorionic Gonadotropin in Mammary Gland Differentiation and Cancer Initiation

Experimental studies have contributed to clarifying the mechanisms of this protection by demonstrating the role played by pregnancy-induced terminal differentiation of the mammary gland on the susceptibility of the mammary epithelium to carcinogenesis (Nandi et al. 1995; Rao et al. 1994; J. Russo et al. 1979, 1982, 1992; J. Russo and I.H. Russo 1980a, b; 1993, 1994). These observations indicate that the terminally differentiated state of lactation should be reached for attaining protection, although other mechanisms have been proposed for the protective effect of early first full-term pregnancy, including the occurrence of sustained changes in the level or regulation of hormones that affect the breast (I.H. Russo et al. 1991; Sinha et al. 1983). Regardless the intervening mechanism, the end result of the first pregnancy is a dramatic modification of the architecture of the breast (Nandi et al. 1995; I.H. Russo et al. 1991; I.H. Russo and J. Russo 1998; J. Russo et al. 1979, 1982, 1992; Sinha et al. 1983).

The induction of mammary cancer in rodents with a polycyclic aromatic hydrocarbon (PAH) such as 7,12-dimethylbenz(a)anthracene (DMBA) requires that the carcinogen be administered to young nulliparous females (J. Russo et al. 1977). In Sprague-Dawley rats, if the females have completed a full-term pregnancy with or without lactation, prior to carcinogen exposure, carcinoma incidence is dramatically decreased (J. Russo et al. 1979, 1982; J. Russo and I.H. Russo 1980a, b, 1994). The inhibitory effect of pregnancy on mammary cancer initiation can be mimicked in virgin rats by treatment with the glycoprotein placental hormone chorionic gonadotropin (hCG). Administration of hCG as a daily intraperitoneal injection for 21 days, followed by a 21-day resting period prior to carcinogen administration results in a dose-related reduction in tumor incidence and number of tumors per animal (I.H. Russo and J. Russo 1993, 1994; I.H. Russo et al. 1990a, b; J. Russo et al. 1977). This phenomenon is in great part mediated by the induction of mammary gland differentiation, the inhibition of cell proliferation, an increase in the DNA repair capabilities

of the mammary epithelium, decreased binding of the carcinogen to the DNA, and activation of genes controlling programmed cell death (PCD) and of tumor suppressor genes (Alvarado et al. 1993, 1994; Mgbonyebi et al. 1996; I.H. Russo and J. Russo 1994, 1996; J. Russo et al. 1982; J. Russo and I.H. Russo 2000; Srivastava et al. 1998; Tahin et al. 1996). Our results indicate that hCG activates physiological and phylogenetically conserved forms of active cell death, such as PCD or apoptosis, which are associated with specific phases of development that control cell proliferation and differentiation (J. Russo and I.H. Russo 2000). Among the tumor suppressor genes activated by hCG is inhibin, a gene product primarily found in testes and ovary (Alvarado et al. 1993). Inhibin-deficient mice homozygous for the null allele identifies α -inhibin as an important negative regulator of cell proliferation. Our results indicate that inhibin mediates the differentiating action of both pregnancy and hCG on the mammary gland, in which it might act as an autocrine and/or paracrine growth regulator (I.H. Russo and J. Russo 1994).

Role of hCG in Breast Cancer Progression

Our studies of the protective effect of hCG-induced differentiation on experimental mammary carcinogenesis led us to postulate the possibility that hCG might be useful in preventing the development of breast cancer in women. The fact that the time of initiation of breast cancer in the female population is not known represented a major drawback for accomplishing the goal of instituting a truly preventative hormonal treatment. Thus, it had to be assumed that all women are at risk of being the carriers of initiated lesions. This assumption requires that before the hormonal treatment is initiated it has to be proven that it either inhibits the progression of putatively initiated cells, or at least does not cause tumor progression. Based upon our previous observations that the chemical carcinogen DMBA induces neoplastic transformation in the mammary gland by acting on the highly proliferating TEBs of the virgin animal (I.H. Russo and J. Russo 1996; J. Russo et al. 1979, 1982), and that once initiated these structures progress to intraductal proliferations (IDPs) within

3 weeks of exposure to the carcinogen (Srivastava et al. 1998; J. Russo et al. 1982), we tested the effect of hCG on tumor progression by administering 8 mg DMBA/100 g body weight to 45-day-old virgin Sprague-Dawley rats. Twenty days later, when IDPs were already evident, the animals were treated with 100 IU/hCG per day for 40 days (DMBA+hCG group). Age-matched untreated, hCG-, and DMBA+ saline-treated rats were used as controls. Tissues were collected at the time of DMBA administration and at 5, 10, 20, and 40 days of hCG injection, and 20 days after cessation of treatment (I.H. Russo and J. Russo 1996; J. Russo and I.H. Russo 2000).

Mammary Gland Development Under the Influence of hCG

The development of the mammary gland in the rat requires the evaluation of changes in the parenchyma of the gland, since, unlike women, no significant external changes occur in this organ after puberty (I.H. Russo and J. Russo 1996). The

six pairs of the mammary gland of the young virgin rat is composed of ducts ending in club-shaped terminal end buds (TEBs), which are multilayered structures measuring 100–140 μ m in diameter. They are lined by a 3- to 10-layer thick cuboidal epithelium that rests on a discontinuous layer of myoepithelial cells (J. Russo et al. 1977; I.H. Russo and J. Russo 1996; J. Russo and I.H. Russo 1980). After the beginning of ovarian function, the mammary ducts undergo further longitudinal lengthening and branching with sprouting of a few alveolar buds (ABs) that progressively evolve to lobular structures (Fig. 1). The lobules found in the rat mammary gland can be classified according to their degree of development as lobule type 1 (Lob 1), which consists of clusters of approximately 10 ± 4 ductules per unit. Individual ductules are lined by a single layer of cuboidal epithelial cells and few myoepithelial cells. With further growth, Lob 1 evolve to lobules type 2 (Lob 2), which are larger and composed of approximately 40 ± 7 ductules; these progress to lobules type 3 (Lob 3), that contain approximately 60 ± 12 ductules or alveoli

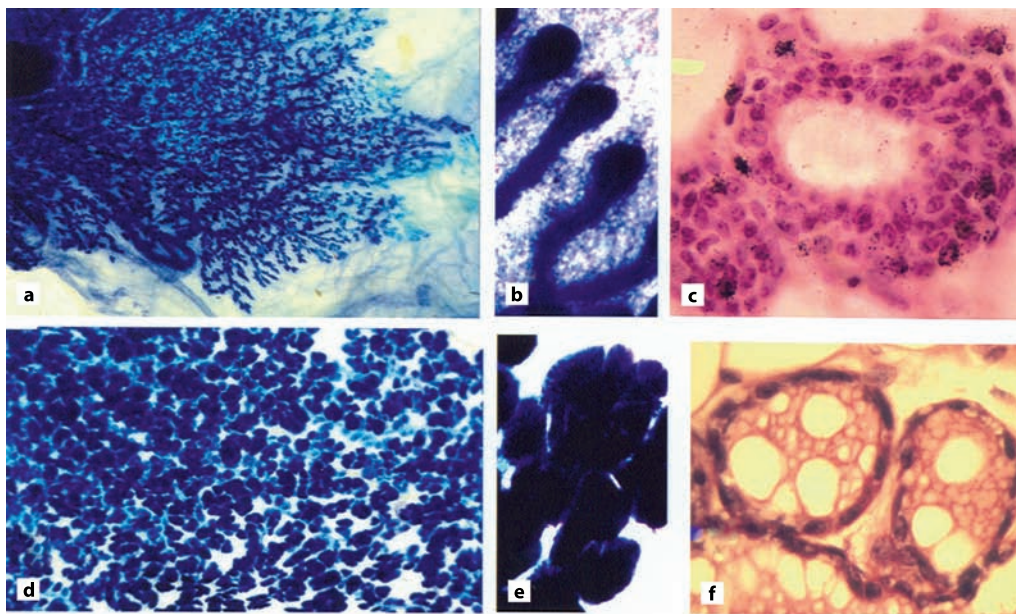


Fig. 1 a Whole mount of a virgin rat mammary gland, 55 days of age (toluidine blue, $\times 2$). b Terminal end buds (TEBs) (toluidine blue, $\times 10$). c TEB is a multilayer structure measuring 100–140 μ m in diameter. The TEB is lined by a three- to ten-layer-thick cuboidal epithelium that rests on a discontinuous layers of myoepithelial cells. d Whole mount of a pregnant rat mammary gland, 75 days of age (toluidine blue, $\times 2$). e Lobule type 3 (toluidine blue, $\times 10$). f Histological section of the ductules of a lobule type 3 containing secretory material (H&E, $\times 40$)

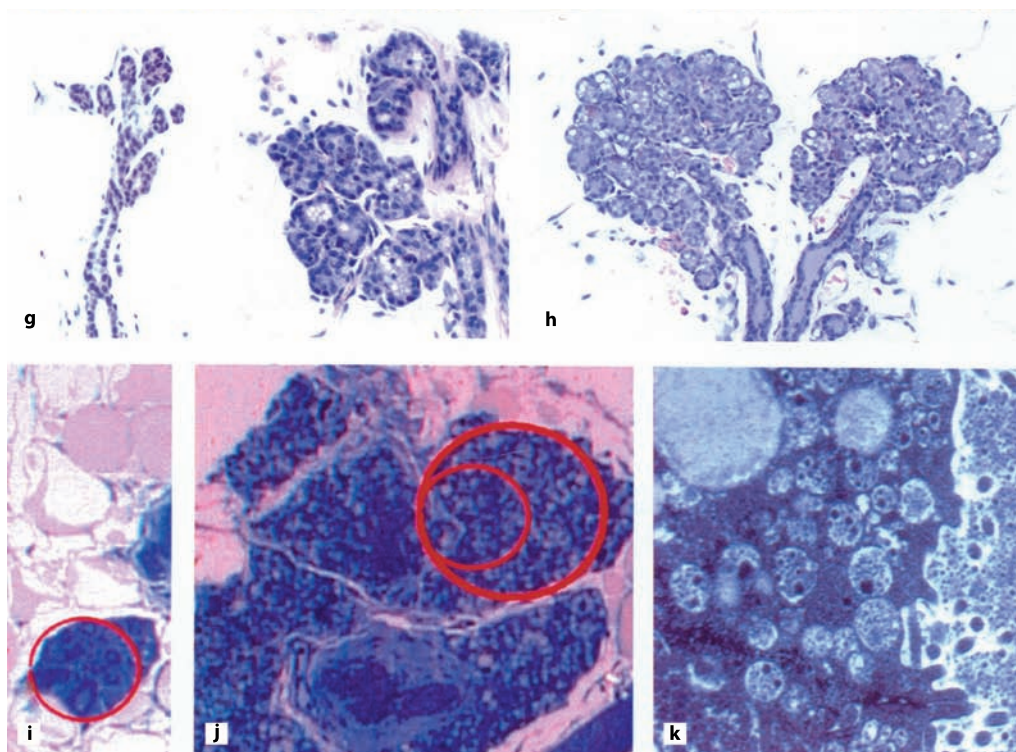


Fig. 1 (continued) g-i The lobules found in the rat mammary gland can be classified according to their degree of development as Lob 1, which consists of clusters of approximately 10 ± 4 ductules per unit (g). Individual ductules are lined by a single layers of cuboidal epithelial cells (ECs) and few myo-ECs. With further growth, Lob 1 evolves to Lob 2, which is larger, and composed of approximately 40 ± 7 ductules (h). Lob 3 contains approximately 60 ± 12 ductules or alveoli per lobule (i); j-l Under the effect of hCG, the mammary gland forms lobules type 4 (j) that are formed by more than 80 ductules per lobular unit containing material in their lumen (k). Electron microscopy section in l, shows the proteinaceous and lipid composition of the milk secretion. (Uranyl acetate and lead citrate, $\times 4,000$)

per lobule (I.H. Russo et al. 1990a). The administration of 100 IU/hCG per day for 40 days to young virgin rats deeply affects the development of the mammary gland, profoundly modifying the relative proportions of Lob 1, Lob 2, and Lob 3. While the concentration of Lob 1 in the mammary gland of untreated or saline-injected control virgin rats decreases slightly as a consequence of aging, in hCG-treated animals the number of Lob 1 decreases slightly by the 10th day of hormonal treatment, and even further between the 20th and 40th days. After cessation of treatment, their number increases sharply, reaching the same values found in control animals. Lob 2 are practically nonexistent in the 45-day-old animals; they first became evident when the animals

reach the age of 75 days, and their percentage increases even further in the next 10 days, reaching its peak in the 85-day-old animals, remaining unchanged thereafter. With hCG treatment, the lobules type 2 develop in a biphasic pattern. Their concentration increases progressively from 70 to 85 days of age, decrease significantly by the time the animals reach the age of 105 days, and increase again after cessation of treatment. Lob 3 formation, on the other hand, starts at the 10th day of treatment, it increases progressively between the 20th and 40th days, decreasing only after cessation of the hormonal treatment due to their regression to Lob 2. The resulting recovery of this type of lobule is absent in control animals.

Effect of hCG on Terminal End Buds, Intraductal Proliferations, and Ductal Carcinomas in Situ

The mammary gland of 45-day-old virgin rats contains the highest number of terminal end buds (TEBs). In animals of the saline control group, the number of TEBs decreased slightly as a function of age, as has been previously described, whereas in the DMBA group their number remained constant. In both hCG-treated groups, a reduction in the relative percentage of TEBs was observed as early as 5 days after the initiation of treatment, and more sharply between the 10th and the 20th days, to reach a plateau thereafter. The percentage of TEBs in these two groups of animals was significantly lower than the values found in the saline control and DMBA groups ($p < 0.01$). A more noticeable effect of the hormonal treatment occurred at the level of intraductal proliferations (IDPs) and ductal carcinoma in situ (DCIS). In DMBA-treated animals, there were 5.80 IDPs per gland when they reached the age of 105 days, that is, 25-fold higher than the values observed in the hCG-treated animals, in which there were 0.23 IDP/gland. These differences were still significant in the 125-day-old animals. The number of DCISs was also higher in the DMBA-treated group, and their number was decreased by 13-fold by hCG treatment. The number of DCISs increased slightly when the animals reached the age of 125 days, averaging 1.76 DCISs/gland; however, it was still significantly lower than that observed in the DMBA group of animals that contained 23 DCISs/gland. Occasional lactating adenomas were observed in both hCG- and DMBA+hCG-treated animals (J. Russo and I.H. Russo 2000).

Effect of hCG Treatment on DMBA-Induced Tumor Progression

While mammary tumors were palpated as early as 25 and 30 days after carcinogen administration in the DMBA+hCG and DMBA groups, respectively, none of the animals in the saline control or the hCG-treated groups developed tumors. In the group of animals treated with DMBA, the number of palpable tumors contin-

ued increasing until the end of the experiment. In the DMBA+hCG group, the number of palpable tumors reached a plateau when the animals were 105 days old, and no additional tumors were detected in the 125-day-old animals. The highest total number of tumors and number of tumors per animal were observed in the DMBA group, while the DMBA+hCG group showed a reduction in the total number of palpable tumors and number of tumors per animal at all the time points studied. The histopathological analysis of both palpable tumors and microscopic lesions revealed that most of them were adenocarcinomas with papillary, cribriform, or comedo features. Only three fibroadenomas developed in the DMBA group and two in the DMBA+hCG group. The hormonal treatment reduced the incidence of adenocarcinomas more noticeably, from 8.3 in the DMBA to 1.8 adenocarcinomas per animal in the DMBA+hCG group (Table 1) (J. Russo and I.H. Russo 2000).

In summary, hCG treatment inhibited the progression of mammary carcinomas by stopping the development of early lesions, i.e., IDPs and carcinomas in situ (CISs). These findings indicated that hCG has a significant potential as a chemopreventive agent not only before the cell is initiated, but also after the carcinogenic process has been initiated and is vigorously progressing. Ours was the first report to indicate that a hormone preventive agent such as hCG is able to stop the initiated cells by inhibiting the formation of the intermediate step represented by the CIS, what ultimately results in a lower incidence of invasive tumors (J. Russo and I.H. Russo 2000).

Direct Effects of hCG on Mammary Epithelial Cells

Inhibitory Effect of DMBA-Induced Mammary Carcinogenesis in Ovariectomized Animals

In order to determine if hCG has a direct effect in the mammary gland, the experimental protocol depicted in Table 2 was utilized. Ovariectomy was performed after DMBA administration (group 3) and compared with intact animals in group 1. As expected, the tumor incidence and the number of tumors per animals were signifi-

Table 1 Effect of hCG treatment on the progression of DMBA-induced mammary tumors

Group/treatment	Age/days of treatment ^a	Tumor incidence			
		No. animals with tumors/total No. of animals ^c	%	No. tumors per animal/total no. tumors ^d	No. AdCa per animal/ total no. Od AdCa ^h
DMBA + saline	70/5	0/11	0.00	0.00/0	0.00/0
DMBA + saline	75/10	4/11	36.36	0.36/4	0.36/4
DMBA + saline	85/20	7/11	63.63	1.45/16	1.45/16
DMBA + saline	105/40	11/11	100.0	7.45/82	7.45/82
DMBA + saline	125/20 ^b	11/11	100.0	8.54/94 ^e	8.27/91
DMBA + hCG	70/5	1/16	6.25	0.06/1	0.06/1
DMBA + hCC	75/10	3/16	18.75	0.18/3	0.18/3
DMBA + hCG	85/20	8/16	50.00	0.87/14	0.87/14
DMBA + hCG	105/40	13/16	81.25	2.00/32 ^f	1.93/31
DMBA + hCG	125/20 ^b	13/16	81.25	1.57/30 ^g	1.81/29

^aAge of the animals (in days) at the time of sacrifice/days of treatment with 100 IU hCG/day

^bTwenty days post-termination of the 40 day-hCG treatment

^cNumber of animals with tumors/total number of animals per group/treatment and age group

^dNumber of tumors per animal/total number of tumors from each specific group/treatment and animal age group

^eThree out of 94 tumors were fibroadenomas

^fOne out of 32 tumors was a fibroadenoma

^gOne out of 30 tumors was a fibroadenoma

^hNumber of invasive adenocarcinomas (Ad-Ca) per animal/total number of adenocarcinomas per group/treatment and animal age group

DMBA 7,12-dimethylbenz(a)anthracene, hCG human chorionic gonadotropin

Table 2 Effect of ovariectomy and hCG treatment in DMBA-induced mammary carcinogenesis

Group	Number of animals	Number of animals with tumor/ number of animals	%	Number of tumors	Tumors/ animal
1. DMBA + saline	18	18/18	100	60	3.30
2. DMBA + hCG	20	9/20	45	20	1.00
3. OV+ DMBA	18	1/18	6	4	0.22
4. OV + DMBA + hCG	20	0/20	0	0	0.00
5. OV + DMBA + EP	18	6/18	33	8	0.44
6. OV+ DMBA + EP+ hCG	20	2/20	10	2	0.10

DMBA 7,12-dimethylbenz(a)anthracene, hCG human chorionic gonadotropin, OV ovariectomy, EP estrogen + progesterone

cantly reduced by ovarian ablation as well as by hCG (groups 2 and 4). Estrogen supplementation in the ovariectomized animals reestablished the tumor incidence and number of tumors per animal (group 5); however, hCG significantly reduced, in those supplemented animals, the number of tumors per animal as well as the incidence (group 6). These data clearly indicate that hCG has a direct effect on the mammary gland independently of ovarian function. This also suggests that hCG could be a tumorigenic agent in postmenopausal women, even in presence of hormone replacement therapy.

Effect on Human Breast Epithelial Cells in Vitro

Treatment of human breast epithelial cells with hCG inhibits the proliferative activity of the cells and induces activation of apoptotic genes. Inhibition of cell growth was observed only in HBEC, whereas the urothelial cells T24 were

not affected by this treatment (Fig. 2). MCF-10F cells exhibited activation of the apoptotic genes TRPM2, ICE, TGF- β , p53, bax, and p21WAF1/CIP1 (Fig. 3). BPI-E cells, derived from BP-transformed MCF-10F cells were also growth-inhibited; however, the pattern of gene activation differed from that exhibited by the parent cells (Fig. 4). BPI-E cells exhibited activation of only ICE, bax, and p21WAF1/CIP1 and significantly downregulated bcl2, but did not modify TGF- β , p53, or c-myc expression. The urothelial cells did not show activation of any of the apoptotic genes. The lack of activation of the genes that control programmed cell death in these latter cells coincides with the selectivity of hCG in the inhibition of in vitro cell proliferation, which was observed only in HBEC, but not in T24 cells (Tahin et al. 1996). This specificity of action might be attributed to a receptor-mediated effect of hCG on human breast epithelial cells, whose presence has recently been reported in rat mammary epithelial cells.

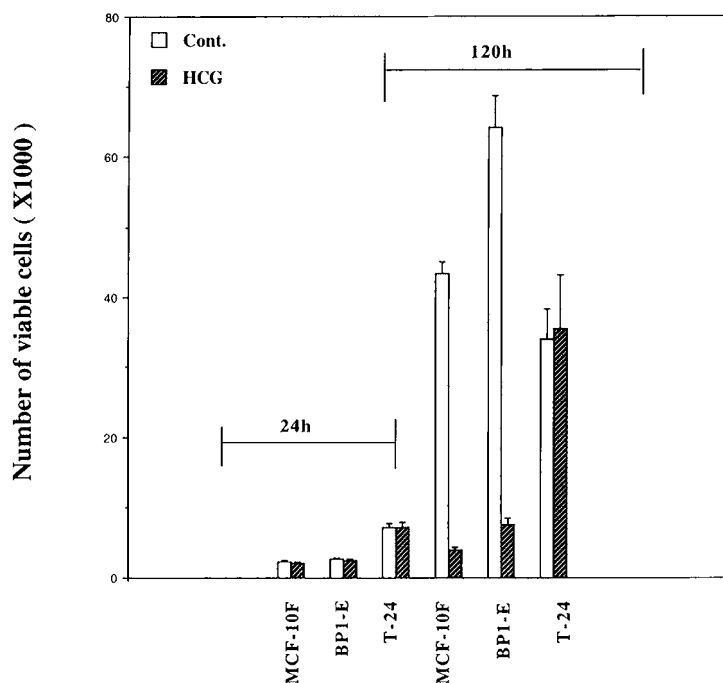


Fig. 2 Effect of hCG treatment on cell growth. MCF-10F, BPI-E, and T24 cells were treated daily with 100 IU/ml hCG and harvested at 24 and 120 h for cell growth determination by WST-colorimetric assay. Control cells were treated with vehicle only. Values represent the mean number of viable cells ($\times 1000$) \pm SD of three wells from two experiments. (Reprinted with permission from: Srivastava et al. 1998)

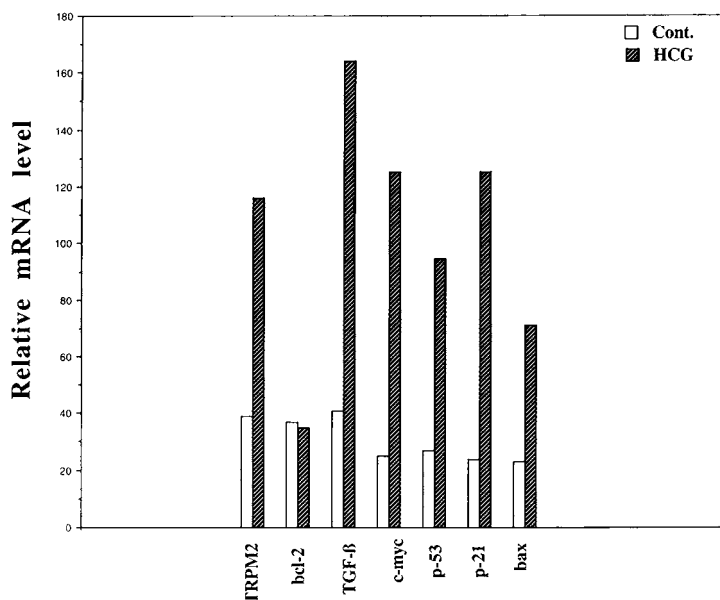


Fig. 3 Histogram showing the expression of TRPM2, ICE, bcl-2, TGF β , c-myc, p53, p21, and bax mRNA relative to their respective controls in MCF-10F cells treated with hCG for 24 h. Relative MRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β -actin. (Reprinted with permission from Srivastava et al. 1998)

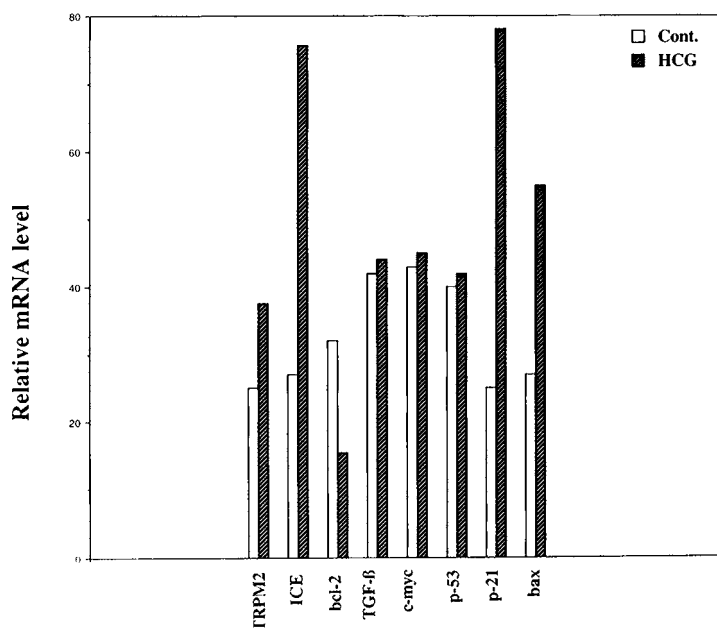


Fig. 4 Histogram showing the expression of TRPM2, ICE, bcl-2, TGF β , c-myc, p53, p21, and bax MRNA relative to their respective controls in BPI-E cells treated with hCG for 24 h. Relative MRNA content was determined by scanning laser densitometry of autoradiographs, and equalized by detection of β -actin. (Reprinted with permission from Srivastava et al. 1998)

Increased expression of TRPM2 and TGF- β genes have been shown during chemotherapeutic regression of a mouse bladder tumor (Buttayan et al. 1989), regressing human breast cancer cells, and in prostatic tumors after hormone withdrawal (Armstrong et al. 1992; Kyprianou et al. 1991). In our experimental model, activation of these genes occurred only in MCF-10F but not in the chemically transformed and T24 cell lines. This observation supports the concept that activation of these two genes might be dependent on specific cell characteristics. The association between the induction of cell growth inhibition and TRPM2 activation has also been reported to be stimulated in MCF-7 cells by 1,25dihydroxyvitamin D3 (Simboli-Campbell et al. 1996). ICE gene expression was increased by hCG treatment in MCF-10F and BPI-E cells by the hormonal treatment. This gene, which belongs to a protease family has been shown to be relevant in the in-

duction of apoptosis (Fig. 5) (Tewari et al. 1995; Fernandes-Ainemri et al. 1995a, b; Harvey et al. 1998). Increases in the levels of ICE (caspase-1) mRNA have been associated with apoptosis in mammary epithelial cells by loss of attachment to extracellular matrix proteins and treatment of some tumor cell lines with chemotherapeutic drugs (Boudreau et al. 1995). Several lines of evidence indicate that the induction of apoptosis can be mediated by both p53 and c-myc, which are the major players in the context of growth arrest and apoptosis (Evan and Littlewood 1993). We have found that hCG treatment significantly induced the expression of p53 and p21WAF1/CIP1 in MCF-10F cells, an observation that suggested that the cell growth arrest was mediated by the tumor suppressor p53 through its downstream target gene p21WAF1/CIP1 (Evan and Littlewood 1993; El-Deiry et al. 1993). Nevertheless, BPI-E cells exhibited an inhibition in their

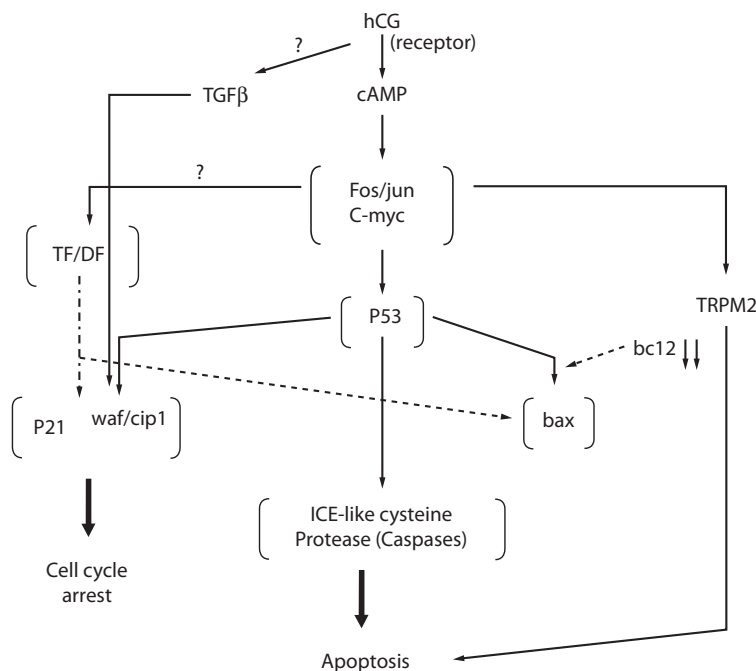


Fig. 5 Postulated model of hCG-induced cell cycle arrest and apoptosis in human breast epithelial cells. In the presence of hCG for 24 h breast epithelial cells bind the hormone to a putative membrane receptor. This triggers a cascade of programmed cell death gene activation through the CAMP-PKA pathway, as well as through activation of TGF- β . HCG treatment activates (upregulates) TRPM2, ICE, TGF- β , p53, and p21 in MCF-10F cells; in BPI-E cells it activates TRPM2, ICE, p21, and bax, but does not activate TGF- β , c-myc, or p53, leading us to postulate that p21 and bax activation in these cells proceeds through an alternative pathway, i.e., TF/DF (bent arrow). (Reprinted with permission from Srivastava et al. 1998)

in vitro growth and induced p21WAF1/CIP1 mRNA, but the expression of c-myc and p53 genes was not modified by the hormonal treatment. These observations might indicate that cell growth and activation of the apoptotic genes have been independently modulated by other genes and/or other external factors. Recent evidence has shown both p53-dependent and p53-independent apoptosis pathways (Sakamuro et al. 1995; Ronen et al. 1996). Thus, our observation that p53 was significantly activated by hCG treatment in MCF-10F but not in BP1-E cells led us to postulate that the activation of apoptotic genes might have occurred through those two different pathways for the inhibition of in vitro cell proliferation (Fig. 5). Our observations suggested that in MCF-10F cells hCG arrested the progression of the cell cycle by inducing (probably through its receptor) the CAMP-PKA and p53, as well as the TGF- β pathways to act on their target gene p21WAF1/CIP1, proceeding then toward cell cycle arrest and apoptosis (Fig. 6). In the case of the chemically transformed cell line, TGF- β , p53, and c-myc did not express any changes in their level of expression, although there was a profound induction of p21WAF1/CIP1 mRNA, thus

suggesting that this gene was induced by hCG independently of p53, probably via transcription factor (TF), differentiation factor (DF) (Fig. 5), as has been shown in other systems (Johnson et al. 1994).

ICE class proteases (caspases) have been shown to play an important role in p53-mediated apoptosis (Harris 1996), though the molecular details are not fully understood. 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. Another possible involvement of p53 in apoptosis is the regulation by members of the bcl2 multiprotein family (Stroebe et al. 1996; Merlo et al. 1997). Some of the members of the bcl2 family such as bcl2 and bcl-XL, are blockers of cell death, while others, e.g., Bax and bcl-XS, are promoters of apoptosis (Stroebe et al. 1996; Merlo et al. 1997). Recent studies have indicated that bax can be activated by both p53-dependent and -independent pathways in different systems (Stroebe et al. 1996). In the present study, hCG treatment induced bax expression in both MCF-10F and BP1-E cells, but it markedly reduced bcl2 expression in BP1-E cells only. The fact that p53, bax, and bcl2 expres-

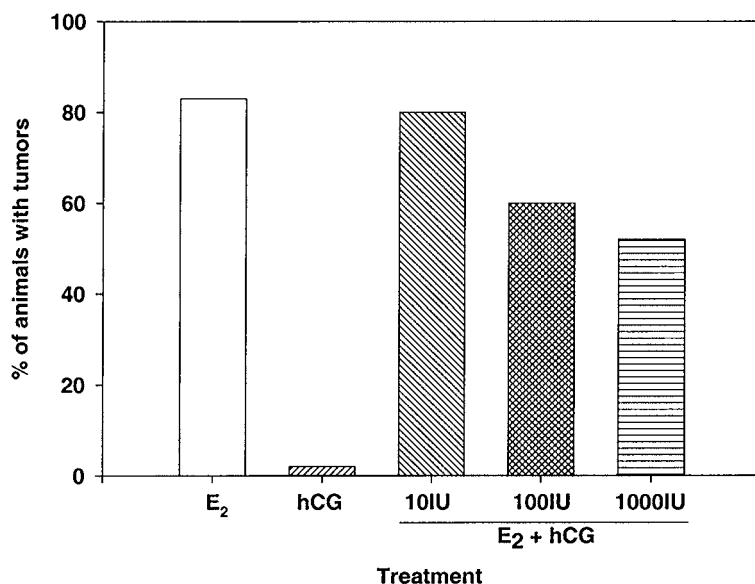


Fig. 6 Effect of hCG treatment on the growth of tumors formed by MCF-7 cells inoculated in nude mice. E₂, animals treated with 17- β -estradiol; hCG, animals treated with hCG alone; E₂+hCG, animals implanted with one pellet of 17- β -estradiol and treated with 10, 100, or 1,000 IU of hCG/day for 50 days. (Reprinted with permission from J. Russo and I.H. Russo 2004)

sion are differently modulated by the hormonal treatment is a strong indication that alternative pathways might be operational in the activation of apoptotic genes by hCG. In these studies, we observed that control cells exhibited an elevation in the level of expression of apoptotic genes. After 120 h in culture, the level of expression of TRPM2, ICE, TGF- β , bax, and p21WAF1/CIP1 genes was increased in MCF-10F. In BP1-E cells, TRPM2, ICE, and p21WAF1/CIP1 were higher at 120 h in culture than at 24 h. The similarities in the activation of gene expression between levels in 24-h-treated and 120-h controls indicate that hCG accelerates the process of gene activation, a phenomenon that has been reported to be associated with confluence (Merlo et al. 1997).

In conclusion, our results demonstrate that the 24-h hCG treatment of immortalized and chemically transformed human breast epithelial cells activates apoptotic genes even before the arrest of cell growth becomes evident. Of relevance is the fact that hCG, which is an inhibitor of *in vitro* cell proliferation and *in vivo* acts as a preventive and tumoristatic agent (I.H. Russo et al. 1990b; Alvarado et al. 1994), may utilize different pathways for activating apoptotic genes, depending upon the degree of expression of neoplastic phenotypes (J. Russo et al. 2001; Calaf and Russo 1993; Mgbonyebi et al. 1997; Albini et al. 1997). Taken together, the results of the present study demonstrate that the growth inhibitory effect of hCG is associated with its ability to activate the expression of apoptotic genes. The importance of our present findings lies in the potential use of hCG as a chemopreventive and chemotherapeutic agent in breast cancer.

Tumoristatic Effect of hCG on Malignant Human Breast Epithelial Cells Transplanted into a Heterologous Host

The observation that hCG had an inhibitory effect on chemically induced rat mammary carcinomas led us to test whether this hormone had an effect on the *in vivo* growth of malignant human breast epithelial cells. For this purpose, MCF-7 cells, a cell line derived from a metastatic breast carcinoma, were injected to Balb/c nude mice (nu/nu). The animals were divided into five groups: the animals of four groups had implanted

a silastic tube containing 5 mg 17- β -estradiol in the interscapular region 5 days after castration, and one group was castrated but did not receive the estrogen supplementation. The cells were injected in the mammary fat pad of mice in all the groups at a concentration of 1×10^6 cells. HCG was administered to the group of animals that did not receive the estrogen at a dose of 1,000 IU/day, and to the three estrogen-supplemented groups at the doses of 10, 100, or 1,000 IU/day. The animals that received estradiol pellets alone had an incidence of 85% tumor formation. The group of animals injected with hCG alone did not develop tumors. Animals that received estradiol pellets and also hCG exhibited a reduction in both tumor incidence and tumor size that were dose-dependent. These studies led us to conclude that the treatment with hCG abrogates the effect of the estrogen growth dependency of MCF-7 cells in a heterologous hosts (Fig. 6).

Genomic Signature Induced by hCG and Pregnancy

RNA was obtained from mammary glands of rats in their 15th and 21st day of pregnancy or hCG treatment, and 21 and 42 days postpartum or post-treatment, respectively. RNAs were analyzed utilizing two membranes for each animal and compared with mRNA of age-matched virgin control rats. RNAs were hybridized to cDNA array membranes that contained 5,800 rat genes (Research Genetics, Huntsville, AL). Cluster analysis was performed using the Jaidexp (Java Analysis information & Data Exploration) specific program, version 1:0 and statistically analyzed. Four clusters of genes were identified (Fig. 7): cluster A shows genes that were over-expressed (threefold) at 15 and 21 days of pregnancy/hCG treatment, but decreased to control values after 21 and 42 days postpartum or post-treatment, respectively. These genes, which included beta casein and alpha lactalbumin, are related to the secretory properties of the mammary epithelium (Mailo et al. 2002). Similar genes were identified using the DD technique, as explained above. Cluster B was composed of genes that were increased threefold at 21 days of pregnancy/treatment and continued rising, reaching the highest peak at 21 days, decreas-

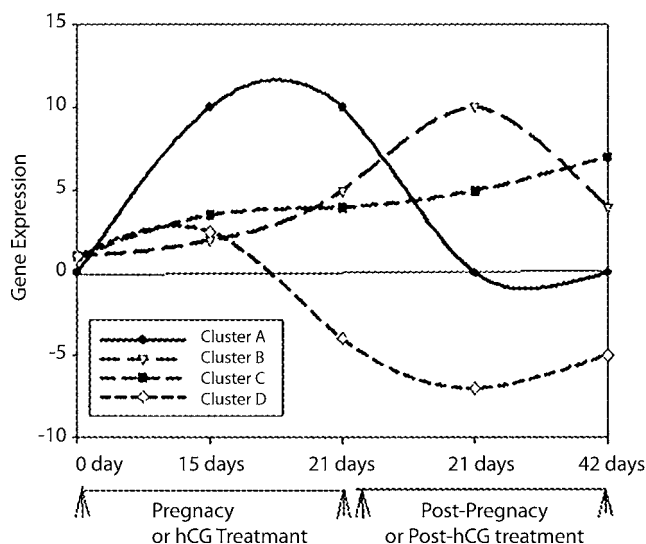


Fig. 7 Cluster analysis of rat mammary gland gene expression during and after pregnancy and hCG. (Reprinted with permission from J. Russo and I.H. Russo 2004)

ing by 42 days postpartum/post-hCG treatment. Among these genes were the fatty acid binding protein, the EST Rn.37635 with high homology to BCL7B gene, catechol-O-methyltransferase, and the EST genes Rn. 5953, Rn.22912, and Rn.4339 (Mailo et al. 2002). The upregulation of catechol-O-methyltransferase is significant because it can be involved in the conjugation of estradiol and catechol estrogens, reducing the carcinogenic effect of these hormones. Genes related to the apoptotic pathways, such as testosterone repressed prostate message 2 (TRPM2), interleukin 1 β -converting enzyme (ICE), bcl2, bcl-XL, bcl-XS, p53, p21, and c-myc were also upregulated from three to fivefold (Mailo et al. 2002) (Fig. 7). We have shown that the activation of programmed cell death genes occurred through a p53-dependent process, modulated by c-myc and with partial dependence on the bcl2-family related genes (Srivastava et al. 1998, 1999; J. Russo and I.H. Russo 2000). In this cluster were also included inhibins A and B, heterodimeric nonsteroidal secreted glycoproteins with tumor suppressor activity (Sun 1984; Vermeulen et al. 1976). We have found that inhibins are not present in the normal resting mammary gland, but are induced by pregnancy or hCG treatment (Alvarado et al. 1993, 1994). We have also shown that hCG has an autocrine or paracrine effect on mammary epithelial cells (J. Russo and I.H.

Russo 2000). HCG also activates the cluster B of genes in DMBA-induced mammary tumors, indicating that this hormone acts through the same pathways for exerting its preventative and therapeutic effects (Fig. 7). Cluster C (Fig. 7) represents genes whose level of expression progressively increased with time of pregnancy or hCG treatment, reaching their highest levels between 21 and 42 days postpartum or after the end of treatment. Among these were known genes such as those coding for a fragment of glycogen phosphorylase, AMP-activated kinase, bone morphogenetic protein 4, and vesicle-associated protein 1 (149). G/T mismatch-specific thymine DNA glycosylase gene, which was observed to be upregulated in the Lob 3 of the human breast, was also increased by fivefold in this model. These data indicate that the activation of genes involved in the DNA repair process is part of the signature induced in the mammary gland by either pregnancy or hCG treatment of virgin animals. These observations confirm our previous findings that in vivo the ability of the cells to repair carcinogen-induced damage by unscheduled DNA synthesis and adduct removal is more efficient in the parous and in the hCG-treated virgin than in the untreated virgin animal mammary gland (J. Russo et al. 1982; Tay and Russo et al. 1981a, b, 1983). Therefore, a principal mechanism mediating the protection from mammary carcinogen-

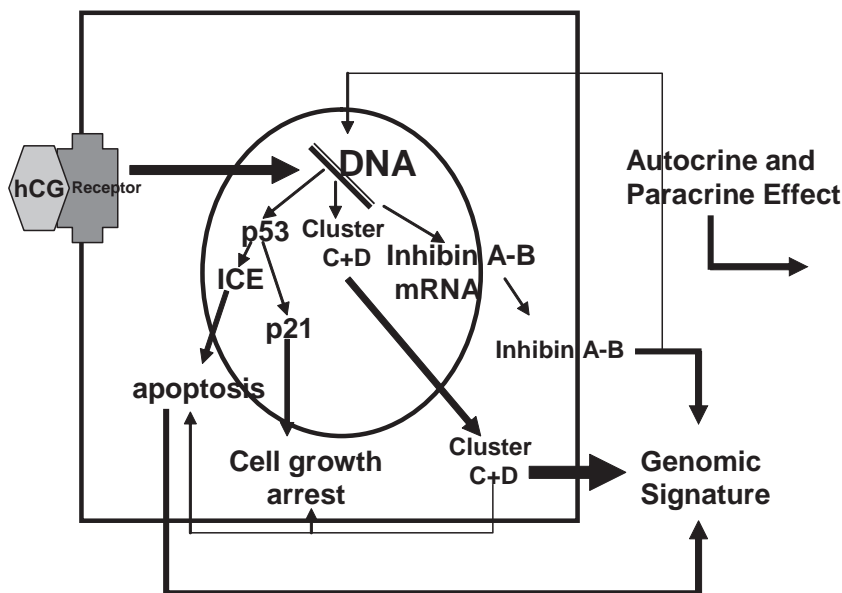


Fig. 8 Postulated mechanism of action of hCG. The hormone binds to a specific membrane receptor, activating genes identified to be specific for pregnancy or hCG-induced differentiation and that have been found to be correlated with the lobular development of the mammary tissue. Thus, a pathway of activation of p53 and ICE may lead to apoptosis or through p21 to cell growth arrest. Activation of inhibin A and B may lead to differentiation through autocrine or paracrine mechanisms. The activation of genes (clusters c and d) are responsible for the refractoriness of the gland to carcinogenesis. (Reprinted with permission from J. Russo and I.H. Russo 2004)

esis conferred by either full-term pregnancy or hCG treatment is the enhancement of the ability of the cells to repair DNA damage, which is in turn the determinant of the lower susceptibility to carcinogenesis. Cluster D consists of genes coding for pro- α collagen III, pro collagen II α 1, BTG1, and thymosin beta 4, which were upregulated more than threefold at the 15th day of pregnancy or hCG treatment, downregulated at the 21st day in both pregnant and hCG-treated animals, and remained downregulated up to 42 days (Mailo et al. 2002) (Fig. 7). Cluster D, in combination with Cluster C, is a component of the signature induced by hCG in the mammary gland (Fig. 8).

These data demonstrate that the genomic signature of the mammary gland induced in virgin animals by exogenous administration of hCG is similar to that induced by pregnancy, and that specific genomic profiles are still manifested by 42 days after termination of treatment. The importance of these specific signatures is highlighted by the fact that administration of car-

cinogen to hCG-treated or control virgin rats whose mammary glands appear morphologically similar will induce a markedly different tumorigenic response, supporting the concept that the differentiation induced by hCG is expressed at genomic level, and results in a shift of the susceptible cells to refractory cells. The permanence of these changes, in turn, makes them ideal surrogate markers for the evaluation of hCG effect as a breast cancer preventive agent.

Unifying Concepts

Based in our knowledge of the pathogenesis of mammary cancer we have tested the effect of hCG hormone on the early phases of tumor progression, namely from TEBs damaged by DMBA to IDPs, in situ carcinomas, and invasive carcinomas, and demonstrate that this hormone inhibits the progression of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors. Treatment of young virgin rats with hCG induced a

profuse lobular development of the mammary gland, practically eliminating the highly proliferating TEBs, with overall reduction in the proliferative activity of the mammary epithelium and induction of the synthesis of inhibin, a secreted protein with tumor-suppressor activity. The hormonal treatment induced differentiation of the mammary gland, which was manifested at morphological, cell kinetic, and functional levels. The morphological changes consisted of progressive branching of the mammary parenchyma and lobule formation. They were accompanied by reduction in the rate of cell proliferation. The functional changes comprised increased synthesis of inhibin, β -casein, and other milk-related bioactive peptides. In addition, hCG increased the expression of the programmed cell death TRPM2, ICE, p53, c-myc, and bcl-XS, also inducing apoptosis and downregulation of cyclins. Programmed cell death genes were activated through a p53-dependent process, modulated by c-myc, with partial dependence on the bcl-2 family-related genes. Of relevance was the observation that lobular development, which reached its maximal expression after the 15th day of hCG treatment, regressing after hormone withdrawal, was preceded by activation of genes associated with the expression of programmed cell death, and furthermore, that the expression of these genes was still elevated 20 days after cessation of treatment. Data generated with the new tools provided by the cDNA microarray techniques have demonstrated that while lobular development regressed after the cessation of hormone administration, programmed cell death genes remained activated, but more importantly a new set of genes (cluster C) reached maximum expression, whereas others (cluster D) are down-regulated. The genes in clusters C and D are those providing the genomic signature that is specific for hCG and pregnancy. The genomic signature is specific for pregnancy and hCG and significantly different from that induced by other hormones such as estrogen and progesterone.

Altogether these mechanisms play a role in the protection exerted by hCG from chemically induced carcinogenesis, and might even be involved in the life-time reduction in breast cancer risk induced in women by full term and multiple pregnancies. The implications of these observa-

tions are twofold: on one hand, they indicate that hCG, like pregnancy, may induce early genomic changes that control the progression of the differentiation pathway; and on the other hand that these changes are permanently imprinted in the genome, regulating the long-lasting refractoriness to carcinogenesis). The permanence of these changes, in turn, makes them ideal surrogate markers of the hCG effect in the evaluation of this hormone as a breast cancer preventive agent.

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