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Hormonal Approach to Breast Cancer Prevention

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Abstract Breast cancer is more frequent in nulliparous women, while its incidence is significantly reduced by full-term pregnancy. The fact that the protection conferred by pregnancy is observed in women from different countries and ethnic groups, regardless of the endogenous incidence of this malignancy, indicates that this protection does not result from extrinsic factors specific to a particular environmental, genetic, or socioeconomic setting, but rather from an intrinsic effect of parity on the biology of the breast. Using an experimental system we have shown that treatment of young virgin rats with human chorionic gonadotropin (hCG), like full-term pregnancy, efficiently inhibits the initiation and progression of chemically induced mammary carcinomas. Treatment of young virgin rats with hCG induced a profuse lobular development of the mammary gland, reduced the proliferative activity of the mammary epithelium, and induced the synthesis of inhibin, a secreted protein with tumor-suppressor activity. HCG treatment also increased the expression of the programmed cell death (PCD) genes testosterone repressed prostate message 2 (TRPM2), interleukin 1-β-converting enzyme (ICE), p53, c-myc, and bcl-XS, induced apoptosis, and downregulated cyclins. PCD genes were activated through a p53-dependent process, modulated by c-myc, and with partial dependence on the bcl-2 family-related genes. The possibility that this hormonal treatment activates known or new genes was tested by differential display technique. We have identified a series of new genes, hormone-induced-1 (HI-1) among them. The characterization of their functional role will contribute to clarify the mechanisms through which hCG inhibits the initiation and progression of mammary cancer. Of great significance was the observation that PCD genes remained activated even after lobular formations had regressed due to the cessation of hormone administration. We postulate that this mechanism plays a major role in the long-lasting protection exerted by hCG from chemically induced carcinogenesis, and might be also involved in the lifetime reduction in breast cancer risk induced in women by full-term pregnancy. The implications of these observations are two-fold: on one hand, they indicate that hCG, as pregnancy, may induce early genomic changes that control the progression of the differentiation pathway, and on the other, 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 hCG effect in the evaluation of this hormone as a breast cancer preventive agent. J. Cell. Biochem. Suppl. 34:1–6, 2000.

Key words: breast cancer; hCG treatment; programmed cell death genes

Breast cancer is the most common malignancy diagnosed in American women, and the number one cause of cancer-related death in non-smokers [Landis et al., 1998]. A major concern in the healthcare community is the gradual and steady increase in the incidence of this disease that has occurred during the last few decades in most Western countries and in societies that have recently become Westernized [King and Schottenfeld, 1996]. While the reasons for this increase are uncertain, epidemiological and clinical evidences indicate that endocrinological and reproductive influences play major roles in this phenomenon [Lambe et al., 1996].

Reproductive Factors and Breast Cancer Risk

It has long been known that the incidence of breast cancer is greater in nulliparous than in parous women, while completion of a full-term pregnancy before 24 years of age effectively reduces breast cancer risk [Kelsey and Horn-Ross, 1993; Lambe et al., 1996; Rao et al., 1994]. Further reduction in the lifetime breast cancer risk has been associated with increasing number of pregnancies [Lambe et al., 1996]. It is of interest to note that the degree of parity-induced protection is observed in women from different countries and from different ethnic

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groups, regardless of the endogenous incidence of this malignancy [Rao et al., 1994]. These observations indicate that the reduction in breast cancer risk associated with early first full-term pregnancy does not result from extrinsic factors specific to a particular environmental, genetic, or socioeconomic setting. Rather it seems to be the result of an intrinsic effect of parity on the biology of the breast, which nevertheless may be modified by environmental, genetic, or other factors [Apter, 1996, Russo and Russo, 1980a,b, 1994; Russo et al., 1977, 1982].

Endogenous Modulators of the Susceptibility of the Mammary Gland to Carcinogenesis

Studies of the pathogenesis of chemically-induced mammary carcinogenesis have demonstrated that the mammary epithelium is susceptible to undergo neoplastic transformation only in the young virgin female, becoming resistant after a full-term pregnancy. These observations have led researchers to hypothesize that the protection conferred by full-term pregnancy is due to a reduction of the susceptibility of the mammary epithelium to carcinogenesis as the result of the terminal differentiation of this organ [Russo and Russo, 1994, 1996]. In women, like in rodents, the differentiation of the breast is a progressive process initiated at childhood that evolves with age and sexual maturation through the elongation and branching of mammary ducts. Lobular development starts at puberty. The formation of lobules evolves from the most undifferentiated lobule (Lob) type 1 present in the breast of young nulliparous females to the more differentiated Lob2, that originates under the cyclic hormonal stimulation of the ovaries. These structures rapidly evolve during pregnancy, forming Lob3, and culminate in the fully differentiated Lob4 present at the end of pregnancy and during lactation [Russo et al., 1992; Russo and Russo, 1996]. There are striking differences in morphology, function, and gene expression among the different lobular types. The Lob1 has a high rate of cell proliferation and a high content of estrogen (ER) and progesterone (PR) receptors, while the more differentiated Lob 2, Lob 3, and Lob 4 show a progressive decrease in both cell proliferation and steroid hormone receptor content [Russo et al., 1998; Russo and Russo, 1998]. Fully differentiated lobules are characterized by the expression of specific gene products, such as inhibin, mammary derived growth inhibitor (MDGI), and serpin [Hu et al., 1997, 1998].

Role of Pregnancy and Chorionic Gonadotropin in Mammary Cancer Inhibition

We have demonstrated that mammary cancer in rodents can be induced with the chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) only in the young nulliparous females. Cancer initiation is inhibited by completion of pregnancy prior to carcinogen exposure. The preventive effect of pregnancy can be mimicked by the exogenous administration of human chorionic gonadotropin (hCG) to virgin rats [Russo J et al., 1979; Russo IH et al., 1991]. Chorionic gonadotropin is a glycoprotein hormone first secreted by the fertilized human egg and later on by the placenta. Its detection in the maternal circulation is the only established way of ascertaining the presence of pregnancy. The main known function of hCG in women is the maintenance of the corpus luteum through its interaction with a receptor shared with the pituitary luteinizing hormone (LH), the lutropin-choriogonadotropin -receptor (LH-CG-R) present in the granulosa and luteal cells of the ovary. Upon interaction with its receptor, CG increases adenylyl 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, with the resulting increases in serum levels of estrogen, progesterone in most species, and of inhibin in women [Russo and Russo, 1994].

Human chorionic gonadotropin obtained from the urine of pregnant women is clinically used for induction of ovulation, among other uses. The fact that its administration to rats inhibits both the initiation and progression of DMBA induced mammary carcinomas led us to test whether the effect of this hormone was specific for hCG, or whether some other compounds also present in the urine of pregnant women were responsible for the effects previously observed in vivo. Towards these purposes we compared vis-à-vis the effects of hCG from various sources with the effect of a recombinant hCG (r-hCG), and in all cases confirmed the similarities among the changes elicited in the mammary glands and ovaries by these various hCGs with those induced by full term pregnancy with lactation. Virgin rats treated for 21 days with a daily intraperitoneal injection of hCG exhibited

a dose-related reduction in tumor incidence and number of tumors per animal, with maximal effect observed with a 100 IU hCG/day [Russo IH et al., 1990a,b,c]. This phenomenon was in great part mediated by the induction of mammary gland differentiation, inhibition of cell proliferation, increase in the DNA repair capabilities of the mammary epithelium, decrease binding of the carcinogen to the DNA, and activation of genes controlling programmed cell death (PCD) [Russo and Russo, 1994; Srivastava et al., 1997].

Role of hCG in Breast Cancer Progression

Our studies on the effect of hCG on the differentiation of the mammary gland led us to postulate the possibility that hCG might be useful for the prevention of cancer development in women. A major drawback for pursuing this goal was posed by the lack of knowledge of when breast cancer initiates in women, what makes it is impossible to determine when to institute a truly "preventative" hormonal treatment. Thus, it had to be assumed that all women are at risk of being the carriers of "initiated" lesions; therefore, any type of treatment has to be proven to inhibit the progression of those putatively "initiated" cells, or at least not to stimulate tumor growth. Based upon our previous observations that the chemical carcinogen DMBA induces neoplastic transformation in the mammary gland through its binding to the highly proliferating terminal end buds (TEBs) of the virgin animal, and that once initiated those structures progress to intraductal proliferations (IDPs) within 3 weeks of exposure to the carcinogen [Russo J et al., 1977, 1979], 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. Treatment with hCG inhibited mammary carcinogenesis by stopping the progression of early lesions, i.e., IDPs and carcinomas in situ (CIS), findings that indicated that hCG has a significant potential as a chemopreventive agent not only before the cells were initiated by the carcinogen, but after the carcinogenic process was vigorously progressing. This is the first report indicating that a hormone preventive agent like 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 [Russo et al., 1996a,b].

Effect of hCG on Programmed Cell Death Gene Expression

Treatment of virgin rats with the placental hormone hCG after the administration of the chemical carcinogen DMBA, as described above, inhibits the progression of mammary carcinomas [Russo IH et al., 1996a,b]. This inhibition has been shown to be associated with the activation of genes known to be responsible of programmed cell death and apoptosis. Northern blot analysis of mammary gland RNA obtained from treated animals using gene specific probes for interleukin-1-β-converting enzyme (ICE), testosterone repressed prostate message 2 (TRPM2), p53, c-myc, and bcl-XS revealed that there was a remarkable induction of these apoptotic genes in the mammary glands of rats treated with hCG, either alone or after DMBA, whereas very little or no significant changes were found in the mammary glands of rats treated with DMBA [Srivastava et al., 1997]. The expression of bcl2, bcl-XL, TGF-α, and TGF-β, on the other hand, was not significantly affected by either hCG or DMBA treatments. The effect of hCG on the activation of programmed cell death genes was specific for the mammary glands, since the hormonal treatment did not modify their expression in the ovary, even though this is the main known target organ of hCG action [Srivastava et al., 1997]. The activation of programmed cell death genes occurred as early as 5 days after the initiation of hCG treatment; they remained activated throughout the treatment period, and some of them were still activated up to 20 days post-cessation of hCG administration. Gene activation was followed by fragmentation of intranucleosomal DNA, detected as the formation of apoptotic bodies, in the mammary epithelium of lobules and in the few tumors that developed. It was concluded that hCG treatment of virgin rats in which the carcinogenic process had been initiated in the mammary gland with the chemical carcinogen DMBA, in addition to inducing differentiation of the mammary gland, it induced activation of programmed cell death genes, cell growth arrest and apoptosis, effects postulated to be p53 dependent, and modulated

by c-myc expression. These results suggest the possibility of the existence of a cell death program that is dependent of the bcl2 family because of the potential involvement of p53, bcl-XS, and Bax in apoptosis [Srivastava et al., 1997].

Effect of hCG Treatment on the Virgin Rat Hormonal Profile

Administration of hCG to young virgin rats either alone or after DMBA treatment raised the serum levels of estrogen and progesterone, while the levels of prolactin, follicle stimulating hormone (FSH), luteinizing hormone (LH), and inhibin were not modified. The increment in estrogen and progesterone levels induced by hCG was accompanied by an increase in the size of the ovaries, due mainly to the enlargement of the corpora lutea. These effects were transient, since ovarian size regressed to normal values as early as 5 days after cessation of the hormonal treatment. The effect of hCG on the mammary gland, however, persisted even after the cessation of hormone administration, an indication that the effect of hCG on the mammary gland persisted and sufficed for differentiating the mammary epithelium and for stopping the progression of the initiated cells. Although the protective effect of hCG was attributed to the differentiation of the mammary gland in great part mediated by elevated levels of estrogen and progesterone, there was also evidence that hCG had a direct effect on the mammary gland, an indication of the presence of an hCG receptor in its epithelium [Russo IH and Russo J, 1994]. In summary, our data indicate that hCG stops the progression of IDPs to carcinomas in situ, and of these to invasive carcinomas. This effect was attributed to the differentiating effect of this hormone on the mammary epithelium, either through a direct effect on the mammary epithelium or an indirect pathway through the ovaries, creating a hormonal milieu that abrogates the progression of the transformation events [Russo IH and Russo J, 1994].

Effect of hCG on Inhibin Synthesis and Early Response Gene Expression

Our observations that the hCG-induced differentiation of the mammary gland is associated with the synthesis of inhibin, a heterodimeric protein that is structurally related to the transforming growth factor-β (TGF-β) family [Alvarado et al., 1993, 1994; Ho et al., 1994], led us to test whether inhibin was also involved in the regression of DMBA-induced rat mammary carcinomas. For these purposes, virgin rats received 8 mg DMBA/100 g body weight when they were 45 days old; 20 days later they were injected daily with 100 IU/hCG for 40 days, as described above. Age-matched untreated, hCG-, and DMBA + saline treated rats were used as controls. Mammary tissues and ovaries were collected at the time of DMBA administration and at 5, 10, 20, and 40 days of hCG injection and 20 days post-cessation of treatment. Total and polyadenylated RNAs were probed for inhibins A and B, c-myc, c-fos, and c-jun. The mammary glands of hCG-treated animals exhibited elevated expression of Inhibin A (1.5- to 4.0-fold) and Inhibin B (1.5 to 3.0-fold), from the 5th day of hCG treatment up to 20 days post-treatment. The expression of these genes was also enhanced by hCG in the DMBA treated group, whereas no changes occurred in the animals treated with DMBA alone. The hormonal treatment markedly increased the expression of c-myc and c-jun by four- to seven-fold and two- to three-fold, respectively. No significant changes were found in the levels of c-fos expression, and DMBA treatment alone did not modify the expression of these genes. Immunohistochemical staining showed a very strong immunoreactivity for inhibin α and β subunits that became evident in the lobular epithelium by the 10^{th} day of treatment, reaching a peak of expression at the 20^{th} day, with a similar pattern of reactivity observed in animals treated with hCG alone or after DMBA. The expression of both subunits remained elevated up to 20 days post-hormone withdrawal, even though the lobular structures had involuted from the well-developed secretory lobules type 3 and 4 to lobules type 2 and 1. The finding that c-myc and c-jun were also elevated at the time of maximal inhibin synthesis indicated that early response genes could be involved in the pathway of hCG-inhibin induced synthesis [Salicioni et al., 1998; Silva et al., 1998].

Effect of hCG on Human Breast Epithelium Cell Cycle Regulators

The observations that the in vitro growth of the normal immortalized and the malignant human breast epithelial cell lines MCF-10F and MCF-7 respectively was inhibited by hCG treatment led us to hypothesize that this hormone might act at the level of specific cell cycle

regulators. We utilized Profasi, an hCG preparation obtained from the urine of pregnant women, for treating the cells with 100 IU hCG/ml for 72, 96, or 120 h. Western blot analysis showed a significant decrease in cyclin D_3; $p27^{KIP1}$ was downregulated by 72 h-treatment; and changes in $p21^{WAF1}$, cyclin A, and its cyclin-dependent kinase (cdk)-2 were downregulated in MCF-10F cells cultured for shorter periods of time. Our observations indicated that the inhibitory effect of hCG on MCF-10F cells was mediated by an alteration in the pattern of protein expression of several cell cycle regulators [Salicioni et al., 1998].

Comparative Effect of hCG and Pregnancy on Gene Expression

Our findings indicate that under in vivo conditions hCG induces a myriad of effects that ultimately result in activation of programmed cell death genes, increased apoptosis, and induction of inhibin synthesis in the mammary epithelium, all phenomena leading to the inhibition of cancer development. Equally important is to determine whether specific genes are differentially expressed in the rat mammary gland of animals treated with hCG or during and after pregnancy. If genes activated by either one or both of these two phenomena remain activated after their physiological effects have ended, it will indicate that an imprinting has occurred in the mammary epithelium, reflecting the higher state of differentiation achieved by this organ after pregnancy or hCG treatment. It remains to be demonstrated whether this imprinting plays a leading role in the inhibition of cancer initiation and progression.

In order to evaluate whether gene activation was a transitory or a long-lasting phenomenon in hCG treated rats, virgin rats that received this hormone for 15 or 21 days were sacrificed after the 15th injection or 40 days after the 21st injection, respectively. Parous animals, used as controls, were either sacrificed at the 15th day of pregnancy or 40 days after weaning of the pups, respectively. Each group was compared with untreated age-matched virgin rats. The permanence of these changes was evaluated by comparison of values obtained at the 15th day of hormonal treatment or pregnancy with those still present 40 days post-cessation of treatment or breast-feeding respectively. The mammary glands of hCG treated animals showed elevated expression of TRPM2 transcripts by 2.5- to five-folds after the 15-day treatment, and remained elevated thereafter. The rate of elevation was similar to that observed in the pregnant animals. The treatment of virgin rats with hCG, as well as pregnancy, induced in the mammary glands the expression of the differentiation genes β-casein and whey acidic protein, two of the major milk proteins in most species, which were absent from the mammary glands of untreated virgin controls [Silva et al., 1998]. The expression of those genes was elevated during both pregnancy and the injection period with the hormone, and it remained activated up to 40 days post-weaning or cessation of treatment respectively. A third cDNA fragment, called hormone-induced 1 (HI-1) was expressed following the same pattern observed in β-casein and whey acidic protein genes. The sequence homology of HI-1 did not match any previously identified genes, appearing to be a novel gene whose function might be related with process of differentiation. Its role in the protection from carcinogenesis is actively pursued in our laboratory. The fact that casein has a strong antimutagenic activity both in vivo in and in vitro [Cassard et al., 1994; van Boekel et al., 1997] indicates that further identification of the functional role of this protein and of others whose synthesis is stimulated by pregnancy and hCG treatment might provide important clues on the mechanisms through which these hormonal and reproductive influences protect the breast from neoplastic transformation.

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