Review

Toward a Physiological Approach to Breast Cancer Prevention

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Abstract

Breast cancer is one of the most frequent malignancies in women, and its incidence is increasing. No reduction in the mortality rate has resulted from advances in early diagnosis and new therapeutic modalities; therefore, new approaches to the understanding of this disease are required. The observation that, in an experimental animal model, full-term pregnancy prior to exposure to a carcinogenic agent protects the mammary gland from malignant transformation led us to study the mechanisms underlying this phenomenon. The protective effect observed after pregnancy did not depend upon gestational or lactational hyperplasia of the mammary gland, but upon structural changes induced in the mammary parenchyma by those processes. Those changes are permanent, since the protective effect is maintained after pregnancy and consists in the complete differentiation of terminal end buds to lobules. The protective effect exerted by pregnancy can be mimicked by treating young virgin rats with a single placent al hormone, chorionic gonadotropin. Since the possibility of preventing breast cancer by treating young nulliparous females with hormones that mimic a full-term pregnancy that results in complete differentiation of the gland is of practical interest to the human female population, we undertook the study of the human breast. The breast of postpubertal nulliparous women is composed of lobular structures reflecting different stages of development. Lobules type 1 (lob 1) are the most undifferentiated ones. Lobules type 2 evolve from the previous ones and have a more complex morphology, being composed of a higher number of ductular structures per lobule. They progress to lobules type 3 and 4, which are present in the pregnant and lactational periods of the mammary gland. In nulliparous women the structure most frequently found at all ages is the lob 1, whereas in parous women, lob 3 is the most frequent one. Lob 1 are considered to be the site of origin of ductal carcinoma in situ, which progresses to invasive carcinoma. Lob 2 originate lobular carcinoma and lob 3 originate adenomas, fibroadenomas, sclerosing adenosis, and apocrine cysts. These observations led us to test if the degree of lobular development influences the transformation of human breast epithelial cells exposed in vitro to chemical carcinogens. We found that primary cultures derived from breast tissues composed of lobules type 1 and 2 express phenotypes of cell transformation which were not observed in cells derived from lobules type 3. These data acquire relevance to the light that women with a history of early pregnancy are at a lower risk of developing breast cancer than nulliparous women, an effect attributed to differences in the degree of differentiation of the breast. Therefore, the stimulus of pregnancy furthers the differentiation of lobules type 1 to lobules type 3 and 4. It is postulated that their more differentiated condition makes them refractory to neoplastic transformation. Overall, the data provide a solid basis for developing physiological means of breast cancer prevention and control.

Current Concepts in Breast Cancer Prevention

Breast cancer is one of the most common neoplasms in women, and its incidence is reaching epidemic proportions in the United States (1). Whereas it is clear that a better understanding of the biology of breast cancer has been accomplished (2), it is a disease with a complexity that is rooted in multifactorial components which comprise the endocrinology of the female, interacting with exogenous factors and lifestyle (3). This complexity has led researchers to place a major emphasis on endogenous and exogenous hormonal factors, the influence of diet, obesity, nulliparity, age greater than 30 years for the completion of the first full-term pregnancy, early menarche, late menopause, and family history as risk factors in breast cancer (3, 4).

Hormones and Breast Cancer

Hormones, especially estrogens, have been linked to breast cancer (4) and their role has been attributed to their ability to stimulate cell proliferation, which in turn leads to accumulation of random genetic errors that result in neoplasia (5). On the basis of this concept, chemoprevention of breast cancer has been mostly aimed at reducing the rate of cell division through administration of antimetabolites (6). The chemopreventive agents most widely considered are antie strogenic compounds such as prostaglandins, tamoxifen, gonadotropin-releasing hormone analogues, and aromatase inhibitors (7). From all these agents, tamoxifen is the one that has been proven to be effective in both primary and advanced breast cancer. Animal studies have shown that tamoxifen interferes with the phases of tumor initiation and promotion (8, 9). Tamoxifen has shown to be species, tissue, and cell type specific (10). In the pubertal rat, tamoxifen is capable of promoting full ductal development in the mammary gland (11). In the mature cycling animal, tamoxifen acts as an antiestrogen causing atrophy of lobular structures (12). In postmenopausal women, tamoxifen treatment results in up-regulation of the proportion of ductal cells expressing estrogen receptor (13). Whereas the use of ta-
tamoxifen has been considered beneficial in reducing blood lipids and bone mineral metabolism, reduction in ischemic disease and osteoporosis (14, 15), a real concern about tamoxifen is that little is known about its effects on normal or premalignant breast tissue (16). The fact that endometrial cancer (17), increased incidence of contralateral breast cancer (18), thromboembolic events (19), ocular toxicity-inducing disabling retinopathy, and keratopathy (20) have been reported raises concerns about the use of tamoxifen as a general chemopreventive agent. Furthermore, the impact of many years of ovarian stimulation by tamoxifen remains to be evaluated (21).

It has been reported that breast epithelial cells undergo variations in their rate of proliferation during the different phases of the menstrual cycle, being relatively low in the follicular phase, when estrogen level prevails, but increasing by a factor of two in the middle to late luteal phase under prevailing progesterone levels. These observations have led researchers to conclude that the combination of both estrogen and progesterone appears to have a greater stimulatory effect than estrogen on the cell division of the mammary epithelium (22–24). This realization led other investigators to postulate the use of gonadotropin releasing hormone analogues that have the property of inhibiting ovulation and decreasing the hormonal levels of premenopausal women to those of postmenopause (25).

**Other Chemopreventive Agents**

Among other chemopreventive agents proposed to be used in breast cancer are monoterpene (26) and retinoids (27–28). The natural and synthetic vitamin A analogues have been shown to inhibit mammary tumors induced by a variety of carcinogens (27, 28). This effect is manifested by a decreased tumor incidence and multiplicity and an increased latency of tumor development (27, 28). At difference of the known cytodifferentiating effect on acute promyelocytic leukemia cells (29), the mechanism of action of retinoids on the mammary gland has not been clearly elucidated. It has been suggested that the chemopreventive efficacy of pharmacological doses of retinoids might relate to their observed inhibition of cell proliferation and the subsequent inhibition of morphological development (28). On the basis of observations in animal models, a randomized clinical trial of retinoid efficacy on breast cancer chemoprevention is currently underway (30).

**Pregnancy as a Protective Factor in Breast Cancer**

Early full-term pregnancy, before age 30 (years), protects the breast from malignancies by reducing in a magnitude of fourfold the overall risk; the protection conferred is for life (3, 31–34). Although the intimate mechanisms by which an early first full-term pregnancy confers protection against breast cancer have not been completely elucidated, its importance lies in the fact that it represents a physiological mechanism which might imprint in the breast special characteristics that make the organ refractory to cancer (35–41). The understanding of this process might have significant implications in our knowledge of the biology of breast cancer and more importantly the development of strategies for breast cancer prevention and cure (36, 37). In our laboratory we have studied the mechanistic basis of breast cancer prevention, first by utilizing the rat as an experimental model and second by studying the developmental pattern of the postpubertal breast and how this pattern relates to the ability of the cells to respond to carcinogens in vitro.

**The Rat as an Experimental Model**

We have determined that differentiation of the mammary gland prior to exposure to a carcinogenic insult protects this organ from malignant transformation (35–37, 42). This postulate is based upon the observation that the administration of the chemical carcinogen DMBA3 to young virgin rats during the period in which the mammary gland contains numerous undifferentiated terminal ductal structures or TEBs actively cleaving into ABs induces the largest number of mammary tumors, mainly in thoracic mammary glands. This period has been designated the “susceptibility window” (Fig. 1) (43–47). The high susceptibility of the TEB to neoplastic transformation is due to the high proliferative rate of its epithelium, as determined by the mitotic and DNA labeling indices (41, 44). These two indices are very high in the TEB and decrease toward the ductal or proximal portions of the gland, and in more differentiated structures such as ABs and lobules (41, 44, 46, 47). TEBs affected by chemical carcinogens enlarge due to epithelial proliferation, progressing to intraductal proliferations (43). Intraductal proliferations become progressively larger and their confluence leads to the formation of microtumors that histologically are classified as intraductal carcinomas and later, adenocarcinomas (Fig. 2). With further growth these tumors develop various patterns, such as cribriform, papillary, and occasionally comedocarcinoma. Benign lesions, such as cysts, hyperplastic alveolar nodules, adenomas, and fibroadenomas originate from more differentiated structures, such as the AB and lobules type 1 and 2 (2, 43, 47–49). The histopathological criteria for the diagnosis of these lesions have been published elsewhere (47).

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1 The abbreviations used are: DMBA, 7, 12-dimethylbenz(a)anthracene; TEB, terminal end buds; AB, alveolar buds; TD, terminal ducts; hCG, human chorionic gonadotropin; DNA-Li, DNA labeling index; HBEC, human breast epithelial cells; BP, benzoylpyrene; NMU, N-methyl-N-nitrosourea; MNNG, methyl-N-nitro-N-nitosoguanidine; lob, lobule.
DMBA-induced mammary carcinogenesis is significantly inhibited in rats which have undergone full-term pregnancy or pregnancy and lactation prior to exposure to the carcinogen (36, 44, 50) (Fig. 2). The protective effect observed after pregnancy does not depend upon gestational or lactational hyperplasia of the mammary gland, but upon permanent structural changes induced in the mammary parenchyma by those processes. These changes consist in the complete differentiation of TEBs to lobules, which show secretory activity during lactation (51). Those glands that between three and nine weeks after weaning have regressed to a pregestational condition do not appear morphologically different from the glands of virgin animals, except for the absence of TEBs, fewer TDs, and more ABs and lobules (36, 38, 39, 43, 44) (Figs. 3 and 4). Administration of DMBA to parous rats when the glands have regressed to such a resting condition induces the lowest incidence of mammary adenocarcinomas (52). The mechanism of the refractoriness of parous females vis-a-vis young virgin and old virgin female rats is due to decrease in cell proliferation (Fig. 5), decreased carcinogen binding, and increased DNA repair capability of the mammary epithelium, all of which are the result of the increased differentiation of the mammary gland (40, 41, 53) (Fig. 6). Whereas other mechanisms for the protective effect of pregnancy, such as maternal stimulation of the immune system with enhancement of the tumoricidal effect of cytotoxic T-cells, have been suggested (54), the observation that the levels of cytotoxic T-cells return to normal values by 42 days postpartum, whereas the protective effect of pregnancy has been detected by 63 days postweaning, indicates that other more permanent mechanisms are operational (40, 41, 44, 51).

On the basis of our studies on the effect of pregnancy as a physiological mechanism of prevention, we have been able to mimic the protective effect exerted by pregnancy by treating young virgin rats with a single placental hormone, hCG (39, 42, 52, 55–58) (Fig. 2). Since the possibility of preventing breast cancer by treating young nulliparous females with hormones that mimic pregnancy but without the need of a full-term pregnancy, is of practical interest to the human female population, we designed experiments geared to answer the following questions: (a) What is the effect of hCG on mammary carcinogenesis? (b) How comparable is the effect of pregnancy and hCG treatment on mammary carcinogenesis? (c) Does hCG exert secondary effects on body weight and endocrine organs? (d) How does hCG treatment affect the mammary gland structure? (e) What effect has hCG treatment on mammary DNA synthesis? (f) Does hCG affect tumor progression? and lastly (g) How does hCG exert its effect on the mammary gland?

**Effect of hCG Treatment on Mammary Carcinogenesis.** The observations that architectural and cell kinetic changes
induced in the mammary gland by pregnancy markedly decrease the incidence of chemically induced mammary carcinomas led us to postulate that differentiation was the underlying mechanism responsible for this protection (36, 42, 43, 46, 59). This hypothesis was confirmed by inducing similar or even greater protection than that afforded by pregnancy through induction of gland differentiation with the placental hormone hCG (36, 42, 51, 55–58). hCG treatment of young virgin rats for 21 days, the length of pregnancy, reduced mammary carcinoma incidence in a dose-dependent manner (36, 42, 43, 46, 59). This hypothesis was confirmed by inducing similar or even greater protection than that afforded by pregnancy through induction of gland differentiation with the placental hormone hCG (36, 42, 51, 55–58). hCG-treated (hCG + DMBA) group virgin rats received 21 doses of hCG 100 international units/day, and were allowed to rest for 21 days prior to DMBA instillation. DMBA + hCG, virgin rats that received DMBA at 55 days of age and 21 days later started receiving 100 international units/daily hCG for 60 days.

![Table](image)

**Fig. 6.** Correlation between type of structure, its DNA-LI, length of the cell cycle in hours (Tc), showing the relative lengthening of Tc due to lengthening of the G1 phase, growth fraction after 5 days of continuous infusion of [1H]thymidine (GF) and nuclear uptake of [1H]DMBA, detected by autoradiography and expressed as the number of grains/nucleus. The lesions depicted in the last column indicate the histological type.

![Histogram](image)

**Fig. 7.** Histogram showing tumor and adenocarcinoma incidence in virgin SD rats treated with DMBA at 55 days of age (DMBA), parous rats (Preg. + DMBA), and animals that following pregnancy and 21 days of rest received DMBA. hCG-treated (hCG + DMBA) group virgin rats received 21 doses of hCG 100 international units/day, and were allowed to rest for 21 days prior to DMBA instillation. DMBA + hCG, virgin rats that received DMBA at 55 days of age and 21 days later started receiving 100 international units/daily hCG for 60 days.

**Fig. 8.** Physiological events in the life span of a woman and their influence in breast development.

Prolonged Protective Effect of hCG Treatment on Mammary Carcinogenesis: Comparative Study with Pregnancy. In order for a hormone preventive agent to protect the breast efficiently, it should inhibit tumor initiation at the same level or better than pregnancy and the protection has to be long-lasting after the termination of treatment. In order to determine whether the protective effect of hCG on mammary carcinogenesis is temporary and decreases with time after the cessation of treatment or is long-lasting like that of pregnancy, 50-day-old Sprague Dawley rats were divided into 3 groups: (a) animals undergoing full term pregnancy; (b) virgin animals receiving 100 international units hCG daily for 21 days; and (c) age-matched virgin control animals. Each group was subdivided into 2 protocols. For Protocol 1, a resting period of 21 days elapsed between termination of pregnancy or hCG treatment and the administration of 8 mg DMBA/100 gbw; for Protocol 2 the three groups of animals were treated as described above, but the resting period was prolonged 63 days after pregnancy or
termination of hCG treatment. Age-matched controls for each subgroup received DMBA only. Tumorigenesis was evaluated 24 weeks postcarcinogen administration (51). Pregnancy and hCG treatment followed by the 21-day resting period prior to DMBA administration significantly depressed both mammary tumorigenesis and carcinogenesis. When the resting period was prolonged to 63 days there was also a significant depression in both tumor and adenocarcinoma incidence. Pregnant and hCG-treated animals still developed significantly fewer tumors than their age-matched controls, indicating that the lengthened time after delivery or termination of hormonal treatment did not ameliorate the protective effect of these two events, at difference of the observed immunological stimulation of the maternal system (51, 54) (Fig. 7).

Effect of hCG Treatment on Body Weight, Estrous Cycle, and Endocrine Organs. It has been shown that human chorionic gonadotrophin has a DNA sequence different from rat CG; however, this difference seems not to affect the biological effect of the hormone (60, 61). hCG treatment modified the estrous cycle by inducing a prolongation of diestrus but cycles became regular by 21 days posttreatment (57). Treated animals did not exhibit significant differences in pituitary and adrenal gland weights and no histological abnormalities were noted in these organs. hCG treatment induced a significant increase (P < 0.01) in ovarian weight and it remained at a higher value than in virgin controls, but the difference at the end of the experiment was not significant. No significant differences in uterine weight were observed between treated and virgin control animals (57). These data indicate that in the rat model the effect of hCG on endocrine organs is reversible.

Effect of hCG Treatment on Mammary Gland Structure. The mammary gland structure of young virgin rats treated with a daily injection of 100 international units hCG was evaluated in whole mount preparations by a count under the stereomicroscope of the number of terminal ductal structures. In control animals the number of TEBs decreased as a function of age, whereas in treated animals there was a sharp diminution between 5 and 15 days of treatment; values reached a plateau thereafter which remained always lower than values in controls. TDs, which in the mammary gland of control animals increased progressively with aging, were decreased by treatment (P < 0.05). The number of ABs in control animals progressively decreased with aging, whereas treatment maintained their number constant between the first and 10th day of injection decreasing sharply between the 10th and 21st days of injection, as the number of lobules started to rise. After cessation of treatment the number of ABs exhibited a recovery due to the regression of newly formed lobular structures, and their number remained significantly higher than in controls up to the end of the observation period. Lobular structures were almost totally absent from the mammary gland at the beginning of treatment, and their number remained almost unchanged in control animals. hCG administration stimulated a burst of lobular formations starting at the 10th day of treatment, reaching a peak by the 15th day. Although their number diminished progressively thereafter, and even more after termination of treatment, it remained slightly higher than in control animals (57). The morphological pattern observed under hCG treatment mimicked quite clearly the effect of pregnancy (51).

Effect of hCG Treatment on DNA Synthesis. Mammary glands collected from animals treated with a daily injection of 100 international units hCG at the times indicated above were processed for DNA-LI determination. The highest DNA-LI was observed in the TEBs of control animals. DNA-LI was considerably lower in ductal and lobular structures present in the same mammary gland (57).

The effect of hCG treatment on the rate of DNA synthesis varied as a function of time of treatment and type of structure considered. Treatment diminished the DNA-LI in TEBs, an effect evident by the fifth day of injection, and more markedly throughout the remainder of the injection period, remaining significantly lower than in control animals up to the end of the experiment (P < 0.001). The response of TDs, ducts, ABs, and lobules to treatment was similar among themselves, and different from that of TEBs; the DNA-LI increased in all of these structures, reaching a peak of maximal activity by the fifth day of injection, then decreased by the 10th day, and more sharply by the 21st day of injection. There was a slight recovery after hormonal withdrawal, but DNA-LI values remained always lower than those of similar structures in control animals. These data also indicate that there is a difference in response for each different compartment of the gland.

Effect of hCG Treatment on Tumor Progression. In order to determine whether hCG inhibits or promotes tumor growth, 100 international units hCG, a dose that induces maximal protection when given before carcinogen administration (51, 57, 59), were utilized for these studies. For this protocol virgin rats received, when they reached the age of 50 days, 8 mg DMBA/100 gbw. Starting 21 days after carcinogen administration, a time at which intraductal proliferations are already present, the animals started receiving 100 international units of hCG for 60 days (40–46). Carcinogen treatment induced 100% tumor incidence in the animals that received DMBA alone, whereas tumor incidence and the number of tumors per animal were significantly decreased in animals treated with hCG after DMBA administration (Fig. 7). No tumors were detected in animals treated with hCG alone or with the vehicle (56).
Our results show that hCG inhibits tumor progression, since treatment with this hormone after carcinogen administration produces a significant reduction in the number of tumors and adenocarcinomas per animal. We concluded that hCG acted on the initiated foci inhibiting their progression, not merely delaying the time of tumor appearance, since the latency period was not modified (57). It is postulated that hCG act on the intraductal proliferation or IDP that is considered to be a preneoplastic lesion in the rat. More studies in this area are required to understand the effect of hCG in these initiated lesions.

**Postulated Mechanism of Action of hCG.** Although up to now it has been accepted that hCG may influence the differentiation of the mammary gland through its effect on the ovary (51, 55-58, 60-70), what is not known is the mechanism whereby hCG significantly decreases overall tumor incidence and tumor burden. The physiological role of hCG is its activity on the granulosa cells of the ovary through a lutropin-choriogonadotrophin receptor (62, 65), although a similar receptor has recently been detected in the thyroid gland (66). In the ovary hCG increases adenylcyclase activity mediated by intracellular membrane associated G proteins which result in cyclic AMP increases leading to steroid and polypeptide hormone synthesis (65). It has been reported that hCG acts as an immunosuppressive agent (67), a mitogenic agent, and a local growth factor or an activator of c-myc and c-fos oncogene (69, 70), which in turn are important in the regulation of cell differentiation and proliferation. Exogeneous administration of hCG affects endocrine organs, the secretions of which directly or indirectly affect the development and function of the mammary gland (57). We do not know if the endocrinological milieu induced by hCG which results in inhibition of tumor progression exerts this effect through inhibition of cell proliferation, induction of cell differentiation, or activation of programmed cell death. Our observation that hCG induces the synthesis of inhibin by the mammary epithelium (71) suggests that this may represent the local regulatory mechanism through which hCG mediates its effect on the mammary gland. This hypothesis is supported by the recently developed inhibin-deficient transgenic mice, in which this deletion results in the development of gonadal tumors, thus demonstrating that inhibin is a secreted protein with tumor suppressor activity (72).

Inhibins are growth factors with structural homology to the transforming growth factor βs and Mullerian inhibiting substance, among others (72). These nonsteroidal glycoprotein hormones are produced by the gonads and the placenta (73-75). They feed back to the anterior pituitary gland to inhibit specifically the production and/or secretion of follicle stimulating hormone (75-80) and in the placenta they regulate the synthesis of hCG (81). The granulosa cells of the ovary in the female have been identified as the site of inhibin synthesis (82, 83). The major form of this protein is a disulfide-linked heterodimer, consisting of an α-chain and one of two highly homologous β chains, designated A and B. The α chain is an M, 18,000 peptide and the β chains are M, 14,000 peptides, which lead to the formation of a M, 32,000 α-β dimer in most species. Depending on the β chain (A or B), inhibin will be named inhibin A or inhibin B (80). Although the highest concentration of inhibin has been found in testes and ovary, it is also expressed in several nongonadal tissues, including brain, pituitary, placenta, and both human and rat mammary epithelium (83-86). The finding of inhibin subunit mRNAs in nonreproductive tissues suggests a role for the inhibin protein outside the reproductive axis as an important regulator of cellular differentiation. The development of gonadal tumors by inhibin-deficient mice homozygous for the null allele identifies α-inhibin as an important negative regulator of cell proliferation (72). Since the synthesis of inhibin by the ovary is stimulated by the gonadotrophic hormones pregnant mare serum gonadotropin and hCG (74, 75, 85, 86), hormones known to have a powerful differentiating effect on the mammary gland (55-58), our work was designed with the purpose of determining whether inhibin played a role in this process. Inhibin was immunocytochemically detected in the mammary gland of virgin and of pregnant control animals and hCG-treated virgin rats using known antibodies against both inhibin A or B (87). The mammary gland of virgin control animals did not show any immunoreactivity (71), whereas under hCG treatment immunocytochemical reactivity was observed in the mammary gland of pregnant and hCG-treated animals, the intensity of the reaction increasing progressively for reaching a peak by the 15th day of either pregnancy or treatment (71, 88). Inhibin or βA were detected by Northern blot of ovaries and mammary glands collected from hCG-treated and control animals (89). Hybridization of ovarian RNA with the α-subunit probe resulted in a single band of hybridization at about 1.5 kilobases. The intensity of the hybridization signal was increased by hCG treatment. Hybridization with the inhibin βA-subunit probe resulted in a predominant signal at 6.7 kilobases, which also showed an increase after hCG treatment. The total RNA of mammary glands hybridized with the α and β subunits, respectively, showing the same 1.5- and 6.7-kilobase bands corresponding to the inhibin α and βA-subunit mRNA present in the ovary (89).

**The Human Breast**

The understanding of the human breast has been a major biological puzzle, mainly due to the fact that the mammary gland seems to be the only organ that is not fully developed at birth (48, 90). No other organ presents such dramatic changes in size, shape, and function as does the breast during growth, puberty, pregnancy, and lactation (48, 90, 91). It is agreed that the developmental phase of the human breast starts as early as the stage of nipple epithelium during embryonic development, continuing steadily with body growth, and undergoing a spurt of growth with lobule formation at puberty (Fig. 8). Four different lobular structures have been characterized in the breast of postpubertal women, each one representing sequential developmental stages (48). Lobules type 1 are the most undifferentiated ones; they are also called virginal lobules, because they are present in the immature female breast before menarche. They are composed of clusters of 6 to 11 ductules per lobule. Lobules type 2 evolve from the previous ones and have a more complex morphology, being composed of a higher number of ductular structures per lobule. They progress to lobules type 3 which are characterized by having an average of 80 ductules or alveoli per lobule; they are frequently seen in the breast of women under hormonal stimulation or during pregnancy. A fourth type of lobule, lobule type 4, has been described as being present during the lactational period of the mammary gland, but it is not found in the breast of nulliparous postpubertal women.
Lobule type 4 is considered to be the maximal expression of development and differentiation (48) (Fig. 8).

The fact that the breast is the source of the most frequent malignancy in the female population, and the knowledge that breast cancer is heavily influenced by the reproductive history of the individual, requires a thorough understanding of the developmental pattern of the breast during the life span of a woman. It is known that women with a history of early full-term pregnancy are at a lower risk of developing breast cancer than nulliparous women (31, 33, 48, 92–96). The protective effect of pregnancy has been attributed to differences in the degree of differentiation of the breast (97). In the comparative study of the pathogenesis of chemically induced mammary carcinomas in experimental animal models with the pathogenesis of human breast cancer (2), it was concluded that the initiation of the neoplastic process, which was inversely related to the degree of differentiation of the mammary gland in the experimental animal, might occur under similar circumstances in women (2, 41, 44–46, 96). The study of the pathogenesis of human breast cancer indicated that the lobules type 1 are the site of origin of preneoplastic lesions such as atypical ductal hyperplasias, which evolve to ductal carcinoma in situ, progressing to invasive carcinoma. Lobules type 2 are postulated to be the origin of atypical lobular hyperplasia and lobular carcinoma in situ (2), and lobules type 3 to be the site of origin of secretory adenomas, fibroadenomas, sclerosing adenosis, and apocrine cysts (2).

These observations indicate that the degree of differentiation or lobular development of the mammary gland influences the type of tumors developed in the human breast (2). Our studies have been aimed at answering the following questions: (a) Which is the influence of age and parity on the development of the human breast? (b) Which are the main morphological differences between the cancerous and noncancerous breast of nulliparous and parous women? and (c) How does lobular differentiation affect the susceptibility of the breast epithelial cells to neoplastic transformation by chemical carcinogens in vitro?

**Influence of Age and Parity on the Development of the Human Breast**

The breast of nulliparous women is predominantly composed of undifferentiated formations, such as terminal ducts and lobules type 1, although occasional lobules type 2 and 3 are present (95, 96). In parous women on the other hand, the predominant structure is the most differentiated lobule type 3. At difference of the lobule type 1, which in the nulliparous woman remains constant throughout the life span, the lobule type 3 in parous women peaks during the early reproductive years, decreasing after the fourth decade of life. In the breast of nulliparous women, lobules type 2 are present in moderate numbers during the early years, sharply decreasing after age 23 (years), whereas the number of lobules type 1 remains significantly higher. This observation suggests that a certain percentage of lobules type 1 might have progressed to lobules type 2, but the number of lobules progressing to type 3 is significantly lower than in the parous woman (Fig. 9). In the case of parous women, it is interesting to note that a history of parity between the ages of 14 to 20 years correlates with a significant increase in the number of lobules type 3, which remain present in the breast as the predominant structure until age 40 (years). After this age, a decrease in the number of lobule type 3 occurs, probably due to their involution to predominantly lobules type 1 (Fig. 10). It has been shown by several authors that postmenopausal involution is accompanied by diminution and atrophy of the parenchymal components (97–100). Among the changes described in the lobular structures is the increase in intralobular fibrosis and hyalinization, which are interpreted as a response to the lack of hormonal stimulation (99). We have observed that lobules type 1 are present in the breast of both postmenopausal nulliparous and parous women, although the lobules type 1 found in the breast of parous women present higher frequency of hyalinization in the intralobular stroma than the lobules of nulliparous women. During the postmenopausal years, both parous and nulliparous women have breasts with a preponderance of lobules type 1. Although ductal breast cancer originates in lobules type 1, or terminal ductular lobular units (2), the epidemiological observation that nulliparous women exhibit a higher incidence of breast cancer than parous women (31, 33) indicates that lobules type 1 in these two groups of women might be biologically different or exhibit different susceptibility to carcinogenesis (101, 102). The presence of lobules type 1 in the breast of parous women has been also interpreted as a failure of the mammary parenchyma to respond to the influence of pregnancy and lactation (23). If this is the case, then parous women could contain in their breast unstimulated as well as regressed lobules type 1. Therefore, the question that remains to be answered is whether the lobules type 1 which are present in the breast of parous women are as sensitive to carcinogenesis as the lobule found in the breast of nulliparous women. Although this question cannot be answered at the present time, it is possible to speculate that the lobules type 1 in the breast of parous women are terminally differentiated structures that are the consequence of a regressive process, as depicted in Fig. 10. This hypothesis is supported by the observation of the presence of intralobular hyalinization in the lobules type 1 of the parous woman's breast, but not in the nulliparous one. In addition, the proliferative activity of the lobule type 1 is lower in those present in the breast of parous women than in those observed in the breast of nulliparous women (103, 104).

The understanding of breast development requires a horizontal study in which all the different phases of growth...
are taken into consideration. For example, the analysis of breast structures at a single given point, i.e., ages 49-53 years, would lead us to conclude that the breast of both nulliparous and parous women appears identical. However, the phenomena occurring in prior years have imprinted permanent changes in breast biology that affect the potential of the breast for neoplasia but are no longer morphologically observable. This horizontal study allowed us to determine that parous women truly underwent lobular differentiation, whereas nulliparous women seldom reached the lobule type 3 stage (Fig. 9). This may further explain why parous women are more protected against carcinogenesis. In parallel studies (see below), it has been shown that lobules type 1, 2, and 3 exhibit different cell kinetic characteristics; lobules type 1 and 2 grow faster in vitro and have a higher DNA labeling index and a shorter doubling time than lobules type 3 (36, 104). They also exhibit different susceptibility to carcinogenesis. Lobule types 1 and 2 express malignant phenotypes when treated with chemical carcinogens in vitro, changes that are not manifested by lobule type 3 (101) (Fig. 11). We have also shown that during the fourth and fifth decades of life there is a decrease in the number of both lobules type 2 and type 3. Since it has been reported that the incidence of atypical lobular hyperplasia decreases significantly with advancing age, it is possible to postulate that the observed diminution in lobules type 2 is responsible for the decreased incidence of this type of lesions (105).

**Differences between Cancerous and Noncancerous Breast of Nulliparous and Parous Women**

The analysis of breasts taking into consideration the presence or absence of cancer in addition to parity, revealed that the breast of nulliparous women free of cancer and of nulliparous women with cancer had a similar architecture, both having as the predominant structure the Lob 1, with a lower percentage of Lob 2 and even lower Lob 3. In both groups the difference in relative percentage between Lob 1 and Lob 3 was highly significant (Fig. 12).

The breast of parous women free of cancer contained the lowest percentage of Lob 1, and a slightly higher percentage of Lob 2. Lob 3 were the predominant structures (40.54%) as opposed to Lob 1 (25.82%) (P < 0.03). Instead parous women with breast cancer had as the predominant structure the Lob 1, whereas Lob 2 and Lob 3 were represented in lower percentages (Fig. 12). Lob 1 versus Lob 3 were significantly different. These results indicated that parous women who developed the disease contained higher numbers of lobules type 1.

The analysis of these samples allowed us to conclude that although parity influences the architecture of the breast, there is a similarity in architecture between the breast of nulliparous women and of parous women with breast cancer. These results confirm our hypothesis that the degree of breast development is of importance in the susceptibility to carcinogenesis and that parous women who develop breast cancer might exhibit a defective response to the differentiating effect of the hormones of pregnancy (96). These findings indicate that the study of breast architecture through the quantitation and characterization of specific lobular types, which are indicators of the greatest level of differentiation achieved by the organ, provides valid endpoints for assessing breast differentiation. These parameters would, in turn, allow researchers to assess the risk of the gland to undergo neoplastic transformation when exposed to given genotoxic agents. In addition, they can be utilized as intermediate endpoints for assessing the effectiveness of chemopreventive or hormone-preventive agents.

**Lobular Differentiation and the Susceptibility of Breast Epithelial Cells to Transformation by Chemical Carcinogens in Vitro**

Although progress has been made in defining some of the critical processes contributing to carcinogenesis, the specific biochemical and molecular mechanisms underlying many of these complex phenomena remain to be elucidated. Recent studies support the hypothesis that the development of cancer involves the activation of protooncogenes and the inactivation of genes which function as inhibitors of carcinogenesis (106-111). Whereas understanding of the mechanism of action of both oncogenes and tumor suppressor genes is mandatory to mechanistically define the neoplastic process, it is also vitally important to understand the biological sequence of events that drive a normal HBEC to neoplastic growth, under the action of known chemical carcinogens. A major stumbling block in the understanding of HBEC carcinogenesis is the fact that breast cells, as other human diploid cells, rarely become immortal, meaning...
acquire an infinite life span, and full transformation by carcinogens alone has not been achieved reproducibly (112, 113). A likely explanation is that cells usually senesce before they can acquire the multiple changes required for immortalization and transformation. Thus, an important first step in developing in vitro transformation systems for HBEC is to understand the mechanism of cell immortalization.

**Influence of Degree of Mammary Gland Development and of Cell Proliferation on the Growth Rate of Breast Primary Cultures.** On the basis of our previous observations that the susceptibility of the mammary gland to neoplastic transformation is related to its degree of development and proliferative activity (2), we studied 15 reduction mammoplasties by determining the degree of breast development, measured based upon the type and number of lobules present (48). This study allowed us to classify the mammoplasty specimens into two types: (a) poorly differentiated breasts, those composed of lobules type 1 and 2; and (b) well differentiated breasts, those composed almost exclusively of lobules type 3 (40). Portions of the same breasts were utilized for measuring the rate of DNA synthesis or DNA-LI by in vitro incorporation of [3H]thymidine and for studying the in vitro growth characteristics of organoids obtained by digestion of the tissue prior to plating. Breasts composed predominantly of lobules type 1 and 2 had a DNA-LI of 1.03 ± 0.48. Cells derived from these samples attached to the culture dish immediately with a high number of doublings (0.64 ± 0.47). Breast tissue composed almost exclusively of lobules type 3, with a DNA-LI of 0.05 ± 0.05, had a significantly lower number of doublings (102). These data indicate that the properties of the cell in vivo are maintained in in vitro conditions and reflect the condition of the host. The proliferative activity of the breast varies depending upon the phase of the menstrual cycle; although there is great variation between specimens, the geometric means of cell proliferation has been shown to be consistently lower in the follicular than in the secretory phase (6, 22, 23). However, no studies have correlated the variations in cell proliferation during the menstrual cycle with the location of proliferating cells within specific lobular types. Since lob type 1 (terminal ductal lobular units), the site of origin of ductal carcinomas, has significant differences in cell proliferation with lobules type 2 and type 3 (2, 48), the use of cell proliferation as an intermediate endpoint for assessing the effect of chemopreventive agents requires determining the exact location of the cells in which proliferative activity is measured. The added advantage that cell kinetic characteristics of the gland observed in vivo are also reflected in the behavior of the cells in vitro validates the usefulness of the in vitro models for testing the response of the mammary epithelium to hormones or genotoxic agents.

**Response of Human Breast Epithelial Cells in Primary Cultures to Carcinogen Treatment.** Based upon the rationale mentioned above, we tested the effect of chemical carcinogens known to induce mammary tumors in rodents (2) on primary cultures of HBEC obtained from reduction mammoplasties. Normal breast tissues were digested for estimation of lobules type 1, 2, or 3, which were plated in culture medium. When the cells formed monolayers, they were passed and then treated with the following carcinogens: DMBA, NMU, MNNG, and BP. BP and DMBA require metabolic activation, whereas NMU and MNNG are direct acting carcinogens (2, 101, 103). After the second or third passage posttreatment, the cells were seeded in agar metho-

![Early Pregnancy or HCG](cancerepidemiology.org)

**Fig. 13.** Lob 1 and lob 2 are the susceptible targets for different etiological events that will lead them to neoplastic transformation. It is postulated that early pregnancy or hCG treatment may lead lob 1 and lob 2 towards the differentiation pathway.

Conclusions and Future Directions

Our experimental system has allowed us to determine that pregnancy induces differentiation of the mammary gland, which results in protection of this organ from chemically induced carcinogenesis. The stimulus of pregnancy can be simulated in virgin animals by treatment with exogenous hormones, mainly the placental hormone hCG. The novelty of our studies is the evidence that hCG protects the mammary gland against carcinogenic initiation and progression, mimicking the physiological process of pregnancy, without crippling other reproductive or endocrine functions (Fig. 13). The importance of both differentiation
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