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Role of Hormones in Mammary Cancer Initiation and Progression

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Breast cancer, the most frequent spontaneous malignancy diagnosed in women in the Western world, is a classical model of hormone dependent malignancy. There is substantial evidence that breast cancer risk is associated with prolonged exposure to female hormones, since early onset of menarche, late menopause, hormone replacement therapy and postmenopausal obesity are associated with greater cancer incidence. Among these hormonal influences a leading role is attributed to estrogens, either of ovarian or extra-ovarian origin, as supported by the observations that breast cancer does not develop in the absence of ovaries, ovariectomy causes regression of established malignancies, and in experimental animal models estrogens can induce mammary cancer. Estrogens induce in rodents a low incidence of mammary tumors after a long latency period, and only in the presence of an intact pituitary axis, with induction of pituitary hyperplasia or adenomas and hyperprolactinemia. Chemicals, radiation, viruses and genomic alterations have all been demonstrated to have a greater tumorigenic potential in rodents. Chemical carcinogens are used to generate the most widely studied rat models; in these models hormones act as promoters or inhibitors of the neoplastic process. The incidence and type of tumors elicited, however, are strongly influenced by host factors. The tumorigenic response is maximal when the carcinogen is administered to young and virgin intact animals in which the mammary gland is undifferentiated and highly proliferating. The atrophic mammary gland of hormonally-deprived ovariectomized or hypophysectomized animals does not respond to the carcinogenic stimulus. Administration of carcinogen to pregnant, parous or hormonally treated virgin rats, on the other hand, fails to elicit a tumorigenic response, a phenomenon attributed to the higher degree of differentiation of the mammary gland induced by the hormonal stimulation of pregnancy. In women a majority of breast cancers that are initially hormone dependent are manifested during the postmenopausal period. Estradiol plays a crucial role in their development and evolution. However, it is still unclear whether estrogens are carcinogenic to the human breast. The apparent carcinogenicity of estrogens is attributed to receptor-mediated stimulation of cellular proliferation. Increased proliferation could result in turn in accumulation of genetic damage and stimulation of the synthesis of growth factors that act on the mammary epithelial cells via an autocrine or paracrine loop. Alternatively estrogens may induce cell proliferation through negative feedback by removing the effect of one or several inhibitory factors present in the serum. Multidisciplinary studies are required for the elucidation of the mechanisms responsible for the initiation of breast cancer. Understanding of such mechanisms is indispensable for developing a rational basis for its prevention and control.

KEY WORDS: Rat mammary carcinomas; steroid hormones; growth factors; antiestrogens; antiprogestins; pregnancy; differentiation.

INTRODUCTION

Breast cancer is the neoplasm most frequently diagnosed in American women. It is responsible for 17 percent of female cancer deaths, and is the number one cause of cancer-related death in non-smoking women (1,2). These dismal statistics are aggravated

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by the facts that the incidence of this disease is steadily rising in most western societies and in Asian countries traditionally known to have a low incidence of breast cancer (3–5), and by the lack of a clear understanding of the cause of this worldwide increase.

Breast cancer was recognized to be a hormone-dependent malignancy as early as 1896, when Beatson reported that the removal of the ovaries caused the regression of disseminated breast cancer (6). The combined clinical and experimental studies of Huggins led him to determine that both hormone deprivation and hormone supplementation had therapeutic value (7). Other factors have been discovered over the years that modify the risk of developing breast cancer. Increased risk is associated with family history of breast or breast/ovarian cancer (8), nulliparity or late first full term pregnancy, early menarche and late menopause, exposure to ionizing radiation at young age and high socioeconomic status (9–13). Although the cause and the time of initiation of the carcinogenic process are not known, epidemiological, clinical, and experimental data have identified the period between menarche and the first full term pregnancy as a "window" of critical importance in the lifetime risk of developing breast cancer (9–15). Thus, a shortening of this "window", such as occurs with late menarche and early full term pregnancy, confers protection, whereas lengthening, i.e., early menarche, late full term pregnancy or nulliparity, increase breast cancer risk (9–12). That window also represents a period of maximal susceptibility to external agents, such as ionizing radiation, that also increase cancer incidence (13).

The understanding of the complex interactions involved in the initiation and progression of breast cancer requires the design of studies in which all possible variables are carefully controlled. Experimental animal models provide the ideal conditions for exploring the role of chemicals, radiation, viruses or genomic mutations (7,8,13,14–23) on the risk of mammary cancer development and allow researchers to dissect the roles of hormones in the neoplastic process. Mammary tumors induced by administration of the chemical carcinogens 7,12-dimethylbenz(a)anthracene (DMBA)[3] or N-methyl-N-nitrosourea (NMU) to young virgin rats (9,14–19) are the two most widely utilized experimental systems. Despite differences in their mechanisms of action, e.g., DMBA is a polycyclic hydrocarbon that requires metabolic activation, while NMU is a direct acting alkylating agent that preferentially induces a G-A mutation and activation of the Ha-ras oncogene, the type and the incidence of mammary tumors, the number of tumors per animal, and the latency period of the tumors induced by these two carcinogens are similar (9,14–19,22,23).

Chemically induced mammary tumors are, like the majority of human neoplasms, hormone-dependent adenocarcinomas, a characteristic that makes them ideal models for the analysis of the role of hormones in the development of breast malignancies (16,17,19–23). In this review we analyze the effects of natural and synthetic estrogens, progestins and their antagonists on chemically-induced mammary cancers. The main questions addressed are how these hormones affect the initiation and progression of mammary neoplasias, and whether these findings relate to epidemiological observations on cancer incidence trends. We also explore the important issue of whether hormones could act as either initiators or promoters of neoplasia, an issue that is still far from being solved (9,23). Since the study of experimental animal models has shown that the level of development and differentiation of the mammary gland at the time of exposure to a carcinogen determines the tumorigenic response elicited (9,13,16), the endocrine influences that control the normal development of the gland are briefly described later.

INFLUENCE OF SEX AND FETO-MATERNAL HORMONES ON MAMMARY GLAND DEVELOPMENT

The development and function of the mammary gland are under the influence of a multiplicity of genetically determined endocrine factors that might act as either stimulators or inhibitors (9,24,25). The development of the mammary gland exhibits sex-related differences during embryogenesis, an indication that the rodent mammary rudiment is sensitive to the influences of gonadal steroids during the prenatal stage (9,24). The maternal environment also constitutes a rich source of hormones and growth factors during embryonal and fetal life, and after birth the newborn is exposed to a variety of endogenous and exogenous maternal hormones secreted in the milk (9,25–28). The role that these factors play in the development, function

[3] Abbreviations: estrogen receptor (ER); progesterone receptor (PR); terminal end bud (TEB); alveolar bud (AB); 7,12-dimethylbenz(a)anthracene (DMBA); N-methyl-N-nitrosourea (NMU); growth hormone (GH); epidermal growth factor (EGF); medroxyprogesterone acetate (MPA).

and neoplastic potential of the human breast remains to be investigated.

The development of the mammary gland in the female is rigorously controlled by the ovary (25,29). Although puberty is often considered to be the point of initiation of ovarian function, the development of the ovary is, in fact, a gradual process initiated several years earlier under the control of pituitary gonadotropins (9,29–33) (See Fig. 1). In the rat ovarian development has been divided into four major phases: 1) the neonatal period, from birth to day seven; 2) the infantile period, from days 8 to 21 of life; 3) the juvenile period, between 22 and 32 days, and 4) the peripubertal period, encompassing the next three days of life (9,29). During the neonatal period the mammary gland is characterized by the lengthening and branching of a primary main lactiferous duct into secondary ducts. The length and the number of ducts progressively increase with the age of the animal. By the second week of life the ducts have sprouted up to a sixth generation of branches that end in club-shaped terminal end buds (TEBs) composed of 3 to 6 layers of medium-sized epithelial cells. The number of TEBs is maximal when the rats are 21 days old. After the beginning of ovarian function TEBs begin to cleave into 3 to 5 smaller buds or alveolar buds (ABs). (9,28). Continuous estrous cycles, initiated when the animals reach the age of 32 to 42 days, stimulate the progressive differentiation of TEBs into ABs and primitive lobules, also called lobules type 1. In the virgin female this process continues

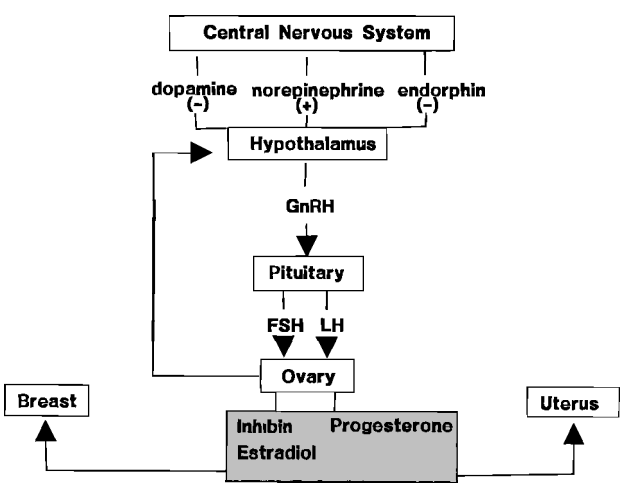
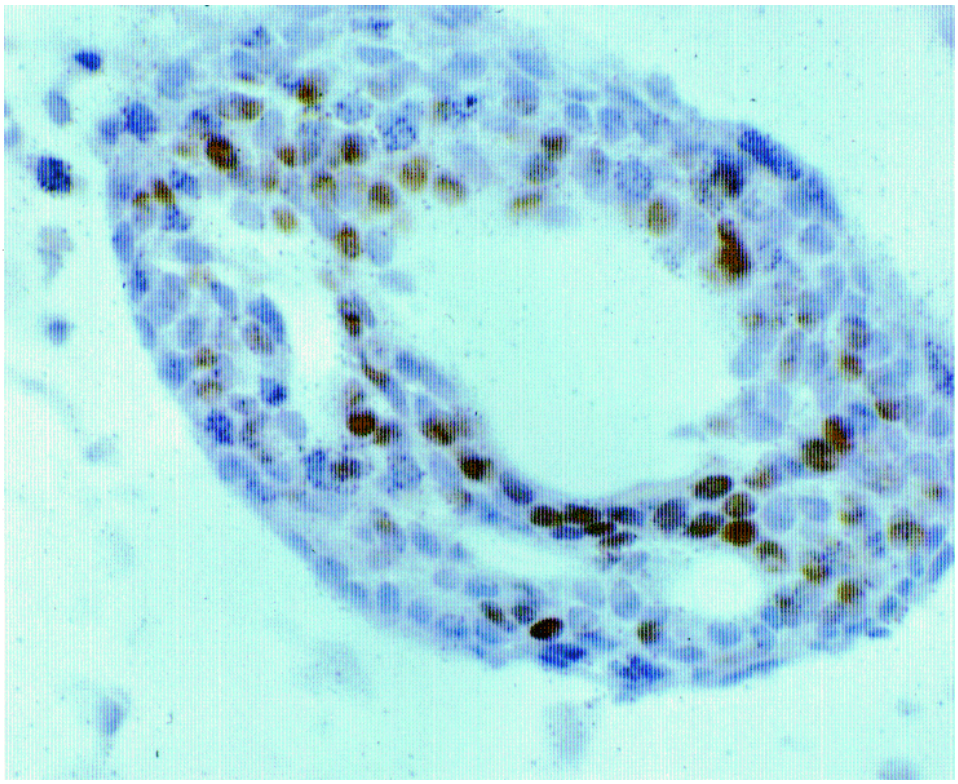


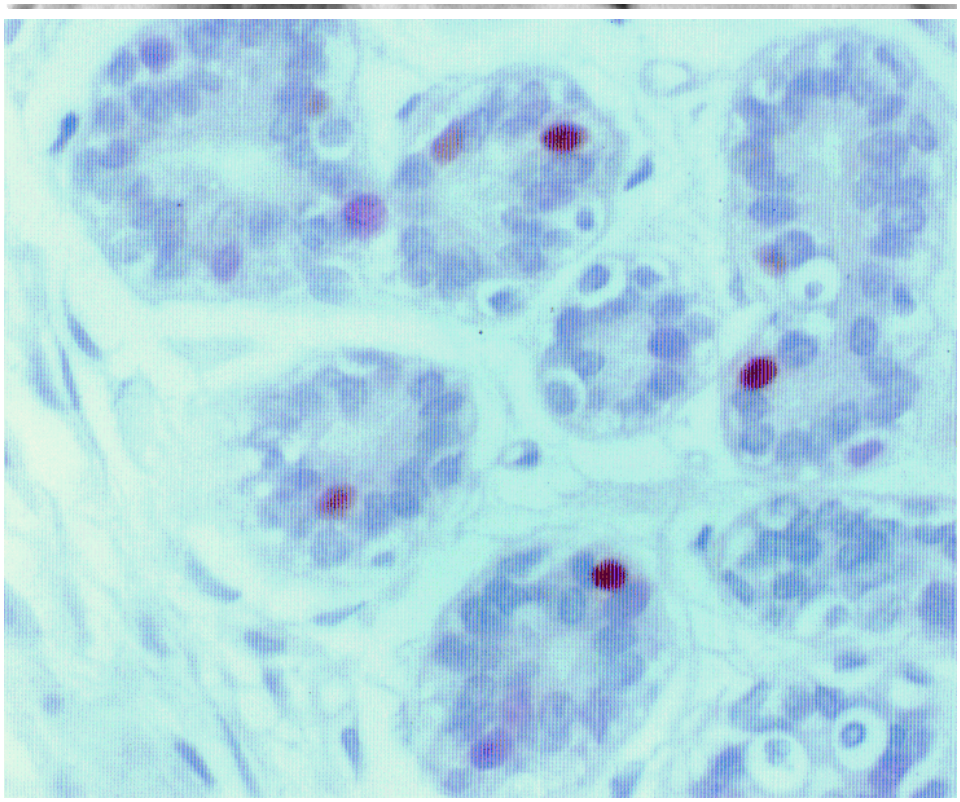
Fig. 1. The central nervous system-hypothalamus interacts with the pituitary through secretion of gonadotropin releasing hormone (GnRH). Secreted follicle-stimulating hormone (FSH) and luteinizing hormone (LH) act on the ovary, which in turn stimulates the breast and the uterus.

during sexual maturity, but after 70 days of age the parenchymal structures start a regressive process. TEBs retain their basic club-shaped morphology, but become smaller. When the diameter of TEBs is less than 100 μm they are called terminal ducts (TDs). As the animal ages the number of TEBs decreases while that of TDs increases. The number of ABs and lobules either remains stationary or slightly decreases by the time the animal reaches the age of 180 days (9).

Ductal branching and elongation occurring during puberty are positively regulated by pituitary growth hormone (GH) (31,34). Although the exact mechanism of action of this hormone is still unclear, it has been reported that it directly stimulates ductal growth in hypophysectomized-ovariectomized rats (31), possibly acting on a plasma membrane receptor (34), or it might well act through its local mediator, insulin-like growth factor 1 (IGF-1) (9,27,31). Normal duct development, however, requires the presence of estrogen (20) and progesterone (21). Estradiol has been shown to act locally in the mammary gland, stimulating DNA synthesis and promoting TEB formation. In the rat mammary gland both estrogen (ER) and progesterone (PR) receptors are present in the epithelial cells lining ducts, TEB and AB (35). We detected simultaneously the presence of ER or PR and of proliferating cells in the mammary gland of virgin rats that had been inoculated with ^{3}H-thymidine one hour prior to sacrifice by combining immunocytochemical techniques and autoradiography. This procedure revealed that most of the cells that incorporated ^{3}H-thymidine during DNA synthesis were hormone receptor negative (Fig. 2). Radiolabeled cells were in general located more to the periphery or basal portion of the ductal epithelium, while the receptor positive cells were more internal. ER positive cells could be seen in close proximity cells synthesizing DNA (Fig. 2), an indication that they represented two different cell populations (35). These observations support the hypothesis that estrogens stimulate secretion of epidermal growth factor (EGF) or other growth factors of mammary or extramammary origin, that might bring about epithelial proliferation (26,27).

It is also known that the prevailing metabolic condition of an individual animal or human may significantly influence mammary gland response to hormones (9,36). The response of the mammary gland to these complex hormonal and metabolic interactions results in developmental changes that permanently modify both the architecture and the biological characteristics of the gland. The mammary gland, in turn, responds selectively to given hormonal stimuli,





depending upon specific topographic differences in gland development, which modulate the expression of either cell proliferation or differentiation (9,37). Thus, administration of DMBA to young virgin rats when the epithelium of TEBs exhibits a high rate of cell proliferation, high growth fraction, and short cell cycle results in a greater uptake of DMBA and less efficient removal of DMBA-DNA adducts. These processes lead to the development of a maximal tumorigenic response (9,16,37).

MAMMARY GLAND DEVELOPMENT AND THE INITIATION OF CANCER

Mammary cancer in experimental models is the result of the interaction of a carcinogen with the target organ, the mammary gland. This target, however, is extremely selective, since only specific structures in the mammary gland respond to the carcinogenic insult. Knowledge of the architecture and cell kinetic characteristics of the mammary gland at the time of carcinogen administration is a necessary initial step to understanding the pathogenesis of the disease. It is also essential in the study of early lesions if those changes induced by the carcinogen are to be distinguished from normal variations in gland development (9,14–16).

The susceptibility of the mammary gland to DMBA- or NMU-induced carcinogenesis is strongly age-dependent. It is maximal, reaching 100% incidence with multiple tumor development per animal, when any one of these carcinogens is administered to intact and regularly cycling virgin females between the ages of 40 to 60 days, that is, soon after vaginal opening and during early sexual maturity, coincident with the period in which the mammary gland exhibits a high density of TEBs (9,14,15). As stated earlier a high rate of proliferation of the glandular epithelium is characteristic of that period, in which there is also a high rate of DMBA activation. The significance of the latter is uncertain, since NMU, which is also most effective at the same age, does not require activation (9,16). The largest number of transformed foci develops when DMBA is administered to virgin rats at the point when TEBs are decreasing in number due to differentiation into ABs. After DMBA administration these structures, instead of undergoing normal differentiation become progressively larger due to proliferation of the luminal epithelial cells. The epithelium becomes multilayered, there is secondary lumen formation and small papillae project into the widened lumen. These early lesions are called intraductal proliferations (9,16). Their confluence leads to the formation of microtumors that histologically are classified as adenocarcinomas. Initially intraductal; these progress to invasive tumors, developing various patterns such as cribriform, comedo, or papillary types (9,14,16,37). Administration of DMBA to older virgin rats elicits a tumorigenic response that is inversely proportional to the age of the animal at the time of carcinogen exposure. Older animals have a much lower incidence of adenocarcinomas and more tubular adenomas, some of which exhibit focal areas of malignant transformation, giving origin to well differentiated adenocarcinomas with a tubular pattern (16,37). Other benign lesions, such as adenomas, cysts, and fibroadenomas arise from mammary gland structures that were more differentiated at the time of carcinogen administration. These observations indicate that the carcinogenicity of DMBA depends on an adequate structural target. The type of lesion that develops is, in turn, dependent upon the state of differentiation of the portion of the mammary gland affected by the carcinogen. Thus, the more differentiated the structure at the time of carcinogen administration the more benign and organized is the lesion that develops (9,14–16,37).

EFFECT OF STEROID HORMONES AND ANTI-HORMONES ON THE INITIATION OF MAMMARY CANCER IN VIRGIN RATS

The response of the mammary gland to a carcinogenic insult depends on the animal's age, reproductive

Fig. 2. *(opposite top)* Terminal end bud (TEB) in the mammary gland of a 45 day-old Sprague-Dawley rat. The animal received 5 μCi/ ^{3}H-thymidine per gram body weight one hour prior to sacrifice. Sections of paraffin-embedded tissues were incubated with photographic emulsion for autoradiography (silver grains), and with anti-progesterone receptor (PR) for immunocytochemistry. PR positive nuclei appear brown. Proliferating cells show stippling. Sections were lightly counterstained with hematoxylin. (×40). (Reprinted from Russo *et al.,* (35) with permission).

Fig. 6. *(opposite bottom)* Section of paraffin-embedded breast tissue of a 33 year old nulliparous woman containing a lobule type 1 doubly reacted with Ki 67 and anti-ER antibody. Ki 67 positive cells stain purplish red, ER positive cells brown. (Reprinted from Russo *et al.,* (35) with permission).

history and endocrinological milieu. Ovarian estrogens, critically involved in mammary gland development, are also essential for eliciting a tumorigenic response with chemical or physical carcinogens (9,14,15). The peripubertal period of gland development is characterized by an active proliferative activity of TEBs and high DMBA activation. Estrogens when administered at low doses stimulate the growth of DMBA and methylcholanthrene induced mammary tumors. However, the development and growth of these adenocarcinomas are inhibited by high doses of 17β-estradiol, estriol, estrone, and diethylstilbestrol (7,9). This biphasic effect might be mediated by prolactin, whose secretion is stimulated by low and inhibited by large doses of estrogens (31,38). Ovariectomy either prior to or very soon after methylcholanthrene or DMBA administration suppresses rat mammary carcinoma development; it also inhibits tumor growth or even causes tumor regression in rats already bearing tumors. Tumor growth can be reactivated by the administration of moderate levels of 17β-estradiol or diethylstilbestrol (7,9,39). For the rat mammary epithelium to be transformed, however, estrogens have to act in combination with the pituitary prolactin, which is essential, since no tumors are induced when the estrogens are administered following hypophysectomy. The stimulatory effect of prolactin on DMBA-induced mammary carcinomas and the inhibition of tumor growth by suppression of prolactin secretion have been well-documented (40–43).

There is considerable evidence that in the rat estrogens are mammary carcinogens because of their stimulatory effects on prolactin secretion (9,31,40–43). The role of these two hormones in tumor growth has been greatly clarified through the use of antiestrogens, such as tamoxifen, ICI 164,384 and ICI 182,780 to examine the mitogenic effect of estrogens on tumor cells (44). The antiestrogen most widely utilized in clinical settings is tamoxifen, which acts as an estrogen antagonist by blocking the ER function. Its role, however, is controversial, since in ovariectomized rodents it acts as an estrogen agonist, stimulating the growth of the uterus and of the mammary ductal tree, respectively. In rats it induces liver tumors, and patients treated with tamoxifen have an increased incidence of endometrial carcinoma (44). ICI 164,384, and ICI 182,780, that completely block all stimulatory effects of estrogens, are considered to be pure antiestrogens (44). Tamoxifen alone or in combination with the retinoid all trans-N-(4 hydroxyphenyl)-retinamide (4-HPR) reduces the incidence of NMU-induced mammary tumors in Sprague-Dawley rats (45,46). Both tamoxifen and ICI 164,384 exert an inhibitory effect on DMBA-induced rat mammary tumors (44). The efficiency of these compounds, however, is lower than that of ovariectomy, an effect attributed to the dependence of rat mammary tumors on pituitary prolactin, whose secretion is not inhibited by these antiestrogens (44).

Progesterone is one of the main hormones produced by the corpus luteum (47). Its role in both normal mammary gland development and in mammary carcinogenesis has not been definitively clarified. Progesterone is considered to be a breast mitogen in premenopausal women, while RU 486, a synthetic progesterone antagonist that binds with great affinity to the progesterone receptor, inhibits PR-mediated growth in breast cancer cells (48). Progesterone increases the frequency of both methylcholanthrene- and DMBA-induced mammary tumors when implanted in either intact or ovariectomized female rats, but does not do so in ovariectomized-adrenalectomized rats (9,39). Chronic administration of progesterone to neonatally androgenized rats at varying times after DMBA treatment causes a marked increase in development and growth of mammary carcinomas; however, moderate to high doses of this hormone in combination with high doses of estrogen inhibit the growth of DMBA-induced rat carcinoma (7,9). The latter treatment causes intense hyperplasia in the normal rat mammae and also ovarian atrophy. It is curious that a moderate dose level of progesterone, by itself a mammary tumorigen, coupled with estrogen at high dose levels, provides a therapeutic hormonal milieu considerably more efficacious than treatment with estrogen alone at high doses (7,9). It is important to notice that moderate dose of progesterone and/or estrogen (natural or synthetic) can enhance the growth of polycyclic aromatic hydrocarbon-induced rat mammary carcinomas. The synthetic progestin medroxyprogesterone acetate (MPA), an effective agent in the treatment of advanced breast cancer, has glucocorticoid, androgenic and progestational activity (48). We have observed that MPA exerts a dual effect on the proliferative activity of the virgin rat mammary gland (49). When 0.5 mg or 5.0 mg MPA pellets were implanted subcutaneously in the interscapular region of animals ranging in age from 35 to 75 days, MPA stimulated the proliferative activity of TEBs in the very young and in the old animals, 35 and 75 days old respectively. This effect was associated with a greater tumorigenic response to DMBA administration 21 days after pellet removal (49). Those ani-

mals that were between 45 and 60 days of age at the time of MPA pellet implant exhibited a lower proliferative response, a greater degree of differentiation and a lower tumorigenic response that the young and old animals (49). The proliferative response was also dose-related, with a greater rate of cell proliferation in TEBs and greater tumorigenic response in those animals receiving the 5.0-mg pellet (49). These observations indicated that both the proliferative or the differentiative response of the mammary gland to this progestational agent was modulated by the priming of the mammary gland epithelium by ovarian estrogens and probably by other growth factors. MPA has also been reported to enhance the tumorigenic potential of DMBA in CD2F mice (50), but it inhibits DMBA-induced mammary tumors when administered to Sprague-Dawley rats alone or in combination with the antiestrogen tamoxifen (51).

INFLUENCE OF PREGNANCY ON MAMMARY GLAND ARCHITECTURE, CELL PROLIFERATION AND RESPONSE TO CARCINOGENS

The most dramatic changes in gland development occur during the first pregnancy, which represents a completely novel endocrinologic experience to the female organism. The earliest hormonal changes occur during cervical stimulation at mating that result in increases in prolactin levels and in the number of ovarian luteal LH receptors, leading to enhanced steroidogenesis (29). The fertilized egg becomes a new endocrine organ soon after conception. Both the developing embryo and the placenta represent a rich source of hormones and growth factors, which enter the maternal bloodstream and influence multiple target organs (9). Early in pregnancy, the combined influences of ovarian estrogen, progesterone, and inhibin (33), with the production of placental rat chorionic gonadotropin (rCG) and placental lactogen (rPL), and pituitary relaxin contribute to stimulate the mammary glands to undergo active cell proliferation (9). Greater activity is observed in TEBs, which rapidly cleave forming ABs, thus diminishing their number. ABs, in turn, progressively differentiate into lobules (9,28). In early pregnancy the estrogen receptor content of the mammary gland becomes significantly higher than that of virgin rats. Immediately following parturition, the estrogen receptor content of the mammary gland increases significantly over a period of two to four days, coinciding with a period of increased DNA synthesis (52). The prolactin receptor content also increases in the mammary gland during lactation, as the levels of prolactin remain elevated (53). Lactation, which lasts up to three weeks, delays the reinitiation of the estrous cycle and ovulation in postpartum rats (29). The mammary gland increases in weight during the first 24 hours post-weaning, declining thereafter. The involution of the mammary gland is accompanied by profound morphological and physiological changes. The secretory alveolar structures collapse, with active removal of cells and secretions by macrophages. There is an increase in the lysosomal enzymes acid phosphatase and aryl sulfatase in both epithelial cells and macrophages (9). The appearance of the mammary gland several weeks post weaning is similar to that of virgin animals, except for the absence of TEBs, fewer terminal ducts and more ABs and lobules (16). All of these structures exhibit a diminished rate of cell proliferation and growth fraction, with a concomitant lengthening of the cell cycle, mainly of the G_1 phase in comparison with similar structures present in the mammary gland of young and age-matched virgin animals (16). The DNA of mammary epithelial cells of parous animals has a lower capacity to bind DMBA and more efficient repair capabilities, all characteristics that potentially play an important role in diminishing the susceptibility of the mammary gland to transformation by a carcinogenic agent (9,14,16,37).

The structural and cell kinetic changes described earlier exert an inhibitory effect on the initiation of chemically induced mammary carcinomas (16,22,54–57). The incidence of mammary carcinomas induced by administration of DMBA during pregnancy is progressively reduced from 96% in virgin controls to 20%, 11%, and 0% in those animals injected with DMBA at the 5^{th}, 10^{th}, or 15^{th} days of pregnancy respectively (22). If DMBA is administered either 21 or 63 days after delivery without lactation a significant depression in tumor incidence in comparison with untreated virgin controls ensues (57). Even though the hormonal levels of estrogen, progesterone and prolactin are identical at the time of carcinogen administration (55), the incidence and number of tumors, namely adenocarcinomas, per animal are significantly reduced in parous animals in comparison with untreated controls (56,57).

EFFECT OF STEROID HORMONES AND ANTIHORMONES ON CANCER PROGRESSION

Analysis of the effect of hormones on cancer progression requires precise determination of the time of exposure to the carcinogenic insult. This requirement can easily be met in the experimental animal model. The fact that in humans the time of cancer initiation is not known makes it necessary, when planning endocrine therapies, to assume that tumors in the breasts of all women have been initiated by a given, though still unknown, carcinogenic stimulus.

Studies of the pathogenesis of DMBA-induced rat mammary carcinomas indicate that cell transformation is manifested morphologically by 21 days after carcinogen administration through the detection of intraductal proliferations (9,14,16,37). The progression of TEBs to intraductal proliferations, and of these to *in situ* and invasive carcinomas are influenced by the same hormones that have been proven to affect its initiation, and probably through similar mechanisms (7,9,22). Dehydroepiandrosterone, an adrenal 17-ketosteroid precursor of testosterone and 17β-estradiol, is a potent inhibitor of NMU induced mammary carcinogenesis, an effect attributed to the induction of differentiation of the mammary parenchyma by this agent (58). MPA has a partial inhibitory effect on DMBA-induced tumor progression, and its effect seems to be inversely related to serum levels of prolactin (59). Estrogen priming seems to enhance the inhibitory effect of this synthetic progestin (59).

HORMONES AND THE DEVELOPMENT OF THE HUMAN BREAST

An important concept in breast development is that this is one of the few organs of the body that is not completely developed at birth, reaching full differentiation only under the stimulus of the hormones of pregnancy and lactation (9,14,16,37). Our studies of the development of the breast as a function of a woman's age and reproductive history showed that the breast of postpubertal nulliparous women is composed of lobular structures reflecting different stages of development. The lobule type 1 (Lob 1), also called terminal ductal lobular unit (TDLU), is the most undifferentiated structure; it is composed of clusters of 6 to 11 ductules per lobule (Fig. 3). It evolves to lobule type 2 (Lob 2) (Fig. 4), which has a more complex morphology, being composed of a higher number of ductules per lobule. Both are present in the breast of mature nulliparous women. During pregnancy, Lob 1 and Lob 2 rapidly progress to lobules type 3 (Lob 3) (Fig. 5), which are characterized by having an average of 80 ductules or alveoli per lobule, and to lobules type 4 (Lob 4), which become present only during the last trimester of pregnancy and the lactational period (9,60). After weaning the parenchymal structures of the mammary gland regress to Lob 3 and Lob 2, acquiring the appearance of Lob 1 after menopause. Lob 1 is the structure most frequently found in the breast of nulliparous women of all ages (50–60% of structures). Second in frequency is the Lob 2 (30–35%), while Lob 3 are the least frequent constituting 5–10% of the total lobular composition (60). In the breast of parous women, on the other hand, Lob 3 predominate, comprising 80 to 100% of the total lobular component. These findings acquire relevance in light of the fact that women with a history of an early pregnancy have a 0.5 relative risk (RR) of developing breast cancer in comparison with nulliparous women (RR = 1.0), an effect attributed to differences in the degree of differentiation of the breast (12,14,37,60). The Lob 1 and Lob 2 present in the breast of nulliparous women also exhibit a proliferative activity higher than that of Lob 3, whereas the breast of parous women, which contains the more differentiated Lob 3, also exhibits a low rate of cell proliferation (60).

Study of the pathogenesis of breast cancer in relation to the lobular composition of the breast led us to identify the Lob 1 or TDLU as the site of origin of the most frequent breast malignancy, the ductal carcinoma (16,37,60). As was observed in the pathogenesis of DMBA-induced rat mammary carcinomas (17), it is possible to postulate that in women the progressive differentiation of lobular structures might be associated with the development of neoplastic lesions with different malignant potential, depending upon the level of differentiation of the site of origin (9,14,16,17).

Even though the breast is also influenced by the myriad of hormones and growth factors briefly outlined for the rodent experimental model, estrogens are considered to play a major role in promoting the proliferation of both the normal and the neoplastic breast epithelium (35,61). The mechanism of action of estrogens has been excellently described in other papers in this issue, therefore no further detail will be added in this manuscript. From the three most frequently

Fig. 3. Whole mount preparation of breast tissue of an 18 year old nulliparous woman showing lobules type 1. Toluidine blue (×25).

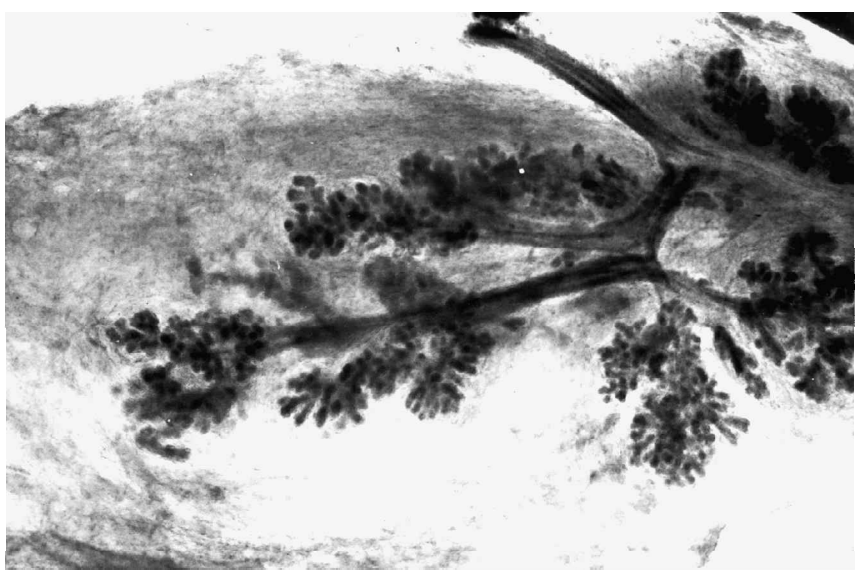


Fig. 4. Whole mount preparation of breast tissue from a 24 year old nulliparous woman showing lobules type 2. Toluidine blue (×25).

accepted mechanisms of estrogen action on the proliferative activity of mammary epithelial cells, a receptor-mediated, an autocrine/paracrine loop, or a negative feedback, according to which estrogens remove the effect of one or several inhibitor factors present in the serum (26,61–63), we concentrated our efforts in determining the distribution of ER in the various lobular compartments of the breast with the objective of correlating the presence of ER with the proliferative activity of each specific type of lobular structure. We collected normal breast tissues from reduction mammoplasties performed in 61 women, ranging in age from 26 to 55 years. Sections of paraffin embedded tissues were incubated with anti-human ER antibody, and in the same samples the proliferative activity was determined using an antibody recognizing the nuclear

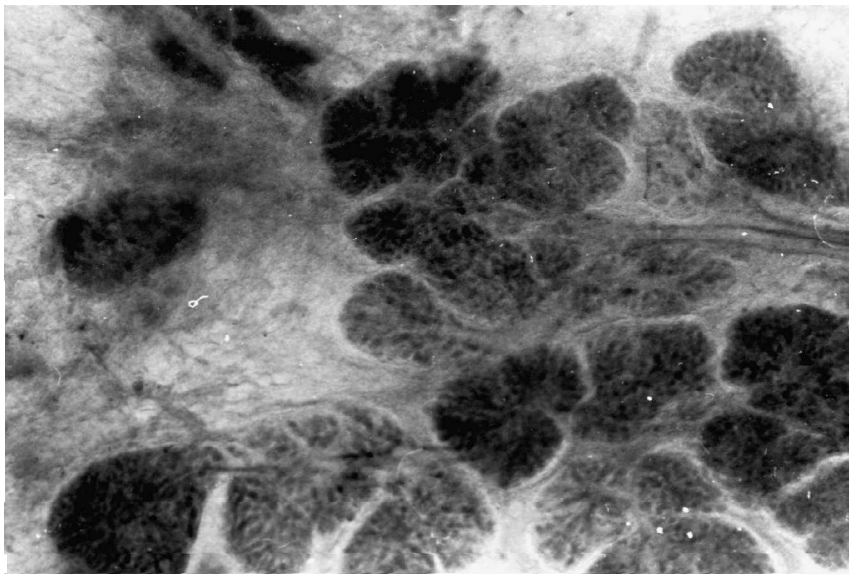


Fig. 5. Whole mount preparation of breast tissue from a 35 year old parous woman showing lobules type 3. Toluidine blue (×25).

antigen ki67, expressed during the S, G_1, and G_2 phases of the cell cycle (64). ER were found to be present in the nucleus of epithelial cells, but their level of expression varied in an inverse proportion to the level of differentiation of the specific structure in which they were detected. Thus, 14% of epithelial cells in Lob 1 consistently contained ER, the values decreasing to 4% in Lob 2 and less than 0.5% in Lob 3. In Lob 4 of the lactating breast the number of positive cells was less than 0.06%. Cell proliferation varied with the differentiation of the structures, as well, and showed a trend correlated to the ER content. Lob 1 expressed the highest level of cell proliferation, decreasing progressively in Lob 2, Lob 3, and Lob 4 (Fig. 6). These data clearly indicated that the degree of differentiation of the breast is an important determinant in the expression of both ER and proliferative activity (35,64–66). After menopause, on the other hand, although the breasts of both nulliparous and parous women contained similar percentages of Lob 1, the proliferative activity was significantly higher in the Lob 1 of the nulliparous women, indicating that pregnancy had imprinted permanent changes in the biological characteristics of these structures. These changes, in turn, may account for differences in the response of the breast to exogenous insults (14,35,60).

The utilization of the double staining procedure allowed us to determine that the cells that were proliferating and those ER positive were not the same, but that they were located next to each other (Fig. 6). These results, also confirmed in the rat mammary gland with ^{3}H-thymidine incorporation (35), indicated there are two different cell populations, one that is the target of the hormonal effect and the other that responds with proliferation. These findings could be explained if a growth factor(s) generated by the differentiated cells are acting on the proliferating or stem cells. Similar results have been shown by Anderson *et al.* (66).

CONCLUSIONS AND FUTURE DIRECTIONS

Experimental studies of rat mammary carcinomas were specifically initiated to answer the questions posed by epidemiological data of why nulliparous women (Fig. 7) have a greater cancer incidence than parous women (Fig. 8). These studies led to the important discovery that the pathogenesis of spontaneous breast cancer in humans is similar to that of chemically-induced rodent mammary cancer. The experimental animal model showed that the undifferentiated gland of young nulliparous females was more susceptible to transformation by chemical carcinogens. Furthermore, these studies clarified how pregnancy, through the induction of differentiation of the mammary gland prevented the initiation of cancer, thus allowing us to identify the "susceptibility window" wherein the mammary gland undergoes either full differentiation or neoplastic transformation, depending

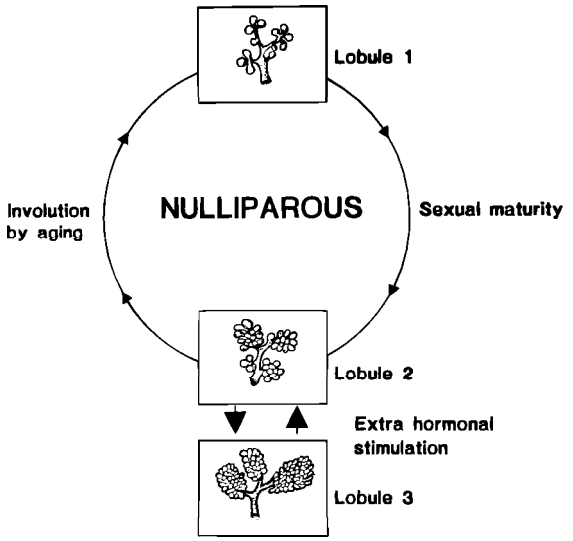


Fig. 7. Life cycle of breast development in the nulliparous woman. The breast is primarily composed of lobules type 1, with some progression to type 2, and only minimal formation of lobules type 3 during sexual maturity, which regress to lobules type 1 at menopause. Reprinted from Russo and Russo (14) with permission.

upon the type of stimulus that first reached the undifferentiated TEB.

Although anatomical studies have identified the undifferentiated Lob 1 as the site of origin of breast cancer, numerous questions remain to be answered. The complexity and multistep nature of the carcinogenic process, the lack of understanding of the mechanism(s) involved in the initiation and progression of the disease, and the lack of identification of specific etiologic agent or agents for human breast cancer are some of the major road blocks in the way of conquering this disease. Nevertheless, the accomplishment of having identified that a "risk window" exists between puberty and the first full term pregnancy might provide a specific period of time in the life of a woman during which interventions that will affect the life time risk of breast cancer development can be made. The protective effect of full term pregnancy indicates that the full differentiation of the mammary gland provides a physiological basis for understanding the reduction in breast cancer risk in women completing a reproductive event at a young age (9,12). Both in humans and in rodents, age and parity history are important modulators of the susceptibility or refractoriness of the mammary gland to carcinogenesis, and both affect two major parameters, differentiation and cell proliferation (7,9,14,15). New advances in molecular and cellular biology, combined with experiments in *in vitro* systems, and the knowledge of the mechanism of action of hormones and growth factors in their interaction with chemicals

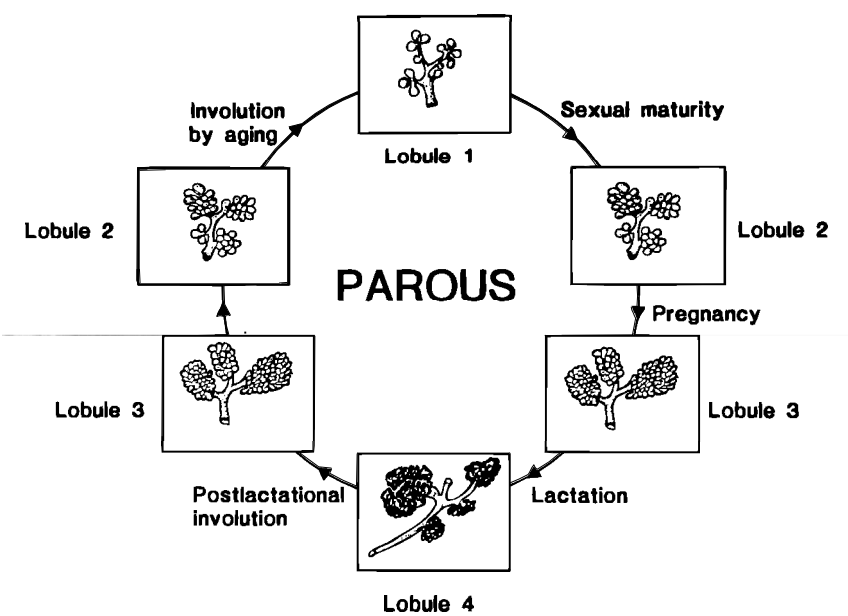


Fig. 8. Life cycle of breast development in the parous woman. The breast undergoes a complete cycle of development through the formation of lobules type 4 with pregnancy and lactation. These regress to lobules type 3 after weaning, and to lobules type 1 at menopause. Reprinted from Russo and Russo (14) with permission.

should provide novel tools for identifying those agents or mechanisms responsible for the initiation and progression of breast cancer and lead to the design of rational methods for its prevention.

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