Hormone-induced refractoriness to mammary carcinogenesis in Wistar–Furth rats

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Introduction

Breast cancer is the most common neoplasm afflicting women of the industrialized nations. It accounts for 32% of all cancers in women and 18% of the cancer-related deaths. About one in nine women in the USA have a lifetime potential of developing breast cancer (1,2).

Some of the risk factors for breast cancer include nulliparity, late parity, early menarche, late menopause and a family history of breast cancer (3–5). In contrast, early parity, late menarche, early menopause, duration of lactation and hormone deprivation provide a protective effect from breast cancer. Therefore, systemic endocrine patterns and reproductive changes occurring in the human breast have important repercussions for breast cancer, and highlight the role of ovarian hormones, estrogen and progesterone, in the development of the disease (6–10). Observations that the same reproductive endocrine events control mammary development and influence breast-cancer risk support the hypothesis that mammary gland development and mammary gland carcinogenesis are fundamentally related. There is ample precedence for the role of estrogen receptor [ER (11)] and progesterone receptor [PR (12)] in mammary gland development from classical endocrinology and current gene knockout experiments, but their respective roles in breast cancer development have been controversial (13,14). Although these hormonal risk factors provide clues to target cell predisposition to breast neoplasia, they have provided little understanding of the underlying mechanisms.

The observation that early full-term pregnancy protects the breast from cancer development is well documented both in humans and rodent models of mammary carcinogenesis. In humans it is an epidemiological observation (15–20); however, induction of mammary carcinomas in rats with chemical carcinogens is a defined model to determine how parity or hormone exposure results in refractoriness to breast cancer (21–31). There are two prevalent hypotheses as to how parity or hormone treatment results in resistance to breast cancer development. According to the first, resistance of the parous gland to development of malignancies is attributed to a greater degree of differentiation (absence of terminal end buds) and reduced rate of cell proliferation in the mammary epithelium (32). These changes reduce the binding of the carcinogen to cellular DNA, lengthen the cell cycle and allow increased DNA repair (33–35). The degree of differentiation also modulates the expression of ER and PR receptor levels, correlating with the level of cell proliferation (32). In this hypothesis, parity results in permanent structural and functional changes in the state of cellular differentiation of the mammary epithelium, affecting the potential of the breast for subsequent response to carcinogen and development of neoplasia. Although this is an attractive hypothesis, key elements have not been tested. As yet, there is no experimental proof that mammary epithelial cells in the involuted gland of the parous animal are intrinsically resistant to carcinogenesis. Indeed, some experiments have not found differences in the structure and proliferative activity, or in carcinogen binding to the DNA of the parous mammary gland compared to the age-matched virgin glands (28,36).

Abbreviations: AMV, age-matched virgin gland; ER, estrogen receptor; PR, progesterone receptor; s.c., subcutaneous; SD, Sprague–Dawley; WF, Wistar–Furth.
A variation of the first hypothesis focuses on a molecular basis underlying the differentiative state resulting from hormone exposure. In this hypothesis differential regulation of the expression of certain genes (37, 38) might determine altered susceptibility to mammary carcinogenesis in the parous and virgin glands based on the role of these proteins in normal mammary gland development. These speculations also have not been documented.

The second hypothesis emphasizes that refractoriness to mammary tumorigenesis in parous rats may be caused by persistent changes in systemic factors such as reduction in the circulating concentration of prolactin (39, 40), growth hormone (31) or concomitant changes in receptor levels, such as ER, PR and/or EGF-R (31, 41). In this model, refractoriness of the mammary gland of parous rats to carcinogen is not intrinsic to the mammary epithelium and can be overcome by treatment with the ovarian hormones, estrogen and progesterone (41). Thus, an inadequate hormonal environment contributes to low ER and PR levels, low levels of proliferation and, consequently, a weak carcinogenic response to MNU. This hypothesis also remains to be tested adequately.

The prevailing hypothesis that hormones of pregnancy induce mammary epithelial target cells to differentiate into a population of mitotically quiescent cells is difficult to reconcile with the fact that such cells retain a normal development capability and can re-enter the proliferative and differentiative pool on subsequent hormone stimulation. The second hypothesis that pregnancy has caused long-term alterations in the systemic environment of the host has not been disproved although specific systemic alterations have not been identified. We have modified the above hypothesis to emphasize that ‘hormones induce a differentiative switch in specific stem cells that result in progeny with persistent changes in the intracellular pathways governing proliferation and response to carcinogens’. These intrinsic differences between the parous and virgin gland may be in signal transduction pathways governed by steroid hormones, in growth factors, in transcription factors or in other unknown regulatory molecules.

As a first step towards identifying these molecular markers of resistance, we have utilized an inbred strain of rats (Wistar–Furth) that allows transplantation studies to examine the permanency of the resistant phenotype. To simplify the model, we have defined a hormone regimen which can reproduce the refractory state induced by a full-term pregnancy. Using just two hormones, estrogen and progesterone, avoids the multiple hormonal changes occurring in pregnancy and focuses attention on possible molecular pathways which might be causative for the refractory state. We also have measured the expression levels of selected genes that might be linked to mammary tumorigenesis in the age-matched virgin (AMV) and hormone-treated gland at the time of carcinogen treatment. Finally, we measured proliferation and apoptosis in these cells both before and after carcinogen treatment.

Materials and methods

Animals

Female Wistar–Furth (WF) rats, 35 days old, were purchased from NCI (Bethesda, MD). The WF female rat is an inbred strain of rats sensitive to both MNU- and DMBA-induced mammary adenocarcinomas (42). The animals were acclimatized to our animal facility for 10 days prior to experimental manipulation. The animals were kept with unrestricted access to food and water, and housed under conditions of a 12 h light–dark cycle.

Preparation of MNU

MNU was chosen as the mammary carcinogen because of its short half-life, ease of delivery and mode of action. It is a direct acting carcinogen not requiring metabolism to its ultimate reactive forms. It was obtained from Ash Stevans (Detroit, MI). The carcinogen was dissolved in phosphate buffered saline, pH 4.0 (acidified with acetic acid), for delivery. Freshly prepared carcinogen solution was injected intraperitoneally (i.p.) at a dose of 50 mg/kg body wt.

Tumorigenesis studies in pregnant rats

For the studies on pregnancy-induced resistance to the carcinogen MNU, WF female rats (20 animals in each group), 70 days old, were mated. After parturition, the mammary glands were allowed to regress for 28 days, so rats 119–122 days old were given two i.p. injections of MNU, 50 mg/kg body wt 1 week apart. An age-matched control (virgin) group of rats also received MNU injections. All mammary tumors appearing in the ensuing 200 days post-carcinogen were collected, and processed for histology and pathological evaluation.

Experimental regimen to mimic pregnancy using E/P

The experimental regimen was designed to mimic pregnancy. In the basic paradigm (Figure 1), 45-day-old female rats which have attained puberty and were cycling regularly were administered a priming dose of 0.1 ml solution of 2.5 µg estradiol benzoate (E2B) in sesame oil (a relatively long-lasting estrogen that will mimic cyclic blood levels) subcutaneously (s.c.). Twenty to twenty-two animals were used in each group. Three days later, the rats received E/P in the form of pellets placed s.c. The pellets were replaced after 10 days to promote a total hormonal stimulation for 21 days. After removal of the second pellet, the mammary gland was allowed to involute for 28 days. Two groups of rats received MNU at 55 days of age as a positive control for MNU carcinogenicity. Tumours were collected for histopathological evaluation. Tumour incidences were statistically evaluated using chi-square tests.
Whole mount morphology of WF rat mammary glands after treatment with different doses of hormones. Abdominal glands were taken after 21 days of hormone stimulation. Depicted are (a) 20 µg E/20 mg P; (b) 10 µg E/10 mg P; (c) 5 µg E/5 mg P; and (d) blank.

Whole mount analysis of mammary glands
For morphological analysis of gland development, either one or both of the abdominal #4 glands were removed by normal surgical procedure and fixed in 10% neutral buffered formalin, defatted in acetone, hydrated, stained in hematoxylin, washed thoroughly in water, dehydrated in graded alcohols, and finally fixed in xylenes and stored in methyl salicylate (43). They were photographed to record development in terms of ductal growth and degree of arborization.

Northern analysis
In a separate group of animals, WF rats were treated with 20 µg E/20 mg P or with blank pellets by the experimental regimen described. At either day 69 (day of pellet removal) or day 97 (day of first MNU treatment), the left abdominal gland (#4) was dissected out using standard surgical procedures and frozen immediately at −70°C for RNA extraction. Five animals were used in each group. The right abdominal gland (#4) was processed for whole mount analysis as described. Total RNA was extracted from the frozen tissues by homogenization in a PT2000 Polytron (Brinkmann, Westbury, NY) with RNAzol B (Tel-Test, Friendswood, TX) or Triazol (Gibco BRL, Bethesda, MD) according to the manufacturers’ recommendations. RNA was quantitated spectrophotometrically and stored at −70°C in water until used. For northern analysis 15–20 µg RNA was resolved in formaldehyde–agarose gels, transferred to Zeta- Probe blotting membranes (Bio-Rad, Hercules, CA) and probed with various radioactively labeled cDNAs as indicated in Figure 6. Cyclophilin was used as the internal standard. Autoradiographs were quantitated using a densitometer (Helena Labs Quickscan, Beaumont, TX).

Cell proliferation using BrdU
In a separate study, WF rats either parous (allowed to nurse their pups for 5–6 days and then involuted for 28 days), exposed to E and P pellets (involuted for 28 days), or age-matched virgins were injected intraperitoneally with MNU (50 mg/kg BW). At various time points after MNU treatment, animals were injected i.p. with BrdU (50 mg/kg BW) and killed 2 h later. The #4 abdominal gland was divided into distal and proximal portions, fixed in Methacarn (methanol:chloroform:acetic acid, 60:30:10), embedded in paraffin and evaluated for BrdU labeling index. BrdU labeling was detected by immunohistochemical staining using the Amersham cell proliferation kit (Amersham International, Amersham, UK). To allow greater access to DNA, the primary antibody incubation was done in the presence of nuclease (44,45). This was followed by secondary antibody incubation and then incubation with DAB substrate. Tissue sections were counterstained with hematoxylin and eosin. The tissues were dehydrated in graded alcohols and xylene, and mounted with Permount. There were five rats per time point in each group. Two thousand cells for each gland portion or 10 000 cells per group were counted.

Differences between groups were tested for statistical significance using the two-sample t-tests. Results were considered significantly different at P < 0.05.

Apoptosis analysis
Standard hematoxylin and eosin (H&E)-stained 4 µm sections, adjacent to sections used for BrdU staining, were evaluated for apoptotic cells by morphological criteria, which has been described and illustrated previously (46). Apoptosis was scored in coded slides by microscopic examination of H&E-stained sections at ×400. Five fields were selected in each specimen, and in each field the numbers of apoptotic cells in mammary ducts were recorded as numbers per 100 nuclei scored and expressed as a percentage or apoptotic index (AI). The AIs reported are therefore based on scoring 500 nuclei for each specimen; a value for AI represents the averages of all specimens in that group. Differences between groups were tested for statistical significance using the two-sample t-tests. Results were considered significantly different at P < 0.05.

Results
Tumor development and characterization in WF pregnant rats
In order to verify that WF rats responded to MNU in a similar manner to Sprague–Dawley (SD) and Fischer rats, 70-day-old female WF rats were mated and treated with MNU. After pregnancy and mammary gland involution, 119–122-day-old parous rats and age-matched virgins received two doses of MNU (50 mg/kg body wt) 1 week apart. At the end of 200 days post-carcinogen treatment, 9/20 (45%) of the virgin rats developed adenocarcinomas whereas 0/20 (0%) of the parous rats had adenocarcinomas. Thus, parity conferred resistance to chemical carcinogen induced mammary tumors in WF rats analogous to that previously observed in SD and Fischer 344 rats.

Differentiation of E/P treated glands
The amount of E/P needed to induce morphological differentiation of the mammary gland equivalent to that obtained in a full-term pregnancy was assessed using seven different hormone combinations. Pellets were implanted s.c. on the dorsal aspect of the back (opposite to the third pair of mammary glands).
glands). To provide a total hormonal stimulation of 21 days, the pellets were replaced after 10 days. In the differentiation study all pellet concentrations listed in Materials and methods were tested; controls received no hormones. Mammary glands from 10- and 21-day pregnant rats were used as positive controls. The two highest doses of hormones resulted in complete alveolar differentiation (Figure 2a), the three intermediate doses stimulated partial alveolar differentiation (Figure 2b), whereas the two lower doses resulted in no significant difference (data not shown) from the glands exposed to blank pellets (Figure 2d). Our results indicated that the effective release of hormones from the pellets occurred over 10 days; therefore it was necessary to replace the pellets after 10 days to achieve consistent hormone stimulation of the gland for 21 days. Based on these results we compared the ability of pellets containing a high concentration (20 µg E/20 mg P), a medium concentration (10 µg E/10 mg P) and a low concentration (5 µg E/5 mg P) to induce a refractory state to MNU-induced carcinogenesis. The low dose (5 µg E/5 mg P) induced only a minor degree of alveolar differentiation (Figure 2c).

Figure 3 contains morphological analyses of glands at 97 days that have undergone treatment with E/P, compared with natural pregnancy. At day 45 when rats are sexually mature and cycling regularly there are abundant distinct terminal end buds. The animals are treated with E/P on day 45. At 97 days, after 21 days of E/P treatment and 28 days of involution, the E/P-treated gland regresses to a morphology similar to the AMV (Figure 3a and b). A similar morphological phenotype is observed when a pregnant gland involutes for the same period of time (Figure 3c and d). The slight differences in the extent of lateral alveoli budding along the ducts reflects a different day of estrus (Figure 3a and c).

Although the general ductal architecture of the fully involuted mammary gland is similar to that of the age-matched virgin, there are several subtle differences between the two glands. First, in the pregnant gland the degree of ductal arborization and the abundance of small alveolar units was greater in the involuted gland than the age-matched virgin (Figure 3c and d). Secondly, at the cytological level, the mammary gland experiencing hormone-induced differentiation and subsequent involution contained lipofuschin pigment in mammary epithelial cells (not shown). A third result from the analysis of these glands was the apparent regional heterogeneity in gland alveolar differentiation in both the age-matched virgin (Figure 4a and b) and the involuted gland (Figure 4c and d). It was apparent that the duct proximal to the nipple and extending half way into the length of the fat pad had a greater degree of alveolar differentiation (Figure 4a and c) than the ducts in the half of the gland distal to the nipple (Figure 4b and d). This observation led us to divide the gland into separate halves for subsequent analyses of proliferation and apoptotic indices.
Tumor incidence

Figure 5 illustrates temporal incidence of mammary tumors in the control and hormone-pelleted rats receiving MNU. Tumor incidences in the two adult groups of age-matched virgin rats (no pellet and blank pellet) were similar and were combined into one group. In these untreated rats, mammary tumor incidence was 56% (26/46) with a mean tumor latent period of 118 days. Rats which received the high dose E/P pellets exhibited a tumor incidence of only 10% (2/20) with a mean latent period of 164 days ($P < 0.05$). Rats which received the medium- and low-dose E/P pellets exhibited a significant reduction in tumor incidence of only 30% (6/20, 6/20) with mean tumor latent periods of 159 and 169 days, respectively ($P < 0.05$). The apparent growth rate of tumors was significantly slower in the E/P treated rats compared with the age-matched virgins. The tumors were measured by vernier caliper for a period of up to 6 weeks. At the third measurement, the mean diameter was 16 mm for 23 tumors arising in age-matched control rats versus only 10 mm for nine tumors arising in the rats which had received the medium- and low-dose hormone pellets.

A positive control of virgin WF rats administered MNU at 55 days was also included, but the data are now shown in Figure 5. These animals responded to MNU with a 95% (21/22) mammary cancer incidence with a mean tumor latency of 118 days. Additionally, multiple mammary tumors were frequently observed in these rats (35 palpable tumors in 21 rats) in contrast with the mature age-matched virgins where only 27 tumors in 23 tumor-bearing rats were noted.

Histology of tumors

Tumors were evaluated by routine H&E pathology. The vast majority of the mammary tumors were adenocarcinomas of the solid or papillary variety. A fibro-adenoma occurred in each of the age-matched controls (no pellet and blank pellet controls). These two tumors were not included in the tumor incidence data.

Northern analysis of gene expression

Several target genes involved in mammary gland development and differentiation are potential targets of differential regulation in the hormone-pelleted and age-matched virgin gland, and could influence susceptibility to mammary carcinogenesis.

Gene expression (mRNA) was examined either in the 69-day-old glands (to assess mRNA at the conclusion of hormone exposure) and at day 97 (to examine persistent changes in the mammary gland before carcinogenic insult). Northern blot analyses of several mRNAs are presented in Figure 6. Progesterone receptor mRNA was three-fold lower in the E/P treated gland compared with the age matched virgin at day 69. This differential expression was not maintained at day 97 and the levels of PR were similar in the hormone-treated and AMV. Estrogen receptor levels were not significantly different at day 69 (not shown) and day 97 between the two glands. On day 69 cyclin D1 was slightly higher (0.5-fold) in the E/P-treated over the AMV whereas the level of cyclin D2 was 2-fold higher in the untreated gland as compared with the E/P treated gland. However, the levels of cyclin D1 and D2 were not significantly different on day 97 in the untreated and hormone treated glands. Changes occurring as a result of hormone treatment were marginal and did not persist in the 97-day-old gland prior to carcinogen treatment. At day 97 the cell cycle inhibitors p16 and p27 were not differentially expressed. The only difference at day 97 between the two glands was an elevation (three-fold) in the levels of p21 in the AMV compared with the E/P treated animal. This is contrary to what one might expect since AMV animals have a higher probability of developing mammary tumors and these glands proliferate more rapidly than the E/P treated animals when challenged with a carcinogen. The tumor suppressor gene p53 also was not differentially expressed at the time of carcinogen challenge. Contrary to what one might anticipate, there were no persistent differences in mRNA levels of any of the target genes examined; namely, PR, ER, cyclin D1, cyclin D2, p16, p27, p53 (shown in Figure 6), and TGFβ1 and EGFR (not shown).

Cell proliferation and apoptosis assays

The terminal end bud is a dynamic structure regulated by a balance between mechanisms of cell proliferation (47) and cell death (48). Both cell proliferation and cell death are coordinately regulated during normal mammary gland morphogenesis and involution. The high levels of proliferation that occur in the large number of terminal end buds (TEBs) in the virgin gland are correlated with increased sensitivity to carcinogenesis. A differential regulation of these processes in
Northern blot analyses of gene expression. WF female rats were treated with 20 µg E/20 mg P or with blank pellets by the experimental regimen described in the text. At either 69 days (21 days of hormone treatment) or 97 days (after 28 days of involution) the left #4 abdominal gland was removed and snap frozen and stored at −70°C for RNA extractions. A sample of 15–20 µg RNA was resolved on formaldehyde–agarose gels, transferred to Zeta-Probe membranes (Bio-Rad, Hercules, CA) and probed with different radioactively labeled cDNAs as indicated in the figure. Cyclophilin was used as the internal control. Each lane contained RNA isolated from mammary glands of different rats.

the parous/hormone-treated gland and age-matched virgin glands could explain the resistance of the parous gland to carcinogenesis. Apoptosis and cell proliferation levels were examined in the parous-involuting gland, the E/P-treated involuted gland and the AMV gland at the time of carcinogen treatment and at intervals up to 8 days after carcinogen treatment.

In these experiments we examined the distal and proximal portions of the gland separately because of the regional differences on morphology described above. Figure 7 shows the data for the distal portions of the mammary gland only as marked differences in proliferation were not observed in the proximal portions of the gland (∗×2.5). The BrdU labeling index (LI) at 97 days (day 0 in Figure 7) in the distal portions of the gland was 0.8% in the parous-involuting gland and 1.8% in the AMV (P < 0.04). Therefore, the proliferation at 97 days was low in both groups of animals. However, at day 8 post-MNU treatment, the LI was still low in the parous-involuting gland (1.3%) and the E/P-treated involuted gland (1.5%), but significantly higher in the AMV (5.7%, P < 0.01).

Among all groups (distal versus proximal, hormone-treated versus AMV, days 0,3,8) there were no differences in apoptotic indices with respect to time after MNU treatment, either in distal or proximal portions of the gland or in age-matched virgin versus hormone-treated glands. The amount of apoptosis was uniformly low with a mode of ~0.5%.

Discussion

One goal in this study was to develop a preventive E/P regimen which mimicked pregnancy in terms of hormone, morphological development of the mammary gland and induction of a refractory state to carcinogenesis. It is well documented that early full-term pregnancy induces a protective effect against tumor development in the mammary gland in both humans (49) and rodents (36). The refractory state can be attained by a single pregnancy with or without accompanying lactation (21,24,28,30) a hormonal regimen comprising the two main steroid hormones of pregnancy, estrogen and progesterone (26,27), or by human chorionic gonadotropin which induces gonadal hormones (29,50,51). The refractory state can be induced in outbred SD rats (32), in inbred Lewis rats (23) and, with the results reported here, in inbred WF rats. A dose regimen which mimics pregnancy closely, both in terms of time and physiological relevance, was extremely effective in preventing MNU-induced mammary cancers in the WF rats.
Hormonal mimicry of pregnancy has been investigated in earlier studies (27). It has been shown that a daily dose of 5 μg E and 2 mg P injected s.c. for 5 days a week for 5 weeks followed by MNU was as effective as high doses consisting of 20 μg E and 4 mg P in inducing resistance to mammary cancer. The lower doses caused complete differentiation of the mammary gland followed by involution. Rats treated with E and P maintained normal estrus cycling, reproduction and lactational activities on cessation of hormone treatment (26,27). The interruption of 2 days after every 5 days daily treatment suggested that the optimal doses and conditions had yet to be determined. The delivery of hormones by slow release hormone—beeswax pellets results in morphological differentiation of the mammary gland over a 21-day period similar to pregnancy with subsequent normal involution of the gland after hormone pellet removal. At this time we have not ascertained the circulating blood levels of hormones concurrent with this delivery method.

Although our working hypothesis predicted that doses of E/P which resulted in complete morphological differentiation of the gland would induce a refractory state, it was surprising that lower doses of E/P were also effective in reducing tumor incidence since these doses of hormones did not induce complete morphological differentiation of the gland. The data in the literature on the relationship between complete morphological differentiation and the attainment of a refractory state are contradictory, with one study (24) suggesting that glandular differentiation was obligatory for refractoriness and a second study (28) that did not support an obligatory relationship. The results of the experiments reported herein support the hypothesis that complete morphological differentiation of the gland induced by hormones is not an obligatory prerequisite for inducing the refractory state. This result is important and suggests that low doses or perhaps short-term exposure (<21 days) would induce a partial refractory state. This knowledge might be helpful for developing strategies for breast cancer prevention in humans.

Earlier studies suggested that hormonal stimulation of mammary gland development induces differentiation of the susceptible mammary cells into a significantly resistant cellular phenotype (32). The glands of the involuted hormone-treated, parous and age-matched virgin are morphologically similar with the exception of a slightly greater number of alveolar buds and side-branching in the involuted gland of the parous rat (indicated by an arrow). All glands are capable of proliferation and differentiation on subsequent treatment with E/P. However, when the glands are challenged with carcinogen, the hormone-treated gland (both parous and E/P treated) is resistant to tumor development, while the AMV is not. The resistant phenotype is unlikely due to the presence of a greater number of functionally differentiated lobuloalveolar cells as this phenotype is strain specific (observed in the SD rat) and was not observed in the involuted gland of the Lewis rat (24). Additionally, this increased alveolar persistence was not observed in the involuted gland of the E/P-treated rats.

An alternative hypothesis is that a specific pathway(s) was altered by E/P administration, and this pathway alteration persisted in the mammary cells and the progeny thereof. We have examined this hypothesis in two ways: first, by assessing selected mRNA markers of proliferation and differentiation; and secondly, by measuring apoptosis and cell proliferation in the hormone-treated and virgin gland. Although the expression patterns of some of these genes changed slightly with hormone treatment (immediate response due to E/P treatment), there were no sustained differences between the hormone-treated and AMV gland after 28 days of involution, the time of carcinogen treatment with one exception. The similarity of mRNA levels applies to steroid receptor genes ER and PR, the cyclin genes D1 and D2, the cell cycle inhibitors p16 and p27, TGFβ1, EGFR and the tumor suppressor gene p53. The exception is p21 where the levels of p21 mRNA were higher in the AMV than the E/P treated rat. At this time, it is difficult to interpret the significance of the result. However, it has been proposed that the stoichiometry of p21 protein—cyclin kinase complex determines the activity of the kinase complex. Both excessive and very low levels of p21 protein are inhibitory to cyclin kinase activity, whereas a normal basal level is necessary for kinase activity (52). Further experiments examining p21-cyclin kinase complexes and cellular localization of p21 might provide information to meaningfully interpret the northern blot results. One previous study examined growth factors in the involuted and nulliparous gland of the mouse and also found no differential expression of TGFβ, cripto-1 and amphiregulin (53). We conclude that constitutive differences in the intracellular loops governing these markers of proliferation (with the possible exception of p21) may not exist or may be too low to be detected. This is substantiated by the observation that the BrdU LI is low in both the AMV (1.8%) and the parous-involuted gland (0.8%) prior to carcinogen treatment. Additional measurements in both parous and E/P-treated rats are needed to confirm this result. Finally, the levels of apoptosis also are low in glands of all three animal groups indicating that this mechanism of regulation of tumor growth was not operative in the hormone-treated gland on carcinogen insult. To date our results are incomplete since we have assessed only a limited number of genes that might regulate cellular control of proliferation or response to carcinogen. Nevertheless,
an important conclusion can be made from our experimental data. The mammary cell in the involuted gland is blocked in its proliferative response to MNU. Previous results have suggested that there are no alterations in the type, amount and persistence of carcinogen adducts in the mammary gland as a consequence of parity (54).

Additionally, the levels of O6-methylguanine-methyltransferase are not increased by pregnancy (55). The recent report by Abrams et al. (41) suggests that cells in the involuted gland of the parous rat are initiated and tumorigenic cells can be recovered by appropriate hormonal stimulation, after MNU treatment. These previous results, along with our results reported herein, favor the hypothesis that the proliferative response to carcinogens of parous cells is blocked by the altered expression of a specific gene(s) or pathway-induced in response to prior hormone treatment. Analyses of the glands by standard northern or RPA methods would be unlikely to detect this alteration in gene expression as only ~6% of the cells are responding to MNU. Analyses of gene expression at the cellular level by immunohistochemistry or in situ hybridization would be more likely to detect altered levels of protein or mRNA, respectively, for relevant regulatory proteins.

In summary, our results demonstrate that both high and low doses of E/P administered to mimic pregnancy are effective in inducing a refractory state to carcinogen-induced tumorigenesis. Our molecular studies suggest that the ability of E/P to induce a refractory phenotype to mammary oncogenesis may not be reflected in sustained alterations of expression of genes commonly involved in the control of proliferation at the time of carcinogen challenge, but rather that E/P may alter the subsequent genetic responses to carcinogen. Additionally, our results indicate that proliferative, not apoptotic, regulatory pathways are altered in response to MNU, as a consequence of prior hormone exposure. Since our current experiments do not rule out systemic alterations which might block proliferative responses, our future experiments will focus on dissecting the relative contributions of systemic and mammary epithelial cell-localized refractoriness.

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