Induction of mammary carcinomas by \(N\)-methyl-\(N\)-nitrosourea in ovariectomized rats treated with epidermal growth factor

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The importance of epidermal growth factor (EGF) in both normal and malignant mammary gland development is presented in these studies. Initial findings demonstrated that in the absence of ovarian hormones, EGF had a significant proliferative effect on mammary epithelial cells. To determine whether mammary epithelial cells grown with EGF, in the absence of ovarian hormones, could be transformed by \(N\)-methyl-\(N\)-nitrosourea (MNU), female ovariectomized Lewis rats were implanted with pellets containing EGF for 1 week and then treated with MNU for initiation. Two days after MNU treatment, ovaries were implanted and EGF pellets were removed from all ovariectomized groups in order to promote carcinogenesis. The mammary carcinoma incidence of the EGF-stimulated group (90%) was not significantly different from the intact group (100%). The mammary carcinoma morphology of EGF-treated carcinomas was either ductal carcinoma or cribriform adenocarcinoma, whereas intact animals developed mainly papillary and occasional cribriform carcinomas. Fifty-eight percent of the carcinomas from the EGF group were ovarian hormone-independent compared with 10% of carcinomas from the intact group. These results demonstrate that EGF-induced proliferation during initiation with MNU was sufficient to induce the transformation of mammary carcinomas in the absence of ovarian hormones. The hormonal dependency of these EGF-induced carcinomas were different compared with MNU-initiated mammary carcinomas in intact rats.

Introduction

Ovarian hormones are important for the normal growth and differentiation of the rat mammary gland (1,2). However, it has been demonstrated that mammogenic hormones, other than ovarian-derived hormones, are also necessary for normal development as well as neoplastic transformation in the mammary gland (3,4). Although numbers of growth factors have been studied for their roles in normal mammary development and epithelial cell proliferation of \(N\)-methyl-\(N\)-nitrosourea (MNU), female ovariectomized Lewis rats were implanted with pellets containing EGF for 1 week and then treated with MNU for initiation. Two days after MNU treatment, ovaries were implanted and EGF pellets were removed from all ovariectomized groups in order to promote carcinogenesis. The mammary carcinoma incidence of the EGF-stimulated group (90%) was not significantly different from the intact group (100%). The mammary carcinoma morphology of EGF-treated carcinomas was either ductal carcinoma or cribriform adenocarcinoma, whereas intact animals developed mainly papillary and occasional cribriform carcinomas. Fifty-eight percent of the carcinomas from the EGF group were ovarian hormone-independent compared with 10% of carcinomas from the intact group. These results demonstrate that EGF-induced proliferation during initiation with MNU was sufficient to induce the transformation of mammary carcinomas in the absence of ovarian hormones. The hormonal dependency of these EGF-induced carcinomas were different compared with MNU-initiated mammary carcinomas in intact rats.

The growth effects of local and/or systemic growth factors alone are sufficient to support mammary gland development or carcinogenesis (3,5). Some of the effects of ovarian hormones on mammary gland development are thought to work through local regulators. For example, ovarian hormones influence systemic levels of epidermal growth factor (EGF) production and secretion from the salivary gland, stimulate mammary epithelial expression of several EGF family members (6–9) and increase the levels of EGF receptors (EGFRs) in the mammary gland (10–12). Therefore, determining the role of EGF in ovarian hormone target organs may provide clues to the mechanisms of normal and neoplastic mammary development.

EGF and its family members have been shown to play a role in mammary development. It has been demonstrated that EGF stimulates lobulo-alveolar development of normal mammary tissues \textit{in vivo} and epithelial cell proliferation of normal and neoplastic mouse mammary tissue \textit{in vitro} and \textit{in vivo} (13–16). Although there is no clear evidence to show that abnormalities in EGF production can be associated with the etiology of mammary carcinomas, human breast cancer studies have indicated that the majority of human ovarian hormone-independent cancers (OHICs) are estrogen receptor (ER) negative, but EGFR positive (17,18). This evidence suggests that EGF/EGFR may be involved in carcinogenesis and in the behavior of mammary carcinomas in human as well as in rodent systems. Normal and neoplastic mammary tissues express EGF (19) and several other EGF family members, including transforming growth factor \(\alpha\) (TGF-\(\alpha\)) (19–23), amphiregulin (23), heregulin (24) and cripto-1 (23). Heregulin \(\alpha\) studies demonstrate that growth factors alone can directly induce ductal growth in the absence of exogenous estrogen and progesterone in prepubertal female mice (24). Further evidence supporting a possible role for EGF in mammary carcinogenesis is found in studies with TGF-\(\alpha\) overexpression. Previous studies have shown that TGF-\(\alpha\) transgenic mice have developed mammary adenocarcinomas with high levels of stromal proliferation. A shorter latency of TGF-\(\alpha\)-stimulated carcinoma formation was found in these transgenic mice. The mammary carcinomas from TGF-\(\alpha\) transgenic mice exhibit an invasive, occasionally metastatic phenotype (12). TGF-\(\alpha\) transgenic mice data suggest that overexpression of other EGF family members may cause aggressive mammary carcinomas in rodents. Therefore, the mitogenic abilities of EGF in mammary carcinogenesis need to be investigated. In addition to growth factor expression in the mammary gland, various related growth factor receptors are expressed either in mammary epithelial or stromal components in rodent (11) and human mammary glands (4,25). These include EGFR, erb-2/HER-2/neu, erbB-3 and erbB-4 (26,27). These findings suggest that the EGF/EGFR pathway may have a possible role in mammary carcinogenesis in rodent as well as in human models.

Proliferation is thought to be essential to support the initiation of mammary carcinogenesis (28). Implants of EGF or TGF-\(\alpha\)
pellets into regressed mammary glands of ovariectomized mice stimulate the reappearance of end buds (19,26). Previously, we showed that implanted EGF pellets in the mouse mammary gland can induce proliferation and morphogenesis of the gland (R.C.Guzman, unpublished data). We also found that EGF administration causes high levels of proliferation in mouse mammary epithelial cells (MECs) (29). This observation supports the idea that the mitogenic abilities of EGF/EGFR-mediated pathways remain functional in the absence of ovarian hormones in rat mammary development.

The mitogenic abilities of EGF, with respect to EGF-induced mammary carcinogenesis, have not been investigated. The main objective in this study was to determine whether systemic treatment with EGF in ovariectomized rats will induce proliferation of MECs and support their neoplastic transformation following N-methyl-N-nitrosourea (MNU) administration. Previously we have demonstrated that different mitogens present during MNU initiation in vitro result in the genesis of mammary carcinomas with different phenotypes in vivo (16,30,31). In the present study, we examined the phenotypes and genotypes of mammary carcinomas, comparing EGF-stimulated MNU-initiated MECs with those initiated in the presence of ovarian hormones. The results demonstrated that systemic EGF is sufficient to stimulate proliferation and support neoplastic transformation of MECs. Furthermore, EGF is effective in supporting chemically induced mammary carcinogenesis and is also important in determining the morphology and hormone dependency of these carcinomas.

Materials and methods

Animal treatment

Virgin Sprague–Dawley rats, purchased from Harlan Sprague Dawley (Indianapolis, IN), were housed in a temperature-controlled room with regulated 12 h light and dark periods. Food pellets (sterilizable rodent diet; Harlan Teklad, Madison, WI) and water were available ad libitum. Six- to seven-week-old rats were ovariectomized under anesthesia using rat anesthesia cocktail (60.6 mg/ml ketamine, 6.06 mg/ml xylazine, 0.91 mg/ml acpecmazine). The mammary gland was allowed to regress for 5 weeks, allowing time for endogenous ovarian hormones to be cleared from the system. Rats were implanted with either five 10 µg EGF (Becton Dickinson Labware, Bedford, MA)/5 mg cholesterol pellets (Hormone Pellet Press, Westwood, KS), 5 mg cholesterol pellets or 20 µg estradiol/20 mg cholesterol (E/C) silastic capsules into their subcapsular fat pads (Figure 1). Five animals from each group were used for the proliferation study. All animals were killed 2 weeks after hormonal treatment. Rats were administered 160 µg/g body wt 5-bromo-2-deoxyuridine (Brdu) (Sigma, St Louis, MO), dissolved in medium 199 with antibiotics, 2 h prior to death to allow for incorporation into proliferating cells. Inguinal mammary glands (no. 4) were collected from animals from the control group and experimental groups and fixed in methacarn. Samples were collected from three different regions of mammary glands, designated as proximal zone (proximal to the nipple, mainly primary and secondary ducts), medial zone (middle area, mainly tertiary and some quaternary ducts) and distal zone (distal to the nipple, mainly terminal end buds and lateral buds) (32). Fixed samples were dehydrated, embedded in paraffin and cut into 5–6 µm thick sections. The sections were then used for immunocytochemistry. Pieces of intestine were also taken to serve as positive controls for immunocytochemistry.

For carcinogenesis studies, inbred female virgin Lewis rats (National Cancer Institute, Frederick, MA) were used to examine the possible direct effect of EGF on MECs under different physiological conditions. One advantage of using inbred Lewis rats is that they can be used for carcino genesis transplantation to determine the transplantability and the hormonal responsiveness of mammary carcinomas. All the groups were treated with MNU (Ash Stevens, Detroit, MI) 1 week after hormonal treatment. Crystalline MNU was dissolved in prewarmed 0.85% NaCl solution (pH 5.5) to a concentration of 10 mg/ml. The dose was 50 mg/kg body wt (33) and was injected i.p. into anesthetized female rats (34). To promote the MNU-initiated MECs, two quarter ovaries were implanted into the subcapsular fat pads. Production of ovarian hormones was confirmed by examining vaginal smears for estrous cycle. Intact animals treated with MNU at the age of 50 days served as a positive control group for these studies. Negative control groups for this study were cholesterol only and ovariectomized alone (Figure 1). Beginning 1 month after carcinogen treatment, rats were palpated for mammary carcinomas once a week. All the experimental animals were observed for a 1 year period and killed at the end of the experiment. Carcinomas were collected and fixed in 10% buffered formalin in phosphate-buffered saline (PBS) (Fisher Scientific, Pittsburgh, PA) at 4°C. Carcinoma samples were dehydrated, paraffinized and sectioned. All carcinomas from each group were evaluated for their hormonal responsiveness by immunocytochemical localization of ER and progesterone receptor (PR) in histological sections. Pieces of uterus were taken to serve as positive controls for immunocytochemistry.

Brdu immunocytochemistry

Brdu immunocytochemistry was as previously described (35). Tissue sections were deparaffinized and treated with 2 N HCl at 40°C for 1 h. After being washed in running distilled water followed by PBS, the slides were incubated with Pierce Blocking Buffer in Tris-buffered saline (Pierce, Rockford, IL) for 30 min to block background staining. Tissues were then incubated with primary mouse monoclonal anti-Brdu antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) diluted 1:5 in blocking buffer overnight at 4°C. Slides were washed in PBS and then overlaid with Pierce Peroxidase Suppressor for 5 min. After washing twice in PBS, slides were incubated in the dark with biotinylated anti-mouse IgG secondary antibody and Vectastain ABC kit (Vector Laboratories, Burlingame, CA). After washing twice in PBS, the Brdu-labeled cells were visualized by staining with a 1 mg/ml solution of 3,3'diaminobenzidine tetrahydrochloride (DAB) (Dako, Carpinteria, CA) and 0.02% hydrogen peroxide in PBS. Cells were counterstained with Mayer's hematoxylin solution (Sigma). To count Brdu-positive cells under a light microscope, only MECs with brown nuclear staining were counted as positive regardless of the intensity of the staining, while those with bluish purple nuclear staining from hematoxylin were counted as negative cells. There were at least 5 animals/group and at least 5000 cells/group counted. The percentage of positively stained cells was determined by dividing the number of positively stained cells by the total number of cells counted and multiplying by 100.

Estrogen and progesterone receptor immunocytochemistry

After the slides were deparaffinized and rehydrated, antigen retrieval was used to improve receptor sensitivity. Slides were soaked in antigen unmasking solution (Vector Laboratories), heated for 21 min and then washed with PBS for 5 min at room temperature to reveal antigens. Sections were incubated with Immuno Pure Peroxidase Suppressor (Pierce) to quench the endogenous peroxidase for 30 min at room temperature. Pierce Super Blocking Solution was then used, at room temperature for 10 min, to block the non-specific background. Sections were incubated with primary antibodies at room temperature for 1 h, followed by three PBS rinses. Anti-ER monoclonal antibodies 1D5 (Dako), diluted 1:30, were used to detect rat ERs (36). Anti-human PR polyclonal antibodies (Dako), diluted 1:100, were used to detect rat PRs. The sections were then incubated with secondary antibodies by Universal Dako LSAB2 labeled streptavidin-biotin peroxidase kit (Dako). Sections were incubated with biotinylated secondary antibodies at room temperature for 1 h, followed by three PBS rinses, then streptavidin–enzyme conjugate at room temperature for 1 h. For color development, sections were treated with a solution of 1 mg/ml DAB chromogen (Dako) and 0.02% hydrogen peroxide. The sections were counterstained with Mayer’s hematoxylin solution (Sigma). After counterstaining, ER/PR-negative nuclei appeared purple-blue and ER/PR-positive nuclei appeared dark brown.

Mutant H-ras PCR–allele-specific oligonucleotide hybridization (ASO) analysis

Isolation of DNA, PCR and Southern transfer conditions were previously described by Zhai et al. (37). Tissue samples (300 mg) were digested in 10 ml of 300 µg/ml proteinase K (EM Science, Cherry Hill, NJ) at 50°C and gently shaken for 4–6 h. The rat c-H-ras allele in 1 µg of genomic DNA was amplified with 1.5 U of Taq polymerase (Perkin-Elmer, Norwalk, CT) and 1.5 mM MgCl2 using 5'-TTG CCT ACT CAT TGG TGG AG-3' as the 5' primer and 5'-CAT ACT CGT CCA AAT GG-3' as the 3' primer to yield a 231 bp product. The PCR conditions were as follows: 95°C for 30 s, 50°C for 30 s, 72°C for 30 s and one cycle at 72°C for 5 min in a Perkin-Elmer Thermal Cycler. PCR amplified DNA products were electrophoresed in 2% NuSieve agarose gels (FMC, Rockland, ME) and blotted overnight onto Nitran membranes (Schleicher & Schuell, Keene, NH) by capillary action in 1.5 M Tris–HCl, 1.0 M NaOH. Membranes were then washed in 1.5 M Tris– HCl, 1.0 M NaCl, pH 7.5. The transferred DNA was fixed by UV irradiation for 5 min. The membrane was pre-hybridized for 2 h in 7% SDS, 0.5 M Na2PO4, 2 mM EDTA and 1% bovine serum albumin. ASO was carried out using [γ-32P]ATP end-labeled 5'-GC CCT GGA GCC GTG GAA AA-3' as
the wild-type and 5'-GC GCT GAA GGC GTG GGA AA-3' as the mutant probes for the rat c-H-ras gene. The probes (10⁶ c.p.m./ml) were added directly to the prehybridization buffer and incubated for 3 h. The blots were washed twice with 1× SSC, 0.5% SDS at 60°C for 20 min. Kodak X-OMat AR film (Eastman Kodak, Rochester, NY) was exposed to the membrane overnight and developed.

**Initial latency, incidence and behavior**

Palpation for mammary carcinomas was performed weekly, beginning 1 month after the administration of MNU. Initial latency was defined as the number of weeks between MNU administration and detection of the first palpable cancer. The location of each cancer, total number of cancers per rat and date of cancer detection were recorded. All the cancers were processed for routine histological evaluation to confirm that they were mammary carcinomas. Carcinoma incidence was calculated as the percentage of rats in a group with mammary carcinomas. Cancer growth dependency on ovarian hormones was determined by two methods: (i) ovariectomy of either experimental rats or syngeneic hosts or nude mice bearing carcinomas which were transplanted into interscapular and cleared mammary fat pads; and (ii) immunocytochemistry for ERs and PRs. Ovarian hormone-dependent cancers (OHDCs) completely regressed after ovariectomy of the host. OHICs continuously grew even after ovariectomy of the host. The percentage of OHDCs was determined for each experimental group.

**Statistics**

The differences between the cell proliferation data for the various animal groups were analyzed statistically by means of the F-test. The differences between the mammary cancer incidence and responsiveness of various experimental groups were analyzed by the χ² test (38). Differences were considered significant when \( P < 0.05 \) was obtained.

**Results**

**Effects of EGF and estradiol on proliferation in the mammary epithelial cells of ovariectomized rats in vivo**

Mammary gland morphology and BrdU labeling index. The examination of whole mounts and sections from mammary glands of control, ovariectomized and EGF-, E/C- and cholesterol-treated animals showed that EGF and E/C predominantly stimulated proliferation of ductal epithelial cells in the proximal areas in the absence of ovarian hormones. Terminal end buds were observed primarily in E/C-treated groups and only occasionally in the EGF-treated group.

Hormonal treatment caused proliferation in different regions of the regressed mammary glands. Comparison of proliferation between different zones within the same group, except for the cholesterol group, indicated that there was statistically significant differential hormonal responsiveness among various zones of the mammary glands (\( P < 0.001 \)). Among all the groups, the proximal zone showed the most response. The proliferation rate in the medial zone was similar to the distal zone. The negative control groups, ovariectomized and cholesterol alone, had significantly lower proliferation in both the distal and the proximal zones (~1%), when compared with intact animals (Table I). The positive control group, E/C, had high cell proliferation percentages, 26.2% in the proximal zone.
Fig. 2. The incidence and initial latency of EGF-stimulated MNU-induced mammary carcinomas in Lewis female rats. The rats were palpated once a week for the appearance of carcinomas and the percent carcinoma-bearing rats were plotted in weeks after MNU treatment. Mammary cancer incidence, number and latency were for a 1 year period. Each group contained 9–15 animals.

Table I. The BrdU labeling index in the mammary gland of EGF-treated ovariectomized rats

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>Proximal zone (%)</th>
<th>Medial zone (%)</th>
<th>Distal zone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>32.7ab</td>
<td>15.9ab</td>
<td>9.3ab</td>
</tr>
<tr>
<td>Ovariectomy</td>
<td>0.5b</td>
<td>2.6b</td>
<td>1.2b</td>
</tr>
<tr>
<td>E/C</td>
<td>26.2ab</td>
<td>5.6b</td>
<td>11.2b</td>
</tr>
<tr>
<td>EGF</td>
<td>17.0ab</td>
<td>8.7ab</td>
<td>3.7ab</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.5a</td>
<td>1.1a</td>
<td>1.0</td>
</tr>
</tbody>
</table>

E/C, 20 µg estradiol/20 mg cholesterol silastic capsule; EGF, 50 µg EGF/25 mg cholesterol pellet; cholesterol, 25 mg cholesterol. All groups were compared statistically and significant differences between groups are indicated below.

$^aP < 0.001$ compared with different groups within the same zone (F-test).

$^bP < 0.01$ compared with different groups within the same zone (F-test).

$^cP < 0.05$ compared with different groups within the same zone (F-test).

$^dP < 0.001$ compared with ovariectomized group ($\chi^2$ test).

$^eP < 0.05$ compared with intact group ($\chi^2$ test).

$^fP < 0.01$ compared with intact group ($\chi^2$ test).

No significance among different zones in the cholesterol group.

and 11.2% in the distal zone. The EGF-treated group had cell proliferation rates of 17.0% in the proximal zone and 3.7% in the distal zone of the mammary glands (Table I). The proliferation of MECs treated with EGF alone, in the absence of ovarian hormones, was significantly greater compared with the ovariectomized or cholesterol-treated groups ($P < 0.001$). EGF-stimulated cell proliferation in the proximal and distal regions was not as great as those in the E/C-treated group. EGF primarily stimulated the ductal structures in the mammary glands of ovariectomized animals. The proliferation rate in lobulo-alveoli and terminal end buds was less stimulated by EGF.

**MNU treatment of EGF-stimulated MECs results in mammary carcinomas**

**Mammary cancer incidence and initial latency.** Mammary cancer incidence and initial latency period for the MNU-initiated animals are presented in Table II and Figure 2. The mammary cancer incidence in the EGF-treated and intact groups were significantly greater than the cancer incidence in the negative control groups. In the absence of ovarian hormones, the stimulation by EGF alone proved to be as efficient as in the intact MNU-initiated group in supporting mammary carcinogenesis. The mammary cancer incidence of the MNU-initiated, EGF-stimulated group was 90%. The incidence in intact MNU-initiated animals was 100%. Mammary cancer incidence in the MNU-initiated cholesterol and ovariectomized negative control groups of rats were 55.6 and 22.2%, respectively. The initial latency in the EGF group ranged from 9 to 50 weeks. Initial latency of both the intact and cholesterol groups ranged between 15 and 37 weeks. The initial latency of the ovariectomized group was from 26 to 32 weeks after MNU administration (Figure 2). The EGF group had 3.7 carcinomas/rat, significantly greater than in the intact group, which had 1.6 carcinomas/rat (Table II).

**Morphology and hormonal responsiveness of rat mammary carcinomas.** The cancer morphology of the EGF group was mainly cribriform or ductal carcinomas (Figure 3). The mammary carcinomas from the EGF group had lymphocytic infiltration, with an occasional invasion into the muscle component. The cancer morphology of the intact group was mainly papillary or occasional cribriform mammary carcinoma. No distinct metastases were observed in any groups.

The hormonal responsiveness and transplantability of the mammary carcinomas was determined either by ovariectomy of the host animal or transplantation of the mammary carcinomas into the cleared mammary fat pads of athymic nude mice or inbred Lewis female rats. The mammary carcinomas from the EGF-stimulated group had a better transplantation efficiency compared with the cholesterol or intact groups (Table III). Fifty-eight percent of the EGF-stimulated mammary carcinomas were OHICs. The EGF-stimulated group had a significantly higher incidence of OHICs when compared with the intact MNU-initiated group (Table IV).

**Immunocytochemistry of estrogen and progesterone receptors.** The ER and PR status in mammary carcinomas from all groups was determined by immunocytochemistry. Intense ER and PR staining was seen in the cholesterol, control and ovariectomized groups. In contrast, the intensity for ER and PR in the EGF group was dependent on whether the mammary carcinomas were ovarian hormone dependent or independent. In OHICs, the EGF-stimulated MNU-induced carcinomas showed weak or undetectable ER and PR immunocytochemical staining.
EGF-stimulated MNU-initiated mammary carcinomas

Fig. 3. The cancer morphology of the EGF-stimulated MNU-initiated mammary carcinomas was mainly (A) ductal carcinomas or (B) cribriform. Hematoxylin and eosin, ×600.

Table III. The transplantation efficiency of EGF-stimulated MNU-initiated mammary carcinomas into the cleared mammary fat pads of athymic nude mice or inbred Lewis rats

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Palpable carcinomas/total transplanted carcinomas (%)</th>
<th>Athymic nude mice</th>
<th>Lewis rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>100 (12/12)</td>
<td>7 (5/7)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0 (0/1)</td>
<td>0 (0/1)</td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>NT</td>
<td>0 (0/1)</td>
<td></td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>NT</td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

NT, not tested.

(Table IV and Figure 4A and B). However, the OHDCs from EGF-stimulated MNU-induced carcinomas and other groups showed intense ER and PR immunocytochemical staining (Figure 4C and D). The percentage of ER- or PR-positive MECs in OHDCs was not significantly different among various groups.

Mutant H-ras analysis. The PCR-ASO analysis detected a frequency of 35% of mutant H-ras in the EGF-stimulated MNU-induced mammary carcinomas. All of the mammary carcinomas from the cholesterol group contained mutant H-ras (100%). There was a lower frequency of mutant H-ras in the intact group (20%) (Table V).

**Discussion**

Various mammogenic growth factors and their receptors are known to be expressed in normal as well as neoplastic mammary tissue. However, the role of these growth factors in mammary carcinogenesis is not completely understood. To study the role of a major growth factor, such as EGF, in carcinogenesis, our laboratory examined two aspects of the role of EGF in the mammary glands. First, the proliferative effect of EGF on the mammary gland of ovariectomized animals was examined. Second, the ability of EGF to support the initiation of mammary carcinogenesis was examined.

Initiation of chemical carcinogenesis requires cell proliferation in target tissues (28). The studies from Russo’s group have also indicated that highly proliferating cells in terminal end buds may serve as primary targets for chemical carcinogen-induced initiation in mammary carcinogenesis (39). Therefore, studying the proliferating potential of EGF in the mammary gland in the absence of ovarian hormones was the first step in this study (32).

EGFs have been demonstrated to stimulate proliferation of normal and neoplastic mouse mammary tissues *in vitro* and *in vivo* (13,14). The results demonstrated that exogenous treatment with EGF, in the absence of ovarian steroids, had a stimulating effect on the proliferation of rat MECs *in vivo*. Previous EGF studies in mouse mammary glands (R.C.Guzman, unpublished data), combined with the results of this study, have demonstrated that EGF has a significant proliferative effect on mouse and rat MECs, either in the presence or absence of ovarian hormones (11). The EGF/EGFR signaling pathway remains functional in the absence of ovarian hormones and may serve as an endocrine or a paracrine mediator in rat mammary development. EGF mainly stimulates the proliferation of MECs of ductal structures, followed by the lobulo-alveolar structures, followed by the terminal end bud structures. Depending on the mammary mitogens used, there appears to be a differential effect between the various regions of the mammary gland. Our observations indicate the proximal region of the mammary gland to be more responsive to mammogenic agents than distal or middle regions. The findings demonstrate that, independent of stimulation by ovarian hormones, EGF has a mitogenic effect on rat MECs. We then investigated the effect of EGF in rat mammary carcinogenesis.

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**Table IV. Hormonal responsiveness and immunocytochemistry of ERs and PRs in the MNU-initiated and EGF-stimulated rat mammary carcinomas**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>OHIC (%)</th>
<th>ER (+ or –)</th>
<th>PR (+ or –)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>58.3 a</td>
<td>– (14/24) b</td>
<td>– (14/24) b</td>
</tr>
<tr>
<td>Intact</td>
<td>10</td>
<td>– (1/10) c</td>
<td>– (1/10) b</td>
</tr>
</tbody>
</table>

aP < 0.05 compared with intact group (χ² test).
bOHIC, ovarian-hormone-independent cancer.
cOHDC, ovarian-hormone-dependent cancer.
Fig. 4. ERs and PRs were localized in the nuclei of mammary carcinoma cells by the streptavidin–biotin technique and counterstained with Mayer’s hematoxylin. (A) ER and (B) PR immunolocalization were found to be either weak or negative in OHICs of the EGF-stimulated rat mammary carcinomas; ×600. ERs (C) and PRs (D) were strongly positive staining in OHDCs of the EGF-stimulated rat mammary carcinomas; ×600. Both ERs and PRs were immunolocalized only in the epithelial cells but not in the stromal cells.

Table V. PCR-ASO analysis of mutant H-ras at codon 12 in EGF-stimulated MNU-initiated mammary carcinomas

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total no. of cancers</th>
<th>Cancers with mutant H-ras (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF</td>
<td>29</td>
<td>35</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>Intact</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Ovariectomized</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

A majority of human breast cancers are classified as OHICs. These OHICs grow in the absence of the major mammogenic hormones, estrogen and progesterone. Immunocytochemical analysis has shown that pathological samples of human OHICs are ER-negative but positive for EGFRs. This observation suggests a possible role of EGF/EGFR in the development of OHICs. Although the role of EGF/EGFR interactions in rat mammary carcinogenesis is unclear, it may be involved in carcinogenesis as well as in determining the morphology and biological behavior of mammary carcinomas in rodents and in humans.

Our studies have shown that EGF-stimulated MEC proliferation in the absence of ovarian hormones supports transformation by MNU in vivo. Beattie et al. suggested that the hormonal environment at the time of cancer initiation modulates the nature of the cancer induced (40,41). In vitro transformation studies demonstrate that MNU-initiated MECs treated with different mitogens result in a variety of transformed phenotypes and genotypes, when transplanted into host mice (42). The current findings, that mammary carcinoma incidence in the EGF-treated group is as high as in the intact group, demonstrates that EGF treatment is as effective as ovarian hormones in the induction of MNU-initiated carcinogenesis. Mammary cancers per rat in the EGF-treated group were significantly greater than in the intact group, therefore, the susceptibility of EGF-treated MNU-initiated MECs is equal to or greater than MECs in the intact group. The first carcinoma in the EGF-treated group appeared 1 month earlier than in other experimental groups. This observation indicates that EGF-treated
MNU-initiated mammary carcinomas may be more responsive to the promotional effects of physiological levels of ovarian hormones. The cancer morphology in the EGF-treated MNU-initiated mammary carcinomas was mainly ductal carcinomas with some stromal proliferation, whereas the cancer morphology in the intact group was mainly papillary with an occasional cribriform. Mammary carcinomas from the EGF group showed more lymphocytic infiltration and carcinoma invasion into muscle components around mammary tissues, which is rarely seen in MNU-initiated mammary carcinomas from intact rats. The EGF/EGFR signaling pathway during the initiation of mammary carcinogenesis may proceed via a different mechanism in ovariecetomized rats treated with EGF, compared with intact rats. Therefore, in rats, elevated levels of circulating EGF can alter the cancer morphology as well as the aggressiveness of mammary carcinomas. The mammary carcinomas induced in the EGF group appear to be more malignant than those in the intact group.

The apparent increase in carcinoma incidence in the cholesterol only group, compared with the incidence in the ovariecetomized group, was not statistically significantly different. The proliferation data from ovariecetomy and cholesterol treatment after ovariecetomy showed a very low BrdU labeling index among MECs of the mammary glands. The carcinoma data indicated that in the absence of ovarian hormones or in rats treated with cholesterol after ovariecetomy, MECs in these mammary glands can be initiated by MNU treatment and promoted with ovarian hormones from transplanted ovaries. Therefore, a very low rate of proliferation in MECs may be sufficient for MNU initiation during mammary carcinogenesis. It is important, however, to have a sufficient level of mammogenetic hormones to promote these MNU-initiated MECs in these animals.

Regarding the ovarian hormonal responsiveness of MNU-initiated mammary carcinomas, several studies have shown that blocking ovarian hormones by treatment with the estrogen antagonist tamoxifen during initiation results in cancers that are primarily ovarian hormone independent (42,43). EGF-treated MNU-initiated carcinomas can be initiated in the absence of ovarian hormones. Carcinomas from the EGF group show not only a unique cancer morphology but also ovarian hormone independence. EGF OHCs lose their ovarian hormone dependency due to the absence of ER and PR expression in the nuclei of MECs. Again, this demonstrates that the hormonal and growth factor environment during the initiation of chemically induced carcinogenesis is important in determining the hormonal responsiveness of mammary carcinomas (42).

EGF-treated MNU-initiated carcinomas have unique biological properties compared with those induced in the presence of ovarian hormones. We examined the genotypic alteration in mammary carcinomas from the EGF-treated and other groups. We have earlier reported that rat exposure to MNU during various stages of the estrous cycle results in mammary carcinomas with different percentages of mutant H-ras activation (44). Also, Zhang et al. showed that a reduction in the frequency of activated ras genes was associated with increasing MNU doses during initiation and also with changing hormonal environment during promotion and progression of rat mammary carcinogenesis (37). Therefore, we studied the mutant H-ras frequency in mammary carcinomas from the EGF group as well as other groups. In our analysis of mutant H-ras with codon 12 G→A transitions, a low frequency of mutant H-ras was found in EGF-stimulated MNU-initiated mammary carcinomas as well as in ovarian hormone-induced MNU-initiated carcinomas. In contrast, a high frequency of mutant H-ras was found in a small number of mammary carcinomas from cholesterol-treated rats. Our data also suggest that the frequency of the specific genetic insult, such as mutant H-ras, during the initiation of mammary carcinogenesis may result from different hormonal environments. Mutant H-ras was detected in only about one third of the EGF-stimulated MNU-initiated mammary carcinomas, compared with all three tumors tested from the cholesterol group. However, due to the availability of only a limited number of tumors in several of the experimental groups, our mutant H-ras activation data were insufficient to arrive at any specific conclusion. Further study of genetic involvement in these EGF-stimulated MNU-initiated mammary carcinomas is required.

These studies provide evidence indicating an important role for EGF in normal and neoplastic mammary development. EGF family members function not only in promoting growth of regressed mammary glands, but also in promoting MNU-induced transformation of MECs. The EGF/EGFR pathway may contribute to an altered responsiveness to ovarian hormones of MNU-initiated cells in rat mammary carcinogenesis. Defining the role of growth factors in mammary carcinogenesis will be an important direction for future studies.

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