Distribution of epidermal growth factor receptors in normal and neoplastic mammary tissues

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Abstract. Epidermal growth factor (EGF) is considered to be mitogenic for proliferation of mammary glands in animals. The action of EGF is mediated by specific EGF receptors (EGF-R). In the present study, we investigated distribution of EGF receptors during various physiological stages of mammary glands, N-methyl-N-nitrosoare (MNU)-induced mammary tumors in rats and human breast cancer samples. EGF receptor concentrations were determined by Scatchard analyses in the membrane fraction of the tissues. Results showed increased EGF receptor levels in the structurally differentiated mammary tissues from pregnant rats; whereas lower concentrations were observed in the functionally differentiated glands from lactating rats. EGF receptors were absent in the majority of the tumors induced by MNU. The loss of EGF receptor was not observed during the first 20 days post carcinogen treatment, but appeared to be correlated with the onset of the tumor. Consistent with the literature, the majority of the steroid receptor positive human breast cancer samples were EGF receptor negative, whereas steroid receptor negative samples contained EGF receptors. These results suggest that the loss of EGF receptors in ovarian hormone dependent mammary tumors does not occur gradually during carcinogenesis but appears to be a characteristic of hormone dependent mammary tumor cells.

Introduction

Epidermal growth factor (EGF) is a 6045 dalton polypeptide with 53 amino acids. The importance of EGF in mammary glands in vivo has been suggested by the finding that sialoadenectomy of mice decreases milk production and increases infant mortality. Moreover, survival rate is increased by administration of EGF to sialoadenectomized pregnant animals (1). This is consistent with the finding that the serum levels of EGF is considerably elevated during pregnancy. It has been well established that EGF has a potent mitogenic activity in diverse cell types including mammary epithelial cells in vitro (2,3). In mammary gland organ culture, EGF is required for a second round of structural differentiation (4). These results suggest a clear physiological role for this growth factor in mammary gland differentiation and proliferation.

The action of EGF is mediated through high affinity EGF receptors (5,6). EGF receptor (EGF-R) is a transmembrane glycoprotein of approximately 170,000 dalton, consisting of a single polypeptide. The EGF receptor can be divided into three functional domains; an extracellular binding domain, a transmembrane domain, and a cytoplasmic domain responsible for tyrosine kinase activity (7). Since EGF receptor is highly conserved in different vertebrate species, it is considered to have an essential function in diverse cell types. Presence of EGF receptors is reported in normal murine mammary cells. Two classes of binding sites, low and high affinity sites have been observed (8). Binding of radioactive EGF to membrane fractions of mammary glands derived from mice during various physiological stages has also been reported. Results from that study indicated that during gestation higher level of EGF receptors are found in the mammary glands as compared to the lactating mice (8).

More recently, it has been reported that steroid receptor positive (ER+, PR+) breast cancer cell lines usually contain lower concentrations of EGF binding sites as compared to steroid receptor negative cells (9,10). Presence of EGF-R is also considered suggestive of poor prognosis for response to therapy in breast cancer patients (10).

Despite the importance of EGF-R in the prognosis and treatment of breast cancer, physiological regulation of these receptors is poorly understood. In this study, we evaluated the distribution of EGF-R in mammary glands during various physiological stages as well as during the process of chemically-induced mammary carcinogenesis in rats.

Materials and methods

Animals. Sprague-Dawley female rats were obtained from Harlen Sprague Dawley Co. Madison, WI. Animals were housed in polycarbonate cages, 3 per cage, in a room artificially illuminated for 14 h each day and maintained at 22°C. They were allowed free access to water and food. Female rats were mated with males, the day sperm appeared in the vaginal wash was considered as day 1 of pregnancy. Days of lactation was monitored as days post partum.

Mammary tumors were induced by a single intravenous injection of 50 mg/kg body weight of N-methyl-N-
nitrosourea (MNU) to 50 day old rats as described previously (11). Mammary glands were obtained after 1, 5, 10, 15, and 20 days post carcinogen treatment. Mammary tumors were obtained from these rats as they appeared. Most of the tumors appearing in rats under these conditions are adenocarcinomas and ovarian hormone dependent.

**Membrane preparation.** Membrane fraction was prepared by a modification of the method described by Edery et al (8). Briefly, mammary tissues were weighed, minced and homogenized in 25 mM Tris.HCl buffer (pH 7.4) containing 10 mM MgCl₂ and 0.1% bovine serum albumin (TMB buffer). The homogenate was centrifuged at 2000 x g for 10 min, the resulting supernatant was further centrifuged at 100,000 x g for 30 min. The membrane pellet was used for EGF receptor assays.

**EGF receptor assay.** Membrane fraction was suspended in TMB buffer so that 40 μl of suspension contained at least 100 μg protein. Assay mixture of 120 μl contained increasing amounts of [¹²⁵I]EGF, in the range of 0.25x10⁻¹⁰ M to 3.0x 10⁻¹⁰ M, either alone or in the presence of 60 ng unlabeled EGF for 16 h at 25°C. After the incubation, the membrane pellet was recovered by centrifugation and washed with TMB buffer twice. Reaction tubes were counted for radioactivity in a Tracor gamma counter. Protein concentrations were measured in the membrane fraction using the Lowry procedure. EGF receptor concentration and binding affinity were determined by Scatchard analysis.

**Results**

Receptor assays for EGF are generally carried out either at 0°C or 25°C. We determined the time and temperature dependence of EGF binding with the membrane fraction of the mammary tissues. Aliquots of membrane fractions prepared from the mammary glands were incubated with 0.36 ng [¹²⁵I]EGF at either 0°C, 25°C or 37°C. A time course of EGF binding was generated for a 16 hour period. Non-specific binding was assessed by incubating the reactions in the presence of excess unlabelled EGF and specific binding was determined as a difference between total and non-specific binding. Results are shown in Fig. 1. At 37°C the binding reached a peak within 2 hours and declined rapidly. However at 0°C there was a steady increase in binding for the first 6 hours and the binding remained unaltered until 16 hours. A similar time course of EGF binding was observed between 0°C and 25°C. All assays were performed at 25°C in the subsequent experiments.

Specificity of EGF receptor binding was estimated by incubating mammary membrane fraction with 0.36 ng [¹²⁵I]EGF either alone or in the presence of increasing concentration of unlabeled EGF, transforming growth factor β, insulin like growth factor, fibroblast growth factor, insulin, or prolactin for 16 hours at 25°C. Specific binding was determined. Only EGF competed for the EGF receptor sites. Other growth factors did not bind with EGF receptors (data not shown).

EGF receptor concentrations and binding affinities were measured in the mammary glands derived from virgin, pregnant and lactating rats. Scatchard plots were generated for all the tissues. Results showed no difference in the affinity of EGF receptor among these three tissues. However, the EGF receptor concentration was found to be the highest for the pregnant animals followed by a dramatic reduction in the EGF receptors in the mammary glands of the lactating rats (Table I). Mammary glands from virgin rats contained 11.3±2.6 fmole/mg protein, as compared to 21.6±5.9 fmole/mg protein for pregnant and 7.4±2.3 fmole/mg protein for lactating rats. The affinity for binding ranged from 1x10⁻¹⁰ to 6x10⁻¹⁰ M in all the tissues.

EGF receptor assays were carried out on MNU-induced mammary tumors. Of the 20 mammary tumors evaluated, the majority had no saturable binding of EGF. Scatchard analyses indicated no EGF receptors in 18/20 tumors (Fig. 2)
and Table I). It has been well established that the majority (approximately 90%) of the MNU-induced mammary adenocarcinomas are ovarian hormone dependent (12). To rule out the possibility that the lack of receptor was not due to preoccupied EGF receptors by endogenous EGF, a denaturation assay was carried out. Membrane fraction was treated with 3 M MgCl₂ for 2 minutes at room temperature followed by addition of 3 ml TMB buffer. The mixture was centrifuged to obtain the membrane pellet. EGF receptor assay was performed as described above. This did not alter the binding capacity in the tumors (data not shown).

The studies described above indicated that MNU-induced tumors, which are largely ovarian hormone dependent, were EGF receptors negative. These results were compared with human breast cancer samples that were either steroid receptor positive (ER⁺ PR⁺) or negative (ER⁻ and PR⁻). As shown in Fig. 3 there was a single class of EGF receptors present in ER⁺ breast cancer samples; whereas ER⁻ samples were largely EGF receptor negative. It was found that only 1/8 ER⁺ breast cancer tissues were EGF receptor positive; whereas all (5/5) ER⁻ samples were EGF receptor positive.

Since normal mammary glands contained EGF receptors whereas mammary tumors did not have saturable EGF binding, experiments were designed to examine the time course of the loss of EGF receptors post carcinogen treatment. Rats were injected with MNU as described in the Methods section and mammary glands were removed for EGF receptor analysis after days 5, 9, 15, and 20 post MNU treatment. The tissues were kept frozen until analyzed. Results showed that the mammary glands from virgin rats treated with MNU contained high affinity EGF receptor binding sites comparable to that observed in the mammary tissues of non-carcinogen treated rats (Fig. 2). The binding increased gradually for 20 days post carcinogen treatment (Table II). Since palpable mammary tumors are normally observed at approximately 30 days post carcinogen treatment, EGF receptors in the mammary glands of carcinogen treated animals were measured only during the first 20 days post MNU treatment.

**Discussion**

The role of EGF in the differentiation of many cell types is well recognized (13). Mammary epithelial cells exhibit enhanced growth in the presence of EGF (2,3,14). Moreover, EGF is reported to maintain lactational state in the nursing
rats. In mammary gland organ cultures EGF is not required for the development of hormone-induced alveolar structures, however the glands do not respond to hormonal stimulus in the absence of EGF for the second round of alveolar growth (4). These results collectively indicate that EGF is required for both differentiation and proliferation of mammary glands. In the present study we measured EGF receptors in the membrane fraction of the mammary gland form virgin, pregnant and lactating rats. In all the tissues EGF bound to the receptors with a single class of binding sites. The affinity of EGF for the binding sites was in the range of 1 x 10^6 to 6 x 10^10 M, which is consistent with that reported in the literature (8). The lactating rats had the lowest level for the EGF receptors. These results suggest that EGF receptor concentration increases during structural differentiation of the gland and decreases during functional differentiation of the gland. Thus, there is a differential distribution of EGF receptors in mammary tissues dependent on the physiological state of the rat. These results suggest a possible hormonal regulation for the expression of EGF receptors. Similar results have been reported for mouse mammary glands in vitro and in vivo (4,8).

Interestingly, the situation is quite different for mammary cancers. Presence of EGF receptor is considered as a poor prognosis for breast cancer patients (10). In general, steroid receptor positive human breast cancer samples have poor EGF receptor levels (9). We observed that the majority of the mammary tumors induced by MNU lack EGF receptors, whereas normal mammary cells and tissues are positive for EGF receptors. The question arises whether this loss of EGF receptor in hormone-responsive mammary tumor tissue is a gradual process during the course of carcinogenesis or a characteristic of the hormone-dependent cancer cell itself. Previous studies from our laboratory as well as by others have shown that 50 mg/kg body weight of MNU induces 100% incidence of mammary tumors in these rats, which makes mammary glands from all rats treated with 50 mg/kg MNU as ‘high-risk’ glands for developing cancer. Since there was no loss of EGF receptors during 20 days post carcinogen treatment, it can be stated that the loss of EGF receptors in the tumors is a characteristic of cancer cells rather than the mammary gland at ‘high risk’ of developing cancer.

The finding that steroid receptor positive human breast cancer samples are often EGF-R negative has a significant clinical implication (9). However, these studies are normally conducted on small tumor biopsies from a large number of samples and often using single point assays (9,14). We analyzed EGF receptors in a small number of steroid receptor-positive and negative breast cancer samples using titration assays and by generating Scatchard plots. Results from Scatchard analyses indicated that only one out of eight ER+ tumor samples (13%) was positive for EGF receptors. It would be of interest to investigate whether ER+, EGF-R+ tumors are less responsive to antihormone treatment as compared to ER+, EGF-R+ tumors. As shown in the sections result, only 10 percent (2/20) of the experimental mammary adenocarcinomas contained EGF receptors. Although we have not examined ovarian hormone-dependent and independent tumor samples in the present study, it has been well established that nearly 90 percent of the mammary tumors induced by MNU in rats are ovarian hormone-dependent (15). Lack of measurable EGF binding in these tumors provide consistent correlation with that observed for the human breast cancers where steroid receptor-positive cancers are usually EGF receptor-negative.

Briefly, in this report we demonstrated that EGF receptor is present in the mammary glands during differentiation and lost only after the tumor phenotype is established. During the process of carcinogenesis, mammary glands treated with carcinogen which are at a ‘high-risk’ of developing tumors do not show reduced EGF receptor concentration.

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References