Role of Estrogen and Prolactin in the Growth and Receptor Levels of N-Nitrosomethylurea-induced Rat Mammary Tumors

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ABSTRACT

Forty-eight of 81 (59%) of N-nitrosomethylurea-induced rat mammary tumors regressed in average to almost one-half of the original size 10 days after ovariectomy (ovax) (hormone responsive), while 33 remained essentially unchanged (hormone resistant). At 20 days after ovax, further decline in hormone-responsive tumors was observed when the rats were treated daily with 0.9% NaCl solution on the tenth day after ovax. Treatment for the same length of time with estrogen either alone or in combination with bromocryptine (to effectively suppress serum prolactin level) prevented tumor regression in hormone-responsive tumors. A similar effect was observed when rats were treated with perphenazine (to stimulate endogenous prolactin secretion) either alone or in combination with the antiestrogen tamoxifen. Estrogen receptors (ERs) significantly declined after ovax. Treatment with estrogen or perphenazine did not have any significant effect on ER levels. Progesterone receptors (PGRs) became virtually undetectable after ovax. Treatments with estrogen, progesterone plus bromocryptine, and perphenazine plus tamoxifen but not perphenazine alone were able to partially restore PGRs although this effect was of borderline statistical significance. ER and PGR levels did not significantly differ between hormone-responsive and -resistant tumors within each group. We conclude that both estrogen and prolactin play a role in the growth of the N-nitrosomethylurea-induced rat mammary tumor. Changes in ER and PGR levels did not correlate with tumor growth under the present experimental conditions.

INTRODUCTION

The vast majority of NMU-induced mammary tumors have been found to be hormone responsive (1, 4, 15). Receptors for estrogen, progesterone, and PRL have been described and characterized in these tumors (1, 17). Recent preliminary data obtained in our laboratory have indicated that both 17β-estradiol and PRL play a role in influencing tumor growth (2). In this experiment, we have investigated the effect of 17β-estradiol and PRL on the growth and hormone receptor levels of the NMU-induced mammary tumor in ovax rats. Since 17β-estradiol is well known to stimulate PRL secretion from the pituitary gland, in order to evaluate the individual effect of 17β-estradiol, we have simultaneously administered BR, which effectively suppresses serum PRL level. Conversely, we have investigated the action of PRL endogenously stimulated by PZ, when 17β-estradiol action was presumably blocked by simultaneous administration of the antiestrogen TAM.

MATERIALS AND METHODS

Materials. NMU was obtained from Sigma Chemical Co. (St. Louis, Mo.). 17β-2,4,6,7-3H]Estradiol (specific activity, 108 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, Ill.) and unlabeled 17β-estradiol was obtained from Sigma. R5020; (specific activity, 87 Ci/mmol) and the corresponding unlabeled hormone were obtained from New England Nuclear (Boston, Mass.). Rat PRLRIA kits were generously supplied by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases. TAM (ICI 46474; pure base powder) was a gift from Imperial Chemical Industries, United States, Inc., Wilmington, Del. Trilafon (aqueous solution of PZ) was obtained from Schering Corp., Bloomfield, N. J. BR was obtained from Sandoz Pharmaceuticals, Hanover, N. J.

Animals and Experimental Design. Mammary tumors were induced in 50-day-old female Sprague-Dawley rats by 2 i.v. injections of NMU (5 mg/100 g body weight) given 1 week apart. Mammary tumors appeared 5 to 15 weeks following the first NMU injection. Tumors were measured twice weekly under light ether anesthesia. Tumor sizes were expressed in sq cm and were derived from the product of the lengths of the 2 major axes, measured with a caliper. Bilateral ovax was performed via the dorsal route under light ether anesthesia. Tumors were defined as hormone responsive if they regressed to ≤75% of the original size 10 days after ovax. When multiple tumors were present in a rat, the growth pattern of each was considered separately.

The following experimental procedures were performed. Seven groups of tumor-bearing rats were used. One group was left untreated and observed over a 20-day period at the end of which the rats were sacrificed. The other 6 groups underwent ovax. Ten days after ovax, one group was sacrificed while the remaining 5 were started on one of the following regimens: 0.9% NaCl solution; 5 µg 17β-estradiol per day; 5 µg 17β-estradiol per day + 1 mg BR twice per day; 1 mg PZ per day; and 1 mg PZ + 200 µg TAM per day. 17β-Estradiol, BR, and TAM were first dissolved in 100% ethanol and then diluted further with 0.9% NaCl solution and given i.m. in a volume of 0.2 ml PZ, in aqueous solution (5 mg/ml), was also given i.m. in 0.2 ml volume. All injections were given between 8 and 10 a.m. An additional dose of BR was given at 5 p.m. The treatment in these 5 groups was continued for 10 days, and the rats were sacrificed 24 hr after the last injection. No significant change in body weight was observed with any of the treatments used. At the time of sacrifice, mammary tumors were removed, immediately frozen in liquid nitrogen, and stored at −70° until the time of receptor assays. In addition, 3 to 5 ml of blood were drawn through an intracardiac puncture just before sacrifice, and the sera obtained were stored at −20° until the RIA for rat PRL was performed.

RIA and Receptor Binding Assays. Standard RIA techniques using the kit and procedure kindly provided by the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases was used in measuring serum rat PRL levels.
The ER level in the cytosol of mammary tumors was measured using the dextran-coated charcoal technique (10). Briefly, the 100,000 x g cytosol fraction was incubated for 16 hr at 4° with serial dilutions of labeled estradiol (from 0.06 to 0.8 nm). The free radioactivity was removed by the addition of dextran-coated charcoal with subsequent shaking for 30 min and spinning at 800 x g for 10 min. Aliquots of the supernatant were then transferred to scintillation vials and counted in a β counter. Scatchard plot analysis (16) was used to determine the number of binding sites expressed as fmol/mg cytosol protein. PGR level in the cytosol was measured using the synthetic progestin R5020 and using the sucrose density ultracentrifugation method (6). Briefly, the 100,000 x g cytosol fraction was incubated for 4 hr at 4° with labeled R5020 (20 nm) in the presence or absence of a 200-fold excess of unlabeled R5020. After removal of the free radioactivity with dextran-coated charcoal, aliquots of bound radioactivity (both specific and nonspecific) were layered on top of a 5 to 20% sucrose gradient and spun for 16 hr at 55,000 rpm. Subsequently, the bottoms of the centrifugation tubes were punctured, and the bound radioactivity was collected in fractions of 6 drops each. The number of specific binding sites was calculated by subtracting the nonspecific binding (both specific and nonspecific) from the total binding and was expressed as fmol/mg cytosol protein. PGRs was observed between hormone-responsive and -resistant tumors. A total of 81 tumors could be evaluated for response to ovax 10 days after castration (Group B). Forty-eight (59%) showed significant regression, as defined above, to a mean of almost one-half of the original size, while 33 (41%) remained in average essentially unchanged and were considered hormone resistant. Twenty days after ovax, when only 0.9% NaCl solution was injected, a further reduction in size of hormone-responsive tumors was observed (Group C versus Group B, p = 0.06). Treatment with 17β-estradiol (Group D), 17β-estradiol plus BR (Group E), PZ (Group F), and PZ + TAM (Group G) was able to prevent the regression of hormone-responsive tumors which was observed in the 0.9% NaCl solution-treated group although the effect of 17β-estradiol plus BR and PZ was of borderline statistical significance (p < 0.08 and 0.06 when comparing Group C with Groups E and F, respectively). Of interest, hormone-resistant tumors too showed evidence of regression 20 days after ovax which appeared to be prevented by the treatments used. However, these changes were not significant, probably due to the small number of tumors and large variation in some groups.

**RESULTS**

**Tumor Size (Table 1).** Tumors in the untreated group (Group A) had grown an average of over 1.5 times their original size at the end of the observation period of 20 days. Since we did not perform any hormonal manipulation in this group, we cannot obviously distinguish between hormone-responsive and -resistant tumors. A total of 81 tumors could be evaluated for response to ovax 10 days after castration (Group B). Forty-eight (59%) showed significant regression, as defined above, to a mean of almost one-half of the original size, while 33 (41%) remained in average essentially unchanged and were considered hormone resistant. Twenty days after ovax, when only 0.9% NaCl solution was injected, a further reduction in size of hormone-responsive tumors was observed (Group C versus Group B, p = 0.06). Treatment with 17β-estradiol (Group D), 17β-estradiol plus BR (Group E), PZ (Group F), and PZ + TAM (Group G) was able to prevent the regression of hormone-responsive tumors which was observed in the 0.9% NaCl solution-treated group although the effect of 17β-estradiol plus BR and PZ was of borderline statistical significance (p < 0.08 and 0.06 when comparing Group C with Groups E and F, respectively). Of interest, hormone-resistant tumors too showed evidence of regression 20 days after ovax which appeared to be prevented by the treatments used. However, these changes were not significant, probably due to the small number of tumors and large variation in some groups.

**Receptors (Table 1).** Since no significant difference in ERs and PGRs was observed between hormone-responsive and -resistant tumors within each group, they are analyzed together. Ten days after ovax, ERs were significantly decreased. No further decline was observed 20 days after castration when only 0.9% NaCl solution was injected. Treatment with 17β-estradiol or PZ did not significantly affect ERs. As expected, ERs were barely detectable when TAM was administered.

PGR declined markedly 10 days after ovax and were undetectable 20 days after castration. Treatment with 17β-estradiol + BR and PZ + TAM was able to partially restore PGRs. Somewhat surprisingly, 17β-estradiol treatment had only a
modest and insignificant effect. No effect on PGRs was observed with PZ administration.

**Serum PRL (Table 1).** As expected, serum PRL level declined although not significantly following castration presumably because of the lowering in serum estrogen. Treatment with 17β-estradiol restored serum PRL to control level, whereas treatment with PZ either alone or in combination with TAM markedly increased serum PRL over base line. It should be noted that, under the present experimental conditions, the administration of BR could completely counteract the stimulatory effect of 17β-estradiol on PRL secretion (Group E).

**DISCUSSION**

Although the hormone responsiveness of the NMU-induced rat mammary cancer is well established (1, 4, 15), the individual role of gonadal and pituitary hormones in supporting tumor growth still remains largely unknown. Our data suggest that both 17β-estradiol and PRL may have a direct effect on tumor growth. Since 17β-estradiol is well known to stimulate PRL secretion from the pituitary, it is essential to evaluate 17β-estradiol action when PRL secretion was virtually totally suppressed by simultaneous administration of BR (Group E). Under these conditions, 17β-estradiol was able to prevent regression of tumor growth which was observed in the 0.9% NaCl solution-treated rats, although this effect was of borderline statistical significance. Likewise, hyperprolactinemia induced by PZ was found to have a similar effect when 17β-estradiol action was presumably blocked by TAM (Group G). In support of a nearly complete blockade of 17β-estradiol action in this group of rats is the finding that ERs were barely detectable. Under these experimental conditions, we did not observe any synergism between 17β-estradiol and PRL, since no enhancement of tumor growth was observed in Group D treated with 17β-estradiol alone where estrogen action was available in the presence of a high to normal circulating level of PRL. It is possible that we might have seen synergism had we treated a group with 17β-estradiol and PZ, which would have caused a much higher serum PRL level.

It is of interest to note that tumors that we called “hormone resistant,” based on lack of regression 10 days after ovx, regressed somewhat 20 days after ovx. In addition, their growth appeared to be affected by the hormonal manipulations used in a similar fashion to the “hormone-responsive” tumors, suggesting that perhaps they had some degree of hormone responsiveness. No firm conclusion can be drawn in this regard, however, since the changes in size were not statistically different, probably as a result of the small number of tumors and large variation in some groups.

Our data are in agreement with recent preliminary experiments performed by us in hypophysectomized rats (2) in which an individual role of 17β-estradiol and PRL in supporting NMU tumor growth was apparent. Under those experimental conditions, we were actually able to demonstrate synergism between the 2 hormones. In agreement with our results, Rose and Noonan (13) observed in hypophysectomized rats that both 17β-estradiol and PRL support the growth of NMU mammary tumors. In contrast, in experiments still conducted in hypophysectomized rats, Pruitt and Rose (11) failed to show a direct effect of 17β-estradiol in supporting the growth of the NMU mammary cancer. In other experiments, however, Pruitt et al. (12) provided evidence for a major role of 17β-estradiol in mediating the growth of this tumor model.

They observed that 17β-estradiol administration after luteinizing hormone releasing hormone analog-induced tumor regression was able to reactivate tumor growth while PZ administration after ovx was ineffective in restoring the growth of regressing tumors.

The endocrinology of the NMU mammary tumor appears to be different from the DMBA mammary tumor which in our experience is PRL dependent and in which we have been unable to demonstrate a direct effect of estrogen on tumor growth (3). Experiments conducted in rats bearing DMBA-induced mammary tumors revealed that, when serum PRL was effectively suppressed with lergotrile mesylate, administration of 17β-estradiol had no demonstrable effect on tumor growth (9). In contrast, the NMU mammary tumor appears to be less exclusively PRL dependent. Further evidence to support this concept has been provided recently by Rose and Noonan (14). These authors observed that PRL-suppressing drugs had a much more potent antitumor effect in the DMBA mammary tumor than in the NMU mammary tumor. Nevertheless, they still demonstrated a role of PRL in the growth of the NMU mammary tumor since PRL administration was able to reinitiate or at least stabilize tumor growth after hypophysectomy-induced tumor regression.

Thus, it is fair to summarize that, whereas the DMBA mammary tumor is PRL dependent, the NMU mammary tumor is both PRL and 17β-estradiol dependent. Since estrogens are likely to stimulate the growth of breast cancer in women (7, 8), the NMU mammary tumor may resemble human breast cancer more closely than DMBA mammary tumor.

In agreement with previous reports (1, 17), ER levels decreased after ovx. Except for the expected suppression in ERs observed in the TAM-treated group (Group G), neither estrogen nor PZ administration had any effect on ERs, whereas they had significant effects on tumor growth. Similarly, we have failed to show any effect of 17β-estradiol and PRL on ER levels of NMU mammary tumors of hypophysectomized rats (2).

As in the DMBA mammary tumor (3), PGR levels were found to be unaffected by PRL (Group F). Of interest, PGR levels were increased when PZ was administered with TAM (Group G). TAM has indeed been found to stimulate PGR under certain circumstances, perhaps as a result of an intrinsic estrogenic activity (5). We were somewhat surprised that 17β-estradiol treatment was not more effective in stimulating PGRs.

ER and PGR levels were not significantly different between hormone-responsive and -resistant tumors within each group. However, since the number of tumors in each subgroup was small, a firm conclusion cannot be drawn in this regard.

**REFERENCES**


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