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Effects of Progesterone Administration on *N*-Nitrosomethylurea-induced Rat Mammary Carcinogenesis*

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Abstract—*N-Nitrosomethylurea (NMU), 4 mg/100 g body wt, was given to female Sprague–Dawley rats by i.v. injection on 2 occasions, 4 weeks apart. One group of 20 animals also received 5 mg of progesterone s.c. on the morning before, of and after each NMU dose (acute progesterone treatment). A second group of 21 rats was given progesterone 2.5 mg twice a day throughout the experiment commencing 7 days before the first dose of NMU (chronic progesterone treatment). The third group of 20 animals comprised the NMU-exposed controls. The latent period for mammary tumor development was reduced and the number of tumors per rat was increased by the acute progesterone treatment. The final mammary tumor incidence for the chronic progesterone treatment group (62%) was lower than that of the controls (85%) and the acute progesterone-treated rats (80%), and tumor multiplicity was less. Estrogen receptor levels were significantly higher in tumors from the chronic progesterone group than in those from the acute progesterone-treated animals ($P < 0.01$), and progesterone receptor levels were lower in comparison to either of the other 2 groups. Serum progesterone concentrations were subnormal in the NMU-exposed controls but the estrogens were unaffected. The acute progesterone-treated rats also had reduced serum progesterone levels when compared with normal animals, although they were significantly higher than those of the NMU-exposed controls ($P < 0.01$). Extremely high serum progesterone levels in rats treated chronically with progesterone were accompanied by reduced estrogen concentrations. Serum prolactin levels were elevated in the NMU-exposed controls and chronic progesterone-treated groups compared with non-NMU-exposed normal rats, while growth hormone concentrations were reduced by progesterone administration. All 3 NMU-exposed groups had elevated serum TSH levels.*

INTRODUCTION

RAT MAMMARY carcinomas induced by *N*-nitrosomethylurea (NMU) are frequently hormone-dependent and undergo regression after ovariectomy [1, 2], antiestrogen therapy [3] or hypophysectomy [4, 5]. That there is a direct requirement for estrogens distinct from that for prolactin is indicated by the arrest of hypophysectomy-induced regressions during treatment with estradiol [4, 6].

Progesterone is a recognized co-carcinogen for chemically induced rat mammary tumors [7–9], but its administration reduced both tumor incidence and multiplicity when commenced 25 days before a single i.v. injection of dimethylbenz(a)anthracene [10]. Sherman and Korenman [11] postulated that inadequate corpus luteal function is a risk factor for human breast cancer because it permits excessive estrogen activity due to a lack of the normal modulating action of progesterone. In support of this hypothesis, low plasma progesterone levels were observed in women at high breast cancer risk [12], and a secretory endometrial pattern was found less

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frequently in breast cancer patients than normal women [13].

We reported previously that female rats exposed to NMU exhibit arrest of the estrous cycle at estrus, endometrial hyperplasia with adenomatous formation, polycystic ovaries and low serum progesterone levels [14, 15]. The purpose of the present study was 2-fold: to determine the influence of progesterone administration on NMU-induced mammary carcinogenesis and also on the endometrial pathology. The protective effect of chronic treatment on the endometrium was reported in an earlier communication [15].

MATERIALS AND METHODS

Female Sprague–Dawley strain rats were purchased from King Laboratories, Oregon, WI at 39 days of age. All rats received 2 i.v. doses of NMU, 4 mg/100 g body wt, the first at age 50 days and the second 1 month later. The controls received no other treatment. The second group was given 5 mg of progesterone (Sigma Chemical Co., St. Louis, MO) dissolved in 0.1 of peanut oil by s.c. injection on the morning before, of and after each of the 2 NMU doses (acute progesterone treatment). The third group commenced treatment with progesterone, 2.5 mg twice daily, 7 days before the first dose of NMU and continued on this regimen throughout the experiment.

All animals were palpated every 7 days for mammary tumors, starting 8 weeks after the first NMU injection. At this time vaginal smears were taken daily for 5 days. The rats were killed and autopsied when they had developed a mammary tumor with a maximum diameter of approximately 2 cm or, if this had not occurred, at 20 weeks from the first dose of NMU. Those treated continuously with progesterone received their last dose 1 day before termination. During the late morning hours, immediately prior to termination, the rats were anesthetized with ketamine, blood samples were taken from the external jugular vein for serum hormone assays and the mammary tumors excised. A portion of each tumor was placed in WARF fixative [1] and the rest frozen in 10 mM Tris, 1 mM EDTA, 0.25 M sucrose buffer, pH 8.0, at -70°C until assayed for estrogen receptor (ER) and progesterone receptor (PgR) content. Fixed tissues were cut into 5-μm sections and stained with hematoxylin and eosin.

Serum for hormone assays was separated immediately and stored at -70°C. Progesterone was extracted from serum with petroleum ether [16] and measured by radioimmunoassay [17] using an anti-progesterone antibody purchased from Accutate Chemical and Scientific Corporation, Hicksville, NY. Serum prolactin, growth hormone and TSH radioimmunoassays were performed using the reagents and procedures provided by the National Hormone and Pituitary Program of the National Institutes of Health. The serum hormone levels were not normally distributed, but became so after conversion to the $\log_{10}$; this was done prior to making statistical comparisons by Student's t test. The ER and PgR analyses of mammary tumors were performed as previously described [14].

RESULTS

The cumulative NMU-induced mammary tumor incidence for the 20 controls, 20 acute progesterone and 21 chronic progesterone-treated rats is illustrated in Fig. 1. When weekly palpation was commenced all of the controls and all but 1 of the rats treated continuously with progesterone were tumor-free, whereas 7 (35%) of the acute progesterone-treated group had already developed a tumor. By 20 weeks, mammary tumors were present in 80% of the controls, 85% of the acute progesterone, and 62% of the chronic progesterone-treated rats.

The cumulative total tumor number for the 3 groups is shown in Fig. 2, and reflects the stimulatory effect of the acute progesterone

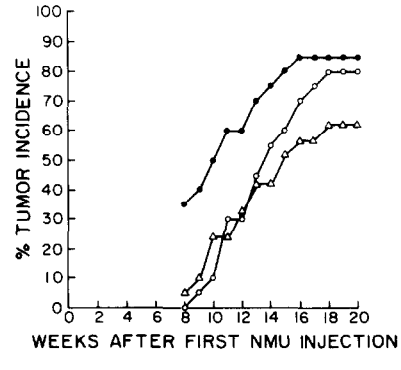

Fig. 1. Cumulative incidence of NMU-induced mammary tumors in control (○), acute progesterone (●) and chronic progesterone (△) treatment groups.

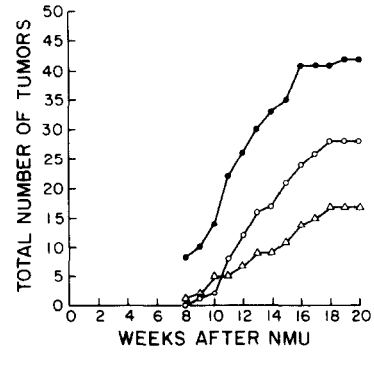

Fig. 2. Cumulative total tumor number after NMU exposure in control (○), acute progesterone (●) and chronic progesterone (△) treatment groups.

regimen and the protective effect of chronic administration when commenced 7 days prior to the first NMU injection. The mean number of tumors per tumor-bearing rat for the acute progesterone-treated group, 2.4 ± 1.2, was higher than that of the chronic treatment group, 1.3 ± 0.5 ($P < 0.01$), while the intermediate value for the controls, 1.7 ± 0.8, did not differ significantly from either of the steroid-treated groups. The final mean body weight of the chronic progesterone-treated animals (292 ± 18 g) was significantly higher than that of the control (257 ± 23 g) and acute progesterone-treated (243 ± 25 g) groups ($P < 0.001$).

All but 1 of the tumors, a fibroadenoma, were carcinomas. The least well differentiated were fibroadenocarcinomas or cystic papillary adenocarcinomas, but with some areas composed of sheets of anaplastic cells showing much mitotic activity. No pure medullary carcinomas were seen. The tumors which did develop in rats treated chronically with progesterone did not differ significantly in their histological appearance from those of the other 2 groups.

The vaginal smears taken for 5 days at 8 weeks from the first dose of NMU showed the effect of continuous progesterone treatment, all but 5 of the 21 rats being acyclic with an absence of endometrial estrogenization. Five of the 20 controls were in an apparent state of continuous estrus, while the acute progesterone-treated group all had normal cycles.

The tumor ER and PgR results are summarized in Table 1. There was a wide range of ER values in all 3 groups. The mean level for the chronic progesterone-treated group was significantly higher than that of the acute progesterone-treated group; a similar trend compared with the untreated controls did not achieve statistical significance. The PgR concentrations were significantly lower in tumors from rats treated chronically with progesterone than in those from the untreated controls ($P < 0.01$) or the acute progesterone-treated group ($P < 0.02$).

The serum hormone levels are summarized in Table 2 and compared with those of 35 normal animals of similar age, housed and maintained under identical conditions, that had not been exposed to NMU. Serum TSH levels were significantly higher in all 3 NMU-exposed groups than in the non-exposed rats ($P < 0.001$), as were the serum prolactin concentrations in the NMU-exposed controls ($P < 0.001$) and the chronic progesterone-treated group ($P < 0.02$). Continuous progesterone administration caused a significant reduction in serum growth hormone compared with both the NMU-exposed controls ($P < 0.001$) and the normal rats ($P < 0.02$). The mean serum growth hormone concentration of the acute progesterone-treated group was also lower than that of the NMU-exposed controls ($P < 0.02$), but did not differ significantly from that of the non-exposed rats. The serum progesterone concentrations were markedly

Table 1. Estrogen receptor and progesterone receptor levels (mean ± S.D.) of NMU-induced rat mammary tumors

Group	No. of tumors	fmol/mg protein ER	PgR
Controls	24	42.4 ± 25.6	211.4 ± 88.0
Acute progesterone-treated	33	40.9 ± 19.2	218.4 ± 127.5
Chronic progesterone-treated	18	60.4 ± 24.5*	122.0 ± 77.2†‡

Significantly different from the acute progesterone-treated group: *$P < 0.01$; †$P < 0.02$.
‡Significantly different from the control group by Student's t test: $P < 0.01$.

Table 2. Serum hormone levels (mean ± S.D.) in normal female rats, NMU-exposed controls and progesterone-treated, NMU-exposed animals

Group	(No.)	TSH (μg/ml)	Growth hormone (ng/ml)	Prolactin (ng/ml)	Progesterone (ng/ml)	Estrogens (pg/ml)
Non NMU-exposed controls	(35)	0.3 ± 0.06	101 ± 83	8.4 ± 10.0	51.1 ± 21.8	117 ± 35
NMU-exposed controls	(20)	0.6 ± 0.3*	145 ± 89	28.8 ± 22.6*	16.9 ± 8.4*	100 ± 25
Acute progesterone-treated	(20)	0.4 ± 0.2*	88 ± 40‖	10.1 ± 13.8§	26.6 ± 10.3*§	101 ± 24
Chronic progesterone-treated	(21)	0.5 ± 0.2*	55 ± 21†‡	19.4 ± 22.1†	216.3 ± 47.8*‡	73 ± 17*‡

Significantly different from the non NMU-exposed controls: *$P < 0.001$; †$P < 0.02$.
Significantly different from the NMU exposed controls: ‡$P < 0.001$; §$P < 0.01$; ‖$P < 0.02$.

reduced in the NMU-exposed controls compared with those of normal rats ($P < 0.001$). While the acute progesterone-treated animals also had subnormal serum progesterone levels, these were significantly higher than those of the NMU-exposed controls ($P < 0.01$). All of the rats treated continuously with the steroid showed the expected extremely high serum progesterone levels, and the serum estrogens were reduced in comparison with the normal rats and NMU-exposed controls ($P < 0.001$).

DISCUSSION

The administration of progesterone may enhance DMBA-induced rat mammary carcinogenesis [7–9] and stimulate the growth of the tumors once they are established [7]. When continuous treatment was started 2 days prior to or 15 days after giving a single dose of the carcinogen, it increased tumor incidence and multiplicity and reduced the latent period [8]. However, when progesterone administration was commenced 25 days before i.v. injection of DMBA there was a reduction in both tumor incidence and multiplicity [10]. In a similar experiment progesterone was shown to be protective when given for 20 days before and 20 days after the injection of DMBA [18]. It was suggested that this inhibitory effect arose because of enhanced development of the mammary glands, with lobuloalveolar growth such that they were resistant to carcinogenic action.

In our study the chronic progesterone treatment was started only 7 days before the first dose of NMU, and yet mammary tumor incidence and multiplicity were both reduced. This inhibitory effect on the carcinogenic process contrasted with the co-carcinogenic activity of the steroid when treatment was begun 2 days prior to DMBA [8].

Progesterone is known to be an antagonist of estrogen action and to inhibit estrogen-mediated growth of target organs [19–21]. Hsueh *et al.* [22] showed that treatment of immature female Sprague–Dawley rats with progesterone decreases the cytoplasmic uterine ER levels and in consequence, the sensitivity of the uterus to estrogen. Other investigators have also produced evidence that progesterone opposes the action of estrogen on uterine tissue by decreasing binding capacity [21].

The NMU-exposed female rat, with low serum progesterone levels, provides a suitable model for progesterone deficiency as an etiologic factor in both carcinoma of the breast and endometrium. Epidemiological studies have shown that these two cancers occur in the same patient with a higher frequency than would be expected from a chance association [23]. Bulbrook *et al.* [12] reported subnormal plasma progesterone levels in pre-menopausal women at high breast cancer risk because of combinations of several recognized risk factors. Also, women with a history of infertility and progesterone deficiency are at an increased risk of breast cancer [24], and low plasma progesterone concentrations occur in patients with fibrocystic breast disease [25], which is itself associated with subsequent malignancy [26]. Analogous to the uterine changes which are seen in rats exposed to NMU [14, 15] are the reports by Grattarola that a high incidence of endometrial hyperplasia, with anovulatory menstrual cycles, occurs in both breast cancer [13] and fibrocystic disease [27].

Progesterone deficiency, with resulting overstimulation by estrogen, has also been associated with endometrial carcinoma and prophylactic progesterone therapy proposed for such patients [28]. We reported elsewhere [15] that the chronic progesterone regimen used in the present study prevents the uterine adenomatous hyperplasia which commonly occurs in rats exposed in NMU. A special clinical situation is the Stein–Leventhal syndrome, in which anovulatory menstrual cycles with low progesterone levels and increased estrone production from androstenedione cause endometrial hyperplasia and a risk of neoplastic transformation in pre-menopausal women [29]. The polycystic ovaries, disturbed estrous cycles, progesterone deficiency and endometrial hyperplasia induced in female rats by NMU combine to provide an appropriate model for Stein–Leventhal syndrome and cancer risk. The only element lacking is an elevation in serum estrogens.

It is not certain that estrogen antagonism was the basis for the protection against NMU mammary carcinogenesis afforded by chronic progesterone administration, although there was a reduction in serum estrogen levels in this treatment group. In contrast to the uterus, an antiestrogenic action of progesterone on rat mammary tissue is not well documented. The tumors which did develop during continuous progesterone treatment generally had high ER levels, perhaps because more unfilled receptor sites were available for assay rather than reduced binding, as occurs in the progesterone-exposed endometrium [22]. The lower levels of cytoplasmic PgR were presumably due to a combination of translocation into the nuclei and occupancy of receptor sites by the exogenous hormone. An alternative mechanism is that the 7 days of progesterone administration before the first dose of NMU was given produced sufficient lobuloalveolar proliferation to protect the mammary epithelium from the carcinogen.

The observed changes in serum peptide

hormone levels were of interest, but do not provide a ready explanation for the protective effect of progesterone. Impairment of growth hormone secretion from the somatotrophs by progesterone has been demonstrated in man [30], and chronic administration suppressed the rat serum growth hormone levels. However, there is no evidence that growth hormone is involved in NMU carcinogenesis, and the serum prolactin levels, suppression of which does inhibit development of these tumors [31], were higher than those of controls not exposed to NMU. Further interpretation of the serum prolactin results is complicated because the blood samples were obtained without regard to the estrous cycle. In fact, this was inevitable because the estrous cycle status of the three groups was different even at 8 weeks: 25% of the controls were in a state of prolonged or continuous estrogen stimulation and most of the chronic progesterone-treated groups were acyclic, while the acute progesterone-treated animals had normal cycles. Elevated serum TSH concentrations in NMU-treated rats have been reported previously together with histological abnormalities similar to those of Hashimoto's thyroiditis [32], but these were uninfluenced by progesterone administration.

We reported previously that reserpine, a drug which elevates the serum prolactin, reduced the number of tumors per rat and increased the proportion of well-differentiated tumors when single doses were administered on the day before, of and after exposure to NMU [33]. It was suggested that the mechanism might involve the induction of a state of diestrus during the time of carcinogenic activity, with a reduced co-carcinogenic effect. Nagasawa *et al.* [34] have reported that the percentage of progressive mammary tumors produced, and their growth rate, was increased significantly when DMBA was administered at proestrus compared with exposure during diestrus. Also, on the second day of proestrus mammary gland DNA synthesis was higher than on the second day of diestrus in 50-day-old rats. In this study the same schedule of progesterone treatment, while having no effect on mammary tumor incidence, reduced the latent period and increased multiplicity. Thus, as in the case of DMBA-induced carcinogenesis [8, 10], progesterone may either protect against tumor induction by NMU or act as a co-carcinogen, depending on the schedule of administration.

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