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Decreasing hormonal promotion is key to breast cancer prevention

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Abstract An early full-term pregnancy in women is highly protective against breast cancer. This protection can be mimicked by short-term treatment with estradiol plus progesterone in nulliparous rats. We determined the effect of long-term hormonal promotion following the protective short-term estradiol and progesterone treatment that mimics parity protection against mammary tumors. Rats were treated with *N*-methyl-*N*-nitrosourea before or after protective hormone treatment. In brief, the animals could be broadly classified into three categories. First, the controls that received no protective hormone treatment, second, the short-term protective hormone treated rats, and third, rats which received the short-term protective hormone treatment plus continuous treatment with estradiol or progesterone or a combination of estradiol and progesterone. Different doses of hormones were used for short-term protective and long-term promotion treatments. The experiments were terminated 9 months post carcinogen treatment. Mammary tumor incidence in all the short-term estradiol- plus progesterone-treated rats was significantly lower compared with controls. Short-term hormone treatment followed by long-term promotion resulted in an increased mammary tumor incidence compared with animals that received only the short-term treatment. Overall, the results demonstrate the importance of the promotional environment in mammary carcinogenesis indicating that the decreased promotional environment could be the reason for protection against mammary carcinogenesis.

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Introduction

Women who have undergone a full-term pregnancy early in life have an approximately 50% reduced risk of developing breast cancer compared to nulliparous women [1–3]. A full-term pregnancy before the age of 20 is the only natural phenomenon known that can drastically reduce the risk of breast cancer in women of all ethnic backgrounds worldwide. Rats and mice that undergo a full term pregnancy also have a greatly reduced susceptibility to chemically induced mammary carcinogenesis as compared with nulliparous animals and are used as experimental models to study the factors involved in protection [4–9]. The physiological mechanisms for the protective effect of pregnancy have not been defined. Currently, there are two theories attempting to explain the phenomenon of parity-induced protection against breast cancer. They are (i) The Terminal Differentiation Theory [10, 11] and (ii) The Reduced Promotion Theory [12–14]. The terminal differentiation theory states that the protective effect is likely due to differentiation by hormones associated with pregnancy of the target structures for carcinogenesis, the terminal end buds (TEBs) and terminal ducts. In contrast, the reduced promotion theory suggests that lowered levels of hormones such as prolactin and growth hormone in parous status are the cause for protection.

Parous women have persistently reduced circulatory levels of prolactin and androgens, increased estriol, as well as elevated levels of sex hormone-binding globulins [14], and parous rats have reduced serum levels of prolactin and

growth hormone compared to their nulliparous counterparts [15–17]. Earlier studies demonstrated that parous BALB/c mice are highly refractory to *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis. However, they become highly susceptible to mammary carcinogenesis by the same carcinogen when exposed to high levels of prolactin during initiation and promotion [18, 19]. These studies also suggested that highly stimulated mammary glands containing secretory lobules and lacking TEBs are fully susceptible to carcinogenesis [18, 19]. Thus, refractoriness of parous mice to carcinogenesis is likely due to the pregnancy-induced permanent reduction in secretion of the mammogenic hormone, prolactin, a situation similar to that observed in parous women. Thordarson et al. [17] have reported earlier that there is a significant persistent decrease in the circulating levels of growth hormone and a decrease in estrogen receptor and epidermal growth factor receptor in the mammary gland of parous rats compared to age-matched nulliparous rats. These systemic changes are thought to be associated with the protective effect of pregnancy. Differential gene expressions have also been reported between nulliparous and parous rats [20]. Recent studies indicate that parity-induced protection against mammary carcinogenesis is not permanent, and this protective effect can be reversed by increasing the promotional environment [21]. Long-term administration of ovarian steroids to parous rats results in a high incidence of mammary tumors in carcinogen-treated parous rats [21]. Furthermore, transplantation studies demonstrate that carcinogen treated mammary epithelial cells from young virgin rats when transplanted to parous host become highly refractory to mammary carcinogenesis [22]. All these data indicate that parity-induced protection against breast cancer is not permanent and that parous mammary cells are susceptible to the initiation by carcinogens. Moreover, the data also indicate that the protective effect of parity against mammary carcinogenesis can be reversed by long-term treatment with exogenous hormones.

Hormonal prevention strategies have used exogenous hormonal treatment to mimic the protective effect of pregnancy against breast cancer in experimental animals [23–30]. We have demonstrated that short-term administration of ovarian steroids for 2–3 weeks before or after MNU treatment could confer long-term protection against MNU-induced mammary carcinogenesis [27]. We have also shown that administration of pregnancy levels of estradiol (E) with or without progesterone (P) for 7–21 days is highly effective in decreasing mammary carcinogenesis in rats [28]. The goal of the present investigation is to understand whether our preventive treatment mimics the parous phenotype with respect to the reversal of the protective effect against mammary carcinogenesis by long-term hormone promotion. In this study, we report that short-term protective hormone treatment-induced protection is very similar to parity-induced protection against mammary carcinogenesis.

Results

Short-term treatment with estradiol and progesterone remarkably decreased mammary tumor incidence confirming our earlier findings. Earlier, we had published the levels of serum estradiol and progesterone in response to these treatments elsewhere [27, 28]. In brief, treatment with 200 μg of estradiol plus 30 mg progesterone resulted in 94.9 ± 17.4 pg/ml of estradiol and 21.6 ± 4.5 ng/ml of progesterone. Treating animals with 30 mg of estradiol and 30 mg of progesterone resulted in 168.8 ± 22.1 pg/ml of estradiol and 25.8 ± 6.6 ng/ml of progesterone. Lower dose of estradiol (30 μg) treatment resulted in 51.4 ± 10.5 pg/ml of estradiol in circulation.

Effect of long-term estradiol plus progesterone treatment on the mammary tumor incidence and multiplicity in rats that received the protective hormone treatment after exposure to MNU

This experiment was conducted to examine the effects of long-term hormonal promotion in rats that had received the protective short-term hormone treatment and the carcinogenic insult. Control rats which received only the carcinogen and vehicle treatment had a 100% mammary tumor incidence. Short-term estradiol plus progesterone treatment drastically reduced the mammary tumor incidence. The rats treated with 200 μg of estradiol plus 30 mg progesterone for 3 weeks had a mammary tumor incidence of 11%. Rats that received long-term treatment with 200 μg of estradiol plus 30 mg progesterone continuously throughout the experimental period had a tumor incidence of 67%. These data confirm our earlier findings that short-term treatment with estradiol plus progesterone induces protection against mammary carcinogenesis. But if the hormone treatments were continued throughout the experimental period the mammary tumor incidence increased remarkably indicating the importance of promotional environment for mammary carcinogenesis (Table 1).

The average number of tumors per tumor-bearing rat was significantly lower in rats that received the short-term protective hormone treatment of 200 μg of estradiol plus 30 mg progesterone. Control rats had a mammary tumor multiplicity of 2.1 tumors/tumor-bearing rat, whereas rats which received short-term protective hormone treatment had a markedly lowered multiplicity of 1 tumor/tumor-bearing rat. Rats that had long-term treatment with 200 μg

Table 1 Effect of long-term hormonal promotion on mammary carcinogenesis in rats protected against mammary cancer development

Treatment	Mammary tumor incidence	Percent of rats with mammary tumor	Average number of mammary tumors per tumor-bearing rat
Control	9/9	100	2.1
200 μg E + 30 mg P (3 weeks)	1/9*	11	1.0*
200 μg E + 30 mg P (3 weeks) plus 200 μg E + 30 mg P long term	6/9*,§	67	2.3§

Effect of long-term treatment with a combination of estradiol plus progesterone on MNU-induced mammary carcinogenesis in rats that received the short-term protective hormone treatment. Rats were treated with MNU at 7 weeks of age and 2 weeks later treated with 200 μg of estradiol and 30 mg of progesterone for 3 weeks by silastic capsule. Half of them continued getting the same treatment for 9 months and in the other half treatment was stopped after 3 weeks. The silastic capsules were changed every 2 months throughout the experimental period. Control rats received only the carcinogen treatment. * $P < 0.05$ (compared to control rats); § $P < 0.05$ (compared to short-term protective hormone-treated rats)

of estradiol and 30 mg of progesterone had a mammary tumor multiplicity of 2.3 tumors/tumor-bearing rat (Table 1).

Effect of long-term treatment with estradiol or progesterone or estradiol plus progesterone on mammary tumor incidence and multiplicity

We carried out this experiment to demonstrate the individual impact of long-term estradiol or progestereone treatment in reversing the protective effect of short-term hormone treatment. Rats treated with 200 μg of estradiol plus 30 mg progesterone for 3 weeks had a remarkably decreased mammary tumor incidence (8%) compared to the controls (100%). Rats which received continuous treatment with a lower dose of estradiol immediately after the protective treatment had a tumor incidence of 36% (Table 2). Continuous treatment with progesterone after the short-term protective hormone treatment resulted in 50% mammary tumor incidence. The combination of lower dose estradiol plus progesterone treatment immediately after the protective treatment increased the tumor incidence to 64% (Table 2). Even though the tumor incidence was increased by long term treatment with lower dose of estradiol or progesterone or a combination of both compared to the short-term protective hormone-treated rats, the incidences were lower than the controls.

Control rats had a mammary tumor multiplicity of 3.8 tumors/tumor-bearing rat, whereas rats treated with 200 μg of estradiol plus 30 mg progesterone for 3 weeks had a drastically lowered multiplicity of 1 tumor/tumor-bearing rat. Long-term treatment with lower dose of estradiol or progesterone also resulted in a mammary tumor multiplicity of 1 tumor/tumor-bearing rat. Lower dose of estradiol plus progesterone treatment for long-term had a multiplicity of 1.6 tumors/tumor-bearing rat (Table 2). Tumor multiplicity was significantly lower in all the hormone-treated rats compared to the controls. Even though there was an increase of mammary tumor incidence in the long-term hormone-treated groups compared to their short-term counterparts, they had a lower incidence compared to the controls.

Table 2 Effect of long-term treatment with estradiol or progesterone or combination of both on mammary carcinogenesis

Treatment	Mammary tumor incidence	Percent of rats with mammary tumor	Average number of mammary tumors per tumor-bearing rat
Control	12/12	100	3.8
200 μg E + 30 mg P (3 weeks)	1/12*	8	1.0*
200 μg E + 30 mg P (3 weeks) plus 30 μg E long term	4/11*,§	36	1.0*
200 μg E + 30 mg P (3 weeks) plus 30 mg P long term	6/12*,§	50	1.0*
200 μg E + 30 mg P (3 weeks) plus 30 μg E + 30 mg P immediate- long term	7/11*,§	64	1.6*

Effect of long-term treatment with estradiol or progesterone or a combination of estradiol plus progesterone on MNU-induced mammary carcinogenesis in rats that received the short-term protective hormone treatment. Rats were treated with MNU at 7 weeks of age and 2 weeks later treated with 200 μg of estradiol and 30 mg of progesterone for 3 weeks by silastic capsule. The hormone capsules were removed after 3 weeks and replaced with 30 μg of estradiol or 30 mg of progesterone or a combination of 30 μg of estradiol and 30 mg of progesterone. The hormone capsules were changed every 2 months throughout the experimental period. Control rats received only the carcinogen treatment. * $P < 0.05$ (compared to control rats); § $P < 0.05$ (compared to short-term protective hormone treated rats)

Effect of long-term estradiol plus progesterone treatment on the mammary tumor incidence and multiplicity in rats that received the protective hormone treatment before exposure to MNU

This experiment was performed to demonstrate that long-term hormone treatment reverses the protective effect in animals which had received the short-term protective hormone treatment before the carcinogenic insult. In this experiment, we had two control groups, one was a young virgin control which received the carcinogen at 7 weeks of age and the other group of controls which received the same carcinogen but at 14 weeks of age. These animals received the carcinogen at 14 weeks to match with the animals that received the short-term treatment prior to carcinogenic insult. The first group of control rats will be referred to as young virgin control and the second group will be referred to as old virgin control. Exposure to short-term protective hormone treatment before MNU treatment significantly lowered the mammary tumor incidence compared to the controls. The young virgin control rats had a mammary tumor incidence of 100% and the old virgin rats had a tumor incidence of 64%, whereas rats treated for 2 weeks with 200 μg of estradiol plus 30 mg progesterone had a mammary tumor incidence of 7% (Table 3). Long-term treatment with 200 μg of estradiol and 30 mg of progesterone to rats which had received the short-term protective hormone treatment had a tumor incidence (67%) similar to the old virgin control rats but lower than the young virgin rats. Mammary tumor multiplicity was the lowest in the short-term protective hormone treatment group (1 tumor/tumor bearing rat) compared to the young virgin controls (2.5 tumors/tumor-bearing rat) and old virgin controls (1.3 tumors/tumor-bearing rat). Long-term treatment with estradiol plus progesterone resulted in 2.5 tumors/tumor-bearing rat (Table 3).

Another group of rats that received 30 mg of estradiol and 30 mg of progesterone for 2 weeks had no mammary tumors. When the short-term protective hormone-treated rats were subjected to long-term exposure to 200 μg of estradiol plus 30 mg progesterone, they had a mammary tumor incidence of 64%. Long-term hormone-treated rats had a tumor multiplicity of 1.7 tumors/tumor-bearing rat (Table 3).

The data indicate that administration of the short-term protective hormone treatment before or after MNU exposure confers protection against mammary carcinogenesis. Increasing the promotional environment reverses the protective effect in both the situations

Discussion

The results from the current experiments emphasize the importance of promotional environment in the development of mammary tumors. We observed from the results in this study similar to our earlier findings [27, 28] that short-term treatment with ovarian steroids, estrogen and progesterone, before or after MNU administration confers a high degree of protection against mammary carcinogenesis. In contrast, continuous long-term treatment with the same ovarian steroids reversed the protective effect and resulted in high mammary tumor incidence similar to the controls. Rats that were treated with the protective hormone treatment before or after carcinogen administration had a low incidence of mammary tumors and also had decrease in the number of mammary tumors. While continuous administration of hormones for promotion increased the tumor

Table 3 Effect of long-term estradiol and progesterone treatment on mammary carcinogenesis in protected rats

Treatment	Mammary tumor incidence	Percent of rats with mammary tumor	Average number of mammary tumors per tumor-bearing rat
Young virgin control	10/10	100	2.5
Old virgin control	7/11	64	1.3
200 μg E + 30 mg P (2 weeks)	1/15*,†	7	1.0*
200 μg E + 30 mg P (2 weeks) plus 200 μg E + 30 mg P long term	6/9*,†,§	67	2.5†,§
30 mg E + 30 mg P (2 weeks)	0/10*,†	0	0*,†
30 mg E + 30 mg P (2 weeks) plus 200 μg E + 30 mg P long term	7/11*,†,§	64*,§	1.7§

Effect of long-term treatment with a combination of estradiol plus progesterone on MNU-induced mammary carcinogenesis in rats that received the short-term protective hormone treatment. Rats were treated at 7 weeks of age with 200 μg or 30 mg of estradiol in combination with 30 mg of progesterone for 2 weeks. Five weeks later, the rats were treated MNU. Each hormone-treated group was divided into two groups; one group received no further treatment and the other received 200 μg of estradiol and 30 mg of progesterone for the entire experimental period. The hormone capsules were changed every 2 months throughout the experimental period. Young virgin and old virgin control rats received only the carcinogen treatment at 7 and 14 weeks of age, respectively. * $P < 0.05$ (compared to young virgin control rats); † $P < 0.05$ (compared to old virgin control rats); § $P < 0.05$ (compared to the respective short-term protective hormone-treated rats)

incidence depending on the strength of promotion treatment.

Our data indicate that though the individual hormones were able to reverse the protective effect of short-term protective hormone treatment, combination of both estradiol and progesterone was more effective. Liu et al. [31] have reported that estrogen replacement in ovariectomized rats results in physiologically significant levels of circulating progesterone, and co-administration of progesterone markedly reduces the circulating estrogen. The combination treatment seems to be more effective in our experiment maybe because of the differences in dynamics of hormone interaction in ovary intact versus ovariectomized rats. Furthermore, it is a well-established fact that these hormones have tissue-specific function and also that both estradiol and progesterone function as mammogenic hormones and synergize in mammary gland development.

Though there are various mechanisms and reasons attributed to phenomenon of parity-induced protection or hormone-induced protection against breast cancers, none of them has been proven to date. Earlier, it has been reported that parous rats when treated with a chemical carcinogen develop many latent mammary tumors [32]. Even though parous rats have decreased mammary tumor incidence, the total number of occult and overt tumors when added together shows no significant difference between nulliparous and parous rats administered with MNU [32]. Abrams et al. [22] demonstrated that when mammary epithelial cells isolated from young virgin rats, previously exposed to MNU are transplanted into the fat pads of isogenic virgin and parous hosts, the tumor development is significantly lower in parous animals compared with young virgin controls. Recently, it has been shown that carcinogen-treated parous animals developed a high incidence of mammary tumors when treated continuously with ovarian steroids [21]. All these findings including the data from this study provide evidence that protection against mammary tumors in parous and short-term protective hormone-treated rats is not permanent. Moreover, it also indicates that initiation occurs as a result of carcinogen exposure before or after the protective treatment, but the initiated cells are not able to progress to form overt mammary tumors due to reduced promotional environment. The data from the current investigation also demonstrates that given continued promotion the short-term protective hormone-treated rats can also support mammary carcinogenesis. Further, both estradiol and progesterone independently or in combination can promote mammary carcinogenesis. All these suggest that protection against mammary cancers in parous or short-term hormone-treated rats is not permanent but plastic.

Our results suggest that in the process of carcinogenesis after the administration of carcinogen, initiation occurs irrespective of the time of carcinogen administration, but promotion and progression to form overt cancers is blocked. This may be due to a reduced promotional hormonal environment for cancer development. It is a well established that estradiol and progesterone actions are mediated by several growth factors. From our findings of this investigation, we can speculate that continuous promotion given in the form of estradiol and progesterone could induce normal mammary carcinogenesis by up-regulating the growth-promoting genes, growth factors and by altering the hormonal environment. The data from this study emphasize the importance of hormones during the promotional period of mammary carcinogenesis. Overall, the data obtained from this study indicate that short-term protective hormone-treated and parous-phenotype are similar; both are not protected from initiation but protected from promotion and progression. Increasing the promotional environment by administration of exogenous hormones increases mammary tumor incidence indicating that parity- or short-term estradiol plus progesterone-induced protection against mammary tumor is not permanent but plastic. In conclusion, the current data suggest that decreased promotional environment could be the reason for protection against mammary carcinogenesis.

Materials and methods

Animals

Virgin Lewis rats were purchased from Harlan Sprague Dawley (Indianapolis, IN and San Diego, CA). The rats were housed in a temperature-controlled room with 12 h light and 12 h dark schedule. They were fed food (Teklad, Madison, WI) and water ad libitum. All the procedures followed Texas Tech University Health Sciences Center Animal Care and Use Committee guidelines.

Carcinogen treatment

A single i.p. injection of MNU (Ashe Stevens, Detroit) at a dose of 50 mg/kg of body weight was given to all the rats. MNU was dissolved in physiological saline that had been adjusted to pH 5.0 [19].

Hormone treatment

The hormones were packed in individual silastic capsules (Dow Corning; size 0.078 inch i.d. $\times$ 0.125 inch o.d., 2 cm in length). All doses of estradiol 17β (Sigma, St. Louis) were packed in the silastic capsules in a cellulose matrix (Sigma) except for the 30 mg estradiol dose that was packed with the hormone alone. Progesterone (30 mg) (Sigma, St. Louis) was packed into the silastic capsules without any matrix.

Control animals received empty silastic capsules. All these capsules were primed by soaking overnight in media 199 (Gibco) at 37°C. Silastic capsules were implanted subcutaneously dorsally. For the long-term hormone-treated groups, silastic capsules were changed every 2 months, throughout the experimental period including the control groups.

Effect of long-term estradiol plus progesterone promotion on mammary carcinogenesis in rats that received the short-term protective hormone treatment after exposure to MNU

At 7 weeks of age, all the rats were treated with a single intra-peritoneal injection of MNU. At 9 weeks of age the rats were divided into three groups: (i) control, (ii) animals receiving 200 μg of estradiol and 30 mg of progesterone for 3 weeks, (iii) animals receiving 200 μg of estradiol and 30 mg of progesterone for 3 weeks; these capsules were removed and were replaced with 200 μg estradiol and 30 mg progesterone continuously until the end of the experiment.

Effect of long-term promotion with estradiol or progesterone or estradiol plus progesterone on MNU-induced mammary carcinogenesis in short-term hormone-protected rats

A single intra-peritoneal injection of MNU was given to 7-week-old rats. At 9 weeks of age, all the rats were treated with 200 μg of estradiol and 30 mg of progesterone for 3 weeks except the control rats that received empty silastic capsules. In this experiment, we gave lower dose of estradiol for promotion and also used estradiol and progesterone individually and in combination. The short-term protective hormone treated rats were divided into the following groups: (i) animals which received no further hormone treatments, (ii) animals which received continuous 30 μg of estradiol treatment immediately following the 3 weeks of protective hormone treatment, (iii) animals which received continuous 30 mg of progesterone treatment immediately following the 3 weeks of protective hormone treatment, (iv) animals which received continuous 30 μg of estradiol plus 30 mg of progesterone treatment immediately following the 3 weeks of protective hormone treatment.

Effect of long-term estradiol plus progesterone treatment on MNU-induced mammary carcinogenesis in the rats that received the short-term protective hormone treatment before MNU exposure

Seven-week-old rats were treated with (i) only empty silastic capsules-controls, (ii) 200 μg of estradiol and 30 mg of progesterone for 2 weeks, and (iii) animals received 30 mg of estradiol and 30 mg of progesterone for 2 weeks. At 9 weeks of age, the silastic capsules were removed. The mammary glands were allowed to undergo regression for 5 weeks, and at 14 weeks of age all the rats were given MNU, and then each hormone-treated group was divided into two subgroups: one which did not receive any further treatment; the other which received 200 μg of estradiol and 30 mg of progesterone continuously in silastic capsules until the end of the experiment (50 weeks). The silastic capsules were changed every 2 months, throughout the experimental period including the control groups. A group of young virgin rats were given MNU at 7 weeks of age and received no further treatment. These rats served as the young virgin controls.

Mammary carcinogenesis

Rats were palpated once every week beginning 1 month after carcinogen exposure for 9 months to monitor for mammary tumor development. Histopathological examination was performed to confirm the carcinomatous nature of all the palpable tumors. Tumors histologically confirmed as carcinomas were used for statistical analysis.

Statistics

The effects of the different hormonal treatments were analyzed by using the χ^2 test using 2×2 contingency tables for mammary tumor incidence. All other statistical analyses were carried out using ANOVA and Fisher's protected least significant difference test. Values of $P < 0.05$ were considered significant.

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