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ORIGINAL ARTICLES

Ovarian hormone dependence of pre-malignant and malignant mammary gland lesions induced in pre-pubertal rats by 1-methyl-1-nitrosourea

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The experiments reported in this study were designed to examine the question of whether a mammary epithelial cell's independence from hormonal requirements is established at the time of carcinogenic initiation, or whether the emergence of hormone independence is associated with the process of tumor progression. A newly developed rat model of mammary carcinogenesis was used in which the latency period to lesion detection is very short and in which the frequencies of both pre-malignant and malignant mammary lesions can be quantified. Two experiments were conducted in Sprague–Dawley rats injected with 50 mg MNU/kg body wt at 21 days of age. In the first experiment 47 animals were ovariectomized after the detection of a mammary tumor of palpable size. Forty-six of the 47 tumors assessed, all of which were subsequently classified as mammary gland adenocarcinomas, regressed to <50% of their initial volume within 14 days of bilateral ovariectomy. However, both pre-malignant and malignant mammary gland lesions were observed when animals were killed. In Experiment 2 a total of 60 rats were ovariectomized 7 days after MNU was injected. At 35 days post carcinogen ovariectomized animals had a higher incidence of intraductal proliferations than sham-operated controls ($P = 0.03$); there was no effect of ovariectomy on the incidence of ductal carcinoma *in situ* or carcinoma. The multiplicity of intraductal proliferations was increased by 58% in ovariectomized rats ($P = 0.12$), but the number of mammary carcinoma per rat was reduced (3.8 vs. 1.57, $P = 0.02$). These data are consistent with the hypotheses that the progression of pre-malignant to malignant lesions is inhibited in the mammary gland by ovariectomy and that the hormone independent phenotype can be conferred at the time of carcinogenic initiation.

Introduction

A clinically important phenotype of a breast cancer is whether or not it requires the presence of hormones, particularly those of the ovary and pituitary, for its proliferation. If hormones are required, the cancer is classified as hormone dependent; whereas, if it does not require these hormones for proliferation, it is classified as hormone independent (1). In women the classification of breast carcinomas based on hormonal requirements is used in deciding on the treatment a patient receives and also is of prognostic importance (2). Despite the significance of this issue, there is little that is known about the mechanisms that account for the hormonal requirements of mammary carcinomas for their maintenance and growth (3). One of several questions that is central to this issue is whether a mammary epithelial cell's independence from hormonal requirements is established at the time of carcinogenic initiation, or whether the emergence of hormone independence is associated with the process of tumor progression (3). Recently, a hypothesis was advanced to explain the origin of the different but predictable frequencies of hormone dependent and hormone independent carcinomas that are observed in humans, mice and rats. That hypothesis proposes that the ratio of replicating luminal mammary epithelial cells that are directly ovarian hormone responsive at the time of carcinogenic insult to the number of luminal mammary epithelial cells that are indirectly ovarian hormone responsive at the time of carcinogen insult, determines the subsequent frequencies of hormone dependent to hormone independent carcinomas (3). The experiments reported in this study were designed to examine this question using a newly developed model of mammary carcinogenesis in which the latency period from carcinogen administration to the detection of mammary carcinomas in all carcinogen-treated rats is ~5 weeks (4). Assessment of the hormone dependency of mammary tumors during this short latency period increases the probability that the hormonal requirements of the tumor for maintenance or growth would be attributable to events associated with initiation rather than progression. Moreover, the frequencies of both pre-malignant and malignant mammary lesions can be quantified using this model system. To our knowledge there are no reports concerning the hormone dependence of pre-malignant stages of chemically-induced mammary carcinogenesis in the rat. Such knowledge could also contribute to understanding the origin of the hormone independent phenotype.

***Abbreviations:** DMBA, 7,12-dimethylbenz[*a*]anthracene; MNU, 1-methyl-1-nitrosourea.

Materials and methods

General procedures

Female Sprague–Dawley rats were obtained from Taconic Farms, Germantown, NY at 20 days of age. At 21 days of age animals were injected i.p. with 50 mg 1-methyl-1-nitrosourea (MNU*)/kg body wt as previously described by our laboratory (4). Rats were housed three per cage in an environmentally-controlled room maintained at 22°C and 50% relative humidity with a 12 h light–dark cycle. They were fed a purified diet (AIN-76A) and distilled water. At necropsy, rats were skinned and the skin was examined under translucent light. The cervical-thoracic glands were evaluated for the presence of grossly detectable mammary tumors. All tumors were excised and processed for histopathological analysis. The abdominal-inguinal mammary glands were carefully excised and spread onto clean 50×75 mm pre-labeled microscope slides. These whole mounts were processed for evaluation as previously described and were photographed at uniform magnification (4). Thereafter all lesions detected by inspection of wholemounts at 2X magnification were dissected using the photograph to provide a permanent identification record of the location and gross morphology of a lesion. Dissected lesions were processed and histologically classified (5). Statistical analyses for significant differences in lesion types among treatment groups were evaluated. Incidence

Table I. Frequency of intraductal proliferations, ductal carcinoma *in situ* and carcinoma in rats ovariectomized after the detection of a mammary tumor of measurable dimensions

Lesion type	Incidence		Multiplicity	
	No. of rats with specified lesion type	%	No. of lesions	Average no. per rat
Intraductal proliferation	14	27.7	17	0.4
Ductal carcinoma *in situ*	8	12.8	8	0.2
Adenocarcinoma	47	100	189	4.0

A total of 47 rats injected with 50 mg MNU/kg body wt at 21 days of age were studied. When a rat was found to have a mammary tumor of measurable dimensions (>1 cm) it was bilaterally ovariectomized and tumor size was measured for 14 additional days or until the tumor regressed to an immeasurable size. The data reported in this table represent the lesions that were found in the mammary glands at necropsy and/or upon inspection of mammary gland wholemount preparations.

was evaluated by the Fisher exact test and differences in tumor count by square root transformation followed by analysis of variance (6).

Experimental design

Two experiments were conducted.

Experiment 1. Fifty rats were injected with MNU. They were palpated three times per week with the intent to detect mammary tumors having dimensions that were measurable using a vernier caliper (tumors were at least 1 cm in diameter). When an animal's mammary tumor attained or exceeded this dimension, the animal was bilaterally ovariectomized, and tumor measurements were performed daily for 14 days. The size of a tumor was determined as its two largest perpendicular dimensions and these data were used to compute volume using the formula for an ellipsoid. The measured tumor was classified as growing if it attained a volume >150% of its maximum pre-ovariectomy volume, as static if its volume was between 50 and 150% of its maximum pre-ovariectomy volume, and regressing if its volume was <50% of its maximum pre-ovariectomy volume. When a rat was euthanized, the tumor that was measured was excised for histological classification. In addition mammary gland whole mounts were prepared and evaluated for additional lesions. The duration over which this experiment extended was 120 days post carcinogen treatment. This extended time frame relative to the protocol used in Experiment 2 (described below) is important to note. Cancer endpoint data between these experiments are not directly comparable for the following reasons. By the nature of the experimental design, ovariectomy was not performed until a mammary tumor of measurable dimensions was detected on each animal. Thus the age of the animal and the time post carcinogen that ovariectomy occurred varied among animals in this experiment. A second major difference in experimental protocols arising from this was that the time post carcinogen that animals were euthanized also varied; the range was 70 days.

Experiment 2. A second experiment was designed to determine how many and what type of mammary lesions would emerge in rats that were ovariectomized one week following carcinogen administration. A similar protocol has been reported by our laboratory and for the investigation of hormone independent carcinomas in rats using the 'conventional' MNU mammary tumor induction protocols (7,8). The most significant difference from that protocol was that 28 day old rats were ovariectomized; female Sprague–Dawley rats of this age do not have fully functional ovaries and are not undergoing regular estrous cycles (9). A total of 60 rats were injected with MNU at 21 days of age. They were then randomized into two groups of 30 rats. At 28 days of age one group of rats was bilaterally ovariectomized whereas the other group (control intact group) underwent either no surgery ($n = 15$) or sham surgery ($n = 15$). Rats were maintained for an additional 28 days and then were euthanized and necropsied as described above.

Results

Experiment 1

Of the 50 rats assigned to this experiment, one died during surgery to remove the ovaries and two animals had tumors that did not reach a measurable size. Thus the effects of bilateral ovariectomy were studied in 47 rats. The first palpable mammary tumor reaching a measurable size in each of these 47 rats was evaluated for its response to bilateral ovariectomy. These tumors were measured daily for 14 days or until a tumor regressed by >50% of its initial volume at which time the animal was euthanized and necropsied. A total of 46 of 47 tumors studied regressed to a size <50% of their initial volume, and thus were categorized as ovarian hormone dependent (data not shown). One tumor grew to a size that exceeded 150% of its initial volume and was classified as hormone independent. Based on histopathological classification, all these tumors were mammary gland adenocarcinomas. At necropsy, all lesions that were detectable were excised and processed for histopathological classification. These lesions were classified as intraductal proliferations, ductal carcinoma *in situ* and carcinoma and the frequency of their occurrence is summarized in Table I. One hundred percent of the animals had carcinomas with an average of four carcinomas per rat. A total of eight ductal carcinoma *in situ* and 17 intraductal proliferations also were observed.

Experiment 2

The purpose of this experiment was to determine the incidence and number of mammary gland intraductal proliferations, ductal carcinoma *in situ* and adenocarcinoma in rats that were either intact or that were ovariectomized 7 days following carcinogen injection. It was also determined within the intact rats whether the sham surgical procedure affected the carcinogenic response. Rats were killed 35 days post carcinogen. All lesions were histopathologically classified. Table II summarizes the response that was observed. The sham surgical procedure resulted in a 22% reduction in the number of mammary lesions observed. The pattern of lesion occurrence indicated that this reduction of lesions was fully accounted for by a decrease in adenocarcinomas; the number of ductal carcinoma *in situ* and intraductal proliferations was unaffected in the sham-treated control rats versus those that did not undergo surgery. Thus for the comparison of the effect of ovariectomy, the sham-surgery control was used as the referent group. As shown in Table II there was a 59% reduction in the number of carcinomas in ovariectomized rats; however, unexpectedly there was a 58% increase in the number of intraductal proliferations in the ovariectomized group. Overall, there was a 22% reduction in the total number of mammary lesions in ovariectomized versus sham-operated rats. The incidence data, also shown in Table II indicate that ovariectomy caused a significant increase in the incidence of intraductal proliferations ($p = 0.03$); the incidence of ductal carcinoma *in situ* or adenocarcinoma was not affected by ovariectomy. The ovariectomized rats in this experiment had a final group mean body wt of 186 + 4 g; both the sham surgery and no surgery controls had a final group mean body wt of 155 ± 4 g. This difference of 20% in body wts between ovariectomized and intact rats was statistically significant ($P < 0.05$).

Table II. Effect of ovariectomy on the occurrence of pre-malignant and malignant mammary gland lesions

Lesion type	No surgery		Sham surgery		Ovariectomy	
	Incidence (%)	Multiplicity No./rat	Incidence (%)	Multiplicity No./rat	Incidence (%)	Multiplicity No./rat
IDP	60	1.33	53.3	1.47	86.7	2.33
DCIS	46.7	0.60	40	0.73	33.3	0.57
AC	100	4.87	80	3.8	80	1.57

Fifteen rats were randomized to each control group and 30 rats to the ovariectomy group. There were no statistically significant differences in the incidence or multiplicity of any type of lesion between the two control groups, although the incidence of carcinomas was reduced by 20% ($P = 0.22$. Fisher exact test) and multiplicity was reduced by 22% ($P = 0.12$, ANOVA on square root transformed data) in the sham surgery group versus the no surgery group. The sham surgery group was used as the referent group in analyzing the effect of ovariectomy. Ovariectomized rats had a higher incidence of IDP ($P = 0.03$) although the 58% increase in multiplicity was not statistically significant ($P = 0.12$). Ovariectomy had no statistically significant effect on the occurrence of DCIS. Ovariectomy significantly reduced the multiplicity of AC ($P = 0.02$) without affecting the incidence of AC.

Discussion

In rat mammary carcinogenesis induction protocols that have been routinely used for the last 30 years, 50 day old virgin female animals are injected with either MNU or 7,12-dimethylbenz[*a*]anthracene (DMBA) (1). At this age the rat is experiencing normal estrous cycles and the mammary gland is rapidly growing and undergoing 'branching morphogenesis' in response to hormonal stimulation (10). Mammary carcinomas emerge over a 6 month period, and depending on the experimental approach used to determine their hormonal requirements, between 65 and 80% are judged to require hormones for maintenance and growth (1,3). Thus chemically induced rat mammary carcinogenesis is considered a good model for this aspect of the human disease, although the proportion of hormone dependent carcinomas in the rat is generally higher than reported in the human populations (3). The model used in the experiments reported in this study differs from the 'conventional rat mammary carcinoma induction model' mentioned above in that rats were injected with MNU at 21 days of age. In Sprague–Dawley rats of this age, vaginal opening has not occurred (vaginal opening occurs at 31.6 days of age [95% CI 30–33 days of age] (9) and the animals do not initiate an estrous cycle for approximately two weeks (first estrus at 33.9 days of age [95% CI 32–35 days of age](9). Despite this, the mammary glands are undergoing rapid extension of the mammary ductal tree into the fat pad. However, since this proliferation is occurring in the absence of mature ovarian function, meaning that levels of ovarian steroids are likely to be lower than those reported in sexually mature rats, it was hypothesized, as suggested in reference 3, that a greater proportion of mammary carcinomas induced in 21 day old rats would be hormone independent.

The fact that the majority of mammary carcinomas assessed in Experiment 1 regressed indicates that mammary epithelial cells initiated by treatment with MNU at 21 days of age give rise to carcinomas that are dependent on ovarian hormones for their maintenance and growth. This observation appears not to support the study hypothesis that initiating rats at 21 days of age would induce more hormone independent mammary carcinomas. However, the fact that there were on average four mammary carcinomas per rat in Experiment 1 suggested the need for a more rigorous evaluation of the study hypothesis before its rejection, and prompted the second experiment. The results of Experiment 2 provided quantitative data concerning the prevalence of populations of pre-malignant and malignant mammary epithelial cells that did not require the presence of ovarian hormones for their maintenance and/or growth. The results of Experiment 2, which are shown in Table II, are to our knowledge the first report of the influence of ovariectomy on the occurrence of pre-malignant mammary gland lesions in the rat. The data presented provide a number of important insights. First, the data in Table II indicate the importance of actually performing a complete sham surgical procedure since failure to do this would result in an overestimation of the protective effect of ovariectomy. Sham surgery itself conferred a protective effect which was manifest primarily on the occurrence of carcinoma (4.87 vs 3.80 carcinomas per rat, $P = 0.12$). While this effect was not statistically significant given the statistical power of the study, it was judged of biological relevance to note the existence of this effect, particularly given that stress of various types has been reported to inhibit mammary carcinogenesis (1). As expected ovariectomy was protective against the occurrence of mammary carcinomas (3.80 vs 1.57) and ductal carcinoma *in situ* (0.73 vs 0.57). However several observations were completely unanticipated. First the incidence of mammary carcinoma was high (80%) and was not affected by ovariectomy. In other work performed by our laboratory in which animals were initiated at 50 days of age and ovariectomized 7 days later, a dramatic reduction in the incidence of carcinoma was observed (100% vs 25%, intact vs. ovariectomized, respectively) (8). Thus this is strong evidence that is consistent with the hypothesis that a greater prevalence of the hormone independent phenotype would occur in rats initiated with MNU prior to sexual maturation. A second unanticipated finding was that the incidence as well as the number of intraductal proliferations occurring in ovariectomized rats was increased in comparison to the sham surgery control (2.33 vs 1.47, respectively). Of the several implications of this finding, one of particular interest is that the conversion of intraductal proliferations to ductal carcinoma *in situ* and carcinomas appears to have been inhibited in the absence of ovarian hormones while the development of initiated cells to morphologically identifiable IDP was not. Whether these observations reflect a requirement for the attainment of a sufficient concentration of locally produced growth factors in emerging clones of transformed cells to progress to a malignant phenotype is not known, but represents one of several hypotheses that can be tested in light of this unanticipated result. Relative to the apparent block in the progression of lesions from the intraductal proliferation stage to a malignant phenotype, the question arises as to whether the block is permanent, or if these intraductal prolifera-

tions ultimately progress to carcinomas. We speculate that pre-malignant lesions will ultimately progress; however, this question is of considerable importance and merits direct experimental evaluation via transplant analysis in appropriate inbred models. If our speculation about the conversion of these hormone independent intraductal proliferations to carcinoma is validated, it would suggest that in addition to prophylactic efforts to manipulate ovarian function in women harboring pre-malignant mammary epithelial lesions, that adjuvant treatment directed against growth factors produced by emerging foci of hormone independent cells might be of additional benefit.

While ovariectomy did result in a lower number of carcinomas and ductal carcinoma *in situ*, the total number of premalignant and malignant mammary gland lesions observed was only reduced 25% by ovariectomy whereas in the work reported in reference 8, a 84% reduction was observed when rats injected with MNU at 50 days of age were ovariectomized 7 days later and subsequently observed for mammary tumor occurrence. Thus the results of Experiment 2 indicate that a greater proportion of mammary carcinomas induced by carcinogenic insult of the immature mammary gland do not require the presence of the ovaries for their maintenance and/or growth. This finding is consistent with the hypothesis of Nandi and coworkers that the ratio of replicating luminal mammary epithelial cells that are directly ovarian hormone responsive at the time of carcinogenic insult to the number of luminal mammary epithelial cells that are indirectly ovarian-hormone responsive at the time of carcinogen insult, determines the subsequent frequencies of hormone dependent to hormone independent carcinomas (3).

In summary the data presented in this study have important practical as well theoretical implications. From a practical standpoint, they show that both ovarian hormone dependent and independent mammary carcinomas are induced in a model that allows the entire carcinogenic process to be studied in a 5 week time span. Theoretically these observations are consistent with the hypothesis that the hormone independent phenotype can be conferred at the time of initiation and is unlikely to be limited to the genetic events that accompany tumor progression. The fact that ovariectomy appears to inhibit a specific step in the carcinogenic process, namely the conversion of intraductal proliferations to ductal carcinoma *in situ*, suggests a previously unappreciated aspect of the hormonal requirements for tumor promotion and progression.

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