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Inhibition of Mammary Tumors by Pretreatment with 17β-Estradiol in F344 Rats Induced with N-Methyl-N-nitrosourea

Akihiro Ito,[1] Taro Okamoto, Nariaki Fujimoto, Peter Osa Ogundigie and Hiromitsu Watanabe

Department of Cancer Research, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734

The influence of 17β-estradiol (E2) and prolactin was studied on N-methyl-N-nitrosourea (MNU)-induced mammary carcinomas (MCAs) in rats. MNU was intravenously injected once into seven-week-old female F344 rats at a dose of 50 mg/kg body weight. Groups of rats also received either 2.5 mg of E2 or a continuous supply of prolactin and/or growth hormone via transplanted MtT/F84 (mammo-somatotropic pituitary tumor). Rats were observed for up to 36 weeks after MNU administration. Although simultaneous administration of MNU and E2 did not much affect the occurrence of MCAs as compared to administration of MNU alone, rats treated with 2.5 mg of E2 for two weeks before MNU administration had significantly reduced occurrence of MCAs compared to those given MNU alone. In contrast, rats with MNU plus MtT/F84 showed high incidence and shortened latency of MCAs and they also had a high incidence of clitorial gland hyperplasias. Average pituitary weights and serum prolactin levels in E2-treated rats were greatly increased compared to those of MNU-alone rats. Average serum E2 levels were about 100 ng/ml in E2-treated rats and 0.05 ng/ml in rats without E2 treatment. Serum prolactin levels were greatly increased in rats with MtT/F84. The results indicated that pretreatment with E2 before MNU administration was inhibitory while increased prolactin caused by grafting MtT/F84 after MNU injection was promotive for the occurrence of MCAs in female F344 rats.

Key words: 17β-Estradiol — Prolactin — Mammary tumor — Rat

Induction of mammary tumors in female rats by chemical carcinogens or by radiation is known to be a suitable model for the analysis of hormonal actions on mammary tumorigenesis.[1] In rat mammary tumorigenesis, roles of estrogen and prolactin in radiation and chemically induced mammary tumors have been documented.[2–5] The importance of estrous state at the time of carcinogenic insult with 7,12-dimethylbenzanthracene or MNU[2] has been established; proestrous and estrous states favor a high frequency of mammary tumors, and the frequency is lower in the diestrous state.[6–9]

We have previously demonstrated that continuous administration of E2 results in mammary tumorigenesis in female Wistar/Furth rats,[10] although the incidence remained low. There is no clear evidence that E2 is carcinogenic to mammary epithelial cells *in vitro*. Even its modifying effect on mammary tumorigenesis is controversial. Estrogen is highly tumorigenic to the pituitary gland in rodents,[11] but no association of tumors in pituitary and mammary glands has been reported in rodents or in human beings. Mammotropic hormone has been shown to be a stronger promoter than estrogen for mammary tumorigenesis in rats,[12] but little evidence is available concerning mammary tumorigenesis by prolactin in humans.

The present study was undertaken to examine the effect of E2, especially the timing of E2 administration, in combination with MNU treatment.

MATERIALS AND METHODS

Animals and chemicals Six groups of female F344 rats (Charles River Japan, Inc.), each consisting of 20 to 30 rats initially, were housed in polycarbonate cages, fed a commercial diet and water *ad libitum* and maintained under a 12-h light/12-h dark lighting regimen. At 7 weeks of age, all rats except those in groups 2 and 3 received a single dose of 50 mg/kg body weight of MNU (Sigma Chemical Co., St. Louis, MO) in physiologic saline intravenously via the jugular vein under light ether anesthesia. In addition to MNU, the rats in groups 2, 4 and 5 received a cholesterol pellet containing 2.5 mg of E2 (Sigma Chemical, E-9750). The rats in group 3 received MtT/F84, induced and maintained with estrogen, as a source for continuous supply of prolactin.[13] The rats in group 4 received E2 throughout the experimental period. The rats in group 5 received E2 for two weeks before MNU injection and throughout the experimental period thereafter, like group 4. The rats in group 6 received MtT just after MNU injection. E2 was mixed with

[1] To whom correspondence should be addressed.

[2] Abbreviations: MNU, N-methyl-N-nitrosourea; E2, 17β-estradiol; DCC, dextran-coated charcoal; MCA, mammary carcinoma; ER, estrogen receptor; MtT/F84, mammo-somatotropic pituitary tumor.

cholesterol powder and pellets were prepared by heating. A pellet containing 2.5 mg of E2 was inserted subcutaneously in the back. It was replaced with a new one every 4 weeks. Blood samples were collected from the abdominal artery under light ether anesthesia at the time when the animals were killed. Collected sera were kept in duplicate in small plastic tubes and stored at −20°C until assays.

The mammary chains were palpated once a week after MNU administration and body weights were measured every other week. Tumor appearance was examined once a week and when the maximal diameter was more than 1 cm, rats were killed. All mammary tumors were examined and a piece of tumor tissue was kept for histological examination.

Serum prolactin and estrogen levels Blood samples were obtained from the abdominal artery when the animals were killed. Serum prolactin levels were determined by a radioimmunoassay technique as described[13] with material supplied by the National Hormone and Pituitary Program, National Insitute of Arthritis, Diabetes and Digestive and Kidney Diseases (rPRL-I-5, anti-rat prolactin serum S-9). MtT cells (about 10^4) were inoculated into a fat pad one week after MNU injection. Growing tumors were surgically resected when they reached about 2 to 3 cm in diameter, leaving a small amount to tumor tissue for the continuous supply of hormones. Serum E2 levels were measured from ether-ethanol-extracted samples by radioimmunoassay. In brief, an individual serum sample was mixed with 3.0 ml of diethyl ether and the organic solution was evaporated under nitrogen gas. The residue was dissolved in ethanol. Recovery of measurable materials was approximately 85 to 92%. E2 solution in 0.1 ml of phosphate buffer was combined with 0.1 ml of rabbit anti-E2 serum (Teikoku Zouki Co. Ltd., Tokyo; its cross reactivity was 3.2% for estrone, 1.8% for estriol) and ^{3}H-E2 solution. The anti-E2 serum was used at 1:45,000 final dilution, and ^{3}H-estradiol (2,4,6,7-^{3}H estradiol: specific activity 101 Ci/mmol, New England Nuclear, Boston, MA) was dissolved in phosphate buffer to give 8,000–10,000 dpm/0.1 ml. Unbound E2 was removed by using the DCC method. As a standard series, E2 solutions of 50–3200 pg/ml were prepared. The ratio of bound ^{3}H-E2 to total counts was 65% to 70% and the standard curve was linear in the range of 5–320 pg/ml.

RESULTS

Experimental schedule and body weights These are summarized in Table I. Intravenous injection of MNU at 50 mg/kg of body weight was not toxic in this strain of rats. The rats given continuous administration of E2 via pellets invariably developed pyometra or hydrometra, but the increase of body weight was not significantly different from that in groups not given E2. However, body weight gain in rats with MtT (groups 3 and 6) was significantly greater than that of rats without MtT/F84, since MtT/F84 has a somatotropic activity.

Incidence of MCA Occurrence, mean number and latencies of MCAs are summarized in Table II. E2 or MtT alone (groups 2 and 3) did not induce MCA. MNU alone (group 1) afforded MCAs in 50% incidence, with an average of 0.95 MCA per rat at termination of the experiment. A combined treatment with MNU and 2.5 mg of E2 in group 4 induced 44% incidence of MCA with an average of 0.44 per rat. Group 5 had only 4% incidence of MCA by 36 weeks after MNU administration. In contrast, rats given MNU and MtT (group 6) showed significantly higher incidence of MCA with much shorter latency compared to the MNU-alone group (group 1).

MNU alone exclusively induced tubulo-papillary type tumors (Fig. 1a). In contrast, a combined treatment with MNU and E2 often yielded not only tubulo-papillary carcinoma, but also compact tubular carcinoma (Fig. 1b)

Table I. Experimental Protocol and Body Weights

Group	Treatment	Observation period (wk)[a]	Effective no. of rats[e]	Body weight (g)	
				Initial	Final
1	MNU	35	20	123±1.0	195±3.1
2	E2	36	19	115±2.5	203±3.7
3	MtT	36	16	121±5.8	265±31.1[f]
4	MNU+E2[b]	34	16	117±1.3	189±2.9
5	E2[c]+MNU	36	28	103±2.3	198±3.5
6	MNU+MtT[d]	16	20	106±10.7	265±31.9[f]

a) Rats were observed maximally for 36 weeks after MNU administration.
b) E2 was given simultaneously with MNU and the pellet was replaced every 4 weeks with a new pellet.
c) E2 was given 2 weeks before MNU administration and the pellet was replaced every 4 weeks with a new pellet.
d) MtT/F84 was grafted 2 days after MNU administration.
e) Animals that died or were killed after the first occurrence of MCA.
f) Significantly higher than group 1 by $P<0.01$.

or cribriform-comedo carcinoma. This type of compact or cribriform tumor is composed of massively arranged epithelial cells with little connective tissue. The rats given MtT showed a marked proliferation of mammary ductules with cystic retention of milk or focal appearance of acinar structure. Thus, a combined treatment with

Table II. Occurrence of MCAs in Female F344 Rats Given MNU, E2 and MtT/F84

Group	No. of rats		Mean no. of MCA/rat	Latency (wk) range (mean)	Pituitary weight (mg) (mean±SD)	Other tumors
	N	with MCA (%)				
1	20	10 (50)	0.95	16–35 (25)	11.0±0.4	3[e]
2	19	0 (0)	0	–	88.0±15.0[b]	0
3	16	0 (0)	0	–	11.4±0.4	0
4	16	7 (44)	0.44	30–34 (32)	78.2±14.3[d]	4[f]
5	28	1 (4)[a]	0.04	36	76.6±16.4[d]	0[g]
6	20	18 (90)[b]	2.10	9–16 (11)[c]	7.9±1.1	0[h]

a) Significantly lower than group 1 ($P<0.01$). *b*) Significantly higher than group 1 ($P<0.05$).
c) Significantly shorter than group 1 ($P<0.05$). *d*) Significantly higher than group 1 ($P<0.01$).
e) One cecal, one thyroid and one mammary tumor. *f*) Two uterine myosarcomas, one mammary tumor and one clitorial tumor. *g*, *h*) These groups of rats had 9 (32%) and 14 (70%) clitorial gland hyperplasias, respectively.

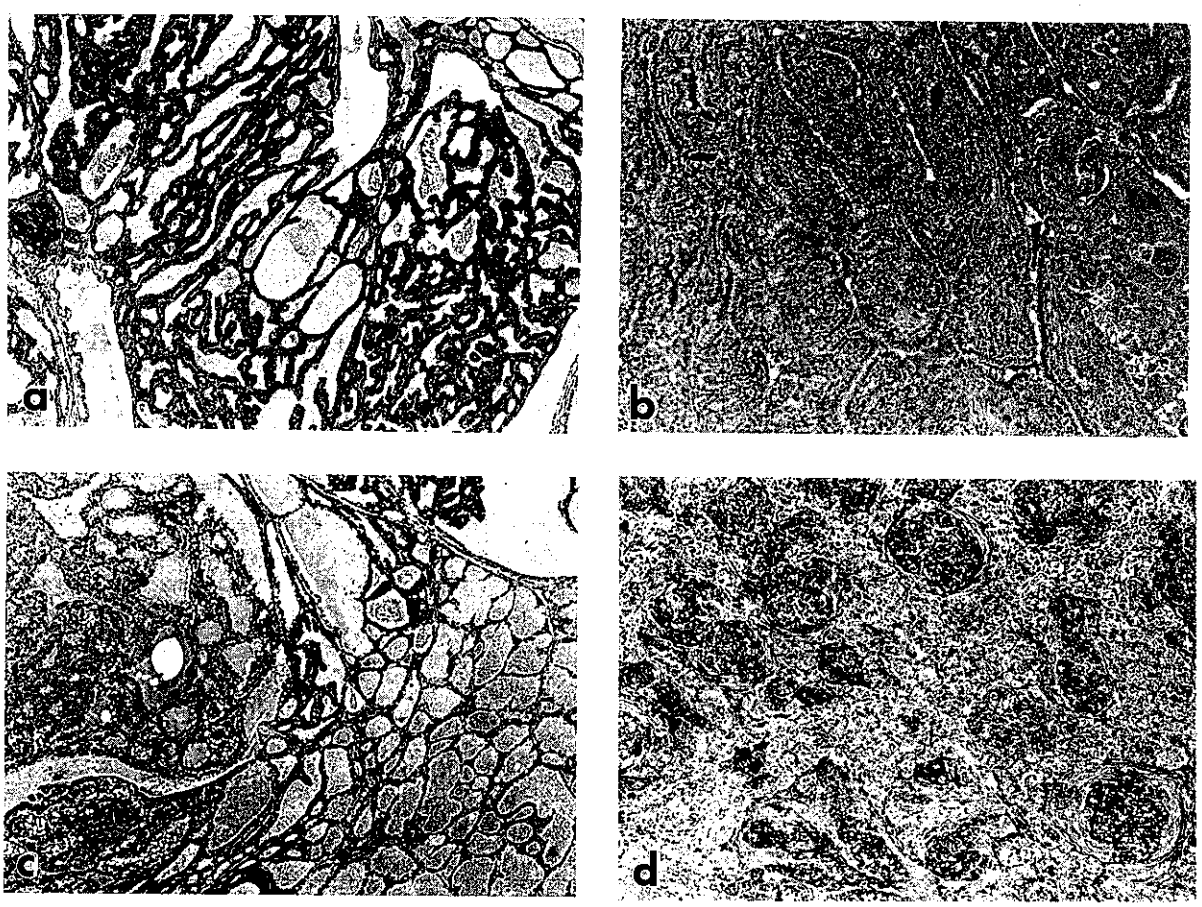

Fig. 1. a. Tubulo-papillary adenocarcinoma induced by MNU alone. b. Compact tubular carcinoma induced by combined treatment with MNU and E2. c. Papillary carcinoma and lobular hyperplasia induced by MNU and MtT. d. Epidermoid hyperplasia at clitorial gland induced by MNU and MtT.

Table III. Serum Estrogen and Prolactin Levels When the Animals Were Killed

Treatment	17β-Estradiol (ng/ml)		Prolactin (ng/ml)	
	No. of samples	Mean±SD	No. of samples	Mean±SD
MNU	6	0.05±0.02	14	45±8.5
MNU+E2	13	94±27.8	13	796±195
E2+MNU	11	108±23.8	16	639±105
MNU+MtT	−	nd	10	31,143±7,300

MNU and MtT exclusively induced tubulo-papillary carcinoma among markedly proliferating mammary tissues (Fig. 1c). The rats in groups 2, 4, and 5, given 2.5 mg of E2, showed enlarged pituitary hyperplasias or tumors with occasional sinusoidal blood cysts or hemorrhage, weighing 88.0, 78.2 and 76.6 mg, respectively, and associated with highly stimulated mammary glands.

Other tumors Besides MCAs, high incidence of clitorial gland hyperplasia was noted in groups 5 and 6 (Table II). They were composed of epidermoid ducts and glandular acini (Fig. 1d). In group 6, grafted MtTs were surgically removed when they were about 1 to 2 cm in diameter. The sizes of mammary tumors and clitorial gland hyperplasias were measured before and 2 weeks after removal of MtTs. A reduction of average tumor size to less than 50% of the original size was defined as an effective diminution of size. The incidence of diminished tumor size was 50% in clitorial gland hyperplasias and 24% in mammary tumors.

Tumors in soft tissue were mainly fibroadenoma or fibroma of mammary tissues. Two kidney tumors were of mesenchymal origin at the cortico-medullary junction. Occurrence of cataracts was sporadic in rats receiving MNU.

Serum E2 level was 0.05 ng/ml in rats with MNU, but increased to about 100 ng after the administration of E2. Serum prolactin levels were also increased by E2 administration and greatly increased by grafting MtT/F84 (Table III).

DISCUSSION

The present study indicated that single intravenous injection of MNU at 7 weeks of age induced 50% incidence of mammary carcinomas in female F344 rats. Simultaneous administration of MNU and E2 did not cause any difference in the development of MCA, but pretreatment with E2 before MNU significantly reduced the incidence of MCA compared to that in rats given MNU alone.

Several studies have indicated that the mammary epithelial cells most susceptible to carcinogenic insult are located in the morphological structures of the mammary glands referred to as "end buds" and "terminal ducts."[14, 15] The administration of 2.5 mg of E2 to female rats resulted in a marked proliferation and differentiation of mammary epithelial cells and ultimately such tissues might be less sensitive to MNU carcinogenesis. It has been reported that pretreatment with E2 is highly effective in preventing mammary carcinomas.[16, 17] This was explained by the fact that highly differentiated mammary glands did not respond to carcinogenic exposure. Grubbs *et al.* also found that pretreatment with E2 slightly reduced the incidence of mammary adenocarcinomas in MNU carcinogenesis.[18] We have previously reported that E2 administration was mammary-tumorigenic in female Wistar/Furth rats,[9] but it was not promotive in female F344 rats in the present study. In contrast, the pituitary gland response to E2 was very high in F344 rats[19] and moderate in W/Fu rats, indicating that increase of serum PRL alone may not be prerequisite for the development of mammary tumors. Histologically, E2-mediated mammary carcinomas in W/Fu rats were exclusively compact tubular carcinomas (unpublished data) and most of those induced in the present study with a combined treatment of MNU and E2 in F344 rats were mixed types of tubulo-papillary, compact tubular and/or comedo-cribriform carcinoma. In our experience, tubulo-papillary types can be readily transplanted, but other types gave little success in transplantation.

In the present experiment, E2 pretreatment was clearly inhibitory for the occurrence of mammary carcinomas. As to the administered doses of estrogen, there was a positive correlation between the size of grafted pituitary tumor (MtT/F84) and E2 pellet size in the range of 0.1 to 12.5 mg in our previous study, and 2.5 mg of E2 seemed to be a suitable dose for the maintenance of estrogen-dependent pituitary tumor.[13] In the present study, almost all of the animals treated with E2 had enlarged pituitary glands with increased serum prolactin levels.

F344 rat is rather resistant to spontaneous or carcinogen-induced mammary tumorigenesis.[20, 21] In our study, susceptibility to mammary tumorigenesis by E2 was 50% in W/Fu and 15% in F344 rats. Combined treatment of X-rays and E2 gave a higher incidence,[10] but it remained only 20% in Sprague-Dawley, and 89% in F344 rats. Latent periods were 77, 86 and 94 weeks, respectively.[21]

In contrast to mammary tumors, pituitary glands were highly susceptible to tumorigenesis in F344 rats and less susceptible in W/Fu rats.[10] These observations indicate that high prolactinemia is important for mammary tumorigenesis, but the occurrence of mammotropic pituitary tumor is not a prerequisite for mammary tumorigenesis. Estrogen may even counteract the mammogenic action of prolactin.

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