Jpn. J. Cancer Res. 82, 1391–1396, December 1991

Rapid Induction of Endometrial Carcinoma in ICR Mice Treated with N-Methyl-N-nitrosourea and 17β-Estradiol

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The present study was undertaken to develop an animal model for endometrial neoplasms. A total of 107 female ICR mice, 10 weeks of age, were used and treated as follows: Group 1 (31 mice) was given intravaginal instillation of N-methyl-N-nitrosourea (MNU) solution (1 mg/100 g body wt.) once a week for three weeks and then fed diet containing 5 ppm 17β-estradiol (E_2) for 20 weeks, starting one week after the last exposure to MNU. Group 2 (30 mice) was given MNU alone. Group 3 (31 mice) was given E_2 diet alone. Group 4 (15 mice) was fed the basal diet alone and served as the untreated control. At the termination of the experiment (week 23), all surviving mice were killed. Histopathological examination revealed that adenocarcinomas in the uterine corpus developed in mice of Groups 1–3, with a high incidence of endometrial hyperplasia. The incidence of endometrial carcinomas in Group 1 (15/31, 48%) was significantly higher than in Group 2 (2/29, 7%, $P<0.001$) or Group 3 (7/31, 23%, $P<0.01$). In the uterine cervix, small numbers of squamous cell carcinomas and preneoplastic lesions (dysplasias and hyperplasias) were also present in mice of Groups 1–3. In Groups 1 and 3, an increased E_2/progesterone (P) ratio was observed. Thus, the results indicated that this medium-term model for endometrial neoplasms is useful for studying the pathogenesis of endometrial cancer and that an increased E_2/P ratio is an important factor for the development of endometrial adenocarcinoma.

Key words: Endometrial carcinoma — N-Methyl-N-nitrosourea — Estradiol — Rapid cancer induction — ICR mice

Endometrial carcinoma is one of the most frequently found neoplasms in women and the incidence of endometrial carcinomas has been increasing steadily in Japan[1] as well as in Western countries. Previously, several animal models for endometrial cancer have been reported.[2–9] However, they presented some problems, i.e., low incidence of malignancy,[2,3,6,8] long delay after carcinogen exposure until tumor development,[2–9] and necessity of complicated surgical methods, such as laparotomy[4,6,8] or mastectomy.[7] Therefore, an effective and simple animal model of endometrial carcinomas is still required for examining the pathogenesis of endometrial carcinoma. The authors have recently reported that application of N-methyl-N-nitrosourea (MNU)[4] into the uterine corpus of aged rats could induce endometrial and cervical neoplasms, but the incidences were relatively low.[10]

The present study was designed to obtain high incidence and rapid induction of uterine cancer in mice treated with MNU and 17β-estradiol (E_2).

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[4] Abbreviations: E_2, 17β-estradiol; P, progesterone; MNU, N-methyl-N-nitrosourea.

MATERIALS AND METHODS

Animals and chemicals A total of 107 female ICR mice, 10 weeks of age, purchased from Charles River Japan, Inc. (Kanagawa), were housed 5 or 6 animals in a plastic cage and kept in an air-conditioned animal room at 25 ± 2°C and 55 ± 5% humidity under a 12-h light 12-h dark cycle during the experiment. The basal diet (Oriental MF, Oriental Yeast Co., Tokyo) and water were available *ad libitum* throughout the experiment.

MNU was purchased from Nacalai Tesque Inc. (Kyoto) and E_2 was obtained from Sigma Chemical Co. (St. Louis, MO).

Treatment Mice were divided into four experimental groups and treated as shown in Fig. 1. Group 1 (31 mice) was given three intravaginal instillations of MNU using a metal syringe (0.1 mm in inner diameter) at a dose of 1 mg/100 g body wt. once a week for three weeks and then fed the diet containing 5 ppm E_2 for 20 weeks, starting one week after the last exposure of MNU. Group 2 (30 mice) was treated with three intravaginal instillations of MNU alone as for Group 1. Group 3 (31 mice) was fed 5 ppm E_2 diet for 20 weeks. Group 4 (15 mice) was fed the basal diet and served as an untreatment control. The experiment was terminated 23 weeks after the start of the study. At the termination of the experiment, all surviving

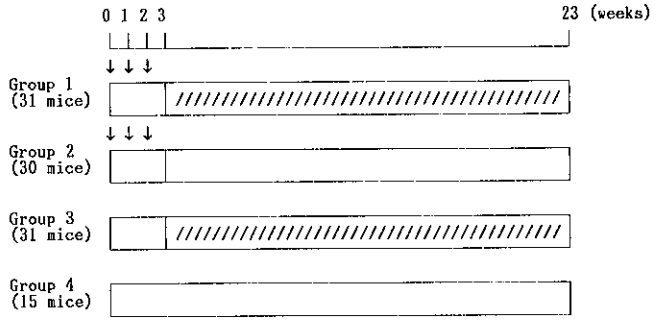

Fig. 1. Experimental design. ↓: vaginal instillation of MNU (1 mg/100 g body wt.). ▨: E_2, 5 ppm in the basal diet. □: Basal diet (Oriental MF).

animals were killed and autopsied. At death, blood was collected from the heart and/or abdominal aorta in four mice of Group 1, three of Group 2, five of Group 3 and four of Group 4. In Groups 2 and 4 in particular, the blood was collected from mice showing a proliferative phase on their vaginal smears. The blood was centrifuged at 3000 rpm for 10 min, and the plasma was stored at −80°C until assayed. All major organs, especially the reproductive organs, were carefully inspected grossly, and the uterus, ovaries, vagina and other lesions suspected of being neoplastic or hyperplastic were taken for histological examination. Tissues were processed for histology by the conventional method, and sections (3 μm in thickness) were stained with hematoxylin and eosin (H-E).

Histology of uterine lesions Uterine endometrial lesions were basically diagnosed according to the WHO criteria[11] and divided into 4 lesions: cystic glandular hyperplasia, adenomatous hyperplasia, atypical hyperplasia and adenocarcinoma. Uterine cervical lesions were basically diagnosed according to the criteria of Muñoz *et al.*[12] and divided into 3 main lesions: hyperplasia, dysplasia and squamous cell carcinoma.

Hormonal assay The plasma values of E_2 and progesterone (P) were estimated by using an estradiol direct radioimmunoassay kit (Sorin Biomedica, Cis, France)[13] and a progesterone (Diagnostic Product Corporation, LA) kit,[14] respectively.

Statistical analysis Statistical analysis was done by using the χ^2 method, Fisher's exact probability test or Student's *t* test.

Table I. Mean Body Weights and Mean Combined Weights of the Uterus and Ovaries in Each Group

Group (Treatment)	Effective number of animals[a]	Body weight (g)	Combined weights of uterus and ovaries (g)
Group 1 (MNU + E_2)	31	32.8±4.0[b]	0.38±0.12
Group 2 (MNU alone)	29	37.0±5.0[c]	0.23±0.12[d]
Group 3 (E_2 alone)	31	33.3±3.6	0.43±0.14
Group 4 (No treatment)	13	32.8±2.6	0.33±0.10

a) Animals that survived more than 10 weeks.
b) Mean±SD.
c) Significantly higher than in the other three groups ($P<0.01$).
d) Significantly smaller than in Groups 1 and 4 ($P<0.05$), and in Group 3 ($P<0.001$).

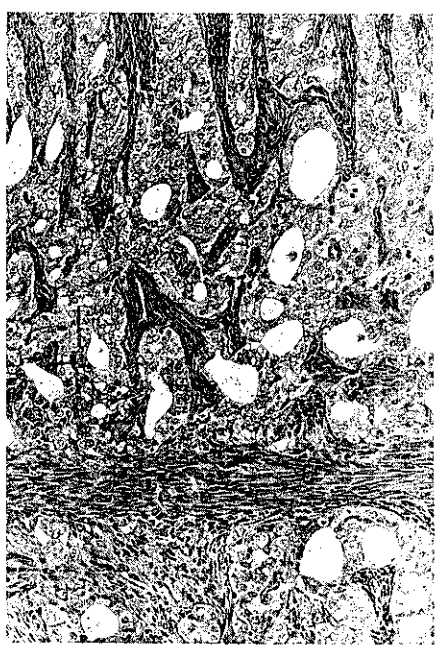

Fig. 2. Moderately differentiated adenocarcinoma of the endometrium in a mouse treated with MNU and E_2. H-E. ×175.

RESULTS

One mouse in Group 2 and two in Group 4 died within 10 weeks after the start of the experiment and no pathological abnormalities other than pneumonia were found. The remaining animals survived until the termination of the experiment and were selected as effective animals.

Table II. Incidences of Preneoplastic and Neoplastic Lesions in the Uterine Corpus in Each Group

Group (Treatment)	Initial number of animals	Effective number of animals[a]	Number of animals with			
			CGH[b]	AdH	AtH	ADC
Group 1 ($MNU+E_2$)	31	31	31[c] (100%)	22[c] (71%)	17[c] (55%)	15[d] (48%)
Group 2 (MNU alone)	30	29	0 (0%)	6 (21%)	4 (14%)	2 (7%)
Group 3 (E_2 alone)	31	31	31 (100%)	21 (68%)	15 (48%)	7 (23%)
Group 4 (No treatment)	15	13	0	0	0	0

a) Animals that survived more that 10 weeks.
b) CGH, cystic glandular hyperplasia; AdH, adenomatous hyperplasia; AtH, atypical hyperplasia; ADC, adenocarcinoma.
c) Significantly higher than in Group 2 ($P<0.001$).
d) Significantly higher than in Group 2 ($P<0.001$) or 3 ($P<0.05$).

Table III. Incidences of Preneoplastic and Neoplastic Lesions in the Uterine Cervix in Each Group

Group (Treatment)	Initial number of animals	Effective number of animals[a]	Number of animals with				
			Hyp[b]	Dys			SCC
				Mild	Moderate	Severe	
Group 1 ($MNU+E_2$)	31	31	29[c] (97%)	4 (13%)	13 (42%) 6 (19%)	3 (10%)	2 (6%)
Group 2 (MNU alone)	30	29	10 (34%)	3 (10%)	12 (41%) 8 (28%)	1 (3%)	1 (3%)
Group 3 (E_2 alone)	31	31	31[c] (100%)	2 (6%)	6 (19%) 3 (10%)	1 (3%)	0 (0%)
Group 4 (No treatment)	15	13	0		0		0

a) Animals that survived more than 10 weeks.
b) Hyp, hyperplasia; Dys, hysplasia; SCC, squamous cell carcinoma.
c) Significantly higer than in Group 2 ($P<0.001$).

The mean body weights, and combined weights of uterus and ovaries are summarized in Table I. The mean body weight in Group 2 was significantly larger than those in the other three groups ($P<0.01$). The mean combined weight of uterus and ovaries in Group 2 was significantly smaller than in the other three groups (vs. Group 1 or 4, $P<0.05$; Group 3, $P<0.001$). Mean consumptions of the diet in Groups 1–4 were as follows: 7.1 ± 0.5, 8.0 ± 0.6, 6.7 ± 0.4 and 7.3 ± 0.5 g/mouse/day. The consumption of the diet in Group 2 was significantly larger than in the other three groups ($P<0.05$).

Histological examination revealed neoplasms in the uterine corpus and cervix in Groups 1–3. In the uterine corpus, all tumors were adenocarcinomas and most of them were well or moderately differentiated (Fig. 2). The incidences of preneoplastic and neoplastic lesions in the uterine corpus are summarized in Table II. The incidence of adenocarcinomas in Group 1 (15/31, 48%) was significantly higher than those in Group 2 (2/29, 7%, $P<0.001$) and in Group 3 (7/31, 23%, $P<0.05$).

The tumors developed in the uterine cervix were keratinizing squamous cell carcinoma. The incidence of neoplasms in the uterine cervix in each group are summarized in Table III. The frequencies of squamous cell carcinomas were 6% (2/31) in Groups 1 and 3% (1/29) in Group 2. Besides such neoplasms, preneoplastic lesions of the uterine corpus and cervix were also found. Atypical hyperplasia (Fig. 3) and adenomatous hyper-

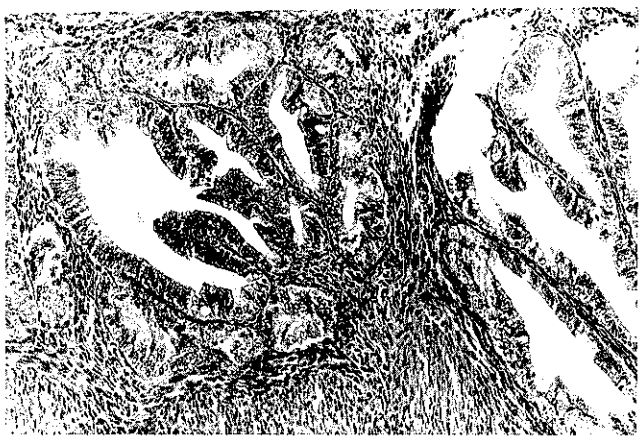

Fig. 3. Atypical hyperplasia of the endometrium in a mouse treated with E_2 alone. H-E. ×175.

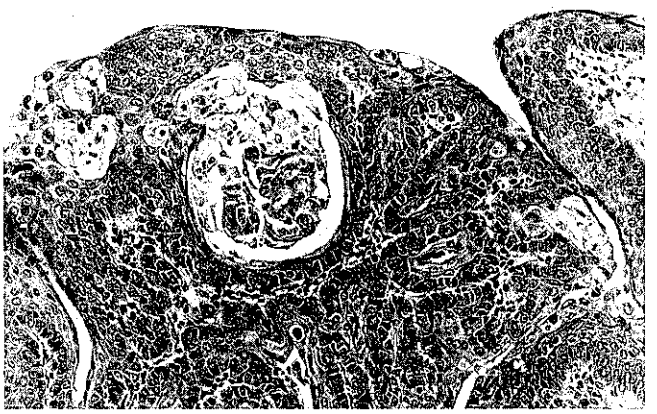

Fig. 5. Severe dysplasia of the uterine cervix in a mouse treated with MNU and E_2. H-E. ×175.

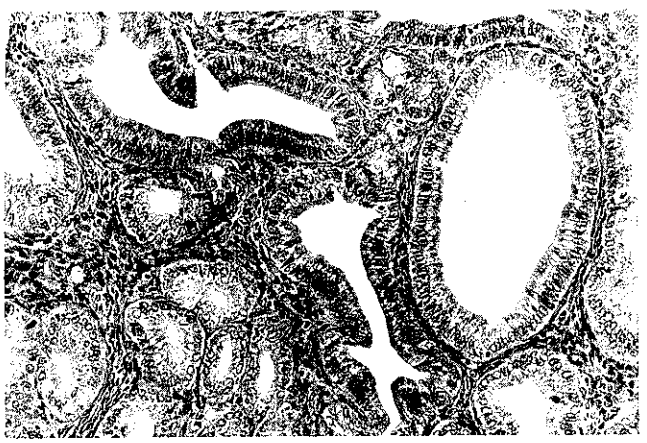

Fig. 4. Adenomatous hyperplasia of the endometrium in a mouse treated with MNU and E_2. H-E. ×175.

Table IV. Mean Plasma E_2 and P Concentrations, and E_2/P Ratio in Each Group

Group (Treatment)	Number of animals examined	E_2 (pg/ml)	P (ng/ml)	E_2/P ratio ($\times 10^{-3}$)
Group 1 (MNU+E_2)	4	56.2±10.2[a, b]	2.5±0.8[c]	29.2±8.3[d]
Group 2 (MNU alone)	3	27.0±7.0	13.5±3.5	2.6±1.0
Group 3 (E_2 alone)	5	46.4±11.5[b]	4.2±1.5[c]	12.9±6.3[e]
Group 4 (No treatment)	4	22.8±4.8	16.6±5.4	3.3±2.5

a) Mean±SD.
b) Significantly higher than in Groups 2 and 4 ($P<0.05$).
c) Significantly lower than in Groups 2 and 4 ($P<0.01$).
d) Significantly higher than in Groups 2 and 4 ($P<0.01$), and in Group 3 ($P<0.05$).
e) Significantly higher than in Groups 2 and 4 ($P<0.05$).

plasia (Fig. 4) of the endometrium were seen in Groups 1–3 (Table II). The incidence of atypical hyperplasia in Group 1 (17/31, 55%) was significantly greater than in Group 2 (4/29, 14%, $P<0.001$). Also, the incidence of adenomatous hyperplasia in Group 1 (22/31, 71%) was significantly higher than in Group 2 (6/31, 21%, $P<0.001$). Cystic glandular hyperplasias were found in Groups 1 and 3. The incidences of cystic glandular hyperplasia in Group 1 (31/31, 100%) and Group 3 (31/31, 100%) were significanty higher than in Group 2 (0/29, 0% $P<0.001$). Hyperplasias in the uterine cervix were found in Groups 1–3. The incidences of hyperplasias in Groups 1 (29/31, 97%) and 3 (31/31, 100%) were significantly higher than that in Group 2 (10/29, 34%, $P<0.001$, Table III). A small number of dysplastic lesions with various degrees of atypia in the uterine cervix (Fig. 5) were seen in Groups 1–3.

In ovaries, follicular cysts were found 6 mice in Group 1, 3 in Group 2, and 4 in Group 3. No pathological changes were seen in other organs, including the vagina.

Mean plasma E_2 and P concentrations, and E_2/P ratios in each group are shown in Table IV. The plasma E_2 values (pg/ml) in Groups 1 (56.2±10.2) and 3 (46.4±11.5) were significantly higher than in Groups 2 (27.0±7.0, $P<0.05$) and 4 (22.8±4.8, $P<0.05$). The plasma P

values (ng/ml) in Groups 1 (2.5±0.8) and 3 (4.2±1.5) were significantly lower than in Groups 2 (13.5±3.5, $P<0.01$) and 4 (16.6±5.4, $P<0.01$). The E_2/P ratio in Group 1 (29.2±8.3) was significantly higher than in Groups 2 (2.3±1.1, $P<0.01$) and 4 (3.4±2.5, $P<0.01$). Also, the ratio in Group 3 (12.9±6.3) was significantly higher than in Groups 2 and 4 ($P<0.05$).

DISCUSSION

In the present study, a high incidence of endometrial adenocarcinoma and hyperplasia could be induced in ICR mice treated with MNU and E_2.

Since Lacassagne first described experimental endometrial carcinoma,[2] several animal models for induction of endometrial carcinomas have been reported.[3-9] However, no simple and useful experimental model is yet available. Thus, we have been trying to develop an effective and simple animal model of endometrial carcinomas. Recently, we have reported induction of endometrial adenocarcinoma by local application of a direct-acting carcinogen, MNU,[15] into the uterine cavity of aged rats.[10] However, the incidence of induced tumors was relatively low. In the present study, MNU and dietary E_2 were given to mice in order to induce rapidly a high incidence of endometrial adenocarcinomas.

In human endometrial carcinomas, two pathogenetic types of the neoplasm have been proposed.[16, 17] One is associated with adenomatous hyperplasia and the other is not.[17] The former type of the disease is considered to be associated with abnormal hormonal manifestations (obesity, infertility and other conditions related to hyperestrogenism) as well as with exogenous estrogen administration.[17] The latter type shows no signs of hyperestrogenism. Therefore, the results in the present study should be useful for evaluating the histogenesis of endometrial carcinomas related to abnormal hormonal manifestations.

Recently, strain differences in the development of spontaneous endometrial carcinoma have been indicated. The incidence of the neoplasms in F344 rats was under 1%,[18] while that in BD/II Han rats[19] or Donryu rats[20] was approximately 35% or 90%, respectively. Hormonal dysregulation, namely increased E_2/P ratio, was found in both strains.[19, 20] In the present experiment, the mice treated with E_2 showed a significantly low P value, probably due to a low gonadotropin level owing to negative feedback. A relatively low level of P in mice treated with MNU is in agreement with the previous data in rats.[21] Therefore, the mice treated with MNU and E_2 showed higher levels of E_2/P ratio than those treated with E_2 alone as well as those with no E_2 treatment. Such a high E_2/P ratio may cause a significantly higher incidence of endometrial cancer in mice treated with MNU and E_2.

In this experiment, the incidences of endometrial adenocarcinomas in mice treated with MNU or E_2 alone were 7% and 23%, respectively, while the incidence in those given combined treatment with MNU and E_2 was 48%. Carcinogenicity of estrogens has been proposed.[3, 5, 9] These results indicated a synergistic effect of MNU and E_2 on endometrial carcinogenesis.[22] The combined treatment with MNU and E_2 was more useful for early development of endometrial adenocarcinoma than treatment with MNU or E_2 alone. Thus, the present experimental method is useful for short-term induction of endometrial carcinoma.

In the present experiment, all mice treated with E_2 alone, as well as with MNU and E_2, had cystic glandular hyperplasias of the endometrium. The mean combined weights of uterus and ovaries treated with E_2 were significantly larger than those in the other groups, possibly due to hyperplasia of the endometrium. The cause of occurrence of cystic glandular hyperplasia is apparently administration of E_2. These findings agree with previous reports.[3, 5, 9] In human endometrium, estrogen-related lesions progress from cystic glandular hyperplasia, through adenomatous hyperplasia and atypical hyperplasia, to adenocarcinoma.[23] However, endometrial preneoplastic lesions have not been studied in animal models. The present study could induce not only a high incidence of endometrial adenocarcinoma but also a relatively high incidence of preneoplastic endometrial lesions. Moreover, the histology of the preneoplastic lesions appeared to be quite similar to those in humans. Thus, the method used in the present experiment should be useful for examining the pathogenesis of endometrial carcinoma. The present model may also be useful to determine the efficacy of anti-tumoral agents against preneoplastic endometrial lesions as well as endometrial adenocarcinomas. An experiment designed to examine the ability of medroxyprogesterone acetate, a well-known anti-cancer drug for endometrial cancer, to inhibit the development of preneoplastic and neoplastic endometrial lesions is ongoing using this model.

ACKNOWLEDGMENTS

The authors thank Dr. S. Morishita at this Department for technical assistance.

(Received April 24, 1991/Accepted August 20, 1991)

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