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High-yield Induction of Uterine Endometrial Adenocarcinomas in Donryu Rats by a Single Intra-uterine Administration of *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine via the Vagina

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A total of 130 female Donryu rats (10-week-old) were divided into two groups; 80 animals in the experimental group were given a single intra-uterine administration of 20 mg/kg *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) dissolved in polyethylene glycol (PEG) via the vagina without laparotomy, and 50 animals in the control group received PEG alone in the same manner. Small numbers of animals in both groups were killed at 3, 6, 9 and 12 months after ENNG treatment for sequential histological and endocrinological examination, and at 12.5 experimental months (15 months of age) all survivors were killed. At the termination, endometrial adenocarcinomas were present in 49% of the experimental group, compared to 0% in the control group. Severe endometrial hyperplasias were also found only in the experimental group and sequential histological examination showed first appearance of hyperplasia at 6 months and adenocarcinoma at 9 months. No tumors other than uterine carcinomas were induced by ENNG and the carcinogen treatment did not affect the endocrine environment of rats, persistent estrus appearing at 6 months after the start and increasing with age in both groups. The estradiol-17β:progesterone (E:P) ratio was also increased after 6 months, with further elevation at 12 months to about 8 times higher than the level at 6 months. These results indicate that an increased E:P ratio might act as a promoter of development of endometrial proliferative lesions initiated by ENNG in this rat strain. The study indicates that the present simple method using Donryu rats provides a good animal model for endometrial adenocarcinoma development in women.

Key words: Endometrial adenocarcinoma — Donryu rat — *N*-Ethyl-*N'*-nitro-*N*-nitrosoguanidine — Intra-uterine administration — Estradiol-17β:progesterone ratio

Endometrial adenocarcinoma is one of the most common malignant tumors in women, and a tendency towards increase in incidence has been observed in Japan.[1] An association between endocrine imbalance and this tumor has been documented in the literature, and it has been pointed out that estrogen may play an essential role in determining occurrence.[2–5]

In rats, while spontaneous occurrence of uterine tumors is generally frequent, the most common lesion is of endometrial stromal polyp type, and endometrial adenocarcinomas are very rare.[6] Many attempts to establish an animal model for human endometrial adenocarcinomas have been made in rats using chemical carcinogens and/or hormones. The incidences of induced tumors, however, have not been satisfactory, as reviewed recently by Anisimov and Nikonov.[6] Uterine tumors were induced by intra-uterine administration of various carcinogens such as polycyclic aromatic hydrocarbons and aromatic amines, with laparotomy, but most were squamous cell carcinomas and only a few were adenocarcinomas.[6] More recently, *N*-nitroso compounds have been applied for induction of uterine tumors and adenocarcinomas have resulted from intra-uterine administration with laparotomy[7,8] or other approaches such as oral,[9] intravenous[10] or intraperitoneal[11] administration. In addition to the disadvantage of low yields, however, intra-uterine application of carcinogen with laparotomy is very complicated, and tumors other than uterine tumors are also induced by the other methods.

Previously, we reported high occurrence of spontaneous endometrial adenocarcinomas in Donryu rats, associated with a hormonal disturbance.[12] In the present report, we describe high yields of endometrial adenocarcinomas in this rat strain after a single intra-uterine administration of *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) without laparotomy, and discuss features making this a useful animal model for endometrial adenocarcinoma development.

MATERIALS AND METHODS

Animals and housing conditions A total of 130 female Crj:Donryu rats, 4 weeks of age, were purchased from Charles River Japan Inc. (Kanagawa) and housed 3 animals in a plastic cage, in an air-conditioned animal room at 21±2°C and 55±10% humidity with a 12-h light-dark cycle. The animals were maintained on basal

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diet CRF-1 (Oriental Yeast Ind. Co., Tokyo) and tap water *ad libitum,* throughout the experiment.

Chemical carcinogen and administration method ENNG, purchased from Nacalai Tesque, Inc. (Kyoto), was dissolved at a 6% concentration in polyethylene glycol (PEG) just before use. Rats were divided into two groups, the experimental (80 rats) and control (50 rats) groups, at 10 weeks of age, and the former animals were given a single dose of 0.33 ml/kg of ENNG solution (20 mg/kg) into a unilateral uterine cavity using a children's otoscope via the vagina without laparotomy. Control animals were given only PEG without ENNG in the same manner. For sequential histological observation and steroid hormone assay, 4–8 animals in each group were killed at 3, 6, 9 and 12 months after the start of the experiment. All surviving animals were killed at 12.5 experimental months (15 months of age).

Histological examination All reproductive and other main organs were removed and fixed in buffered 10% formalin. Each uterus was cut into 6 specimens from both uterine horns and the corpus uteri, being sectioned transversely and/or longitudinally. Sections were routinely prepared, stained with hematoxylin and eosin (H-E), and examined microscopically.

Hormonal assay Estrous cycles were checked in rats scheduled to be killed in both groups, and animals at diestrus or persistent estrus were killed by decapitation between 10:00–11:00 h. Blood was centrifuged at 3000 rpm for 10 min, and stored at −80°C until assay. The serum values of estradiol-17β (E) and progesterone were measured by radioimmunoassay. Specific sheep anti-E serum (GDN 244), kindly supplied by Dr. C. D. Niswender (Department of Physiology and Biophysics, Colorado State University, Colorado, USA), and Progesterone Daiichi II kit (Daiichi Radioisotope Labs. Ltd., Tokyo) were used.

RESULTS

The mean body weights did not differ between groups throughout the experimental period. Spontaneous mortalities (6 animals) were limited to the experimental

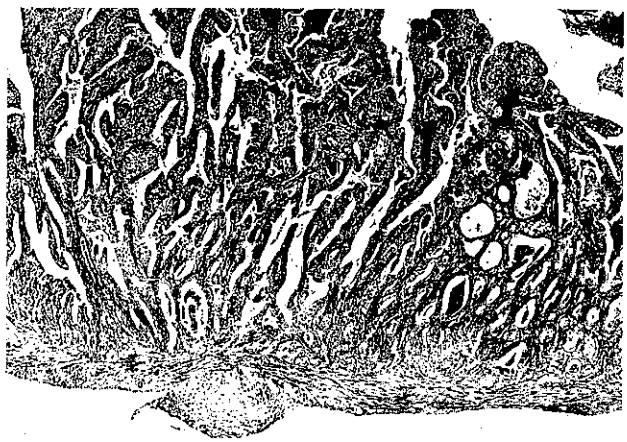

Fig. 1. Moderately differentiated endometrial adenocarcinoma of a Donryu rat killed at 12 months after intra-uterine administration of ENNG. Irregular proliferation of atypical glands in the endometrium and tumor cell invasion into the muscularis are prominent. H-E, ×128.

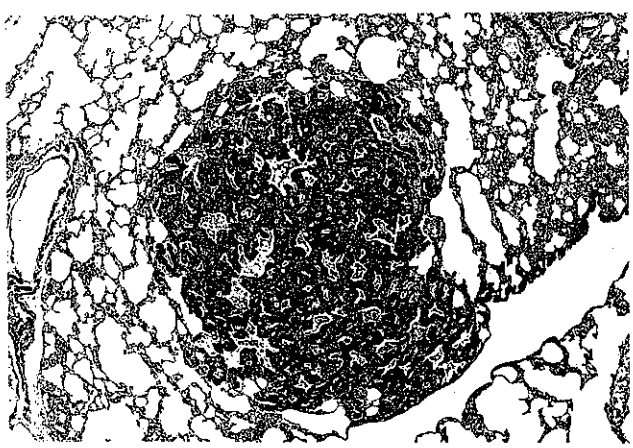

Fig. 2. Lung metastasis of the same tumor as in Fig. 1. H-E, ×96.

Table I. Sequential Changes in the Uterine Endometrium of Donryu Rats after ENNG Treatment

	Experimental group[a]				Control group[a]			
	3	6	9	12 (months)	3	6	9	12 (months)
Number of rats examined	6	6	6	8	4	4	4	8
Hyperplasia +	0	1	2	1	0	0	0	1
++	0	0	1	1	0	0	0	0
+++	0	0	1	2	0	0	0	1
Adenocarcinoma	0	0	1	4[b]	0	0	0	0

a) At 3, 6, 9 and 12 months after the start of experiment, 4–8 animals in each group were killed.
b) One of the adenocarcinomas had metastasized to the lung.

Table II. Proliferative Lesions in the Uterus and Vagina at Termination of the Experiment

	Experimental group		Control group	
		(%)		(%)
Number of rats examined	49		30	
Uterus				
Endometrial hyperplasia +	7	(14)	7	(23)
++	7	(14)	6	(20)
+++	6	(12)	0	
Endometrial adenocarcinoma	24	(49)	0	
Stromal polyp	1	(2)	3	(10)
Stromal sarcoma	1	(2)	0	
Hemangioendothelioma	1	(2)	0	
Vagina				
Squamous cell papilloma	0		1	(3)

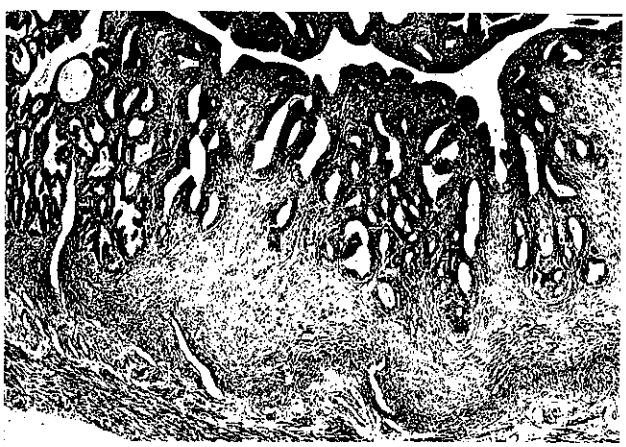

Fig. 3. Severe endometrial hyperplasia (+++) of a Donryu rat killed at 12.5 months after ENNG treatment. Proliferation of atypical glands is found in the endometrium, without invasion into the muscularis. H-E, ×102.

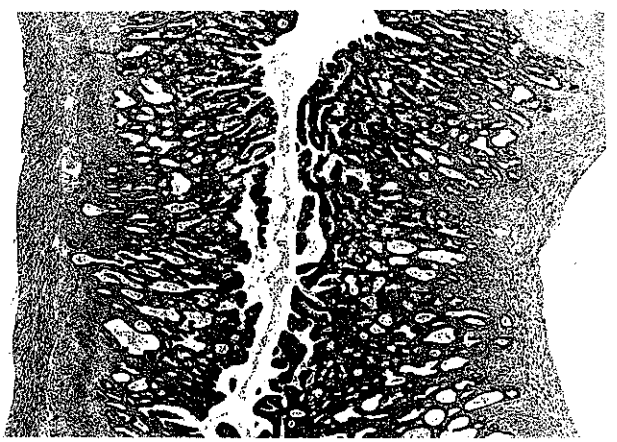

Fig. 4. Well-differentiated adenocarcinoma of a 15-month-old Donryu rat in the experimental group. Diffuse proliferation of atypical glands in the endometrium and tumor cell-invasion into the muscle layer are obvious. H-E, ×60.

group. In the present study, uterine endometrial proliferative lesions were histologically classified into hyperplasia and adenocarcinoma categories. Endometrial hyperplasias were classified into three degrees on the basis of atypia and size: slight (+), moderate (++) and severe (+++), according to the criteria described in our previous report,[13] and adenocarcinomas were diagnosed mainly on the basis of cellular and/or structural atypia, size of the lesions and tumor cell-invasion into the muscularis. Slight/moderate endometrial hyperplasias refers to increased numbers of glands with slight to moderately atypical cells in focal and/or diffuse areas of the endometrium. Severe hyperplasias were composed of irregular proliferation of atypical glands, which were characterized by crowding, stratification and irregularity of cells with back-to-back disposition. Adenocarcinomas were composed of irregular proliferations of atypical cells consisting of one or more layers of cuboidal or columnar cells, with invasion of tumor cells into the muscularis.

Table I summarizes the results of sequential histological observation of uterine endometrium. At 6 months after the start, in the experimental group, endometrial hyperplasia was observed in one rat, and thereafter the incidence and degree of this lesion increased time-dependently. The first adenocarcinoma was found at 9 months in the experimental group and thereafter the tumors also increased in this group with age. One adenocarcinoma observed at 12 months showed lung metastasis (Figs. 1 and 2). In controls, only a few hyperplasias were detected at 12 months of age, and no adenocarcinomas were found.

Table III. Sequential Changes in the Serum Levels of E and P after ENNG Treatment[a]

	Months after the start of experiment			
	3	6	9	12
E (pg/ml)	14.44±4.1[b]	18.62±4.2[b]	10.73±4.7[b]	21.99±4.6[b]
P (ng/ml)	17.78±4.6	8.15±3.9[c]	10.94±10.6	3.05±3.3[d]
E/P ($\times 10^3$)	0.84±0.26	3.15±2.76	1.88±1.67	27.49±30.43

a) The data are combined from both experimental and control groups.
b) Mean±SD.
c) Significantly different from the value at 3 months ($P<0.05$).
d) Significantly different from the value at 3 months ($P<0.01$).
E, estradiol-17β; P, progesterone; E/P, estrogen: progesteron ratio.

Table II summarizes data for proliferative lesions observed in the uterus and vagina at the termination of the experiment. In the experimental group, endometrial adenocarcinomas were found in 24 out of 49 rats (49%), the incidence being significant, as compared to 0/30 (0%) in the control group. In addition, severe hyperplasias were found only in the experimental group (Fig. 3). Almost all adenocarcinomas were macroscopically very small. Histologically, all adenocarcinomas were well-differentiated, showing irregular proliferation of atypical glands (Fig. 4). All adenocarcinomas were detected in the uterine horn treated with ENNG. A few endometrial stromal polyps and/or sarcomas were detected in both groups, the incidences not being significant. One hemangioendothelioma was found in the experimental group. No carcinomas were detected in the cervix. In addition to these lesions, a few cases of uterine hemorrhage and/or hematoma and a vaginal squamous cell hyperplasia were observed in the carcinogen-treated animals.

Table III shows the serum levels of steroid hormones at 3, 6, 9, and 12 months after the start. The ENNG-treatment did not affect the endocrine environment of rats, because the mean estrogen and progesterone levels did not differ significantly at any time point between treated and control groups, though there was a wide variation in individual values. Therefore, the data shown in the table are combined from both groups. The estrogen level did not change during the experimental period, while the progesterone level was decreased after 6 months, with a pronounced drop after 12 months. Thus, the estradiol-17β:progesterone (E:P) ratio was markedly increased, being about 8 times higher at 12 months than at 6 months.

With regard to tumors other than uterine lesions, only mammary and pituitary tumors were observed in both groups. Mammary fibroadenomas were found in 12/49 (24%) and 6/30 (20%) of the experimental and control groups, respectively. In addition, 3 pituitary adenomas in the experimental group and 1 in the control group were evident. In addition to these two tumors, some hyperplastic lesions were found in the thyroid, adrenal and thymus of both groups, the incidences not being significantly different.

DISCUSSION

Tanaka and Mori[7] reported induction of uterine endometrial adenocarcinomas in ACI rats by intra-uterine administration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine with laparotomy. Subsequently, tumors were also induced in F344 rats by similar administration of ENNG, when the animals were observed for an extended period (570 days).[8] However, the incidences of endometrial adenocarcinomas in these two studies were not high. In contrast, the present study featured high yields of severe hyperplasias and adenocarcinomas, when animals were observed until 15 months (about 450 days) of age. Endometrial hyperplasias in rats are considered to be preneoplastic,[13] as in humans. In this strain of rats, the spontaneous occurrence of endometrial adenocarcinomas is relatively high, but most such malignancies have been found at 2 years of age or thereafter.[12, 14] The lack of tumors in the present control group at the termination of the experiment (15 months of age), therefore, is in line with the literature, with the early and high yields of uterine adenocarcinomas in the experimental group clearly depending on the intra-uterine administration of ENNG.

Previously, we reported that a hormonal imbalance indicated by persistent estrus and an increased E:P ratio may play an important role in the spontaneous occurrence of endometrial adenocarcinomas in Donryu rats.[12, 13] In the present study, ENNG administration did not appear to affect the endocrine environment, because there were no inter-group differences in the results for sequential estrous cycle examination and steroid hormone assays. Therefore, an increased E:P ratio with age, similar to that in previous reports,[12, 13] might act as a promoter of endometrial adenocarcinoma development

initiated by ENNG treatment. In a study using the same approach with F344 rats, no increase of E:P ratio was detected during the experimental period, and no increase in endometrial adenocarcinomas was evident after dosing with ENNG (unpublished data). The F344 rat is known to have a low incidence of spontaneous endometrial adenocarcinomas,[12, 15, 16] and in a previous study also no clear hormonal changes with age were detected by sequential analysis of plasma gonad steroids.[13] The available results thus support the possibility that an increased E:P ratio acts as a promoter in Donryu rats treated with ENNG.

In the present case, a single dose of carcinogen was administered into a unilateral uterine cavity directly via the vagina, in contrast to all previous studies where intra-uterine administration of carcinogens was performed with laparotomy, as mentioned above. The uterine tumors did not include any of squamous cell carcinoma type, and, in addition, no induction of tumors other than uterine lesions was observed. The finding of a few cases of hemorrhage or hematoma of the uterus suggests that these might have been the main cause of the observed mortalities. The endometrial stromal polyps/sarcomas, mammary tumors, pituitary tumors, and hyperplastic lesions found in other organs in both groups of the present study can all be considered as spontaneous, since there were no inter-group differences in their incidences and they are well known as common lesions in this rat strain.[14] It has previously been stressed that the histological appearance, biological behavior and histogenesis of endometrial adenocarcinomas in Donryu rats are essentially similar to those in women.[12, 13] The results thus indicate that the present simple experimental method is a good animal model for endometrial adenocarcinoma development in human beings.

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