Original

Transplacental Carcinogenicity of N-Ethyl-N-Nitrosourea in Sprague-Dawley Rats

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Abstract: N-Ethyl-N-nitrosourea (ENU) is known to induce a wide spectrum of tumors in various organs in adult experimental animals. The renal and neuroectodermal tumors are known as representative lesions by transplacental exposure to ENU in the offspring animals. However, little information is available about tumorigenicity in other organs and tissues in offspring when their mother animals are treated with ENU during gestation. Thus, the purpose of this study was to evaluate the effects of transplacently treated-ENU on the various organ tumorigenicity in offspring rats. ENU was injected intraperitoneally to female Sprague-Dawley (SD) rats with a single dose at 50 mg/kg on the 18th day of gestation. After spontaneous delivery, 44 male and 64 female offspring, including moribund and dead after birth, were subjected to the evaluation of carcinogenicity. At the 54th to 55th week of birth, all surviving offspring were euthanized under ether anesthesia for histopathology. ENU showed a wide spectrum of transplacental tumorgenesis in the kidney, central nervous system, peripheral nervous system, thyroid gland, and teeth. The tumors of the thyroid and teeth were characteristic in particular in this study. The thyroid tumors included various histopathological types (follicular cell adenoma and adenocarcinoma, C-cell adenoma and carcinoma, squamous cell carcinoma, and fibroma). As a characteristic tumor of the teeth, ameloblastic odontoma was detected in 3 (one male and 2 females) of 108 offspring. In conclusion, the results indicate that the transplacental exposure of a single ENU dose induces various types of thyroid gland tumors and odontogenic tumors as well as the renal or neuroectodermal tumors in rat offspring.

Key words: transplacental carcinogenesis, ENU, SD rat

Introduction

N-Ethyl-N-nitrosourea (ENU), one of the oncogenic N-nitroso compounds, is a potent carcinogen, and induces a wide spectrum of tumors in experimental animals. The transplacental exposure to ENU is known to induce not only nephroblastoma¹–⁴ and renal tubular cell tumor¹,² in the kidney, but also neuroectodermal tumors⁵–⁷ such as various kinds of gliomas in the central nervous system (CNS) and schwannoma in the peripheral nervous system (PNS). Although these studies have examined the carcinogenicity of transplacental ENU in selected organs or tissues individually, little is known about the details of transplacental carcinogenesis of ENU covering as many organs as possible at a time throughout the whole body.

Diwan and Rice¹ exposed rat fetuses transplacentally to ENU on days 14, 16, or 18 of gestation and observed that the highest incidence of tumors was induced in the offspring by ENU treatment on day 18 of gestation. We, therefore, evaluated the transplacental nephroblastoma induction of ENU in rats by examining offspring after spontaneous delivery from pregnant rats treated intraperitoneally with a single dose of ENU on day 18 of gestation, as suggested from the study by Diwan and Rice¹.

In addition to nephroblastoma and neuroectodermal tumor reported so far as common tumors with transplacental ENU, we confirmed a diversity of tumor cell in the thyroid gland, induction of ameloblastic odontoma in the mandible, and some others, which may provide new insights for future transplacental carcinogenicity studies of ENU or other chemicals.

Materials and Methods

Animals and chemical

Twenty-three female Sprague-Dawley (SD) rats aged 10 weeks and 11 male SD rats aged 12 weeks were purchased from Charles River Japan Inc. (Kanagawa, Japan). After 2 weeks of quarantine/acclimation, they were subjected to experiments. The animals were maintained on a
commercial diet (CRF-1, Oriental Yeast Co., Ltd, Tokyo, Japan) and ultraviolet irradiated tap water *ad libitum*, and housed at a temperature of 21.7 – 24.7°C and a humidity of 43 – 70%, with 12 hours of daily lighting (lighting from 7:00 to 19:00).

ENU (Lot No. V8K5782) was purchased from Nakarai Tesque, Inc. (Kyoto, Japan), and stored at –20°C in a light-protective place. ENU was dissolved, just before use, in injection-grade distilled water (Otsuka Pharmaceutical Co., Ltd, Tokyo, Japan) at a 0.5% concentration.

**Experimental design**

One female rat was placed in one cage together with a male rat overnight. Females were examined on the next morning for vaginal plugs and smear, and pregnancy was confirmed by the presence of sperm in vaginal smear. The day when sperm was detected was designated as Day 0 of gestation. The pregnant rats were randomly allocated to 2 groups of 11 or 12 rats each and identified with ear punch. Then, each animal was housed and cared in an individual polycarbonate cage under the above-mentioned conditions.

A single dose of ENU (50 mg/kg) was injected intraperitoneally on Day 18 of gestation. Eight of 12 ENU-treated rats were delivered of a total of 44 male and 64 female offspring spontaneously. Separately, a total of 56 treated rats were delivered of a total of 44 male and 64 female control offspring on Day 18 of gestation. The pregnant rats were randomly allocated to 2 groups of 11 or 12 rats each and identified with ear punch. All offspring were weaned at the age of 3 weeks and, after identifying their sex, they were housed in individual cages under the same environmental conditions as above. All the surviving offspring were evaluated at the 54th to 55th week after the last delivery. For routine recording, the day of the last delivery was designated as Week 0 of birth.

When found moribund, the animals were euthanized in line with the standard operational procedures for the institutional animal care management. The animals found dead or euthanized were autopsied immediately. All surviving animals were euthanized under ether anesthesia at Week 54 to 55 of birth.

**General appearance, body weight, and histology**

Throughout the study period, all offspring were daily observed for general appearance and clinical signs, and weighed once a week from Week 3 to 20 of birth and every 2 weeks thereafter. The kidneys, brains, spinal cords, and macroscopic lesions/masses were removed and fixed in buffered neutral formalin for the conventional histology. The sections were stained with hematoxylin and eosin (HE) for histological evaluation.

**Statistical analysis**

Categorical data, expressed as incidence or frequency, were analyzed with Fisher’s exact test. For all statistical tests performed, probability of less than 0.05 was considered statistically significant.

### Results

**Survival curves, body weight, and general appearance**

The survival curves for males are shown in Fig. 1 and those for females in Fig. 2. Only three of 44 males (6.8%) exposed transplacentally to ENU survived throughout the entire period of observation, and 52 of 56 control males (92.9%) survived. Eleven of 64 females (17.2%) exposed to ENU survived, and 58 of 61 control females (95.1%) survived (Table 1). Most of the treated rats died or were sacrificed because of tumors of the nervous system, which were observed evenly throughout the observation period. Two male rats died of excessive hemorrhage from the very large nephroblastomas, showing a higher malignancy and rapid growth. One male and one female rats were sacrificed because of loss of appetite and emaciation caused by the large mass of the mandible.

**Body weight curves** are illustrated in Fig. 3. Significantly reduced weight gain was observed both in males and females exposed transplacentally to ENU.

In general appearance, decreased locomotor activity and abnormal gait appeared in ENU-exposed rats from Week 16 (females) or Week 17 (males) of birth. After that, soiled perineal region owing to urinary incontinence was sporadically observed. In ENU-exposed rats, masses were formed in the subcutis (chest, back, and abdominal region), orbit, and mandible in 4 males and 10 females of ENU-exposed rats. In the control rats, enormous masses were formed in the subcutis in 2 of 61 females.

**Histological classification**

Table 2 summarizes the incidence and histological types of neoplastic and pre-neoplastic lesions. Tumors of the kidney: Twenty-six rats (17 males and 9 females) exposed to ENU had a nephroblastoma each. The tumors appeared grossly as spherical, white, solid or occasionally cystic masses. Nine of the 26 tumors were varied in size, and detectable macroscopically, but the others were identified under a microscope. Their margins were usually sharp, and compressed the surrounding normal renal tissue. Some large tumors, measuring 29 to 62 mm in length or diameter, occupied the most part of the renal tissue, with hemorrhage or necrosis around the center of the cut surface. Histologically, most of the tumors were an admixture of epithelial, blastemal and stromal elements. Neoplastic cells frequently showed epithelial differentiation, indicating a

### Table 1. Survival Rates in SD Rats after Transplacental Exposure to ENU

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival</td>
<td>Dead*</td>
<td>Survival</td>
</tr>
<tr>
<td>Control</td>
<td>52 (92.9)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>ENU, 50 mg/kg</td>
<td>4 (7.1)</td>
<td>41 (93.2)</td>
</tr>
</tbody>
</table>

*: Animals which were dead or euthanized within an experimental period, ( ): %.
tubular-like structure with occasional glomeruloid differentiation (Fig. 4). Mitotic figures were most common among the cells of blastemal element. Metastasis was not detected in these cases.

A total of 6 tubular cell tumors were detected in rats exposed to ENU: adenoma in 3 of 44 males and 1 of 64 females, and adenocarcinoma in 2 females. These tumors were composed of large polyhedral cells rich in clear or eosinophilic cytoplasm and took a dense or alveolar structure (Fig. 5). Tumor cells in some adenomas showed papillary or layered growth of the lining epithelium into the cysts (Fig. 6). Mitotic figures were not frequent. Neither metastasis to other organs nor infiltrative growth was seen.

As tubular cell tumor-related lesions, hyperplasias were observed in 3 (1 male and 2 females) of 108 rats exposed to ENU. The affected tubules were two to three times larger in diameter than normal tubules, and the hyperplastic cells were slightly atypical. The above-mentioned tumors and hyperplasias were not observed in the kidney of the controls.

Tumors of the CNS: Since gliomas consisted of neoplastic astrocyte and oligodendroglia in different proportions, we diagnosed the tumors according to their predominant cell type.

Astrocytomas were characterized by relatively high cellularity and poorly-defined margins. Tumor cells were often uniform in size, rounded or elongated in shape, and
relatively rich in bright pink or clear cytoplasm with hyperchromatic and/or euchromatic nuclei (Fig. 7).

Oligodendrogliomas were composed of a sheet-like arrangement of uniform cells with small, round, densely stained nuclei. Individual cells usually had perinuclear halos, thus providing the tumors with a honeycomb appearance (Fig. 8).

Mixed glioma, also called mixed oligo-astrocytoma, consisted of neoplastic oligodendroglia and astrocyte in almost equivalent proportions.

We used the term “glioma (NOS)” for diagnosis when the cell type could not be identified due to postmortem changes.

Tumors of the PNS: Intracranial trigeminal nerve was the most common site for tumors of the PNS in ENU-exposed rats. Grossly, large tumors were soft, grayish-white, and often appeared as cystic masses. Occasionally, pituitary glands were enveloped in the tumors and could not be confirmed at necropsy.

Microscopically, all of the PNS tumors in the ENU-exposed rats were schwannomas. They were composed of small, oval and hyperchromatic nuclei with abundant and eosinophilic cytoplasm. Nuclei of adjacent cells were arranged in rows like palisades (Fig. 9). Cysts containing eosinophilic material were often recognized in the intracranial schwannomas.

Fig. 3. Body weight changes in SD rats after transplacental exposure to ENU.

Tumors of the thyroid gland: Thyroid tumors appeared with a higher incidence in the ENU-exposed rats than in the control rats, and were characterized by the diversity of the tumor types (Fig. 10). The tumors included 9 adenomas and 2 adenocarcinomas originating from the follicular cell, and one each of C-cell adenoma, C-cell adenocarcinoma, squamous cell carcinoma, and fibroma.

Tumor of the teeth: A total of 3 ameloblastic odontomas were detected in one male and 2 females of the ENU-exposed rats. Swelling on the mandible was first observed at Week 12 of birth in one male and one female rat, and progressively increased in size until necropsy at Week 18 (male) and Week 33 (female) of birth. The tumor in the other rat was first detected at Week 24 of birth, and this rat was necropsied at Week 54 of birth according to the study design.

Necropsy revealed hard, whitish bony masses (25 – 40 mm in diameter) on the mandible. Histologically, most part of the tumors was composed of irregular trabeculae of dentin, abundant mesenchyme, linearly arranged ameloblasts, and enamel caps (Fig. 11). In addition, the remaining mandibular bones were slightly destroyed at the peripheral area of the tumors, but no indication of tumor invasion was present.

Tumors of the mammary gland: Benign mammary tumors were either adenomas or fibroadenomas consisting of
fibrotic encapsulated masses with various degrees of intercalated glandular structures. Adenoma was detected in five females, and fibroadenoma in one male and two females. All the eight benign tumors occurred in the ENU-exposed rats.

Malignant mammary tumors were adenocarcinoma consisting of irregular ductular and acinar structures filled with eosinophilic materials. The tumor cells exhibited a high rate of mitosis and pleomorphism. These adenocarcinomas were detected in one female control rat and three female ENU-exposed rats. Metastasis to the lung was observed in one ENU-exposed rat.

Other tumors and pre-neoplastic lesions: In addition to the tumors described, several neoplastic and pre-neoplastic lesions were detected in other organs and tissues. The lesions included hepatocellular adeomas and foci of cellular alteration in the liver, bronchiolar/ alveolar adenoma and adenocarcinoma in the lung, and acinar cell and ductal adenoma in the pancreas. The incidence of neoplastic or pre-neoplastic lesions in the pituitary gland was lower in the ENU-exposed group than that in the control group, probably because the rate of dead or moribund rats was very high in the ENU-treated group during the early phase of the observation period.

Non-neoplastic lesions: As one of non-neoplastic lesions, cyst formations in the kidney were observed in 17 of 44 males and 20 of 64 females exposed to ENU, and in 2 of 56 males and 2 of 61 females in the control group. These lesions were variable in size and lined by a monolayer of flattened cells, and probably induced by ENU, though their pathogenesis remains unclear.

Moreover, chronic nephropathy and its related lesions in the kidney were observed sporadically in ENU-exposed and control groups, and their incidence in males was higher than that in females.

**Discussion**

The transplacental exposure of a chemical compound during gestation is becoming increasingly important as an administration route for carcinogenesis studies. Transplacental tumorigenesis has been studied with dimethylnitrosoamine, 3,3-dimethyl-1-phenyltriazene, azo- and azoxyethane, and ENU. Almost all of these

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**Table 2. Incidence of Neoplastic and Preneoplastic Lesions in SD Rats after Transplacental Exposure to ENU**

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>ENU, 50 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>F</td>
</tr>
<tr>
<td>No. of rats examined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nephroblastoma</td>
<td>17</td>
<td>9 *</td>
</tr>
<tr>
<td>Adenoma, renal tubule</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Adenocarcinoma, renal tubule</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mesenchymal tumor (suspicious)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hyperplasia, renal tubule, atypia #</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>15</td>
<td>14 *</td>
</tr>
<tr>
<td>Mixed glioma</td>
<td>7</td>
<td>7 *</td>
</tr>
<tr>
<td>Glioma (NOS)</td>
<td>3</td>
<td>13 *</td>
</tr>
<tr>
<td>Oligodendrogloma</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Spinal cord</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>8</td>
<td>19 *</td>
</tr>
<tr>
<td>Oligodendrogloma</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Schwannoma</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Mixed glioma</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Glioma (NOS)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Peripheral nerve</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Schwannoma</td>
<td>11</td>
<td>9 *</td>
</tr>
<tr>
<td>Pituitary gland</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chromophobe adenoma</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Adenoma, mixed cell</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adenoma (NOS)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Chromophobe hyperplasia #</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Thyroid gland</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenoma, follicular cell</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Adenocarcinoma, follicular cell</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C-cell adenoma</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>C-cell carcinoma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fibroma</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

M: Male, F: Female, NOS: Not otherwise specified, #: Preneoplastic lesions, *: Significantly different from control, P<0.05 (Fisher’s exact test).
Fig. 4. Nephroblastoma induced by transplacental ENU consisting of undifferentiated epithelial and stromal cells, and of irregular-shaped tubules suggestive of immature formation of glomeruli. HE stain. Original magnification ×33.

Fig. 5. ENU-induced renal tubule adenocarcinoma showing solid pattern. Note the central necrosis (N) and a few mitotic figures (arrows). HE stain. Original magnification ×33.

Fig. 6. Renal tubule adenoma with large cystic space. Note partial papillary (P) and solid (S) patterns. HE stain. Original magnification ×33.
Fig. 7. Astrocytoma induced by transplacental ENU consisting of closely packed cells with moderate amounts of granular cytoplasm, and oval to round nuclei. HE stain. Original magnification ×33.

Fig. 8. ENU-induced oligodendroglioma consisting of diffuse sheets of uniform tumor cells. HE stain. Original magnification ×33.

Fig. 9. Area resembling Antoni type A pattern in a ENU-induced schwannoma of the trigeminal nerve. HE stain. Original magnification ×33.
studies, however, have reported their tumorigenesis in selected organs or tissues, such as the renal tumors\textsuperscript{10–14}, including nephroblastoma\textsuperscript{1–3,15}, and neuroectodermal tumors\textsuperscript{5–7,16–19}. There are few reports on transplacental carcinogenesis, for example, of ENU covering as many organs as possible. We, therefore, evaluated such carcinogenesis of ENU, as a tool compound, in the offspring from female rats exposed to a single transplacental dose of 50 mg/kg on Day 18 of gestation.

In the kidneys from the rat offspring exposed transplacently to ENU during gestation, the tumors were noted as nephroblastoma in males (17/44, or 38.6%) and females (9/64, 14.1%) with a slight difference between sexes, and renal tubule adenoma or adenocarcinoma in each 3 rats in both sexes. Pre-neoplastic tubular cell lesions, such as atypical renal tubule hyperplasia, were observed in 3 (one male and two females) of 108 ENU-exposed rats. Neither tumors nor hyperplasia of these types were present in the controls. Our findings are in agreement with the results that transplacental exposure to ENU induced nephroblastoma and renal tubular cell tumors in the kidney\textsuperscript{1–4}.

As neuroectodermal tumors, astrocytoma, oligodendroglioma, and mixed glioma were found in the CNS, and shwannoma in the PNS of the ENU-exposed rats. These gliomas were present in the brains of males (28/44, 63.6%) and females (39/64, 60.9%), or in the spinal cords of males (15/44, 34.1%) and females (21/64, 32.8%). Shwannoma was noted in males (11/44, 25.0%) and females (9/64, 14.1%). Thirty-seven of 44 males (84.1%) and 55/64 females (85.9%) had one or more tumors of these kinds in the nervous system. These findings are again in agreement with transplacental induction of neuroectodermal tumors\textsuperscript{5–7} as various kinds of gliomas in the CNS and schwannoma in the PNS.
The changes in gross behavior or general appearance included decreased locomotor activity and abnormal gait in the ENU-treated rats on and after Week 16 (female) or Week 17 (male) of birth. These changes were considered to appear as the consequences of the preceding tumor(s) in the CNS. After that, some rats sporadically showed their perineal region soiled by urinary incontinence. Thus, onset of the incontinence was probably related to the preceding development of the CNS tumors.

Common tumors reported so far with transplacental ENU are nephroblastoma and neuroectodermal tumor. In addition, however, we confirmed a diversity of the tumor cell types in the thyroid gland (follicular cell adenoma and adenocarcinoma, C-cell adenoma and carcinoma, squamous cell carcinoma, and fibroma). Stoica and Koestner reported that 3 follicular carcinomas and 4 follicular adenomas were detected in 60 male SD rats treated with ENU (45 mg/kg, 90 mg/kg) intraperitoneally at 30 days of age, and they concluded that malignant neoplasias of the thyroid gland in the ENU treated groups were treatment-related. However, they did not refer to a diversity of the tumor cell types in the thyroid gland. Squamous cell carcinoma in the thyroid gland has been very rarely reported except for occasional reports on the development of such carcinoma with a genotoxic carcinogen. In particular, no reports are available on chemically induced fibroma in the thyroid gland. We believe, therefore, our present findings will provide new insights into the transplacental tumorigenesis of ENU in the thyroid gland.

A total of 3 ameloblastic odontomas appeared in the ENU-exposed rats. Ameloblastic odontoma is a very rare tumor in laboratory animals, and only one study has reported the spontaneous development of mandibular ameloblastic odontoma in Fischer 344 rat. As an example of chemical induction, Stoica and Koestner reported that a single high dose (180 mg/kg) of intraperitoneal ENU caused ameloblastic odontoma in 30-day-old male rats. Moreover, Frank et al. described that odontomas in the dentitious tissue occurred in 3/18 male SD rats following a transplacental exposure to 3,3-dimethyl-1-phenyltriazene on days 16, 18, and 20 of gestation. No data are available on ameloblastic odontoma with ENU in other species, though methyl nitrosourea caused this tumor in Syrian golden hamsters. To our knowledge, therefore, our study is the first to report that the transplacental exposure to ENU induced ameloblastic odontoma in the mandible.

In conclusion, the present evaluation confirms that the transplacental exposure of a single ENU dose on Day 18 of gestation induces various types of thyroid gland tumors and odontogenic tumors as well as the renal or neuroectodermal tumors in rat offspring. Therefore, it may be necessary to further evaluate transplacental tumorigenesis with chemical compounds at certain intervals during gestation so that their tumorigenic spectrum as well as the mechanism and sensitivity of tumorigenesis should be characterized.

References