Induction of Hepatic Tumors by Diethylstilbestrol in N-Nitrosobutylurea-initiated Female Rats

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The carcinogenic effect of estrogens, diethylstilbestrol (DES) and 17β-estradiol (E2), and its modification by N-nitrosobutylurea (NBU) were studied in female W/Fu rats. Multiple mammary tumors (MT) of medullary carcinoma type developed at a high rate following prolonged treatment with estrogens. All MTs were located adjacent to the nipple and were slow-growing. The induction rate, multiplicity and size of estrogen-induced MTs were not influenced by pretreatment with a small amount of NBU, which alone did not induce any tumor. Ten of 12 rats (82%) receiving combined treatment with NBU and DES developed hepatic tumors (HT), while no rats in other treatment groups developed HT. All HTs were multiple nodules of various sizes bulging from the liver surface, and were considered to be neoplastic nodules. A high frequency of HT development was unexpected, because independent treatment with NBU or DES alone did not induce HT in female rats. It appears that DES played a role as a carcinogen, inducing MT and pituitary tumor (PT) through its estrogenic potency (like natural estrogen, E2), while it also acted as a promoter or co-carcinogen in the induction of HT through its pharmacologic effects. These findings may be relevant to an increased frequency of liver neoplasm among women taking oral contraceptives containing synthetic estrogens.

Key words: Diethylstilbestrol — N-Nitrosobutylurea — Hepatocarcinogenesis — Mammary carcinogenesis — Female rat

We have reported that mammary and pituitary tumors (MT and PT) are easily induced in female W/Fu rats by prolonged treatment with estrogen, and that progesterone and tamoxifen showed protective effects against diethylstilbestrol (DES)-induced mammary carcinogenesis.1 The inhibitory effect of progesterone and tamoxifen is attributable to interference with estrogen binding to estrogen receptors on the target cells.

There have been many reports suggesting the causative association of the use of contraceptive steroids with the development of liver neoplasm.2–4 One synthetic estrogen (DES) was shown to be carcinogenic to the mammary and pituitary glands, and weakly carcinogenic to the liver in castrated male rats.5 We have previously reported the induction of hepatic tumors (HT) in castrated male rats treated with N-nitrosobutylurea (NBU) and DES.6,7 In the present experiment, we investigated the induction of hepatic tumors by DES in NBU-pretreated female W/Fu rats.

MATERIALS AND METHODS

Inbred Wistar/Furth (W/Fu) rats were propagated in our laboratory, and were housed in plastic cages (4 or 5 rats/cage) in an air-conditioned room. They were given commercial diet (Oriental MF; Oriental Yeast Co. Ltd., Tokyo) and tap water ad libitum.

At 50 days of age, female rats were given a 250 ppm solution of NBU (Iwai Chemical Co., Ltd., Tokyo) as the drinking water ad libitum for 14 days. A fresh solution was prepared every other morning. The water bottles were wrapped with black vinyl tape to minimize the degradation of NBU through exposure to light. Three days after the termination of the NBU treatment, all rats were divided into three groups. Group I was given no further treatment. In groups II and III, a cholesterol pellet containing 5 mg of DES or 17β-estradiol (E2) was inserted subcutaneously in the
HEPATIC AND MAMMARY TUMORS BY NBU AND DES

back of the rats. In groups IV and V, a pellet containing 5 mg of DES or E2 was inserted at 50 days of age without NBU pretreatment. The pellet was renewed every three months thereafter.

All moribund or dead animals were subjected to a complete autopsy. At twelve months after initiation of estrogen treatment, all surviving rats were killed. The mammary glands, pituitary gland, liver, their tumors and other major organs were removed, weighed and fixed in 10% formalin. Paraffin sections were routinely stained with hematoxylin and eosin, and were examined histologically.

Group comparisons were made by using Student's t-test or $\chi^2$ analysis. Data are presented as mean values ± SD.

RESULTS

The frequency of MT, PT and HT development, and the mean weight of pituitary glands of rats in each group are shown in Table I. None of the rats treated with a small amount of NBU alone (group I) developed a tumor. Many MTs and PTs were developed in rats given estrogens, in groups II, III, IV and V. As shown in Table II, estrogen treatment was followed by the development of multiple MTs in female rats. Both the total number of MTs per rat and the size distribution of MTs were of the same order in groups II, III, IV and V. Moreover, there was no difference in the frequency of PT development or the mean weight of the pituitary glands in these groups. These results indicated that the carcinogenic potencies of DES and E2 on mammary and pituitary glands seem to be comparable to each other, and no synergistic or additive effect of NBU was evident in the present experiment.

Interestingly, every MT that developed following prolonged estrogen treatment was located adjacent to the nipple and was slow-growing. Histologically, all the MTs were either intraductal or medullary carcinomas, composed of cells with uniform and gentle morphology. Mitotic figures were seldom seen. PTs were dark red and presented the appearance of a hemorrhagic adenoma. Morphologically, PTs were composed of chromophobic cells and were diagnosed as adenomas.

Ten of 12 (82%) rats receiving both NBU and DES (group II) developed HT, while no HT developed in rats of any other treatment

Table I. Induction of Mammary (MT), Pituitary (PT) and Hepatic (HT) Tumors in Variously Treated Female W/Fu Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Effective No. of rats</th>
<th>No. of rats with tumor</th>
<th>Weight of pituitary gland (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>MT (%)</td>
<td>PT (%)</td>
</tr>
<tr>
<td>I</td>
<td>NBU</td>
<td>13</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>II</td>
<td>NBU + DES</td>
<td>12</td>
<td>7 (58)*</td>
<td>9 (75)*</td>
</tr>
<tr>
<td>III</td>
<td>NBU + E2</td>
<td>14</td>
<td>12 (86)*</td>
<td>11 (79)*</td>
</tr>
<tr>
<td>IV</td>
<td>DES</td>
<td>11</td>
<td>10 (91)*</td>
<td>5 (45)*</td>
</tr>
<tr>
<td>V</td>
<td>E2</td>
<td>13</td>
<td>12 (92)*</td>
<td>6 (46)*</td>
</tr>
</tbody>
</table>

Table II. Size Distribution of MTs Induced by NBU and Estrogens in Female W/Fu Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Effective No. of rats</th>
<th>No. of MT per rat with following diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt; 2 mm</td>
</tr>
<tr>
<td>I</td>
<td>NBU</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>II</td>
<td>NBU + DES</td>
<td>12</td>
<td>4.00 ± 4.00</td>
</tr>
<tr>
<td>III</td>
<td>NBU + E2</td>
<td>14</td>
<td>2.79 ± 2.08</td>
</tr>
<tr>
<td>IV</td>
<td>DES</td>
<td>11</td>
<td>5.18 ± 3.34</td>
</tr>
<tr>
<td>V</td>
<td>E2</td>
<td>13</td>
<td>5.69 ± 3.54</td>
</tr>
</tbody>
</table>
group (Table I). All HTs induced by NBU and DES presented as a well-circumscribed but nonencapsulated, tan to red brown in color, multiple nodular mass in an otherwise normal liver. The lesions lay subcapsularly, bulging from the liver surface, and varied in size. Five of 10 rats bearing HT possessed large HTs over 20 mm in diameter, and the liver weight of the 5 rats bearing large HTs was over 6 g/100 g body weight (data not shown). Microscopically, HTs consisted of sheets or trabeculae of normal to slightly atypical hepatocytes. Tumor cells were more eosinophilic than surrounding normal hepatocytes, and in some tumors displayed extreme vacuolation, presumably due to glycogen storage. Mitotic figures were seldom seen. The border with the surrounding parenchyma was rather clear and the surrounding hepatocytic cords were compressed by the expansive growth of HTs. There was no evidence of invasion or distant metastasis. All HTs that developed in the present experiment were interpreted histologically as being neoplastic nodules.

Preliminary studies showed that these DES and NBU-induced HTs were not or were only poorly transplantable. Four such HTs, which were taken from five large HTs of over 20 mm in diameter, were transplanted subcutaneously into the back of either intact or DES-treated young syngeneic female rats. None of the HTs have grown during an observation period of twelve months. These histological and biological characteristics of NBU and DES-induced HTs suggested that these HTs were benign neoplasms.

**DISCUSSION**

It was demonstrated in our previous studies that combined treatment with NBU and DES was highly effective in inducing HT in castrated male W/Fu rats. In those studies, in contrast to the present one, NBU and DES were given together for a long period of time. NBU is thought to be a directly acting carcinogen, but many carcinogens require metabolic activation. DES is metabolized in the liver into various oxidative metabolites that directly interact with DNA. The coexistence of NBU and DES might influence their metabolic activation and thus their carcinogenicity might be altered. Since NBU is considered to be a short-acting carcinogen, the effect of NBU on target cells may already be negligible at three days after the termination of NBU treatment. Accordingly, in the present study, estrogen treatments were started at three days after the termination of NBU treatment in an attempt to analyze the roles of the two agents separately.

We have reported that a high frequency of MT and PT development occurred in female W/Fu rats after prolonged treatment with estrogen, and that there was a synergistic effect of chemical carcinogens or irradiation in the induction of MT by estrogen. In the present experiment, a small amount of NBU alone was not tumorigenic in female rats. No increase of frequency or size distribution of MTs was observed in intact or NBU-treated rats given estrogens. MTs and PTs that developed in rats given combined treatment with NBU and estrogens showed similar biological and pathological characteristics to those induced by estrogen alone. Thus, the small amount of NBU used in the present study had no apparent influence on the induction or growth of MTs in estrogenized female rats.

A high frequency of HT occurrence was demonstrated in DES-treated female rats if the rats were given a small amount of NBU prior to DES treatment. Although NBU has been shown to be leukemogenic and mammary-carcinogenic in both intact rats and mice, the liver was not considered to be a target organ of NBU. In our later studies using variously conditioned animals, however, it was demonstrated that NBU possesses much wider organotropism than had been believed. A few cases of hepatoma occurred in hormonally conditioned NBU-treated male W/Fu rats. Furthermore, it was shown that more than 30% of male ICR/JCL mice receiving a single dose of NBU by intragastric intubation developed hepatomas if NBU treatment was preceded by a subcutaneous injection of carbon tetrachloride (CCL) 24 hr previously.

Weak carcinogeticity to the liver was observed in castrated male rats, but not in female rats. It is well known that a sex difference exists in some metabolic activities.
DES is metabolized into an active form.\textsuperscript{7,8} Although no evidence is yet available, we assume that the metabolic activation of DES may be insufficient in female rats and therefore no HT developed. The present experiment shows that the effects of DES in hepatocarcinogenesis can be attributed not to estrogenic potency but to the pharmacologic effect, comparable to the action of DES to induce unscheduled DNA synthesis (UDS), sister chromatid exchange (SCE) or polyploidy in human lymphocytes and Syrian hamster embryo cells.\textsuperscript{16-18}

The results of the present study suggest that NBU might cause certain damage to DNA of hepatocytes, creating either cancer-prone or preneoplastic cell populations in the liver. DES has been shown to promote a proliferation of initiated hepatocytes obtained from enzyme-altered foci induced by hepatocarcinogens.\textsuperscript{19} It can be speculated that DES might act on these dormant initiated cells induced by NBU, causing them to proliferate into overt HT.

An etiological association of the use of oral contraceptive steroids with the development of liver neoplasm was first suggested by Baum \textit{et al.}\textsuperscript{20} Since then there have been many reports supporting such an association,\textsuperscript{1,4} and now it is generally accepted. Although one should be cautious in relating experimental animal findings to human data, the results of the present experiment are suggestive concerning the mechanism of the development of such liver neoplasm in humans.

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