Mammary Tumor Induction by N-Methyl-N-nitrosourea in Genetically Resistant Copenhagen Rats

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ABSTRACT

The Copenhagen rat is completely resistant to mammary cancer induction by N-methyl-N-nitrosourea (MNU) when the carcinogen is administered during sexual development, a period when other strains of rats are normally susceptible to mammary gland carcinogenesis. Here we administered 30 mg/kg MNU i.p. to two groups of neonatal (2-3-day-old) Copenhagen rats. One group (group B, 18 animals) received no further treatment, while the other group (group C, 17 animals) received a second dose of 30 mg/kg MNU via the tail vein at 30 days of age. About 30% of the rats in group B and about 70% of those in group C developed mammary carcinomas before they were 1 year of age. About one-half of the tumors in both groups were cribriform adenocarcinomas and one-half were adenosquamous carcinomas. The latter tumor type has not been observed previously in susceptible rat strains. The ability to induce these mammary tumors in the Copenhagen rat suggests that the putative mammary carcinoma suppressor gene is functionally inactive in neonatal animals or is inactivated when these animals are treated with MNU.

INTRODUCTION

Susceptibility to the development of carcinogen-induced mammary carcinomas varies considerably among different strains of rats (1-3). Highly susceptible strains, including inbred Bu/N, Wistar-Furth, Osborne-Mendel, Lewis, and inbred and outbred Sprague-Dawley rats, develop multiple mammary carcinomas with a short latency following even a single exposure to any of a number of carcinogens (1-6). Strains of intermediate susceptibility, such as inbred Fischer 344, August, and ACI rats develop less than one carcinoma per rat on average, with a relative long latency period (1-4). In contrast to all these strains, the inbred Cop rat is completely resistant to mammary carcinogenesis by a variety of routes of exposure (1, 2, 4, 7, 8). However, this resistance is tissue specific, since Cop rats do develop tumors at other sites following carcinogen exposure (4, 7-9). A number of breeding experiments in which resistant Cop rats were crossed with highly susceptible inbred strains suggest that a single autosomal dominant mcs gene in the mammary parenchyma of the Cop rat confers resistance in the F1 hybrids (1, 2, 10, 11). The putative mcs gene has been found to be functionally inactive in the germ lines of highly susceptible strains (10). In addition, there is evidence that highly susceptible animals carry several independently segregating dominant autosomal susceptibility genes, while strains with intermediate susceptibility carry neither the mcs gene nor susceptibility genes (3, 4, 11). Both the mcs gene and susceptibility genes appear to be active only within the mammary parenchyma (2, 10, 11).

The Ha-ras oncogene, activated by a G to A transition at the second nucleotide of codon 12, is found in a majority of MNU-induced mammary carcinomas (5, 12). Ha-ras activation is probably the initiating event that occurs as a direct result of MNU exposure (13, 14). In a series of experiments designed to investigate the molecular basis of the resistance of the Cop rat to mammary carcinogenesis by MNU, we have demonstrated that the kinetics of formation and repair of the promutagenic DNA lesion O6-methylguanine in DNA isolated from mammary epithelial cells are essentially identical in susceptible Bu/N and resistant Cop rats following a single MNU exposure (15). Furthermore, we have shown that the extent of methylation by MNU of a restriction fragment containing exons 1-4 of the Ha-ras gene in mammary gland DNA and the level of Ha-ras expression in mammary tissue are not different for the two strains (15). These results suggested that the Ha-ras gene in Cop mammary glands is not protected from methylation by MNU and should, therefore, be susceptible to mutational activation. Recently, using a sensitive method involving the polymerase chain reaction, we have indeed demonstrated the presence of activated Ha-ras alleles in the mammary glands of both Bu/N and Cop rats 30-60 days after MNU treatment (16). These results indicate that the resistance of Cop rats to mammary carcinogenesis is not due to a defect in initiation but rather appears to be due to the suppression of the growth of initiated cells. Estrogens are promoters of mammary carcinogenesis (17-21), but they are present at similar levels throughout the estrous cycle in both resistant and susceptible animals (2).

While it appears, therefore, that the uncharacterized mcs gene is responsible for the resistance of the Cop rat, it is unclear how this gene is protected from inactivation by carcinogen exposure, especially since it has been shown that multiple treatments with MNU do not lead to tumors (1). Indeed, our finding that Cop rats are initiated by MNU treatment suggested that there may be treatment regimes that lead to the inactivation of the mcs gene with subsequent tumor growth.

Carcinogenesis studies have shown that the age of rats at the time of carcinogen exposure is critical for mammary tumor induction (18, 22-25). An inverse relationship between the age after puberty and susceptibility has been well documented by several investigators using a number of carcinogens (18, 22, 23). Newborn animals, however, are usually more susceptible than adult animals to tumor induction in several organs by a diverse class of carcinogens (26). Indeed, neonatal rats have been shown to be equally if not more susceptible than pubescent rats to mammary carcinogenesis (19, 21, 24-27). However, attempts to induce mammary tumors in Cop rats have focused on pubescent animals. We decided, therefore, to investigate the effect of MNU on Cop rats that were 2 to 3 days old at the time of treatment. Our results show for the first time that mammary carcinomas can be induced in the resistant animals by this experimental regimen. Here we document the formation and characteristics of these tumors and compare them to those induced in susceptible Bu/N rats by similar treatments.
MAMMARY TUMOR INDUCTION IN COPENHAGEN RATS

MATERIALS AND METHODS

Animals. Inbred Buf/N and Cop rats (males, ~300 g; females, ~200 g) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Animals were housed in a room lighted 12 h/day and maintained at 24 ± 3°C (SD) at 45-55% relative humidity. They were fed rat-mouse chow (6% fat; Teklad Mills) and acidified distilled water ad libitum throughout the experiments.

One week after their arrival, males and females of each strain were mated. The offspring from each strain were randomized into three groups and exposed to the carcinogen as described below. After weaning, males were sacrificed and females were housed three to a cage. They were palpated twice weekly for the detection of mammary tumors.

The time of detection and location were recorded for each tumor that developed in each rat. A complete autopsy was performed on each rat at the time of spontaneous death, when moribund, or at 365 days of age. At the time of autopsy, all grossly detectable tumors were removed and fixed in 10% buffered formalin. Paraffin sections were prepared and stained with hematoxylin and eosin. Some sections were also stained with hematoxylin-phloxin-saffron-alcian green for clarification of tumor type. Each tumor was classified histopathologically according to previously published criteria (28-31).

Carcinogen Exposure. MNU (Sigma Chemical Co., St. Louis, MO) was dissolved in 0.05% acetic acid in normal saline to a concentration of 10 mg/ml and used immediately. Neonatal animals (48 to 72 h old) in group A received i.p. injections of vehicle only, and animals in groups B and C received injections of 30 mg/kg MNU. At 50 days of age, group C received an injection of 30 mg/kg MNU in the tail vein, and groups A and B received a similar injection of vehicle only. As a positive control (group D), female Buf/N rats at 50 days of age were given a single injection of 30 mg/kg MNU into the tail vein.

RESULTS

In an attempt to induce mammary tumors in genetically resistant Cop rats, we have investigated the administration of MNU to neonates and to pubescent animals. Neonatal rats are known to be sensitive specifically to mammary carcinoma induction (18, 22, 23). We chose a dose of 30 mg/kg MNU that is known to produce a high incidence of mammary adenocarcinomas in susceptible rats when administered at 50 days of age (1, 2, 5, 6). Groups of Buf/N and Cop rats were given MNU either only as neonates (group B) or as neonates and then again at 50 days of age (groups C). As a positive control, we treated Buf/N rats with a single dose of MNU at 50 days of age (group D), a regimen known to produce a high incidence of mammary tumors (1, 2, 5).

The mammary tumor incidences and latent periods in the various groups are summarized in Table 1 and Fig. 1. Treatment of Buf/N rats both as neonates and at 50 days (group C) produced tumors in essentially all the animals with a very short latency. One-half of the animals developed tumors within 50 days after the second injection of MNU and almost 100% within 120 days. In the Buf/N rats given a single MNU injection at 50 days of age (group D), the tumor incidence, as expected (1, 2, 5), was also 100%, but the latency period was longer. These animals, however, had more tumors per tumor-bearing rat than the animals that received two MNU treatments. The Buf/N rats in group B that received a single injection of MNU as neonates had only a 20% incidence of mammary tumors (Table 1; Fig. 1).

It is clear from Table 1 and Fig. 1 that Cop rats treated with MNU as neonates and again at 50 days of age (group C) developed a high incidence (71%) of mammary tumors. These tumors generally developed more slowly than in similarly treated Buf/N rats, although the first tumor actually appeared earlier in the Cop rats. In group C of the Cop rats, five animals died at 130-230 days of age, apparently due to paralysis of the hind quarters (one of these animals had a mammary tumor). In contrast, none of the Buf/N animals in any of the groups showed this effect. A single administration of MNU to neonatal Cop rats (group B) also induced about a 30% incidence of mammary tumors, although these tumors developed more slowly than in the two-dose regimen.

We made the qualitative observation that the growth rates of mammary tumors in the two strains were different. Generally the tumors in the Buf/N rats grew faster than in the Cop rats. One or two weeks after detection by palpation, the Buf/N tumors had grown to a size that caused the death of the animals.

Table 1 Induction of mammary tumors by MNU in Buf/N and Cop rats

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Treatment</th>
<th>No. of treated rats</th>
<th>Incidence (%)</th>
<th>D1 (days)</th>
<th>D50 (days)</th>
<th>Average (Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buf/N</td>
<td>A</td>
<td>Veh</td>
<td>12</td>
<td>0</td>
<td>NA  &lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>MNU</td>
<td>15</td>
<td>20</td>
<td>130</td>
<td>NA</td>
<td>1.3 (1-2)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>MNU</td>
<td>12</td>
<td>92</td>
<td>90</td>
<td>100</td>
<td>2.4 (1-6)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>MNU</td>
<td>12</td>
<td>100</td>
<td>170</td>
<td>230</td>
<td>3.7 (3-6)</td>
</tr>
<tr>
<td>Cop</td>
<td>A</td>
<td>Veh</td>
<td>14</td>
<td>0</td>
<td>NA  &lt;sup&gt;c&lt;/sup&gt;</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>MNU</td>
<td>18</td>
<td>28</td>
<td>115</td>
<td>NA</td>
<td>1.8 (1-4)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>MNU</td>
<td>17</td>
<td>71</td>
<td>80</td>
<td>180</td>
<td>2.1 (1-4)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Treated with 30 mg/kg MNU or vehicle (veh) i.p. as neonates.
<sup>b</sup> Treated with 30 mg/kg MNU or vehicle via the tail vein at pubescence (50 days of age).
<sup>c</sup> Age at which first mammary tumor appeared.
<sup>d</sup> Age at which 50% of rats had mammary tumors.
<sup>e</sup> NA, not applicable.
In the Cop rats, most tumors grew slowly over a period of a month without causing death. The distribution of mammary tumors among the mammary glands is shown in Table 2. The glands in the right and left sides were equally susceptible in both Buf/N and Cop rats. Buf/N rats in group D developed more tumors in the thoracic glands (pairs 1–3) than the inguinal glands (pairs 4–6). When Buf/N rats were treated both as neonates and at 50 days of age (group C), however, the tumors were equally distributed in the thoracic and inguinal glands. In contrast, Cop rats treated by either regimen developed somewhat more tumors in the inguinal glands.

Histopathology. Mammary tumors produced in the Buf/N rats by all of the treatments (group B, C, and D) were almost uniformly cribriform adenocarcinomas (Table 3), as observed in previous studies with this and other susceptible strains (20, 30). The tumors consisted of coalescing nests of malignant cells with numerous cribriform spaces. Each nest of cells contained multiple round or irregularly shaped glandular spaces filled with acidic mucopolysaccharides as demonstrated by a positive Alcian green stain.

In Cop rats, about one-half of the tumors in both groups B and C were cribriform adenocarcinomas (Table 3). Most of the rest were coalescing solid sheets of tumor tissue that resembled baseloid squamous carcinomas (Fig. 2). At the transition region between these tumors and the surrounding stroma, the epithelium often displayed a palisade pattern (Fig. 2a), although scant, often minute glandular spaces were present (Fig. 2b) that could be more readily visualized using alcin green stain. Furthermore, these tumors displayed scant, focal, squamous differentiation, characterized by the formation of malpighian cells, maturing into keratin pearls (Fig. 2a). On the basis of these findings, we have classified the tumors as adenosquamous carcinomas. In addition to the adenocarcinomas and adenosquamous carcinomas that were similar in groups B and C, there were two carcinosarcomas and one fibrosarcoma in the Cop rats of group C that were not found in group B (Table 3).

Only a small percentage of the mammary carcinomas in either the Buf/N or Cop rats were invasive, and no tumors were metastatic at the time they were harvested. Tumors induced in tissues other than the mammary gland are summarized in Table 4. It is clear that the animals treated with MNU as neonates developed tumors at a number of sites in addition to the mammary gland.

DISCUSSION

In these experiments we have demonstrated for the first time that mammary carcinomas can be induced in genetically resistant Cop rats by administration of MNU. A single administration of MNU to neonates or administration to neonates and again at 50 days of age both gave mammary tumors, although the latter produced a higher yield with a shorter latency. Compared to the susceptible Buf/N rats in which mammary tumors are uniformly adenocarcinomas, the tumors in the Cop rats are heterogeneous, consisting of about 50% adenocarcinomas and 50% adenosquamous carcinomas. The latter tumor type has not previously been observed in rats that are susceptible to MNU-induced mammary carcinogenesis, including the Buf/N rats used in this experiment (20, 28, 31). Adenosquamous carcinomas, however, have been shown to be one of the most frequent tumors induced in mouse mammary glands by chemical agents (32–34). A difference in tumor histology analogous to the difference we observed in mammary tumors of Cop and Buf/N rats has been observed in bladder tumors induced in Cop and Fischer 344 rats by intravesical administration of MNU (9). All the Fischer 344 rats developed transitional cell carcinomas. Only 38% of the Cop rats developed similar tumors, but 50% of the Cop bladders developed either squamous cell carcinomas alone or in combination with transitional cell carcinomas.

There are at least two possible explanations for the lower tumor incidence and somewhat longer latency period in Cop compared to Buf/N rats for the same carcinogen treatment. First, since the putative mes gene is inactive in Buf/N rats, one less genetic event may be necessary for tumor production in this strain. Second, Gould (3) has postulated the existence of tumor susceptibility genes that may accelerate the development of mammary tumors in highly susceptible strains.

Our results demonstrate that the distribution of mammary tumors among individual mammary glands depends on both strain and treatment regime. Buf/N rats treated during puberty are known to produce an excess of tumors in the thoracic compared to the inguinal glands (20), and our results confirm this distribution (Table 2). When the Buf/N rats were treated both as neonates and at 50 days of age, however, there was almost an equal distribution of tumors in thoracic and inguinal glands. In Cop rats, both treatment regimes (B and C) gave more inguinal than thoracic gland tumors. The reasons for these differences are unclear.

Table 2 Distribution of mammary tumors among individual mammary glands

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Total tumors</th>
<th>Left glands</th>
<th>Right glands</th>
<th>Thoracic glands</th>
<th>Inguinal glands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buf/N</td>
<td>B</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>26</td>
<td>12</td>
<td>14</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>44</td>
<td>20</td>
<td>24</td>
<td>32</td>
<td>12</td>
</tr>
<tr>
<td>Cop</td>
<td>B</td>
<td>9</td>
<td>6</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>25</td>
<td>12</td>
<td>13</td>
<td>9</td>
<td>16</td>
</tr>
</tbody>
</table>

* Gland pairs 1–3.

Table 3 Histopathological classification of mammary tumors induced by MNU in Buf/N and Cop rats

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Total tumors</th>
<th>Adenoca*</th>
<th>Adenosquaca*</th>
<th>Adenoca in situ</th>
<th>Carcasa</th>
<th>Fibroadb</th>
<th>Fibrosarc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buf/N</td>
<td>B</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>26</td>
<td>26</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>44</td>
<td>38</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cop</td>
<td>B</td>
<td>9</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>25</td>
<td>9</td>
<td>11</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

* Adenocarcinomas.

b Adenosquamous carcinomas.

c Carcinosarcomas.

d Fibroadenomas.

e Fibrosarcomas.
MAMMARY TUMOR INDUCTION IN COPENHAGEN RATS

It is unclear at this time how this gene is protected from inactivation in pubescent rats, although this may be related to mammary gland growth and development. The growth of rat mammary glands can be divided into at least two phases (18, 23). The first phase occurs from birth to 5 weeks of age, during which time growth is rapid, ductal in type, and dependent of ovarian hormones. Mammary epithelial cells are immature and undifferentiated during this phase. When the rats reach 35 days of age, the second phase starts, during which the estrous cycle begins and the final phase of mammary gland development occurs. This phase is strongly hormone dependent. During these phases, different patterns of gene expression could clearly influence carcinogenesis. Indeed, it has already been shown that when neonatal Sprague-Dawley rats are treated with MNU, mammary tumors may contain either Ha-ras or Ki-ras oncogenes (19, 21), whereas only Ha-ras activation is found in tumors produced in the same animals by treatment at 50 days of age (13). It is possible that the mcs gene in the Cop rat is a negative regulator of mammary epithelial cell division that is not expressed during hormone-dependent gland development. We have recently shown that quiescent genes may be less susceptible to damage by chemical carcinogens, including MNU, than genes that are actively transcribed (35, 36).

Very recently, Wang et al. (37) have demonstrated induction of mammary carcinomas in 50–60-day-old Cop rats by direct introduction of the v-Ha-ras oncogene into mammary epithelial cells in situ, using a replication-defective retroviral vector. It is unclear in this model, however, how the v-Ha-ras gene was able to overcome the mcs gene activity. Neither the double mutation in the v-Ha-ras gene nor its overexpression provides an adequate explanation (37). It will be interesting to determine the pattern of oncogene activation in the Cop rat tumors and whether different genes are activated in the tumors of different histological types.

In summary, we have shown that mammary carcinomas can be induced in genetically resistant Cop rats when newborns are treated with MNU. These results suggest that the putative mcs gene is inactive in neonatal animals or is inactivated when these animals are treated with MNU.

**ACKNOWLEDGMENTS**

The authors thank Jayne E. Chaulk and Valentia Lee for excellent technical assistance.

**REFERENCES**


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Table 4 Tumors at sites other than the mammary gland induced in Buf/N and Cop rats by MNU

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buf/N</td>
<td>A</td>
<td>No tumors</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1 hematoma, 1 neurofibroma, 3 abdominal carcinomas, 2 abdominal sarcomas, 1 hemangioma, 1 colon fibrosarcoma</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1 hepatocarcinoma, 2 abdominal carcinomas, 2 abdominal sarcomas</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>No other tumors</td>
</tr>
<tr>
<td>Cop</td>
<td>A</td>
<td>No tumors</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>1 small cell eye tumor, 1 heart sarcoma, 6 abdominal sarcomas</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>1 lymphoma, 3 leiomyosarcomas, 2 colon adenomas, 2 colon adenocarcinomas, 1 kidney adenocarcinoma</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>1 spindle-shaped eye tumor, 1 lung neurofibrosarcoma, 2 abdominal sarcomas, 1 skin (nose) squamous carcinoma, 2 skin trichoepitheliomas, 2 colon leiomyosarcomas, 1 colon adenocarcinoma</td>
</tr>
</tbody>
</table>

* Abdominal mass of undetermined origin.

In our previous studies we have shown that MNU induces activating Ha-ras mutations in the mammary glands of pubescent Cop rats (16). The ability to induce mammary tumors in the Cop rat by our new treatment regimes, therefore, suggests that the putative mcs gene is functionally inactive in neonatal animals or is inactivated when these animals are treated with MNU.

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**Fig. 2.** Histology of MNU-induced mammary adenosquamous carcinomas in the Cop rat. a, squamous malpighian cells (s), keratin pearls (k), organization of epithelial cells at the transition between connective tissue stroma in a pattern of palisades (p), and attempt at small gland formation (g). In b, gland formation (g) is more prominent, and squamous differentiation (t), keratin pearl formation (k), and palisades (p) are again in evidence. Hematoxylin-phloxin-saffran stain, a, × 2500; b, × 1000.
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