Morphogenesis of Neoplasms Induced in the Hamster Trachea with N-Methyl-N-nitrosourea

Sherman F. Stinson and Juliet C. Lilga

INTRODUCTION

Development of a defined reproducible system with a predictable dose response is a prerequisite for carcinogenesis studies. Induction of tracheal and lung tumors with benzo(a)pyrene-ferric oxide in the Syrian hamster (11) has been the most widely used experimental model for lung cancer. Although the induced neoplasms are very similar morphologically to those observed in the human lung (2), differences in carcinogen distribution and activity due to varying of the physical properties of the carrier particle (4, 11) make the effective dose as well as the localization and incidences of tumors difficult to predict and control.

To overcome these and other problems, Schreiber et al. (11, 13) developed a cannula system for the instillation of water-soluble carcinogens in a localized region of the hamster trachea. Further studies have shown that, with MNU, the system is very reproducible with a clearly defined dose response (15, 16).

During preliminary experiments designed to investigate the potential usefulness of this technique for testing suspect respiratory carcinogens and for studies on the modulation of carcinogenesis, we observed some interesting lesions which had not been described in the literature. Inasmuch as these lesions appeared to be associated with the early stages of carcinogenesis in the trachea, the present study was initiated to more clearly define the morphogenesis of the neoplasms induced by using the described system.

MATERIALS AND METHODS

Ninety-five male 15-week-old (110- to 125-g) Syrian golden hamsters (ARS/Sprague-Dawley Division, The Mogul Corp., Madison, Wis.) were used as vehicle controls. Hamsters were housed 3/cage in polycarbonate cages with metal tops. Cages were placed on racks in a room with a 12-hr light-dark cycle. The temperature was maintained at 21 ± 2°C, and the humidity was 50 ± 5%. The animals were given Wayne laboratory meal (Allied Mills, Chicago, Ill.) and fresh water ad libitum.

MNU (Ash Stephens, Inc., Detroit, Mich.) was dissolved in 0.01 M acetate buffer, pH 5.0, at room temperature, and the concentration was adjusted to 1.0% as determined from the molar extinction coefficient at 234 nm. (The molar extinction coefficient measured at 234 nm in our laboratory was 6040.) The MNU concentration was measured before and after each series of exposures to assure accurate dosing. Under these conditions, the MNU was stable for at least 8 hr. Carcinogen solutions were used for a maximum of 3 hr.

Hamsters were anesthetized with a 3 to 5% mixture of halothane (Abbott Laboratories, Inc., Chicago, Ill.) in air, within an anesthesia apparatus in which the atmosphere was continuously recirculated through soda lime to remove carbon dioxide. When deep narcosis was achieved (6 to 10 min), the animals were removed and fixed to a slanted board, and the carcinogen or vehicle solution was instilled intratracheally with a specially prepared cannula (fabricated by I.I.T. Research Institute, Chicago, Ill., after the design of Schreiber et al.) (12, 13). The cannula was designed to expose a 7-mm section of the tracheal mucosa, approximately 10 mm distal to the vocal cords, to a flowing stream of the carcinogen or vehicle solution for a total exposure time of 5 sec and to effectively aspirate the fluid. Preliminary tests with dye solutions indicated that no fluid penetrated distal to the bifurcation. The hamsters were exposed once each week for a maximum of 15 weeks.

Twenty hamsters were killed 1 week after the 8th carcinogen treatment, and 2 and 12 weeks after the 15th carcinogen treatment. Five to 7 vehicle controls were also killed at each of these times. The larynx, trachea, and bifurcation were removed in toto and fixed in Zenker's acetic acid formalin. Each trachea was cut transversely into 6 pieces which were embedded in glycol methacrylate. Cross-sections (1.5 μm thick) were taken at 3 levels, 100 to 150 μm apart, from each of the pieces. Slides were stained with hematoxylin and eosin.

Received July 10, 1979; accepted November 27, 1979.

1 To whom requests for reprints should be addressed.
2 The abbreviation used is: MNU, N-methyl-N-nitrosourea.

MARCH 1980

609
Hamsters that became moribund or died following the 15-week treatment period were necropsied, and the tissues were processed as described above. Three vehicle control and 11 dosed hamsters that died during the treatment period were not used for histopathological evaluation.

RESULTS

All proliferative lesions were localized in the central one-third of the trachea, the site of the carcinogen application. Some epithelial flattening, however, was observed proximal and distal to this zone. No alterations attributable to carcinogen administration were found in the peripheral lung.

The spectrum of epithelial changes that were observed in this study are summarized in Table 1. Epithelial flattening was a consistent finding in exposed animals at all sacrifice times. The entire epithelium of each tracheal ring in the exposed regions was affected from 9 to 18 weeks. A progressively smaller proportion was involved as the experiment proceeded, until by 27 weeks from 25 to 50% of the ring was flattened. Cells making up the epithelium ranged from low cuboidal to flattened; their nuclear axes were rotated so that they were aligned parallel rather than perpendicular to the wall, and cilia were absent. Focal areas of degeneration and necrosis with cells containing intracytoplasmic vacuolization and pyknotic nuclei were common. For the most part, the epithelium was regular and well differentiated, but in animals killed from 9 to 5 cell layers could be distinguished. In these areas, the cells lacked orientation, and cells and nuclei showed a relative variation in size and shape (Fig. 1). Cells in the lower layers were polygonal, whereas those in the surface layers tended to be flattened and elongated. Occasionally, some of the cells contained large, pleomorphic, hyperchromatic nuclei. These atypical areas were found in 10% of the animals at 9 weeks and in 50% at 16 weeks, but at later times they were observed only in association with neoplasms.

Following exposure, focal hyperplastic lesions were also a very common finding. These foci consisted of nonciliated cuboidal to columnar cells 4 to 10 layers thick or of metaplastic nonkeratinizing stratified squamous cells. Usually, the areas were regular and well differentiated, but in animals killed from 16 through 21 weeks varying degrees of dysplasia were frequently observed (Fig. 2). Severely dysplastic foci showed a lack of cellular orientation and nuclear and cellular pleomorphism. Intraepithelial cysts and cellular vacuolization were also present in these foci.

Beginning at 16 weeks, invaginations of the epithelium into the submucosa were seen (Fig. 3). The invaginations varied in size and appearance. Some appeared almost completely enclosed and cyst-like; their lumina contained lymphocytes and neutrophils as well as sloughed cellular debris. Others were open to the tracheal length. The size ranged from a few cells in diameter to over one-half of the circumference of the tracheal cross-section. The inner surface of all of the invaginations, as well as the leading portions of the overlying tissue projections, were lined by a single to double layer of flattened epithelial cells. The epithelial lining of the lower part of the invaginations were invariably in close proximity to the tracheal rings. Focal proliferative lesions were very common in regions of the epithelium that were adjacent to, as well as within, the invaginations (Fig. 4). Small focal carcinomas were frequently found in the portion of the epithelial lining of the invaginations that was closest to the tracheal rings; at 27 weeks, 5 of the 7 invaginations contained poorly differentiated carcinomas of relatively small size (less than 50 cells/cross-section) that had invaded into the tracheal cartilage (Figs. 4 and 5). These small carcinomas will be described in more detail later.

The first neoplasms were found 16 weeks after the initiation of treatment, but high incidences were not observed until after 22 weeks. Neoplasms were usually solitary. Only 1 hamster was found with multiple tracheal neoplasms, a papilloma, and a carcinoma at 27 weeks. Papillomas were usually sessile, were composed of well-differentiated nonkeratinized squamous cells, and were vascular (Fig. 6). Their size was variable, but most ranged from one-quarter to one-half of the cross-sectional area of the tracheal lumen.

Carcinomas exhibited predominantly intramural growth, although some intraluminal proliferation was also usually present. The smallest cancers consisted of minute foci of poorly differentiated polygonal to elongated angular cells, interpreted as squamous cells (Fig. 5). The foci were frequently closely associated with the tracheal rings, and invasion into the cartilage was evident. Larger cancers involved from one-quarter to the entire circumference of the trachea (Fig. 7). Tracheal rings were distorted and displaced by the disorganized masses of cells. Invasion of the wall was extensive, and cells commonly penetrated through the muscle layers. Invasion into the outer layers of the esophagus was seen in 2 cases, but no evidence of metastases was found. The cancers were composed of squamous cells with varying degrees of differentiation, but little keratinization was observed. Some of the carcinomas showed areas with a glandular pattern containing epithelial as well as intraepithelial cysts (Fig. 8). As the major cell type in these

<table>
<thead>
<tr>
<th>Schedule of sacrifice (wk)</th>
<th>No. of tracheas</th>
<th>Diffuse epithelial flattening</th>
<th>Atypical area</th>
<th>Focal mucosal hyperplasia</th>
<th>Focal squamous metaplasia</th>
<th>Dysplastic focal hyperplasia</th>
<th>Epithelial invagination</th>
<th>Papilloma</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>20</td>
<td>20 (100)a</td>
<td>2 (10)</td>
<td>5 (25)</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>1 (5)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>20</td>
<td>20 (100)</td>
<td>10 (50)</td>
<td>12 (60)</td>
<td>18 (80)</td>
<td>16 (80)</td>
<td>18 (80)</td>
<td>1 (5)</td>
<td>1 (5)</td>
</tr>
<tr>
<td>18-26b</td>
<td>24</td>
<td>23 (96)</td>
<td>0</td>
<td>12 (50)</td>
<td>20 (83)</td>
<td>4 (17)</td>
<td>9 (39)</td>
<td>3 (13)</td>
<td>10 (42)</td>
</tr>
<tr>
<td>27</td>
<td>20</td>
<td>18 (90)</td>
<td>0</td>
<td>8 (40)</td>
<td>16 (80)</td>
<td>1 (5)</td>
<td>7 (35)</td>
<td>6 (30)</td>
<td>15 (75)</td>
</tr>
</tbody>
</table>

Numbers in parentheses, percentage of tracheal ring.

* Hamsters found dead or killed when moribund.
areas appeared to be squamous, the cancers were classified as squamous cell carcinomas.

**DISCUSSION**

The decrease in the incidence of dysplastic focal hyperplasias from 16 through 27 weeks associated with an increase in the incidence of carcinomas over the same period is consistent with the conclusion that the carcinomas developed from the dysplastic foci. Further support for this hypothesis is provided by the observation that the earliest carcinomas were cytologically identical and of size similar to those of the foci. These small cancers are very interesting in that distinct invasion into the cartilage rings was demonstrated by minute foci of only a few cells. To our knowledge, these are the earliest respiratory cancers that have been reported in experimental animals or humans.

Another previously unreported finding is the presence of epithelial invaginations into the submucosa. The observation that a high percentage of these were associated with small carcinomas and dysplastic proliferative foci makes an understanding of their genesis particularly important. One possibility is that the invaginations may represent dilated hypertrophied submucosal glands. This carries interesting implications as to the origin of the tracheal neoplasms. Another possibility is that they may be sites of prior mechanical and chemical trauma which the epithelium has since covered. Alternatively, they may be caused by hyperplasia in adjacent areas forcing epithelial projections over the existing lining. The fact that the invaginations lie close to the cartilage rings makes the first 2 possibilities most likely.

Epithelial flattening was followed by development of areas of metaplastic stratified squamous epithelium that has been reported by other investigators using this system (15, 16). Similar morphological findings have also been found in hamsters given intratracheal instillations of benzo(a)pyrene (1) and in rat tracheal grafts exposed to benzo(a)pyrene (9). This reaction is apparently an early and intermediate regenerative response of the squamous tracheal epithelium to injury and can be induced by simple mechanical damage (7). Production of high incidences of neoplasms in a controlled, reproducible region of the respiratory tract has been a persistent problem in respiratory carcinogenesis studies. The traditional inhalation and intratracheal injection routes (5, 10, 14) for carcinogen administration do not meet these criteria, as they produce low or unpredictable yields of tumors, the localization of which is uncertain. Implantation of carcinogen pellets in bronchi (3) or in tracheal grafts (6, 8, 9) are more easily controlled but have the disadvantage of blocking the airways, which may interfere with further experimental procedures. The localization of proliferative lesions in the median portion of the trachea, in the present study, attests to the usefulness of this technique for producing neoplasms in a reproducible, well-circumscribed region of the respiratory tract. Also, the tumor incidence is high, with a short induction time.

**ACKNOWLEDGMENTS**

The authors gratefully acknowledge the assistance of Dr. Michael B. Sporn for coordinating much of the study, Dr. Robert A. Squire for aid in interpreting some of the lesions, Joseph M. Smith for expert technical support, and Joan O'Brien and Ann Johnson for typing the manuscript.

**REFERENCES**

Morphogenesis of Neoplasms Induced in the Hamster Trachea with $N$-Methyl-$N$-nitrosourea

Sherman F. Stinson and Juliet C. Lilga