A Method of Producing Carcinoma in Upper Aerodigestive Tree and Esophagus of the Syrian Golden Hamster Using Wounding and Instillation of \(N\)-Methylnitrosourea

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Abstract

Details of a method for producing carcinoma of the aerodigestive tree of the Syrian golden hamster and the use of this model to evaluate putative agents for chemoprevention of these carcinomas are described. The method produces a majority of squamous carcinomas of the trachea and glottis that follow squamous metaplasia of respiratory epithelium. In addition, seen are adenocarcinomas arising in glands of the respiratory tree. Squamous carcinomas of the digestive epithelium arise in primary squamous epithelium. These tumors of digestive epithelium have a growth pattern that differs from that of the respiratory epithelium in that they grow and invade without filling the epithelial layer with tumor cells. (Cancer Epidemiol Biomarkers Prev 2007;16(8):1644–50)

Introduction

Carcinoma of the larynx (9,510), pharynx (8,950), and esophagus (7,250) affected \(\sim 25,700\) patients in the United States in 2005 (1). Cancers with an epicenter within the esophagus (7,250) affected a majority of squamous carcinomas of the trachea and glottis that follow squamous metaplasia of respiratory epithelium. In addition, seen are adenocarcinomas arising in glands of the respiratory tree. Squamous carcinomas of the digestive epithelium arise in primary squamous epithelium. These tumors of digestive epithelium have a growth pattern that differs from that of the respiratory epithelium in that they grow and invade without filling the epithelial layer with tumor cells.

New models of prevention and treatment paradigms need to be introduced and developed to improve the outcome of cancer of the upper respiratory tree as well as cancer of the esophagus. To date, there are no standard chemoprevention strategies directed exclusively at upper respiratory or esophageal carcinoma. In this article, we extend the development of a hamster model for tracheal and laryngeal cancer (6) to include esophageal cancer.

Schriber et al. (7) initially described a method using a circumscibed wounding and carcinogen application in hamster trachea as a way to study the effects of topicaly applied anticarcinogenic agents or co-carcinogens. Moon et al. (8) briefly reviewed several methods of carcinogenesis in the hamster respiratory tree in 1992. They concluded that the likelihood of reproducing a model of respiratory cancer in different laboratories was remote. Moon et al. also used a local application of \(N\)-methylnitrosourea (MNU) to the hamster trachea using a specially designed catheter. This produced up to a 68% incidence of cancer in a 6-month period with low toxicity. The results of this model of carcinogenesis were difficult to reproduce in our laboratory; however, we developed a method that reliably caused carcinomas of the trachea, larynx, pharynx, and esophagus. In addition to the instillation of MNU, we added a wounding procedure. Wounding has long been thought to be a part of carcinogenesis (9) and has proved carcinogenic in mouse skin (9). In addition to the upper airway, our method also produced carcinoma of the esophagus as well as squamous carcinoma of the forestomach of the hamster. The pattern of increased cancers of the upper aerodigestive tree and esophagus produced in the hamster resembles that seen in human smokers and suggests that the model may serve as a system for testing chemopreventive or chemotherapeutic agents for tumors of these areas.

In the following sections, we describe methods of treatment and carcinogen application that reproducibly produce carcinomas of the trachea, larynx, pharynx, and esophagus. In addition, we indicate methods of tissue harvest, fixation, sectioning, scoring, and evaluation of sectioned material that are suitable for use in studies of chemoprevention.

Briefly, the model uses wounding the hamster trachea twice under visual control and three tracheal instillations of a solution of MNU separated by an interval of 2 weeks. Further instillation of MNU occurred at weekly intervals for up to six total administrations of MNU.

Materials and Methods

Animals. Male Syrian golden hamsters obtained from Charles River Laboratories (Kingston, NY) were used. They were shipped at 7 weeks of age and held for 2 weeks before initiation of experiments.

Procedure of Wounding and Carcinogen Administration. The standard hamster protocol requires two wounding procedures and five or six administrations of MNU.
Hamsters are anesthetized with 1% Brevital sodium by i.p. injection. The dose is based on animal weights (1.0 mL/140 g). Anesthetized hamsters are placed on a board slanted at an angle under a dissecting scope. Animals are held in place by a copper wire attached to the board from which the hamsters are suspended by their upper teeth. This causes the lower jaw to fall open naturally.

The wounding procedure is done with a curved curette (at 135 degrees) inserted into the trachea of the anesthetized hamster. The curette is pulled out gently while making a mild abrasion on the interior ventral side of the trachea. The following day, the “wounded” hamsters are given MNU. This is done with the same board and dissecting scope. The MNU procedure is similar to wounding, except a curved gavage needle is used to administer 0.05-mL MNU to the wounded area of the trachea.

The hamsters are given a week to recuperate and then the wounding and MNU procedures are repeated, only this time 48 h apart. The hamsters are again given a week to recuperate. A third MNU administration was given a week later. Then, MNU was given weekly until the experimentally required number of MNU doses has been given. Hamsters are anesthetized for each wounding and MNU administration. All procedures are done in a fume hood.

**Hamster Tissue Processing.** Hamster lung, trachea tissue, and esophagus are removed en bloc and fixed in zinc formalin. The formula for this fixative is ZnSO4 7H2O (67.84 g), formalin solution (37% formaldehyde; 360 mL), and H2O (3,240 mL). The solution is not buffered or pH controlled. After the trachea is dissected from the lungs, it is decalcified in a solution consisting of 10% formic acid and 10% formalin for 18 h to soften the cartilaginous tissue for cutting on the microtome.

Dehydration is done using a botanical technique that does not shrink or harden the tissue (10). Specimens are washed in distilled water to remove decalcifying solution, and then dehydrated using graded increasing concentrations of t-butyl alcohol. We added a final three anhydrous solutions consisting of 90% t-butyl alcohol and 10% tetrahydrofuran to the above dehydration technique. After dehydration, the trachea is cut into ten to twelve 3- to 4-mm tubes as seen in Fig. 1. The tissue tubes are embedded in Paraplast X-tra (melting point, 51°C). They are set upright on the base mold embedding dish in sequential order to allow cross sections to be cut. Each tube is embedded from cephalad to caudad with the exception of the first section containing the epiglottis/hypopharynx. Due to its shape, this part is embedded the reverse of all other segments to allow the flattest side to be placed downward.

**Histologic sections are cut at 6 μm. Three consecutive sections are mounted on a single slide, one of which will be used in the scoring process described later. The next 27 sections are discarded, and then three more are cut and mounted. This process is repeated until a total of 10 slides have been completed. Slides are stained with H&E and coverslipped.**

**Tumor Data Processing and Scoring.** Ten microscopic slides processed as above are scanned on an Epson 2450 photo scanner at 1,200 dpi and enlarged so that one section

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**Table 1. Divisions of the upper airway**

<table>
<thead>
<tr>
<th>Name</th>
<th>Location description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supraglottis</td>
<td>From top of epiglottis to base of epiglottic cartilage</td>
</tr>
<tr>
<td>Glottis</td>
<td>From base of epiglottis to cricoid cartilage</td>
</tr>
<tr>
<td>Subglottis</td>
<td>Start of the cricoid cartilage</td>
</tr>
<tr>
<td>Trachea</td>
<td>Below cricoid cartilage to carina</td>
</tr>
</tbody>
</table>

**Table 2. Tumor classification of upper airway (glottis through trachea)**

<table>
<thead>
<tr>
<th>Squamous and notation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Lower third of epithelium</td>
</tr>
<tr>
<td>D1</td>
<td>Middle third of epithelium</td>
</tr>
<tr>
<td>D2</td>
<td>Upper third of epithelium</td>
</tr>
<tr>
<td>D3</td>
<td>In situ</td>
</tr>
<tr>
<td>C0</td>
<td>Minimally invasive</td>
</tr>
<tr>
<td>C1</td>
<td>Involves submucosa</td>
</tr>
<tr>
<td>C2</td>
<td>Involves muscle or cartilage</td>
</tr>
</tbody>
</table>

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**Figure 1.** Demonstration of block technique and resulting microscopic slides. The fixed trachea is seen with lines indicating plane of gross section, resulting here in 12 tissue tubules to be embedded in a single block. Seen beneath is a single section of the block as it may appear on a resulting H&E microscopic slide.

**Figure 2.** Scan of microscopic slide with locations and diagnoses of lesions. Scan of one microscopic slide cut from a single block from a hamster. Pieces are numbered according to Fig. 7 (see Fig. 8 for explanation of T pieces). Lines to the lesions indicate the diagnosis and location of each lesion as classified in Tables 1 to 3. Ten such slides are produced and scored for each animal, representing 10 levels 160 μm apart. Results are recorded in a summary sheet as pictured in Fig. 8.
nearly fills one half of an 8.5 × 11 in. sheet (see Fig. 2). The anatomic divisions used in the scoring method are determined as summarized in Table 1. Parts of the “pieces” in a section are labeled with diagnoses from dysplasia or precarcinoma to invasive carcinoma based on the criteria in Table 2 for the upper airway and Table 3 for the esophagus. An example of each diagnosis may be seen in Figs. 3 to 6.

Scans of control hamster sections have been reproduced and the boundaries of pharynx, supraglottis, glottis, subglottis, and trachea determined to use as a numbered “master piece list” (Fig. 7). By comparing experimental sections with this master list, we can determine the three-dimensional geographic location of each tumor from section to section and animal to animal. Using the master piece list and the scored blow-ups of the scanned slides, the location of each lesion (piece number, dorsal, ventral, right, left) and its diagnosis are entered into a database or spreadsheet such as Microsoft Excel (Fig. 8).

Whereas the nature of “field carcinogenesis” precludes exact multiplicity counts, interruption of tumors/dysplasia by a lesion-free zone does allow an estimate of multiplicity.

Results

Results for each animal are recorded in three categories: absence of cancer, presence of in situ cancer, and presence of infiltrating cancer. The percentage of animals in each category is compared with carcinogen-treated controls to determine the effect of preventive agents. An example of this method has been reported in brief in (6). Each group contains up to 33 animals. Control group includes absolute controls (carcinogen only) and solvent controls (carcinogen plus exposure to aerosol solvent control) when aerosol delivery is used. Animals that die before the planned sacrifice date are included as a separate category.

Of animals that survive to scheduled sacrifice, the method produces an average of 88.2% with cancer in absolute controls and solvent controls for experiments lasting ~30 weeks after the last dose of MNU. As seen in Table 4, results of three protocols lasting an average of 30.5 weeks produced 85.7% to 89.5% of animals with cancer in the respiratory tree in controls. In 20 weeks post-carcinogen, 42% to 57% of the animals are cancer-free (6). In controls, the protocols above produced an average of 78.2% of animals with cancer of the esophagus (Table 4).

We have observed that dysplasia squamous lesions that arise in metaplasia of respiratory epithelium in the glottis and below show a marked difference from the precancerous lesions seen in the primary squamous epithelium of the supraglottis, pharynx, and esophagus. These differences are

Table 3. Classification of supraglottis/esophageal tumors

<table>
<thead>
<tr>
<th>Classification and notation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precarcinoma 1</td>
<td>Two or more shallow undulations into submucosa</td>
</tr>
<tr>
<td>Precarcinoma 2</td>
<td>Several deeper undulations into submucosa*</td>
</tr>
<tr>
<td>Carcinoma</td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>Minimal invasion†</td>
</tr>
<tr>
<td>C2</td>
<td>Invasion into submucosa</td>
</tr>
<tr>
<td>C3</td>
<td>Involves muscle or cartilage</td>
</tr>
</tbody>
</table>

*The depth of invasion in the submucosa of the esophagus is less than the thickness of overlying surface mucosa.
† The depth of invasion in the submucosa of the esophagus is deeper than the thickness of overlying surface mucosa.

Figure 3. Samples of tumor classification for tumors of glottis and below. Samples of lesions classified using the criteria of Table 2. Magnifications are as listed. A. Dysplasia 1 (×25). B. Dysplasia 2 (×25). C. Dysplasia 2 and dysplasia 3 (×25). D. Carcinoma in situ (×50).

Figure 4. Samples of tumor classification for tumors of glottis and below. Samples of lesions classified using the criteria of Table 2. Magnification, ×25. A. Carcinoma 1. B. Carcinoma 2. C. Carcinoma 2. D. Carcinoma 3.

Figure 5. Samples of tumor classification for tumors of epiglottis, pharynx, and esophagus. Samples of lesions classified using the criteria of Table 3. Magnifications are as listed. A. Epiglottis, carcinoma 3 (×10). B. Pharynx, carcinoma 1 and carcinoma 2 (×10). C. Esophagus, precarcinoma 1 (×25). D. Esophagus. Between arrows, precarcinoma 2 (×25).
reflected in the classifications seen in Tables 1 and 2. Lesions in the supraglottis, pharynx, and esophagus arising in this primary squamous epithelium, almost without exception, show invasion of the submucosa before the overlying epithelium is filled with dysplastic cells. In fact, the epithelium rarely shows dysplastic cells above the lower one third of this layer. Thus, dysplasia 3 and carcinoma in situ were not seen in lesions in the hamster arising in primary squamous epithelium, and the definition of dysplasia for this epithelium is different than that for respiratory epithelium. We recognize these differences by designating these early lesions in primary squamous epithelium as precarcinoma rather than dysplasia. We have separated precarcinoma into two stages, precarcinoma 1 and precarcinoma 2, based on the depth of invasion compared with the thickness of the overlying epithelium (see Table 3 and Figs. 5 and 6).

**Discussion**

The smoking of tobacco produces carcinomas of the aerodigestive tree, the lungs, and other organs. Multiple tobacco-associated carcinogens are involved and perhaps by many routes of exposure (i.e., carcinogens in the airstream, mucociliary blanket, digestive tract, and blood stream). To our surprise, exposure of the trachea to MNU by gavage produced not only local lesions (both adenocarcinomas and squamous cell carcinomas) in the trachea but also lesions of the glottis, pharynx, epiglottis, esophagus, and forestomach. Tatsumura et al. (11) have shown that 5-fluorouracil instilled in the trachea is carried to the glottis and beyond, most likely via the mucociliary blanket. The appearance of cancers in the glottis, epiglottis, pharynx, esophagus, and forestomach of the hamster treated with MNU is likely to be the result of transport of and exposure to MNU by the same mechanism. We do not claim that these MNU-induced lesions have a similar molecular etiology to those produced by smoking tobacco. However, we are presently investigating dysplasias, precancers, and carcinomas of the aerodigestive tree in the MNU-treated hamster to see if there are common molecular alterations in these lesions and tobacco smoke–induced carcinomas.

Because these lesions are produced by instillation of carcinogen in the trachea, it is likely that preventive or therapeutic (11) agents delivered in the trachea may have a similar distribution and may be effective on carcinoma at one or more of these locations.

The broader question of effectiveness of preventive or therapeutic agents against cancer produced by MNU will have to be shown by direct experimentation. Ultimately, we hope that this model system will be useful in developing single agents or combinations of agents that are effective in preventing or treating human cancer in the aerodigestive tree.

**Table 4. Data summary for control groups including only animals that survived to scheduled sacrifice**

<table>
<thead>
<tr>
<th>Protocol number</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>807*</td>
<td>19</td>
</tr>
<tr>
<td>806</td>
<td>29.9</td>
</tr>
<tr>
<td>796*</td>
<td>89.5</td>
</tr>
<tr>
<td>Animals with carcinomas in the upper respiratory tract (%)</td>
<td>89.5</td>
</tr>
</tbody>
</table>

*Control diet and solvent control.

† Absolute control (control diet, no solvent involved).
Figure 7. Master piece list used to identify location of experimental histologic sections scanned at 1,200 dpi into Adobe Photoshop and numbered in anterior to posterior order. Some anatomic structures are labeled.
Figure 8. Spreadsheet (in Microsoft Excel) used to record and accumulate locations and diagnoses of lesions from a single hamster using 10 graded slides such as that pictured in Fig. 2. Lesions are assumed to be tracheal unless prefixed with H- (for pieces 1-12) or E- (for pieces 13+). Because the pieces after #38 are indistinguishable, the sections containing distal trachea are numbered in order as T1 through T6. *, this row lists the animal’s ID number and the major pathology found.
References

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