Carcinogenesis in Heterotopic Respiratory Epithelium in Canine Subcutaneous Bronchial Autografts

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ABSTRACT

Short bronchial segments obtained by pneumonectomy were implanted, 9-12 per dog, in the subcutaneous tissues of the back of seven dogs. These subcutaneous bronchial autografts (SBA) became vascularized, and they contained viable, histologically normal respiratory epithelium 4 wk after implantation. From 1-3 mo after implantation, 10% methylcholanthrene in steroid suspension medium was instilled into 21 SBAs, and 10% methylcholanthrene in a silicone polymer sustained release implant was placed in 22 SBAs. Ten SBAs were left carcinogen free as controls.

SBA contents were examined cytologically at 3-mo intervals. Biopsies were done from 2-32 mo after bronchial implantation. Progressive preneoplastic changes were noted in all five dogs which received carcinogen. Curetments of five SBAs after 14-mo exposure to methylcholanthrene yielded $10^4-10^5$ cells from each SBA; 40-70% of the cells obtained were at the same stage of atypical squamous metaplasia. At least one SBA in each dog yielded cancer cells by cytological criteria by 19-29 mo after instillation. Biopsy of a grossly abnormal SBA revealed well-differentiated epidermoid carcinoma at 32 mo.

The multiple SBA method provides isolated portions of canine respiratory epithelium for the study of chemical carcinogenesis and for the production of sizable preneoplastic cell populations.

INTRODUCTION

In the study of respiratory carcinogenesis, heterotopic respiratory epithelium has been utilized in a number of models. Nettlesheim et al. (1) used syngeneic tracheal transplants in rats. The utility of this model is limited by the small amounts of available neoplastic tissue. In dogs, we made bistomal tracheal pedicle grafts which could be returned to the orthotopic position in staged fashion, thereby allowing for assessment of reversibility of epithelial change (2, 3). Others found tracheal grafts to be susceptible to chronic inflammation without regularly producing cancers (4, 5). Tracheal xenografts in athymic nude mice have been used (6, 7); their epithelial cancers were tiny, and there was a high incidence of sarcomatous tumors of host origin. Kobayashi et al. (8) produced cancers using single canine SBA; this provided only one site per animal for carcinogenesis and so is not more cost effective than our method of orthotopic endobronchial carcinogenesis in dogs (9).

Seeking increased cost effectiveness of canine lung cancer models, we have modified the SBA method to provide multiple predetermined sites for respiratory epithelial chemical carcinogenesis in single animals. We are reporting results to date because theft of 8 dogs undergoing SBA experiments interrupted an ongoing, more complete study, and we wish to make available the data obtained with this unique model while we proceed with new animals.

MATERIALS AND METHODS

Mongrel and closed-colony beagle dogs of both sexes, 2-6 yr old, were used.

Under general anesthesia, a pneumonectomy was done, the lung was placed in normal saline containing streptomycin (2 mg/ml), and the bronchi were cleared of vessels and parenchyma by sharp dissection. The bronchial tree was then cut into 10-16 segments, 6-15 mm long.

After closure of the thoracotomy incision, 2 short midline incisions were made on the back in the interscapular and lumbar regions. By blunt dissection, 6 or more subcutaneous tunnels were made radially from each incision. A single bronchial segment was placed at the periphery of each tunnel.

Under general anesthesia, a short skin incision was made over each SBA. Through a 1-2 mm stab incision into the lumen of the autograft, a 14 or 16 gauge short plastic tube was inserted, and a purse string suture was tightened around it. This allowed for leak-proof aspiration or injection of materials.

The carcinogen was 10% MCA, either in CRY or in a SRI made according to our previously described method (10, 11). By either CRY or SRI method, 0.2-1.0 ml of the carcinogen-containing material (40-200 mg of MCA) were injected until the SBA was full.

Fine needle aspiration biopsies were performed as previously described (12). Open biopsies were obtained by partial excision of the SBA wall. Selected SBAs were aspirated, washed, and then opened, and the mucosal lining was curetited bluntly, but gently. The curetments were aspirated into a flowing stream of buffered saline solution. SBA defects were closed with a continuous suture.

Aspirates and curetments were examined cytologically with the Papnicolaou stain. Biopsies and excised autografts were fixed in formalin and prepared in the usual fashion, stained with hematoxylin-eosin, and examined microscopically. Total cellular DNA content of aspirated cells prepared for cytological examination was measured by the image analysis method previously described (13).

Pilot studies to evaluate the feasibility of the SBA method were done in 7 dogs, including 6 mongrels and 1 beagle. Twelve SBAs without carcinogen were placed in 2 mongrels; 2 SBAs were resected from each dog at 2, 3, and 4 wk after placement. To evaluate the effectiveness of the method, a total of 53 SBAs was placed in 5 dogs. From 1-3 mo later, 43 SBAs were surgically exposed, and inspissated mucus was removed. By the SRI method, 10% MCA was injected into 22 SBAs. Crystalline 10% MCA in steroid suspension medium was injected into 21 SBAs. Ten SBAs were left carcinogen free as controls. From 2-4 mo after instillation of carcinogen, and at approximately 3-mo intervals thereafter, the contents of approximately one-half the SBAs in each dog were removed for examination. The remaining carcinogen-containing SBAs were sampled at the next time interval. The mean duration of cytological follow-up of the SBAs was 21 (19-29) mo.

To evaluate the relative effectiveness of CRY versus SRI in 8 dogs, carcinogen was instilled into 96 SBAs, leaving 12 SBAs as concurrent controls. The choice of CRY versus SRI was by random assignment.

RESULTS

No dogs succumbed to this experimental method, but on December 9, 1984, 8 beagles undergoing SBA experiments according to the random assignment of CRY versus SRI method
were stolen. Therefore, the following findings derive from the 7 dogs which had pilot or feasibility studies and from 1 set of observations 5 mo after the onset of the CRY versus SRI protocol experiments.

At 2, 3, and 4 wk after grafting, SBAs without carcinogen all contained clear mucus and were about the same size as at original placement. The ends of each segment had sealed over, leaving a cystic structure with a cartilaginous wall and respiratory epithelial lining. After 2 wk, there were some necrosis and disorganization of the epithelial lining. After 3 wk, the epithelium was returning toward normal; after 4 wk, there was a normal, well-vascularized epithelial lining with a normal distribution of cell types, including viable ciliated epithelium (Fig. 1). In 6 control SBAs from other dogs, examined after 4.5–5 mo, there were no cytological abnormalities in the aspirates; the only histopathological abnormality noted was mild cystic dilatation of some submucosal glands.

SBAs which contained SRIs usually expanded 2–5 times their initial size to a diameter of 2.3 cm within 2–4 mo. The SBAs in which CRY was placed increased to no more than twice their initial diameter. After 4 mo, little additional increase in size occurred in either case. Control SBAs without carcinogen remained unchanged in size (Fig. 2).

The entire lining of all SBAs consisted of respiratory epithelium. The character of the mucus within the SBAs was variable: SBAs with SRI produced thin, watery mucus which flowed easily; those with CRY produced thick, tenacious mucus; and controls produced gelatinous, inspissated mucus. Although useful as controls, SBAs less than 4-mm initial diameter were too small for repeated studies with carcinogens. For satisfactory serial sampling of SBAs only 4–6 mm in diameter, at least 12–15 mm of length at the time of grafting were needed.

Serial changes in the respiratory epithelium of SBAs as studied by cytology of aspirates are summarized in Table 1. The aspirates showed progressively more severe atypical squamous metaplasia somewhat in proportion to the duration of continued exposure of the epithelial lining to carcinogen. By 19–29 mo after carcinogen implantation, 13 aspirates contained cancer cells by cytological criteria (Fig. 3), and at least 1 SBA from each dog had yielded cancer cells on cytological examination of aspiration biopsies. There was no evidence to suggest that either SRI or CRY is more effective, and there was no difference in response to carcinogen attributable to initial bronchial level of the SBA.

In each of 5 of 7 curetments, obtained from 7 SBAs in 1 dog after 14 mo of carcinogen exposure, $10^4$–$10^5$ cells were present; 2 SBAs were curet too cautiously, and only a meager cell yield was obtained. From 40–70% of the cells in the curetments showed the same degree of atypical squamous metaplasia as was present in the washings aspirated prior to the curetment.

Table 1 Cytological findings in washings from subcutaneous bronchial autographs

<table>
<thead>
<tr>
<th>Mo after carcinogen instillation</th>
<th>2</th>
<th>4</th>
<th>6–7</th>
<th>9–11</th>
<th>12–14</th>
<th>16–18</th>
<th>20–23</th>
<th>26–29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal or benign</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Regular squamous metaplasia</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Atypical squamous metaplasia</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>5</td>
<td>10</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Severe</td>
<td>2</td>
<td>2</td>
<td>10</td>
<td>6</td>
<td>8</td>
<td>4</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Epidermoid carcinoma</td>
<td>4</td>
<td>4</td>
<td>10</td>
<td>7</td>
<td>15</td>
<td>9</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>No. of SBAs sampled</td>
<td>9</td>
<td>19</td>
<td>24</td>
<td>22</td>
<td>27</td>
<td>27</td>
<td>19</td>
<td>12</td>
</tr>
<tr>
<td>No. of dogs sampled</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

* Each aspirate is noted according to the most severe abnormality seen.
Fig. 3. Representative abnormalities of respiratory epithelium from SBA. Intracellular vacuoles in C represent early cytoplasmic degeneration. Regular squamous metaplasia: A, cytology; B, histology. Moderate atypical squamous metaplasia: C, cytology; D, histology. Epidermoid cancer: E, cytology; F, histology. Cytological specimens stained by Papanicolaou method, × 500. Histological sections stained with H & E, × 300. Bars, 20 μm.
Table 2  Mean total cellular DNA values as related to cytological diagnosis

<table>
<thead>
<tr>
<th>Cytological diagnosis</th>
<th>No. of cells measured</th>
<th>Total cellular DNA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regular squamous metaplasia</td>
<td>137</td>
<td>1.43 ± 0.34</td>
</tr>
<tr>
<td>Mild atypical squamous metaplasia</td>
<td>128</td>
<td>1.98 ± 0.46</td>
</tr>
<tr>
<td>Moderate atypical squamous metaplasia</td>
<td>127</td>
<td>2.95 ± 0.58</td>
</tr>
<tr>
<td>Severe atypical squamous metaplasia</td>
<td>130</td>
<td>3.70 ± 0.81</td>
</tr>
<tr>
<td>Epidermoid carcinoma</td>
<td>272</td>
<td>4.51 ± 0.96</td>
</tr>
</tbody>
</table>

* Values = integrated absorbance of abnormal cell

Table 3  Comparison of SBA versus orthotopic endobronchial carcinogenesis

<table>
<thead>
<tr>
<th>SBA</th>
<th>Orthotopic endobronchial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxic effects are limited to SBA without hazard to the host's life</td>
<td>Toxic effects upon functioning lung are a potential hazard to the host's life</td>
</tr>
<tr>
<td>Multiple (10—12) sites are available for carcinogenesis in each animal</td>
<td>Not more than 2 sites are useful for carcinogenesis in each animal</td>
</tr>
<tr>
<td>Large number of preneoplastic or cancer cells can be harvested from up to 12 readily accessible subcutaneous bronchial segments</td>
<td>Restricted number of preneoplastic or cancer cells can be harvested from small samples obtained by bronchoscopic biopsy or washings</td>
</tr>
<tr>
<td>The model can potentially be used to evaluate the effects of therapeutic regimens upon cells in various stages of the neoplastic continuum</td>
<td>The model can be used to explore diagnostic or therapeutic methods upon endobronchial cancers similar to those in humans</td>
</tr>
</tbody>
</table>

Estimated cost/cancer = $230—290

Cost/cancer induced with MCA is about $1000

When the same SBAs were again aspirated at 2 or 5 mo after curetage, cytological examination revealed the same degree of atypia noted in the curetments, indicating that the regenerating epithelial lining retained its abnormality.

To date, 14 histological examinations of SBAs containing carcinogen have been made. Open biopsy samples have been taken from 11 SBAs containing MCA (7 SRI, 4 CRY). Ten samples were obtained 6.5—7 mo after placement of carcinogen, and one sample was taken from an enlarged, irregular SBA 32 mo after placement of carcinogen. Three SBAs were excised 2 and 12 mo after placement of carcinogen, respectively. Histological findings in the excised grafts were confirmatory of the cytological findings. Epidermoid cancer was noted 32 mo after placement of SRI in the palpably abnormal SBA. Fig. 3 shows representative cytological abnormalities together with the corresponding histological findings.

Mean values for total cellular DNA are shown in Table 2. For each cytological diagnosis, cellular DNA was measured in material from each dog. A progressive increase in hyperploidy with increasing cytological abnormality was noted, corresponding to the findings in cells exfoliated from canine bronchial epithelium during orthotopic endobronchial carcinogenesis (14).

DISCUSSION

Our observation that total DNA content in bronchial epithelial cells was increased in proportion to the degree of metaplasia or atypia (14) indicates that genomic alteration occurred during carcinogenesis. The unique importance of the SBA method is the feasibility of obtaining preneoplastic bronchial epithelium at specific stages during the transitional neoplastic continuum. We have reason to believe that preneoplastic epithelial cells from SBAs will be available in amounts sufficient to assess gene expression and to correlate molecular biological findings with morphological phenotype. Moreover, one can repetitively harvest biochemically useful amounts (>0.5 g) of lung cancer cells without a major operation or sacrificing the host.

In the SBA preparation, it is impractical to take serial open biopsies to identify the site(s) of origin of the first cancer cells detected by exfoliative cytology. In our previous studies with endobronchial carcinogenesis in dogs (9), and from subsequent data (5), we know that progressively abnormal cytological findings and hyperploidy regularly precede histologically confirmed lung cancer in dogs. This experience, plus the confirmatory biopsies taken periodically from SBAs, makes us confident that the finding of cancer cells by cytological criteria in the SBAs indeed reflects the presence of focally microinvasive cancers.

On the basis of our previous experience with histologically proven orthotopic endobronchial carcinogenesis using MCA (9, 15, 16), we anticipated overt cancer in SBAs approximately 2.2—2.8 yr after initiation of exposure to carcinogen. It has taken about 2.7 yr to produce the first histologically confirmed cancer by the SBA approach.

Table 3 compares features of bronchial carcinogenesis in the orthotopic endobronchial location to the SBA approach. Relative costs were calculated according to the formula

\[\text{initial cost/dog} + (\text{mo required for cancer} \times \text{monthly cost/dog})\]

\[= \text{no. of cancers achieved/dog}\]

Our most successful method for endobronchial canine carcinogenesis has required 1.5—2.0 yr for invasive cancer to occur. Current data suggest that the SBA approach will require about 8 mo additional animal housing cost as compared to orthotopic methods. However, the endobronchial approach yields only fragments of preneoplastic tissue harvested by bronchoscopy, usually from a single site per dog. With the SBA approach, we expect to produce 10-12 separate carcinogenic foci per dog. Thus, the total cost per cancer by the endobronchial method is estimated to be about $1000, while via the SBA approach, the cost per cancer is expected to be about $230—290. No comparative cost estimate can be assigned to the unique opportunity to obtain serial samples of preneoplastic cells and tissues from SBAs, because neither cell culture techniques nor samplings from patients can currently provide such biological material reliably and in significant amounts. We believe that the ability to produce these quantities of epidermoid tumor and precursor bronchial cells in vivo provides a very useful tool for studies of cell biology pertaining to bronchogenic cancers.

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