ABSTRACT

Epithelial cell lines derived from rat tracheal implants 2 and 9 months after a 4-week exposure to 200 µg dimethylbenz(a)anthracene-beeswax pellets, and previously assayed for growth in soft agarose and tumorigenicity, were tested at the same time of subculture for cytochalasin B-induced multinucleation to determine the relationships between anchorage-independent growth, uncontrolled nuclear division, and tumorigenicity. The relationships among the three phenotypic markers could be separated into five distinct groups. Group I cell lines showed no growth in agarose, showed no cytochalasin-induced multinucleation, and formed no tumors in nude mice. Group II cell lines exhibited anchorage independence but were negative for the other markers. Group III cell lines were anchorage independent and exhibited a positive response to cytochalasin B (more than 10% of the cells had three or more nuclei), but were tumor negative. Group IV cell lines were positive for all three markers. Group V cell lines grew in soft agarose, were cytochalasin B negative, but formed tumors only 4 months after the cell inoculations. The 20 cell lines generated 2 months after carcinogen exposure distributed in the groups as follows: Group I, 20%; Group II, 20%; Group III, 50%; Group IV, 15%; and Group V, 10%. The 27 cell lines generated 9 months after carcinogen exposure distributed among the groups as: Group I, 4%; Group II, 18%; Group III, 26%; Group IV, 52% and Group V, 0%. The results indicate that: (a) anchorage independence precedes the two other markers of growth autonomy (b) uncontrolled nuclear division appears as a separate step after anchorage-independent growth and before tumorigenicity; (c) tumorigenicity appears preferably in the cell populations that exhibit anchorage independence and uncontrolled nuclear division; and (d) progression in growth autonomy occurs in the tracheal implants in vivo which can be detected in vitro as an increase in cell lines positive for the three phenotypic markers.

INTRODUCTION

Human clinical studies (e.g., Refs. 1–3) and a wide range of experimental animal studies (e.g., Refs. 4–6) strongly support the hypothesis that cancer development is a multistep process. Studies of carcinogenesis in in vitro systems, particularly those utilizing primary and early passage cell cultures, have given further credence to this view (7–9). In the in vitro systems, the progression of cancer development after carcinogen treatment is seen first as focal morphological transformation in the cell cultures, followed by immortalization, anchorage independence, and ultimately tumorigenicity upon continued cultivation of the cells. We use an in vivo-in vitro experimental approach, the tracheal implant-cell culture model (10–13), to study the development of cancer in the respiratory tract. In this approach, carcinogen exposure and neoplastic progression takes place in the animal for the desired lengths of time before in vitro markers of neoplasia are looked for in cell cultures generated from the exposed tracheas. We have found that a very early marker of cancer initiation in rat tracheal cells was their ability to survive in medium without added pyruvate, a supplement required by normal tracheal cells in culture (14). The next indication of increased growth autonomy in the surviving cells was their capacity to withstand dissociation and subculture into cell lines, i.e., the acquisition of immortality (11). Further alteration in the growth control of these cells during subculture was evident as growth in soft agar. This anchorage-independent growth was found long before tumorigenicity in many of the cell lines (11).

Recently we completed a study in which we tracked the progression of neoplasia in cell populations isolated from tracheal implants at increasing lengths of time after exposure to different doses of DMBA (13). Because we followed both classical morphological markers and changes in the in vitro growth behavior of carcinogen-altered cell populations isolated from the same tracheas, direct correlations between in vivo and in vitro markers of cancer progression could be looked for and indeed were found. The findings in that study most pertinent to the present report are the following. Extending the time interval between the end of carcinogen exposure and generation of primary cell cultures and subsequent cell lines increased the percentage of lines that exhibited anchorage-independent growth. Extending the time interval also increased the number of anchorage-independent cell lines that tested positive for tumorigenicity. These results clearly showed that progressive changes take place in the carcinogen-exposed tracheal epithelium in vivo that can be detected subsequently as quantitative increases in growth parameters in vitro. During the course of the same study we also began testing cell lines for their ability to form multinucleated cells in the presence of CB. Although a subject of controversy (15, 16), multinucleation in the presence of this fungal metabolite is believed to be a reflection of altered control of nuclear division and has been correlated with the uncontrolled growth of tumorigenic cells (17, 19). It was our specific aim to determine whether CB-induced multinucleation occurs in the tracheal cell lines and whether there is any correlation with anchorage-independent growth and/or tumorigenicity. Our results indicate that uncontrolled nuclear division appears as a separate step after anchorage-independent growth but before tumorigenicity in rat tracheal cells.

MATERIALS AND METHODS

Cell Lines. All 53 cell lines used in this study came from specific-pathogen-free Fisher 344 rat tracheal implants exposed to DMBA-beeswax pellets by reported procedures (20, 21). Passages 9 and 20 of cell line 1000W came from an early study in which tracheal implants were exposed for 2 weeks to 640 µg DMBA (11). Cell line 1000WT was established from a tumor which developed after inoculation of cell line 1000W into an irradiated isogenic host. Twenty of the cell lines were established from tracheal implants 2 months after a 4-week exposure to 200-µg DMBA pellets. Twenty-seven cell lines were estab-
lished from tracheal implants 9 months after a 4-week exposure to 200-μg DMBA pellets. The remaining 4 cell lines came from tracheal implants exposed for 9 months to the 200-μg DMBA pellets. The procedures for developing and maintaining these rat tracheal epithelial cell lines have been described in detail in previous reports (10–13). Most pertinent to note concerning these procedures are the following. The cell lines originated as epithelial outgrowths from pieces of the carcinogen-exposed tracheal implants. Three epithelial outgrowths were generated from each piece. The carcinogen-altered cells in these primary populations were selected out by culture for 2 weeks in an enriched Waymouth’s 752/1 medium which lacked pyruvate and insulin supplements. Once the surviving populations reached approximately 4 × 10⁶ cells, they were subcultured and tests for anchorage-independent growth and tumorigenicity were carried out at about the third passage. Relationships between the epithelial cell origin and growth rates, anchorage-independent growth, and tumorigenicity for one cell line from each explant were reported in the earlier publications (12, 13). In the present study we have included all the cell lines established from the explants at 2 and 9 months post-DMBA exposure to maximize the samples of lines tested and have classified the lines into groups according to relationships among the markers of neoplasia including CB-induced multinucleation which was assayed at the same time as growth in agarose and tumorigenicity.

Anchorage Independence and Tumorigenicity Assays. Anchorage-independent growth was tested by dispersing, in duplicate 4 × 10⁶ cells in 2.0 ml of 0.33% agarose (Sigma, type II). This was layered over 5.0 ml of 0.5% agarose in 60-mm tissue culture dishes embossed on the bottom with 2-mm grids. After 3 weeks, the cells which formed colonies were stained by adding 1.0 ml of a vital tetrazolium dye, following the procedure of Schaeffer and Friend (22). Colonies of 8 or more cells were counted 2 days after adding the stain. A colony of 8 cells was chosen as a minimum because it indicates that at least 3 cell divisions occurred. These colonies were easily distinguished from cell clumps by this method. Cultures were considered positive if at least one colony was found per dish (0.01%).

The ability of cells to form tumors was tested by inoculating 1 × 10⁶ cells s.c. on the backs of four 5- to 7-week-old C3H-10 nude mice which had been irradiated with 400 rads within 24 h of inoculation. The animals were palpated for tumors weekly for 180 days. Carcinoma verification was made in slices of the tumors fixed in Bouin’s solution and embedded and stained in hematoxylin and eosin.

Multinucleation Assay. The multinucleation of cells in the presence of CB was measured as described by O’Neill et al. (15). Cells were stained by adding 1.0 ml of a vital tetrazolium dye, following the procedure of Schaeffer and Friend (22). Colonies of 8 or more cells were counted 2 days after adding the stain. A colony of 8 cells was chosen as a minimum because it indicates that at least 3 cell divisions occurred. These colonies were easily distinguished from cell clumps by this method. Cultures were considered positive if at least one colony was found per dish (0.01%).

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Of the 4 cell lines derived from rat tracheas, 2 cell lines (2-12b, 2-12c) were CB positive and 2 cell lines (2-10b, 2-10c) were CB negative. The 2 CB-positive cell lines were positive for anchorage-independent growth and tumorigenicity, and the 2 CB-negative cell lines were positive for anchorage-independent growth and negative for tumorigenicity. The results of these experiments are presented in Table 1.

Table 1 CB-induced multinucleation and anchorage-independent growth in tumorigenic tracheal epithelial cell lines

<table>
<thead>
<tr>
<th>Cell line designation</th>
<th>Growth in agarose (%)</th>
<th>Multinucleation (%)</th>
<th>Tumorigenicity in nude mice (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CB</td>
<td>3 × CB</td>
</tr>
<tr>
<td>T-1a</td>
<td>23.77</td>
<td>1.1</td>
<td>17.0</td>
</tr>
<tr>
<td>T-2a</td>
<td>0.08</td>
<td>1.8</td>
<td>26.6</td>
</tr>
<tr>
<td>T-2b</td>
<td>0.82</td>
<td>3.2</td>
<td>25.7</td>
</tr>
<tr>
<td>T-1b</td>
<td>21.13</td>
<td>3.1</td>
<td>34.8</td>
</tr>
<tr>
<td>1000W p9</td>
<td>0.02</td>
<td>0.6</td>
<td>9.7</td>
</tr>
<tr>
<td>1000W p20</td>
<td>1.98</td>
<td>0.5</td>
<td>39.9</td>
</tr>
<tr>
<td>1000W T</td>
<td>16.07</td>
<td>1.1</td>
<td>78.5</td>
</tr>
</tbody>
</table>

- The tumor-derived cell lines are numbered consecutively according to CB-induced multinucleation. A line is given the same number and the letter b if it is a second cell line in the list from the same explant.
- The number of cells forming colonies of 8 or more cells from the 4 × 10⁶ cells seeded per 60-mm dish.
- The CB concentrations were 1 or 2 μg/ml medium or 3 times this amount.
- p, passage number; ND, not determined.

The data in Table 1 indicated a significant relationship between CB-induced multinucleation and tumorigenicity in the tracheal cell lines. This prompted us to measure CB-induced multinucleation at the same time that we were testing for anchorage-independent growth and tumorigenicity in cell lines established from tracheal implants 2 months (Table 2) and 9 months (Table 3) after a 4-week exposure to DMBA. The relationships among these 3 phenotypic markers could be separated into 5 distinct groups. Cell lines placed in Group I did not grow in soft agarose, did not show significant CB-induced multinucleation, and were not tumorigenic. Cell lines in Group II exhibited anchorage-independent growth but were negative for the 2 other markers. Group III cell lines all exhibited anchorage-independent growth and were classified CB positive (10% or more of the cells have 3 or more nuclei when cultured in CB-supplemented medium). The cell lines designated Group IV were positive for all 3 markers, i.e., for anchorage independence and CB-induced multinucleation, and were tumorigenic when inoculated into nude mice. Only tracheal cells from the 2-month post-DMBA exposure had cell lines in Group V (Table 2). The 2 cell lines (2-12b, 2-12c) making up many cell lines under fairly constant assay conditions. Therefore, a cell line was classified as CB positive if 10% of the countable cells had 3 or more nuclei. This percentage was chosen because it was above control levels in the tracheal cell lines and in the control cell cultures used in other studies (18, 19).

RESULTS

First of all, 4 tracheal epithelial cell lines generated from pieces of carcinogen-exposed tracheal implants bearing carcinomas were tested for multinucleation in the presence of CB to determine if there was a direct correlation between multinucleation and their tumor origin (Table 1). All 4 of the cell lines exhibited 3 or more nuclei in 14% or more of the cells. Also, all the cell lines were tumorigenic when inoculated back into nude mice and were anchorage independent when tested for growth in agarose. Secondly, a cell line, 1000W, previously shown to become anchorage independent and tumorigenic during subculture (11), was tested at a relatively early and late passage for CB-induced multinucleation (Table 1). At passage 9, the cells exhibited low growth in agarose but had less than 10% multinucleation, while at passage 20 when the cells were tumorigenic anchorage-independent growth and multinucleation had increased markedly. The percentage of cells showing these 2 phenotypic markers was even higher in the cell line derived from the tumor.

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this group exhibited anchorage-independent growth and were tumorigenic but did not form multinucleated cells in the presence of CB above control levels. However, the tumors formed by the 2 cell lines were not palpable until 126-145 days after inoculation. This contrasted markedly to the short time to tumorigenicity of the 3 cell lines in Groups III and IV, which were CB positive and this distributed as 26% in Group III and 52% in Group IV. These data strongly suggest that over all, 50% of the cell lines from the 2-month post-DMBA-exposed tracheas were CB positive and 35% of these were in Group III while 15% were in Group IV. In contrast 78% of the cell lines from the 9-month post-DMBA-exposed tracheas were CB positive and this distributed as 26% in Group III and 52% in Group IV. These data strongly suggest that over time cells first acquire uncontrolled nuclear division and later become tumorigenic.

The degrees of multinucleation in the individual cells of the Group III and Group IV cell lines were compared to determine if the nontumorigenic lines had only a limited capacity to divide or if the nontumorigenic lines had a direct relationship with increase in culture time (more proliferation) and progression of the markers of neoplasia.

The most surprising finding in this study was the detection of a number of cell lines that were able to form multinucleated cells in the presence of CB but were not tumorigenic (Group III). Overall, 50% of the cell lines from the 2-month post-DMBA-exposed tracheas were CB positive and 35% of these were in Group III while 15% were in Group IV. In contrast 78% of the cell lines from the 9-month post-DMBA-exposed tracheas were CB positive and this distributed as 26% in Group III and 52% in Group IV. These data strongly suggest that over time cells first acquire uncontrolled nuclear division and later become tumorigenic.

The degrees of multinucleation in the individual cells of the Group III and Group IV cell lines were compared to determine if the non-tumorigenic lines had only a limited capacity to divide in the presence of CB. Fig. 1 shows the percentage of distribution of the number of nuclei per cell for a cell line from Groups III and IV from both the 2-month and 9-month post-carcinogen-exposed tracheas. Although the 2 cell lines from the 9-month post-DMBA-exposed tracheas had a higher percentage of cells with 7 or more nuclei, all the lines had some cells with large numbers of nuclei. This was generally the case for all the cell lines in Groups III and IV from both exposure conditions (data not shown).

**DISCUSSION**

In this study, we showed that an alteration in the control of nuclear division appeared after anchorage-independent growth but before tumorigenicity in epithelial cell cultures generated...
from DMBA-preexposed rat tracheal implants. This uncontrolled nuclear division reveals itself as continued replication of nuclei in the presence of cytochalasin B, which binds actin in the cytoskeletal network thereby inhibiting cytoplasmic cleavage (23). Many investigators have shown that tumorigenic cell lines will form multinucleated cells in the presence of cytochalasin B while nontumorigenic cells do not (17-19). The interpretation has been that cytoplasmic and nuclear division are coordinately regulated in normal cells and that this tight regulation is lost in tumor cells. Because CB-induced multinucleation was not found in all tumorigenic cell lines, the relationship between uncontrolled nuclear division and tumorigenicity has been questioned by some investigators. The most notable lack of correlation is found in RNA virus-transformed cells and in some tumorigenic liver cell lines reported by San et al. (24) cannot be clearly inter-pretation has been that cytoplasmic and nuclear division are coordinately regulated in normal cells and that this tight regulation is lost in tumor cells. Because CB-induced multinucleation was not found in all tumorigenic cell lines, the relationship between uncontrolled nuclear division and tumorigenicity has been questioned by some investigators. The most notable lack of correlation is found in RNA virus-transformed cells and in some tumorigenic liver cell lines reported by San et al. (24) cannot be clearly inter-

REFERENCES

CB-INDUCED MULTINUCLEATION IN TRACHEAL CELLS


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Ann C. Marchok and Donald H. Martin


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