Differential Response of Cultured Mouse Mammary Cells of Varying Tumorigenicity to Cytochalasin B

Marion R. Steiner, Betty Altenburg, Carolyn S. Richards, Jaquelin P. Dudley, Daniel Medina, and Janet S. Butel

ABSTRACT

Cultured BALB/c mouse mammary gland epithelial cells of varying oncogenic potential in vivo have been examined for their ability to multinucleate in the presence of cytochalasin B (CB). Highly tumorigenic cell lines derived from mammary tumors with hormonal, viral, or chemical carcinogen etiologies were extensively multinucleated when cultured in CB-supplemented medium. Normal mammary gland cells from either pregnant or lactating animals were predominantly mono- or binucleate under comparable conditions. At low passage levels after cloning, cell lines derived from a chemical carcinogen-induced mammary tumor were weakly oncogenic and remained largely mono-, or binucleated when cultured in CB-supplemented medium. At higher cell passage levels, both the ability to produce tumors in vivo and the degree of multinucleation in CB-supplemented medium increased dramatically with the clonal cell lines. Thus, the response of cultured mouse mammary gland epithelial cells to CB in vitro may be useful as a marker of the oncogenic potential of such cells.

INTRODUCTION

Cultured mouse mammary gland epithelial cell lines of varying oncogenic potential have been described recently (3). Growth properties such as high saturation density and ability to grow in semisolid medium, which often have been positively correlated with transformation of fibroblasts, did not appear to correlate well with the in vivo tumorigenicity of mouse mammary gland cells (3, 17). Moreover, epithelial cells derived from other tissues or from other species often have been observed not to exhibit a positive correlation between growth properties and in vivo tumorigenicity (2, 6, 9, 12). There is currently no in vitro marker that can reliably and readily predict the tumorigenic potential in vivo of cultured epithelial cells.

A differential effect has been described for the mold metabolite CB on normal and transformed cells. Normal cells cultured in medium supplemented with CB remain mono- or binucleated, whereas some transformed cells become highly multinucleated under the same culture conditions (1, 10, 11, 14, 15, 18). The effect of CB on cultured mouse mammary gland cells and its potential as a marker of tumorigenicity in this system is reported here.

MATERIALS AND METHODS

Cell Culture. The ESD/BALB, MTV-L/BALB, and DMBA/BALB cell lines were derived from BALB/c mouse mammary tumors with hormonal, viral, and chemical carcinogen etiologies, respectively (3). The DMBA-2/BALB cell line was derived from a carcinoma that arose from a fourth transplant generation of a carcinogen-induced hyperplastic alveolar nodule. Primary cells from tumors and from normal mammary glands were prepared by brief trypsinization of minced, washed tissue, followed by treatment with collagenase (2 mg/ml) (type III; Worthington Biochemicals Inc., Freehold, N. J.) or, in the case of mammary glands from lactating animals, by direct treatment with collagenase (type II). Cells were cultured in Dulbecco's modified medium supplemented with 10% fetal bovine serum, antibiotics [either penicillin (100 units/ml)-streptomycin (100 µg/ml), or gentamicin (50 µg/ml)] and bovine insulin (5 µg/ml) (Sigma Chemical Co., St. Louis, Mo.). In addition, the culture medium for the DMBA/BALB and DMBA-2/BALB cells was supplemented with hydrocortisone (5 µg/ml) (Sigma). Tumorigenicity in vivo was tested in syngeneic mice by transplantation s.c., as previously described (3) or by transplantation of 10^5 cells in cleared mammary fat pads (7).

Multinucleation Assay. The multinucleation of cells in the presence of CB was measured as described by O'Neill et al. (15). Cells were seeded onto glass coverslips in plastic tissue culture dishes. After 24 hr, when the cells were 50 to 75% confluent, fresh medium containing 1.5 µg CB per ml (Sigma) was added. Effective concentrations of CB ranged from 1.0 to 3.0 µg/ml and have been noted to vary among different lots of CB. The experiments reported here were performed with a single lot of CB. Since stock solutions of CB (1.0 or 1.5 mg/ml) were prepared in dimethyl sulfoxide, parallel solvent controls were performed. After 5 days of incubation, the cells were fixed, stained with hematoxylin, and counterstained with eosin, and the number of nuclei per cell was determined in a representative number of cells (200 or more cells per coverslip).

RESULTS AND DISCUSSION

Highly tumorigenic mammary tumor cell lines with hormonal (ESD/BALB), viral (MTV-L/BALB), or chemical carcinogen (DMBA-2/BALB) etiologies were extensively multi-
nucleated (≥3 nuclei/cell) after culture in CB-supplemented medium (Chart 1). In contrast, normal mammary gland epithelial cells derived from either pregnant or lactating animals were predominantly binucleated under similar conditions (Chart 1). The cells derived from the mammary glands of lactating animals included both epithelial and fibroblastic morphologies; however, when the number of nuclei per cell was considered for only the epithelial cells, the results were the same as those illustrated in Chart 1F. Since the normal epithelial cells were primary cultures, primary cells from tumors induced by the injection of established cell lines were also examined. Such primary tumor cells were also highly multinucleated when cultured in CB-supplemented medium (e.g., cells from a tumor produced by the DMBA-2/BALB cells, as in Chart 1D).

Supplementation of culture medium with steroid hormones did not appear to affect the response of the cells to CB. For example, when the multinucleation assay was performed in medium also containing hydrocortisone (5 μg/ml) and estradiol (0.5 μg/ml), 3% of the cells from normal lactating mammary glands had ≥3 nuclei, while 96% of the MTV-L/BALB cells had ≥3 nuclei. The DMBA/BALB and DMBA-2/BALB cells were routinely subcultured in medium supplemented with hydrocortisone (5 μg/ml). All normal and tumor cell cultures incubated in medium containing dimethyl sulfoxide, i.e., solvent controls, were greater than 85% mononucleated.

While the majority of the mouse mammary tumor-derived cell lines examined were found to be highly tumorigenic (3), low-passage cells of both DMBA/BALB clonal lines 1 and 2 were weakly tumorigenic in vivo and remained predominantly mono- or binucleated when tested by the CB multinucleation assay (Table 1). At higher cell passage levels,

### Table 1

<p>| CB-related multinucleation and tumorigenicity of cultured mammary cells |
|-------------------------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>The CB multinucleation assay was performed as described in &quot;Materials and Methods.&quot; For the transplantability assay, 1 or 2 × 10⁶ cells were injected s.c. into 8- to 10-week-old BALB/c mice, and the animals were palpated weekly.</th>
<th>CB multinucleation assay (%) cells with ≥3 nuclei/cell</th>
<th>No. of animals with tumors/No. of animals injected</th>
<th>Latent period (wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>No. of</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESD/BALB clone 3</td>
<td>53</td>
<td>3/3</td>
<td>3</td>
</tr>
<tr>
<td>Passage 12a</td>
<td>79</td>
<td>5/5</td>
<td>2</td>
</tr>
<tr>
<td>DMBA-2/BALB clone 4</td>
<td>70</td>
<td>5/5</td>
<td>3</td>
</tr>
<tr>
<td>Passage 10</td>
<td>90</td>
<td>3/3</td>
<td>2</td>
</tr>
<tr>
<td>DMBA/BALB clone 1</td>
<td>93</td>
<td>5/5</td>
<td>3</td>
</tr>
<tr>
<td>Passage 13</td>
<td>10</td>
<td>1/5</td>
<td>8</td>
</tr>
<tr>
<td>Passage 15</td>
<td>19</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>Passage 18</td>
<td>17</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>Passage 20</td>
<td>33</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>Passage 27</td>
<td>69</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>Passage 70</td>
<td>90</td>
<td>ND</td>
<td>3</td>
</tr>
<tr>
<td>DMBA/BALB clone 2</td>
<td>7</td>
<td>1/5</td>
<td>7</td>
</tr>
<tr>
<td>Passage 8</td>
<td>26</td>
<td>ND</td>
<td>28</td>
</tr>
<tr>
<td>Passage 14</td>
<td>70</td>
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<td>2</td>
</tr>
<tr>
<td>Passage 15</td>
<td>7</td>
<td>5/5</td>
<td>2</td>
</tr>
</tbody>
</table>

a Passage number indicates passage number after cloning. The ESD/BALB, DMBA-2/BALB, and MTV-L/BALB parental cell lines were cloned at approximately passage 30; DMBA/BALB passage 40.

b ND, not done.

c Passages 63 and 71 were highly tumorigenic in vivo.

d When the proportion of tumorigenic versus weakly tumorigenic cells with ≥3 nuclei/cell is compared, p < 0.005.
both the tumorigenicity and the extent of multinucleation increased dramatically with both clonal lines. These data with the DMBA/BALB clones 1 and 2, which have been subcultured extensively, suggest that a marked increase in CB-related multinucleation in vitro is associated with a parallel increase in tumor production in vivo.

Additional clones of the DMBA/BALB cells have been prepared from the parental DMBA/BALB cells at passage 16. Five of these clones (DMBA/BALB clone 16a to 16e) have been examined with respect to CB-related multinucleation and tumorigenicity in vivo. Eight passages after clonning, the percentage of CB-treated cells with \( \geq 3 \) nuclei/cell ranged from 2 to 8% (average, 5.4%). No tumors were observed 13 weeks after transplantation of the DMBA/BALB clone 16a to 16e cells into the cleared mammary fat pad (transplantation into 6 fat pads/clone). DMBA/BALB clone 1 cells at passages 20 and 71 produced tumors in 2 weeks in the cleared mammary fat pads. Furthermore, no tumors were observed 27 weeks after s.c. injection of passage 5 DMBA/BALB clone 16a to 16e cells (10\(^6\) cells/animal; 5 animals given injections per clone). In addition, 2 of the DMBA/BALB clone 16 clones have been further subcultured. At passage 19 the cells were tumorigenic (tumors observed 2 weeks after s.c. injection of 10\(^6\) cells) and were multinucleated in the CB assay system (clone 16a, 45%; clone 16b, 55% cells with \( \geq 3 \) nuclei/cell). Thus, the degree of CB-related multinucleation appears to correlate with tumorigenicity in vivo in a qualitative manner. Since both the degree of CB-related multinucleation and tumorigenicity may change very rapidly with the DMBA/BALB cells, the absolute level of multinucleation in the CB assay system (clone 16a, 45%; clone 16b, 55% cells with \( \geq 3 \) nuclei/cell) may be of importance in determining tumorigenicity in vivo.

The properties of cells derived from a DBMA-induced rat mammary carcinoma have been reported to be altered after multiple subculturing (16). There were changes in the cloning efficiency, growth requirements, and trypsin sensitivity; however, tumorigenicity was not examined. The instability exhibited by the rat mammary carcinoma cells may be a phenomenon that is similar to the change observed in this study with the DMBA/BALB cells. The alteration in oncogenicity of the DBMA/BALB clonal cell lines suggests that they represent a system useful in identifying those changes that may be of importance in determining tumorigenicity of mammary epithelial cells.

A high degree of CB-induced multinucleation has been observed previously in many DNA virus-transformed fibroblasts (10, 11, 14, 15, 18), in cultured human tumor cell lines (5, 13), and in some sarcoma virus-transformed cells (1, 15). At the levels of CB used in this study, CB has been found to inhibit cytokinesis while karyokinesis may continue (4, 8). Cytokinesis and karyokinesis may be coordinated in normal and weakly oncogenic cells; this coordination appears to be lost in the highly tumorigenic cells.

The data presented in this report indicate that the ability of mouse mammary epithelial cells to multinucleate when cultured in CB-supplemented medium appears to be positively correlated with the degree of tumorigenicity of the cells. Therefore, the CB multinucleation assay may be useful as an in vitro method for estimating the oncogenic potential of mouse mammary epithelial cells.

REFERENCES

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