Transformation of Rat Embryo Cells in Vitro by Chemical Carcinogens

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SUMMARY
A cell line derived from Fischer rat embryo was treated in vitro with various levels of polycyclic aromatic hydrocarbons [3-methylcholanthrene, 7,12-dimethylbenz(a)anthracene, and benzo(a)pyrene or dimethyl sulfoxide (control)]. Cells treated only with the carcinogens underwent transformation in vitro and produced progressively growing transplantable tumors when injected into homologous hosts.

INTRODUCTION
The in vitro transformation of mouse and hamster cells by chemical carcinogens has been described and well established. (1–5, 8, 13, 19, 20). Most in vitro transformations of cell cultures by chemical carcinogens yielded sarcomas upon reinsertion into syngeneic hosts. Transformation of rat embryo cells by 4-nitroquinoline 1-oxide (15) and fluorenylhydroxamic acids (7) has been reported recently. However, there is no evidence for the transformation of rat embryo cells by polycyclic aromatic hydrocarbons. This communication reports results of experiments showing that rat embryo cells can be transformed in vitro by polycyclic aromatic hydrocarbons and that these transformed cells produce tumors when injected into newborn rats.

MATERIALS AND METHODS
Cell Cultures and Media. Continuous rat embryo cell line (S-1193h) was established from primary cultures of Fischer rat embryo cells. Approximately 20 ml of primary rat embryo cell suspension (1 X 10^6 cells/ml) in EMEM + 10% FBS, 2 mM glutamine, and antibiotics was planted in a B flask (approximately 50-sq cm surface area) and incubated at 37° under 5% CO₂ in air. By the 3rd day after planting, a complete monolayer was obtained. Fourteen days after incubation, the cultures were subdivided by trypsin treatment. This was repeated every 7 to 10 days. The medium was renewed at 3- to 4-day intervals. For the 1st 4 subcultures, the cells were maintained in Eagle’s minimum essential medium with 0.1 mM calcium, supplemented with 5% dialyzed calf serum, 2% fetal bovine serum, 2 mM glutamine, 0.1 mM nonessential amino acids, and antibiotics (6). Thereafter, the cells were maintained in the medium (EMEM + 10% FBS) described above. Rat embryo (1193h) cells from the 26th to 31st subcultures were used.

Chemicals. Aromatic hydrocarbons were obtained from Eastman Organic Chemicals, Rochester, N. Y., and were dissolved in 1 ml of DMSO to yield a stock solution of 100 μg/ml, which was stored in the dark at 4°. The desired concentration was prepared by diluting stock solution with the medium (EMEM + 10% FBS).

Toxicity Tests. One day after planting 5 X 10^4 cells/ml from the 26th subculture in Falcon plastic Petri dishes, the medium (EMEM + 10% FBS) was removed and fresh medium containing various concentrations of chemical carcinogens in 0.5% DMSO was added. The control medium contained 0.5% DMSO. The media with carcinogens were changed twice for 7 days, and then the attached cells were trypsinized and counted.

Transplantability of Transformed Cells. Newborn Fischer rats were inoculated s.c. with various numbers of freshly trypsinized cells in order to determine the transplantability of the transformed cells.

RESULTS
One day after rat embryo cells, at 26th subculture, were plated at a density of 5 X 10^6 cells/ml, the medium (EMEM + 10% FBS) was removed and replaced with media containing 3MC at concentrations of 1.0 and 0.5 μg/ml in 0.5% DMSO. The control media contained 0.5% DMSO. After 7 days of treatment with the chemical carcinogen, 2 dishes from each group were trypsinized and the cells were counted. The results in Table 1 show that these cells were susceptible to the toxicity of 3MC because they grew more slowly than the DMSO controls. It was also evident that these cells grow well in the presence of 0.5% DMSO.

In the above experiment, after incubation of the cells with 3MC for 7 days, replicate dishes were washed and refed with carcinogen-free growth medium. Every 2 to 3 days, fresh 3MC-free medium (EMEM + 10% FBS) was supplied. Ten days later the cultures were subdivided by trypsin treatment. This was repeated every 7 to 10 days thereafter for additional subcultures. In the 3MC-treated rat embryo cells, morphologi-
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cal alteration of cells and an abnormal pattern of the growth were noted in the 4th subculture 40 to 43 days after treatment; a similar change was not observed in the rat embryo cells treated with DMSO. This change was first observed in the rat embryo cells treated with 3MC, 1.0 µg/ml. Foci of transformed cells consisted largely of criss-crossing, randomly oriented, spindle-shaped cells with nuclear and cytoplasmic overlapping which stained heavily with Giemsa (Fig. 1A). In contrast, the cellular morphology remained unchanged in the untreated rat embryo cell line, which continued to grow in monolayers of normal-appearing, fibroblast-like cells (Fig. 1B) despite the fact that they were subcultured 10 times during the ensuing 84 days after the chemical treatment.

Increased growth rate was observed in the 3MC-transformed cells, as indicated by the fact that they grew faster than untreated cells and had to be subcultured much more frequently. The growth rates compared under identical conditions of culture indicated that rat embryo cells transformed by 3MC grew at about 2 to 3 times the rate of the normal rat embryo cells (Table 2). The inhibitory effect of 3MC, 1.0 µg/ml, on growth rate of transformed cells for 7 days indicated that the transformed cells were more resistant to the toxicity of 3MC than were the untransformed rat embryo cells.

For determination of tumorigenicity of the transformed cells, cells were trypsinized, centrifuged, suspended in growth medium, and injected s.c. into newborn Fischer rats (Table 3). All the inoculated rats given 1.0 to 3.2 X 10⁶ cells developed fibrosarcomas (Fig. 1C) within 8 days at the site of inoculation. The injection of 10⁴ cells/rat led to more than 50% tumor incidence, and the tumors appeared within 40 days. No tumors developed during observation over a period of 3 months in the group of rats inoculated with cell suspensions prepared from the untreated, untransformed cultures. The tumors were progressive, reaching 8 cm in diameter within 10 weeks after inoculation and before all the animals died from their tumors. Metastasis was not observed; however, the tumor cells were readily established in several subpassage transplants to newborn and adult rats. Cells established from the tumors resembled the original transformed cells (Fig. 1D).

An additional experiment to test the transformation effects of DMBA, 0.01 µg/ml, and BP, 10 µg/ml, was carried out with rat embryo cells at 31st subculture. The method of DMBA and BP treatment was the same as described above. The results

### Table 1

<table>
<thead>
<tr>
<th>Addition to media</th>
<th>Initial cell no./ml</th>
<th>Final av. cell no./ml</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% DMSO control</td>
<td>5 x 10⁴</td>
<td>120 x 10⁴</td>
<td>0</td>
</tr>
<tr>
<td>0.5% DMSO + 3MC, 0.5 µg/ml</td>
<td>5 x 10⁴</td>
<td>81 x 10⁴</td>
<td>33</td>
</tr>
<tr>
<td>0.5% DMSO + 3MC, 1.0 µg/ml</td>
<td>5 x 10⁴</td>
<td>94 x 10⁴</td>
<td>22</td>
</tr>
</tbody>
</table>

* a Cells were treated with 3MC for 7 days.
* b S-1193h line at the 26th subculture.

### Table 2

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Passage</th>
<th>Initial cell no./ml</th>
<th>Final av. cell no./ml at Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% DMSO</td>
<td>7</td>
<td>5 x 10⁴</td>
<td>1.5 x 10⁵ 3.4 x 10⁵ 5.2 x 10⁵ 9.4 x 10⁵</td>
</tr>
<tr>
<td>0.5% DMSO + 3MC, 0.5 µg/ml</td>
<td>7</td>
<td>5 x 10⁴</td>
<td>2.1 x 10⁵ 4.3 x 10⁵ 1.1 x 10⁶ 2.3 x 10⁶</td>
</tr>
<tr>
<td>0.5% DMSO + 3MC, 1.0 µg/ml</td>
<td>7</td>
<td>5 x 10⁴</td>
<td>2.4 x 10⁵ 5.7 x 10⁵ 2.1 x 10⁶ 3.1 x 10⁶</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Subculture after 3MC or DMSO treatment</th>
<th>Days after 3MC removed</th>
<th>No. of cells inoculated/rat</th>
<th>Days after inoculation</th>
<th>No. of tumors⁡/no. inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>6</td>
<td>51</td>
<td>3.2 x 10⁴</td>
<td>111</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>3MC, 1.0 µg/ml</td>
<td>6</td>
<td>51</td>
<td>3.2 x 10⁴</td>
<td>6</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>3MC, 0.5 µg/ml</td>
<td>6</td>
<td>51</td>
<td>3.2 x 10⁴</td>
<td>6</td>
<td>5/5</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>8</td>
<td>69</td>
<td>1.0 x 10⁴</td>
<td>103</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>3MC, 0.5 µg/ml</td>
<td>8</td>
<td>69</td>
<td>1.0 x 10⁴</td>
<td>8</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>3MC, 0.5 µg/ml</td>
<td>8</td>
<td>69</td>
<td>1.0 x 10⁴</td>
<td>30</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>3MC, 0.5 µg/ml</td>
<td>8</td>
<td>69</td>
<td>1.0 x 10⁴</td>
<td>40</td>
<td>3/5</td>
</tr>
</tbody>
</table>

* a The tumors grew progressively and were sectioned and diagnosed as fibrosarcomas by Dr. L. Rabstein.
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Table 4

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Transformation</th>
<th>Subculture after chemical or DMSO treatment</th>
<th>No. of cells inoculated/rat</th>
<th>Days after inoculation</th>
<th>No. of tumors(^a)/no. inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>7</td>
<td>(3.5 \times 10^6)</td>
<td>80</td>
<td>0/5</td>
</tr>
<tr>
<td>DMBA 0.01 (\mu g/ml)</td>
<td>+</td>
<td>7</td>
<td>(4.7 \times 10^6)</td>
<td>12</td>
<td>5/5</td>
</tr>
<tr>
<td>BP, 10 (\mu g/ml)</td>
<td>+</td>
<td>7</td>
<td>(3.3 \times 10^6)</td>
<td>8</td>
<td>5/5</td>
</tr>
</tbody>
</table>

\(^a\) The tumors were sectioned and diagnosed as fibrosarcomas by Dr. L. Rabstein.

were similar: (a) similar morphological changes were observed in the rat embryo cells treated with DMBA or BP but not in the untreated rat embryo cells (Fig. 2); (b) or cells treated with DMBA or BP grew faster than the nontransformed rat embryo cells; (c) transformed cells produced tumors when inoculated into newborn homologous rats (Table 4).

**DISCUSSION**

The experiments described herein indicate that treatment of chemical carcinogens induced in vitro transformation of rat embryo cells. The rat cells transformed by chemical carcinogens had the following properties: (a) the carcinogen-treated cells were morphologically altered and grew as randomly oriented multilayers; (b) the treated cells showed loss of contact inhibition and increased growth rates; (c) the 3MC-treated transformed cells were more resistant to the toxic effect of 3MC; (d) when inoculated into newborn rats the transformed cells produced progressively growing serially transplantable tumors.

Namba et al. (15) recently observed the transformation of rat embryo cells by 4-nitroquinoline 1-oxide. However, successful transformation was achieved only by 19 repeated intermittent treatments given over a period of 125 days. Gutmann et al. (7) obtained malignant transformation by exposure of rat embryo cells to carcinogenic aryl amides or their activated metabolites up to 4 times at 3- to 5-day intervals. Induction of carcinoma has been reported by in vitro exposure of epithelial-like cells from rat liver to various doses of chemical carcinogens for various intervals (22). Various treatments of carcinogens were with aflatoxin B1 (0.05 to 0.1 mg/liter, 24 hr), \(N\)-hydroxy-\(N\)-2-fluorenylacetamide (1 mg/liter, 22 weeks), or DMBA (0.1 mg/liter, 26 weeks, or 1 mg/liter, 4 weeks). We recently obtained in vitro transformation by exposure of rat kidney cells to DMBA once for 7 days (17).

The mechanisms of chemical carcinogenesis are at present unknown. Three cellular mechanisms have been proposed: (a) the chemical carcinogen selects malignant cells through clonal selection of preexisting potential malignant cells (16); (b) the carcinogen transforms the cells by derepressing natural host cell oncogenes (14); (c) there is activation of the latent oncogenes of RNA tumor virus genomes (12, 18, 21). Huebner et al. (9–11) recently concluded that inherited transmitted type C RNA virus genomes possess characteristics which implicate them as basic determinants of chemically induced tumors. Although the exact mechanism of the in vitro transformation process remains unknown, the data described herein suggest that chemical induction of oncogenesis can be studied in cell cultures derived from rat embryos.

Studies are in progress to quantitate further the chemical in vitro transformation of the rat embryo cell system described above.

**ACKNOWLEDGMENTS**

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**REFERENCES**


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Fig. 1. A, transformed rat embryo cells 45 days after exposure to 1.0 µg of 3MC for 7 days, showing criss-crossing, spindle-shaped cells with nuclear and cytoplasmic overlapping. Giemsa stain, x 70. B, rat embryo cells 45 days after exposure to 0.5% DMSO for 7 days. Giemsa stain, x 70. C, fibrosarcomas induced in a newborn Fischer rat by implantation of 1.0 × 10⁴ cells transformed in vitro by 3MC, 1.0 µg/ml. Giemsa stain, x 70. D, typical field of a culture originated from primary tumor in C (unstained, x 70). Cells resembled the original transformation (A).
Fig. 2. **A**, transformed rat embryo cells 48 days after exposure to 0.01 μg of DMBA for 7 days. Giemsa stain, X 42. **B**, rat embryo cells 48 days after exposure to 0.5% DMSO for 7 days. Giemsa stain, X 42.
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