IN VITRO CARCINOGENESIS OF HEPATOCYTES OBTAINED FROM ACETYLAMINOFLUORENE-TREATED RAT LIVER AND PROMOTION OF THEIR GROWTH BY PHENOBARBITAL*1

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The hepatic cells of rats being fed acetylaminofluorene were transferred into culture at various times. Proliferative hepatocytic foci were obtained from animals treated with the carcinogen for more than 9 weeks. These hepatocytic foci became transplantable and capable of growth in soft agar after 4 to 12 months in culture. Phenobarbital markedly enhanced the growth of these hepatocytes in culture.

Key words: Hepatocarcinogenesis — Acetylaminofluorene — Promotion — Phenobarbital — In vitro carcinogenesis — Rat

The wide validity of the concept of two-stage carcinogenesis, originally based on observations in mouse skin carcinogenesis, has now been confirmed in various experimental systems.29) To achieve a comprehensive understanding of carcinogenesis, the features and mechanisms of both initiation and promotion should be studied analytically in appropriate systems. Hepatocarcinogenesis in rodents is one such system, because of the availability of promoters such as phenobarbital (PB) and also the presence of enzyme-altered islands (EAI) as a practical indicator for quantitative assessment of initiation and promotion.12, 14, 15, 24, 26, 32, 34, 37) EAI's of hepatocytes have been considered to be progenies of initiated cells, and therefore important precancerous lesions.10, 24, 36

The mechanism of promotion by PB is still not clear. Peraino23) suggested that the promotive effect of PB is not likely to be associated with immunosuppression, stimulation of cell proliferation or facilitation of the initiation process, but most probably with anabolic action. While this is a reasonable postulation, and we have observed a selective growth-enhancing action of PB on EAI-cells,11, 13) one can still argue that PB may not act directly on EAI-cells and that the promotive effect of PB may arise from selective toxicity on normal hepatocytes, a mechanism which has been shown to be important for the enhancement of hepatocarcinogenesis in vivo.31, 35)

In an attempt to establish a suitable system for analyzing the mechanism of promotion, we adapted the in vivo—in vitro system of hepatocarcinogenesis, modifying previous trials.5, 13, 19, 27, 30) The present paper describes the general features of our experimental system and the growth-promotive effect of PB on cultured EAI-cells.

MATERIALS AND METHODS

Animals and Carcinogen Feeding Male Donryu rats weighing about 150 g were purchased from Nihon Rat Co., Urawa. They were kept in an air-conditioned room. The rats were fed a diet (CE-II, CLEA Japan Inc., Tokyo) containing 0.03% 2-acetylaminofluorene (AAF) (Tokyo Kasei Co., Tokyo) for the initial...
6 weeks and then a diet containing 0.06% AAF for 6 weeks. On this regimen the rats developed multiple EAIs during 6–9 weeks and macroscopic hyperplastic nodules by 12 weeks. Isolated hepatocytes were obtained from animals at 0, 6, 7, 9, 12 and 24 experimental weeks by the perfusion method.

**Perfusion Technique** The enzymatic perfusion technique originally developed by Berry and Friend\(^1\) and applied in a previous experiment\(^13\) was used with further modification. The animals were anesthetized with sodium pentobarbital (5 mg/100 g body weight) and injected with 100 units of heparin intraperitoneally. Two-thirds partial hepatectomy was carried out just before starting perfusion, because the anterior large lobes of carcinogen-treated animals were difficult to perfuse well. The liver was perfused via the vena cava with 30 ml of Swim’s 77 medium for 1 min initially and then with 200 ml of Swim’s 77 medium containing 0.05% collagenase (Worthington Biochem. Co., N.J.) for 20 min. The perfused liver was minced on a No. 120 wire mesh and isolated cells were obtained by filtration. Approximately 10\(^6\) cells were plated on a No. 120 wire mesh and isolated cells were obtained by filtration. About 2 \times 10\(^6\) cells were injected sc into syngeneic newborn rats within 24 hr after delivery. The inoculated animals were observed for up to 12 months for development of tumors. The developed tumors were studied histologically on routine paraffin sections and also histochemically for activities of glucose-6-phosphatase by the method of Wachstein and Meisel\(^36\) and \(\gamma\)-glutamyl transpeptidase by the method of Rutenberg et al.\(^26\).

About 10\(^4\) cells were seeded in a 4 ml overlay composed of 0.4% agar and 20% fetal calf serum in Waymouth’s medium on top of a 5 ml underlay containing 0.5% agar and 10% fetal calf serum in Waymouth’s medium in a 60 mm Petri dish.

**Promotion by Phenobarbital** Hepatocytes obtained from animals at 12 weeks of AAF feeding were plated onto 40 dishes in every experiment. Half of them were cultured with the medium containing 1.5 mmol of PB, the rest being cultured without PB. The medium was changed twice a week. At the end of the 12th week of culture, the dishes were stained with 4% Giemsa and the number and size of epithelial cell foci were scored.

### Table I. Duration of AAF Feeding and Establishment of Transplantable Epithelial Cell Lines in Culture

<table>
<thead>
<tr>
<th>Weeks on AAF diet</th>
<th>No. of perfusions (No. of rats)</th>
<th>No. of perfusions giving transplantable epithelial cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>12</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td>12 (+12)*</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

* 12 weeks on AAF diet and then 12 weeks on basal diet
varied from a few to many. Several representative epithelial cell foci were selected from every perfusion and subcultured after about 3 months, depending on the growth rate. With repeated subcultures, the growth rate of epithelial cells increased. When a sufficient number of cells became available, the transplantability and the growth-capability in soft agar were tested. About 40 foci from 10 different perfusions were studied in this manner.

In the earliest case, transplantability was seen at the beginning of the 4th month of culture. Most of the foci became transplantable during 5–12 months. The transplantability and growth-capability in soft agar correlated quite well.

Some representative histologies of the transplanted epithelial cells are shown in Figs. 4 and 8, together with their morphology in culture plates (Figs. 3 and 7). There is no question that they are hepatocellular carcinomas of either trabecular type or mixed trabecular and glandular type. They invariably showed activity of γ-glutamyl transpeptidase and occasionally that of glucose-6-phosphatase both in vivo (Fig. 5) and in vitro (Fig. 6).

Promotion of Growth of Epithelial Cells by Phenobarbital The results of 4 separate experiments are listed in Table II. Representative plates with or without PB treatment and after staining with Giemsa are shown in Fig. 9, and representative microscopic features of epithelial cell foci stained with Giemsa are presented in Figs. 10 and 11. It is apparent that the number and size of epithelial cell foci are much greater in PB-treated plates. In perfusions 2 and 4, epithelial cell foci appeared only in PB-treated plates by the end of the 3rd month.

DISCUSSION

The present experiments have clearly shown the growth-promoting action of PB on cells from EAI, or progenies of AAF-initiated cells, in culture. The statement that the proliferative epithelial cell foci observed in this experiment are derived from EAI-cells is warranted by the following observations. (1) The proliferative epithelial cell foci were obtained only from animals fed AAF for more than 9 weeks, when AAF-induced EAI become florid.10,30 (2) Cells selectively obtained from EAI or hyper-
Figs. 3~6. Morphological and biochemical features of a hepatocytic line: Fig. 3. Morphology in culture plate at passage 18. Phase contrast, ×90. Fig. 4. Section of a tumor arising from sc injection of the cells, showing a trabecular pattern of hepatocellular carcinoma. Hematoxylin and eosin, ×180. Fig. 5. Histochemical section of the tumor showing marked activity of glucose-6-phosphatase, ×90. Fig. 6. Glucose-6-phosphatase activity in the cultured cells. ×36.
Fig. 7. An epithelial cell focus with some irregularity in arrangement in culture at passage 6. Phase contrast, ×90.

Fig. 8. Section of a tumor arising from sc inoculation of the cells shown in Fig. 7. Note glandular formation of tumor cells with goblet cell metaplasia. Hematoxylin and eosin, ×90.

Table II. Promotion by Phenobarbital of the Growth of Epithelial Cells Transferred into Culture Systems from the Livers of Rats Fed AAF for 12 Weeks

<table>
<thead>
<tr>
<th>Perfusion No.</th>
<th>PB</th>
<th>No. of plates</th>
<th>1~4</th>
<th>5~9</th>
<th>10~19</th>
<th>20~</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+</td>
<td>15</td>
<td>0.40</td>
<td>0.73</td>
<td>1.07</td>
<td>0.20</td>
<td>2.40</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>10</td>
<td>0</td>
<td>0.40</td>
<td>0.70</td>
<td>0.60</td>
<td>1.70</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>12</td>
<td>0.92</td>
<td>0.67</td>
<td>0.25</td>
<td>0</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>20</td>
<td>2.84</td>
<td>0.58</td>
<td>0.21</td>
<td>0.16</td>
<td>3.79</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>11</td>
<td>0.09</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.09</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>19</td>
<td>1.42</td>
<td>0.84</td>
<td>0</td>
<td>0</td>
<td>2.26</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>16</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>+</td>
<td>66</td>
<td>1.48</td>
<td>0.70</td>
<td>0.35</td>
<td>0.09</td>
<td>2.62</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>48</td>
<td>0.02</td>
<td>0.08</td>
<td>0.15</td>
<td>0.13</td>
<td>0.38</td>
</tr>
</tbody>
</table>

* Checked at the end of the 3rd month of culture
Fig. 9. Plates stained with Giemsa showing the promoting effect of PB on the growth of epithelial cell foci at the 12th week of culture. Upper two; PB-treated plates showing two large epithelial cell foci. Lower two; control plates showing only a tiny epithelial cell focus (far right).

Figs. 10 and 11. Microscopic features of the large epithelial cell foci shown in Fig. 9. Giemsa. Fig. 10, × 36, Fig. 11, × 90.
plastic nodules can survive in culture systems much longer than normal hepatocytes.\(^{13}\)

(3) They were positive for $\gamma$-glutamyl transpeptidase activity.\(^ {17}\) It may reasonably be concluded, therefore, that PB also acts in vivo on cells of EAI, directly resulting in promotion of carcinogenesis.

Previously Guillouzo et al.\(^ {6}\) observed increased multiplication of explanted hepatocytes from newborn rat liver in the presence of PB in the culture medium. In the present experiment PB did not stimulate replicating activity of normal hepatocytes from adult rat liver in culture. In vivo, replication-stimulating action of PB on normal hepatocytes was observed, but only transiently.\(^ {11,22}\)

An in vitro system has advantages for detailed investigation of the various phases of carcinogenesis. Models of two-stage carcinogenesis in vitro have been introduced by utilizing fibroblasts\(^ {18,20}\) or epidermal cells\(^ {4}\) as target cells and phorbol esters as promoters. However, no experimental model of two-stage carcinogenesis of hepatocytes in vitro is available so far, although there have been many publications on in vitro carcinogenesis with established epithelial or epithelial-like cell lines obtained from normal rat liver.\(^ {2,5}\)

The present in vivo–in vitro system of hepatocarcinogenesis is probably more useful for studying the effects of various types of chemicals, including promoters, on initiated cells than the systems utilizing established "hepatocyte" lines which have actually deviated substantially from normal hepatocytes, at least functionally. It is noteworthy that the histology of the tumors of transplanted cells in the present study (Figs. 4 and 8) generally showed much greater histological similarity to rat hepatic carcinomas chemically induced in vivo than to those derived from in vitro-transformed "normal hepatocytes" reported in the literature.\(^ {5,21,36}\)

References