Promoting Effect of 12-O-Tetradecanoylphorbol-13-acetate on the in Vitro Malignant Transformation of Fetal Rat Brain Cells Exposed in Utero to Ethylnitrosourea

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ABSTRACT

In order to investigate the possibility that the theory of two-stage carcinogenesis can be applied to neurogenic carcinogenesis, we analyzed the promoting effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on the in vitro malignant transformation of fetal rat brain cells exposed in utero to ethylnitrosourea (ENU). Rat brain cells were transferred to a cultured system at 72 h after a single pulse of ENU (50 mg/kg body weight) to pregnant SD-JCL rats on the 18th day of gestation. The positive findings of glial fibrillary acidic protein and S-100 protein in primary cultured cells by the analysis of immunohistochemistry revealed the neuroglial origin of transformed cells. These cells were divided into 12 groups and were treated twice a week with or without TPA at concentrations from 0.1 to 50.0 ng/ml. From the results of cellular morphology, Concanavalin A agglutinability, colony forming capacity in semisolid soft agar, and tumorigenicity in vivo, malignant transformation of fetal rat brain cells appeared earlier in the ENU group treated with TPA than in the untreated ENU group. On the basis of these observations, it is suggested that TPA might be effective as a tumor promoter on ENU-induced neurogenic carcinogenesis.

INTRODUCTION

The evidence of two-stage carcinogenesis has been most clearly established in mouse skin in vivo (1). Initiation is presumed to involve damage to DNA, and in the promotion phase, the expression of malignancy may be brought about the continuous addition of an agent that is not carcinogenic and mutagenic by itself (2–6). The most potent promoters in mouse skin are esters of the diterpene alcohol phorbol isolated from Croton triglum plants. The most active agent of the phorbol esters is TPA,2 which has been extensively studied in experimental carcinogenesis (4, 7–9). Although studies of promoting agents against various organs (7, 10, 11) and culture systems (9) and of transplacental carcinogenesis (8) have been described, there is no evidence that the theory of two-stage carcinogenesis was applied to neurogenic carcinogenesis in in vitro or in vivo studies.

ENU is well known as a most potent experimental carcinogen in the nervous system (12, 13). Recently, attention has been directed to the use of in vivo-in vitro assay for the analysis of ENU induced transplacental carcinogenesis, because it is cheaper and quicker than in vivo studies (14).

MATERIALS AND METHODS

Reagents. TPA, ENU, Con A (type V), and α-methylmannoside were obtained from Sigma Chemical Co., St. Louis, MO.

Treatment of Animals with ENU. Pregnant SD-JCL rats on the 18th day of gestation were given injections i.p. with a single pulse of freshly prepared ENU (50 mg/kg body weight) dissolved in 0.1 M phosphate buffer, pH 6.0. The controls were given injections i.p. with an equal volume of 0.1 M phosphate buffer.

Transfer of Fetal Rat Brain Cells to a Cultured System. At 72 h after the ENU pulse in vivo, 7 to 8 fetal cerebral hemispheres were freed from meninges, mechanically dissociated into fragments about 0.5 mm³, incubated for 30 min at 37°C in Disperse solution (1000 units/ml) (Godo Susco Co., Tokyo, Japan), and passed through a 100-μm stainless-mesh. Single cell suspensions were plated on 60-mm Falcon plastic dishes (Falcon Plastics, Oxford, CA).

Conditions of Tissue Culture. Cultured cells were maintained in modified Eagle’s medium with Earle’s salt supplemented with fetal bovine serum (10%), L-glutamine (398 μg/ml), and antibiotics (penicillin G (100 IU/ml) and streptomycin (100 μg/ml)) and were grown by monolayer culture at 37°C in a humidified chamber with 5% carbon dioxide and 95% air atmosphere. These cultured reagents were obtained from Flow Laboratories, Irvine, Scotland. The control cells or the ENU-exposed cells at primary culture were divided into 6 groups, as follows: (a) without treatment; (b) with acetone; (c) with TPA (0.1 ng/ml); (d) with TPA (1.0 ng/ml); (e) with TPA (10.0 ng/ml); and (f) with TPA (50.0 ng/ml). TPA was dissolved in acetone and diluted in growth medium. Final concentration of acetone was 0.01% (v/v). TPA or acetone was added twice a week into cultured medium beginning 4 days after the transfer of fetal rat brain cells to culture. These agents and growth medium were renewed every 3 or 4 days. Confluent cells were harvested by trypsinization with 0.25% trypsin in PBS for 10 min and diluted to the cell concentration of 5 × 10⁶ cells/ml for passaging. At least 3 dishes were used in each experimental group.

Immunohistochemistry for GFAP and S-100 Protein. Cultured cells were fixed with pre-cooled McClean’s paraformaldehyde-periodate-lysins fixative for 30 min and were rinsed with PBS at 4°C. The peroxidase-antiperoxidase immunohistochemical method of Sternberger et al. (15) was used for qualitative analysis of GFAP and S-100 protein in the cells using rabbit anti-GFAP and rabbit anti-S-100 protein serum (Dako Corp., Santa Barbara, CA).

Colony Forming Assay in Semisolid Agar Medium. The procedures were based on the methods of MacPherson and Montagner (16). Single cells (1 × 10⁵) of each experimental group that were in various culture stages and were suspended in 4 ml of 0.33% Special Noble Agar (Difco Laboratories, Irvine, Scotland).
Laboratories, Detroit, MI) supplemented with growth medium were plated on growth medium containing 0.5% soft agar. At 14 days after cell plating, colonies consisting of over 50 cells were counted, and plating efficiency under 0.01% was evaluated as meaning no ability for colony formation. Experiments were done at least three times in each experimental group.

Agglutinability to Con A. The procedures were based on the methods of Kakizoe et al. (17) with our modifications.

Agglutinability was assayed in a final volume of 40 μl of PBS containing 1 x 10⁶ cells/ml and Con A (200 or 400 μg/ml) with or without α-methyl-D-mannoside (100 μg/ml), which was used as a competitive inhibitor to Con A. After gentle mixing on a micromixer for 30 min at 37°C, the number of aggregates was counted in a hemocytometer. An aggregate of more than 3 cells was estimated as positive agglutination, regardless of the number of cells in it, and the percentage of these aggregates per 200 single cells was determined.

Tumorigenic Growth of Cultured Cells in Vivo. Single cells (1 x 10⁵ cells/10 μl of growth medium) of each experimental group at various intervals after culture were injected into the right parietal lobe of 4-week-old male SD-JCL rats. The rats with growing tumor showed signs of decreased body weight, hemiparesis, hemiconvulsion, and/or paraparesis first at 26 days after implantation. At 30 days after implantation of cultured cells, all animals were sacrificed and examined for the growth of tumor at the implanted site macroscopically and microscopically.

RESULTS

Morphological Changes of Fetal Rat Brain Cells after Culture. At primary culture, fetal rat brain cells of the control group (Fig. 1A) were composed of flattened large epithelial cells mixed with a few small cells containing fine long cytoplasmic processes; they did not differ from the ENU-exposed cells (Fig. 1B). These primary cultured cells of the control group and the ENU group were stained positively for GFAP (Fig. 2A) and S-100 protein (Fig. 2B). Although the ENU-exposed cells treated with TPA showed the typical “piled up focus” at 30 days after culture (Fig. 3A), the ENU-exposed cells showed the regular pavement growth pattern at confluency (Fig. 3B). The appearance of “piled up focus” in the ENU-exposed cells did not become evident until 120 days after culture. At 90 days after culture, the ENU-exposed cells treated with TPA showed the small appearance of cytoplasm with long bipolar cytoplasmic processes (Fig. 4A), in contrast to ENU-exposed cells (Fig. 4B), the appearance of which resembled the primary cultured cells. These morphological changes observed in the ENU-exposed cells treated with TPA were more obvious at the 1.0 ng/ml concentration of TPA than at other concentrations. During culture, control cells treated with TPA did not show any distinct morphological changes in comparison with untreated control cells (Fig. 1A), and ENU-exposed cells treated with acetone did not show morphological changes in comparison with ENU-exposed cells. At later stages of culture, morphologically transformed cells treated with or without TPA showed positive staining for GFAP and S-100 protein by immu-
Fig. 3. Morphologies of cultured cells at 30 days after culture. A, ENU-exposed cells treated with TPA (1.0 ng/ml); B, ENU-exposed cells. Phase contrast, x 400.

Fig. 4. Morphologies of cultured cells at 90 days after culture. A, ENU-exposed cells treated with TPA (1.0 ng/ml); B, ENU-exposed cells. Phase contrast, x 400.

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nohistochemistry (data not shown).

Colony Forming Assay in Semisolid Agar Medium. At 40 days after culture, plating efficiency in the ENU group treated with TPA was 2.50 ± 0.72% (SD), in contrast to 0% plating efficiency in the ENU group (Table 1). Although the plating efficiency in the ENU group was 1.80 ± 0.51% at 150 days after culture, this value was significantly lower than that of the ENU group treated with TPA (1.0 or 50.0 ng/ml). The ENU group treated with acetone did not show any significant differences in plating efficiency compared with the ENU group throughout culture. The control group with or without TPA did not show any growth of colony on the semisolid agar during culture.

Agglutinability to Con A. As for the agglutinability to Con A (200 μg/ml) at 37°C for 30 min, progressive increase of percentage of Con A agglutinability was shown in the TPA-treated ENU group beginning 30 days after culture, whereas Con A agglutinability in the ENU group was not evident until 90 days after culture (Fig. 5). No significant differences between the Con A agglutinability in the untreated ENU group and the ENU group treated with acetone were found throughout culture. The Con A agglutinability in the control group with or without TPA was 0% during culture.

Tumorigenic Growth of Cultured Cells in Vivo. Although the ENU-exposed cells treated with TPA showed tumorigenicity at 60 days after culture, the ENU-exposed cells showed tumorigenicity at 150 days after culture (Table 2). At 150 days after culture, percentages of tumorigenicity in ENU-exposed cells treated with TPA (1.0, 10.0, or 50.0 ng/ml) were 100, 70, and 60%, respectively, in contrast to 10% tumorigenicity in ENU-exposed cells. Macroscopically, at 30 days after implantation of cultured cells, a large tumor with central necrosis was formed at the implanted site, with clear demarcation from surrounding brain tissue. Microscopically, tumor tissue that was formed with implantation of the ENU-exposed cells treated with TPA (Fig. 6A) consisted of a compact cellular component with many mitotic figures, depending on the concentration of TPA, but that seen in the ENU group (Fig. 6B) consisted of more loose cellular components with many giant multinucleated cells and necrotic areas.

DISCUSSION

The presented data showed that TPA promoted the in vitro malignant transformation of fetal rat brain cells exposed to ENU in vivo. Various criteria for the malignant transformation of normal cells in culture have been described, such as morphological "piled up focus" (18, 19), growth in suspension of a semisolid medium (1, 16, 20), increase of Con A agglutinability (21, 22), increased saturation density (5), induction of plasminogen acti-
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Table 1

Changes in colony forming efficiency in semisolid soft agar medium at various intervals after culture

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>0 day</th>
<th>10 days</th>
<th>20 days</th>
<th>40 days</th>
<th>80 days</th>
<th>120 days</th>
<th>150 days</th>
<th>180 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENU only</td>
<td>0.04</td>
<td>0.021</td>
<td>0.087</td>
<td>1.8</td>
<td>3.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENU plus TPA (0.1 ng/ml)</td>
<td>&lt;0.01</td>
<td>0.008</td>
<td>0.006</td>
<td>0.026</td>
<td>0.037</td>
<td>0.042</td>
<td>0.031</td>
<td>0.088</td>
</tr>
<tr>
<td>ENU plus TPA (1.0 ng/ml)</td>
<td>2.08</td>
<td>3.10</td>
<td>3.41</td>
<td>4.02</td>
<td>5.06</td>
<td>6.02</td>
<td>7.01</td>
<td>8.02</td>
</tr>
<tr>
<td>ENU plus TPA (10.0 ng/ml)</td>
<td>6.03</td>
<td>8.52</td>
<td>9.31</td>
<td>10.0</td>
<td>11.0</td>
<td>12.0</td>
<td>13.0</td>
<td>14.0</td>
</tr>
<tr>
<td>ENU plus TPA (50.0 ng/ml)</td>
<td>3.97</td>
<td>5.25</td>
<td>6.31</td>
<td>7.59</td>
<td>8.71</td>
<td>9.82</td>
<td>10.92</td>
<td>12.0</td>
</tr>
<tr>
<td>ENU plus acetone (0.01%)</td>
<td>0.030</td>
<td>0.053</td>
<td>0.061</td>
<td>0.093</td>
<td>0.135</td>
<td>0.230</td>
<td>0.330</td>
<td>0.430</td>
</tr>
</tbody>
</table>

% Plating efficiency (%) in semisolid soft agar was calculated as follows:

\[
\% = \frac{\text{No. of colony}}{\text{No. of plated cells}} \times 100
\]

* + SD.
** Differs from the ENU group at \( p < 0.005 \).
* Differs from the ENU group at \( p < 0.05 \).
* Not significant.

Table 2

Tumorigenicity of cultured cells in vivo

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>60 days</th>
<th>90 days</th>
<th>120 days</th>
<th>150 days</th>
<th>180 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENU only</td>
<td>0/8(0)</td>
<td>0/10(0)</td>
<td>0/9(0)</td>
<td>1/10(10)</td>
<td>2/10(20)</td>
</tr>
<tr>
<td>ENU plus TPA (1.0 ng/ml)</td>
<td>2/8(25)</td>
<td>8/10(80)</td>
<td>9/9(100)</td>
<td>10/10(100)</td>
<td>5/5(100)</td>
</tr>
<tr>
<td>ENU plus TPA (10.0 ng/ml)</td>
<td>1/8(12.5)</td>
<td>5/10(50)</td>
<td>7/10(70)</td>
<td>7/10(70)</td>
<td>4/5(80)</td>
</tr>
<tr>
<td>ENU plus TPA (50.0 ng/ml)</td>
<td>1/5(20)</td>
<td>6/8(75)</td>
<td>6/10(60)</td>
<td>6/10(60)</td>
<td>5/5(100)</td>
</tr>
<tr>
<td>ENU plus acetone (0.01%)</td>
<td>0/6(0)</td>
<td>0/5(0)</td>
<td>0/6(0)</td>
<td>0/10(0)</td>
<td>1/5(20)</td>
</tr>
</tbody>
</table>

* Numbers in parenthesis, percentage of tumorigenicity was calculated as follows:

\[
\% = \frac{\text{No. of rats that formed tumors}}{\text{No. of rats for implantation}} \times 100
\]

Fig. 5. Changes of percentage of Con A agglutinability in each experimental group at various intervals after culture. O, ENU group; A, ENU group treated with TPA (1.0 ng/ml); C, ENU group treated with TPA (50.0 ng/ml); D, ENU group treated with acetone. Points, mean of 3 replicate cultures; bars, SD.

Fig. 6. Photomicrographs showed the histologies of growing tumors at 30 days after implantation of cultured cells at 150th day after culture. A, ENU group treated with TPA (1.0 ng/ml); B, ENU group. H & E, x 400.

Correlated with the malignant transformation of normal cells in culture (16, 25). Laerum and Rajewsky (14) reported that the malignant transformation of fetal rat brain cells after a single transplacental pulse of ENU (75 mg/kg body weight) occurred at 100 and 200 days after culture and was characterized by the
findings of capacity to form colonies in semisolid agar medium and of tumorigenicity in vivo. Our results seen in the ENU group treated with or without acetone showed almost the same results as those shown in the previous report (14). In contrast to the ENU group, the continuous treatment with TPA of the ENU-exposed cells caused an earlier appearance of "piled up focus" [about 90 days (Fig. 3)] and the earlier occurrence of colony formation in semisolid soft agar [about 110 days (Table 1)].

Finally, tumorigenicity in the ENU group treated with TPA appeared earlier (about 90 days) than it did in the ENU group (Table 2). The frequency of tumors at 150 or 180 days after culture was increased with the treatment of TPA, in contrast to initiation by ENU only. Moreover, with the analysis of plant lectin Con A agglutinability, which was also thought to be an indicator of in vitro malignant transformation and a probe in studies on changes of the cell surface of transformed cells (21, 22), the addition of TPA caused the Con A agglutinability at more early cultured stage than that in ENU initiation only (Figure 5). Morphologically, primary cultured cells exhibited large, flattened epithelial cells mixed with a few small cells that were thought to be immature glioblasts (26). These cells were considered to be neuroglial cells from the evidence of the positive stain for GFAP and S-100 protein (Fig. 2, A and B) (27–29). The theory of two-stage carcinogenesis in neurogenic carcinogenesis might be suggested because TPA acted as a tumor promoter against rat brain cells in culture after the intraperitoneal exposure of ENU.

As for the effect of TPA on the nervous system, even though it was reported that TPA caused the blockage of spontaneous neurite formation in mouse neuroblastoma cell (30) and the inhibition of nerve growth factor-induced neurite outgrowth in embryonic chick ganglion cells (31), there has been no evidence of the existence of promoting agents of neurogenic carcinogenesis except for the report of the posttreatment of gonadectomy after the exposure of carcinogen (32). While this report showed that orchietomized male progeny caused a carcinogenesis except for the report of the posttreatment of the endocrine system than did intact males, any findings of tumor promotion multiplicity and greater frequency of peripheral tumors in nervous system has not been seen in the previous reports. Our results showed that orchiectomized male progeny caused a carcinogenesis in END group (Table 2).

In conclusion, TPA promoted the in vitro malignant transformation of fetal rat brain cells pretreated with the transplacental carcinogen ENU. Further studies to determine the exact mechanism of this promoting action of TPA on neurogenic carcinogenesis are necessary.

REFERENCES

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