GROWTH STIMULATION OF HAMSTER EMBRYONIC FIBROBLASTS BY PHORBOL ESTERS*1

Kazuo UMEZAWA and Sanae FUJIE

Department of Molecular Oncology, Institute of Medical Science, University of Tokyo*2

The growth of hamster embryonic fibroblasts was highly dependent on the serum concentration in the medium. A tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), enhanced the growth of hamster embryonic fibroblasts in the medium containing low concentrations of serum. Among phorbol derivatives, phorbol-12,13-didecanoate and 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate also showed growth stimulation, but 4α-phorbol-12,13-didecanoate, phorbol-12,13-diacetate and phorbol did not. TPA increased the accumulation of 2-deoxyglucose into the cells at low serum concentrations. Thus, hamster embryonic fibroblasts were sensitive to phorbol esters as growth stimulators, and TPA reduced the serum requirement of the cells.

Key words: Tumor promoter — Serum requirement — 2-Deoxyglucose transport — Epidermal growth factor

12-O-Tetradecanoylphorbol-13-acetate (TPA) was isolated from croton oil as an active tumor-promoting principle. In cell cultures, TPA induces morphological alteration,3 inhibits terminal differentiation,1 and increases the syntheses of ornithine decarboxylase11 and plasminogen activator.10 It also increases the saturation density of 3T3 cells2 and of human diploid fibroblasts.2 TPA appears to mimic malignant transformation in cultured mammalian cells. However, one of the characteristics of transformed cells is that they have a reduced serum requirement for growth.6 We have therefore investigated the growth-promoting effect of TPA and its effect on hexose transport in hamster embryonic fibroblasts at various concentrations of serum.

Materials and Methods

Materials Syrian golden hamsters were purchased from Tokyo Experimental Animals Co. Ltd., Tokyo. TPA, phorbol-12,13-didecanoate (PDD), 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate (4-O-methyl-TPA), 4α-phorbol-12,13-didecanoate (4α-PDD), phorbol-12,13-diacetate (PDA) and phorbol were purchased from Consolidated Midland Corporation, Brewster, N.Y. Epidermal growth factor (EGF) and 125I-EGF were purchased from Collaborative Research, Inc., Waltham, Mass. 2-[3H(G)]-deoxy-D-glucose (8.26 Ci/mmol) was purchased from New England Nuclear, Boston, Mass.

Cell Culture A primary cell line was prepared from Syrian golden hamster embryos at 13 days of gestation. The cells were obtained by trypsinization of embryos after removal of the head and viscera. The cells were cultured for 2 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (Microbial Associates, Walkersville, Md.). Batches of 5 × 10⁶ cells were sealed in ampoules with 8% dimethyl sulfoxide and stored in liquid nitrogen until use. The cryopreserved cells were thawed and cultured for 3 days in medium with 20% fetal bovine serum. After trypsinization, samples of 10⁵ cells were inoculated into 25 cm² culture flasks with 4 ml of medium containing the indicated amount of fetal bovine serum and chemicals, and incubated in 10% CO₂ in air at 37°. The cell number was counted with a hemocytometer after trypsinization.

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*2 Shirokanedai 4-6-1, Minato-ku, Tokyo 108 (柴崎一夫, 藤江幸苗).
Hexose Transport Assay Subconfluent cultures of hamster embryonic fibroblasts in 35 mm culture dishes were incubated in DMEM containing various concentrations of serum for 2 hr, then TPA was added and incubation was continued for another 2 hr. Cell monolayers were washed once with Dulbecco's phosphate-buffered saline (PBS) at 37°C, and incubated for 10 min at 37°C in 0.5 ml of PBS with 2 μCi/ml of 2-[^3H](G)-deoxy-d-glucose. The cells were then washed 3 times with ice-cold PBS and solubilized in 1 ml of 1% Triton X-100, 5 mg/ml trypsin, 2 mg/ml EDTA. The cell lysate was removed and the dishes were rinsed with 1% sodium dodecyl sulfate.

EGF Binding Assay A subconfluent culture of hamster embryo cells was washed once with PBS, then 2 ml of serum-free DMEM containing 1 mg/ml bovine serum albumin and 0.05 μCi of ^125I-EGF were added. Chemicals were added just prior to the addition of ^125I-EGF. The plates were incubated for 2 hr at 37°C in a 10% CO₂ atmosphere. The medium was then removed, and the cells were washed 3 times with ice-cold DMEM and solubilized with 1 ml of 1% Triton X-100, 5 mg/ml trypsin, 2 mg/ml EDTA. After incubation for one hour, the cell lysate was removed and 0.5 ml of 1% sodium dodecyl sulfate was added. The radioactivity of the total lysate was counted by liquid scintillation. The value of nonspecific binding (200~300 cpm) was subtracted from all binding assay data.

Results

The growth of hamster embryonic diploid fibroblasts depends on the serum concentration in the medium, being rapid at high serum concentrations. Hamster embryonic cells did not grow in medium containing 2% fetal bovine serum, but addition of TPA at 0.1 μg/ml to the medium induced cell division (Fig. 1A). In medium with 5% or 10% serum, the addition of 0.1 μg/ml of TPA enhanced the growth of the cells, as shown in Fig. 1B and 1C. In medium with 20% serum, however, TPA did not increase the growth of the cells, as shown in Fig. 1D. The effect of the concentration of TPA on the cell saturation density in medium with 5% serum is shown in Fig. 2. The maximal effect was obtained with TPA at 0.01~0.1 μg/ml. Increases of cell saturation density induced by diterpene derivatives are shown in Table I. 4-O-Methyl-TPA and PDD increased the cell saturation density to the same extent as TPA (P<0.001), but 4α-PDD, PDA and phorbol had no effect.

Surface binding of EGF was inhibited by TPA in hamster embryonic fibroblasts; the 50% inhibitory dose was 0.06 μg/ml (Table I). PDD and 4-O-methyl-TPA also inhibited EGF binding at 0.08 μg/ml and 0.50 μg/ml, respectively. 4α-PDD, PDA and phorbol did not inhibit EGF binding below 1 μg/ml.

Addition of 0.05 μg/ml of TPA enhanced the accumulation of 2-deoxyglucose in hamster embryonic fibroblasts at serum concentrations of 0, 2, 5 and 10%, but not at 20%.
Fig. 2. Dose–response relation for the growth-stimulating activity of TPA

Cells (10⁶) were incubated in 4 ml of medium containing 5% serum with the indicated concentrations of TPA. The medium was changed every other day. On day 6, the cells were trypsinized and counted. Points represent means of duplicate samples.

Fig. 3. Stimulation of hexose transport by TPA

Hamster embryonic fibroblasts were incubated with (●) or without (○) 0.05 μg/ml of TPA for 2 hr. Values are means of duplicate samples.

Table I. Growth Stimulation and Inhibition of Epidermal Growth Factor (EGF) Binding by Phorbol Esters

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Cell number on day 6(a) (No. of cells × 10⁵/flask)</th>
<th>ID₅₀ for EGF binding(b) (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.0 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>14.0 ± 0.9</td>
<td>0.06</td>
</tr>
<tr>
<td>PDD</td>
<td>13.5 ± 0.5</td>
<td>0.08</td>
</tr>
<tr>
<td>4-O-methyl-TPA</td>
<td>14.2 ± 0.4</td>
<td>0.50</td>
</tr>
<tr>
<td>4α-PDD</td>
<td>8.9 ± 0.4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>PDA</td>
<td>8.5 ± 2.2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Phorbol</td>
<td>8.5 ± 0.1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

(a) Cells (10⁶) were incubated with 0.1 μg/ml of a designated chemical in 4 ml of medium containing 5% serum. The medium was changed every other day, and on day 6 the cells were trypsinized and counted. Values are means for 4 replicate samples ± standard deviation.

(b) Details are given in “Materials and Methods.” The control value was 2300 cpm/dish.

(Fig. 3). The effect of TPA was most prominent in the absence of serum.

DISCUSSION

It has been reported that TPA slightly inhibits logarithmic growth but increases the saturation density of C3H 10T1/2 cells. However, we found that TPA slightly enhanced the logarithmic growth rate and increased the saturation density of primary cultures of hamster embryo cells in medium with a low serum concentration. Since TPA had no effect on cell growth at a high serum concentration, it appears to reduce the serum requirement of the cells. The two protein growth factors, EGF and fibroblast growth
factor (FGF), stimulated the growth of hamster embryo cells at 0.025 μg/ml, EGF causing a slightly greater increase in the cell saturation density than FGF when the serum concentration was low (data not shown). The effects of TPA in stimulating cell division were similar to those of EGF and FGF.

In vivo studies on structural analogs of TPA showed that PDD has tumor-promoting activity on mouse skin, while 4-O-methyl-TPA, 4α-PDD, PDA and phorbol have little or no activity. Therefore, the effects of these chemicals in stimulating the growth of cultured cells were roughly correlated with their in vivo tumor-promoting activities.

It has been reported that EGF binding in HeLa cells is blocked by TPA and related tumor promoters. Although 4-O-methyl-TPA showed growth stimulation in the same way as TPA, a much higher concentration was required to inhibit EGF binding than in the case of TPA. The growth stimulating effect of phorbol esters may be saturated at 0.1 μg/ml.

The decrease of serum requirement by TPA was also reflected in 2-deoxyglucose uptake. Activation of the uptake of hexose from the medium may contribute to the growth-stimulating effect of TPA.

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REFERENCES