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AGGREGATION OF HUMAN LYMPHOBLASTOID CELLS BY TUMOR-PROMOTING PHORBOL ESTERS AND DIHYDROTELEOCIDIN B

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SUMMARY: Human lymphoblastoid cells transformed by Epstein-Barr virus aggregated rapidly in the presence of tumor-promoting phorbol esters and dihydroteleocidin B. Cell aggregation was almost complete after incubation for 6 hours. In amounts of a few ng, they induced significant aggregation. Their abilities to aggregate cells could be measured quantitatively and correlated well with their effects in promoting skin tumors.

INTRODUCTION

Tumor-promoting agents are themselves noncarcinogenic, but they can induce tumors in animals previously exposed to limited amounts of carcinogens. The tumor promoters that have been studied most extensively in vivo and in vitro are phorbol esters (1-3). We found recently that dihydroteleocidin B (4) was a naturally occurring potent tumor promoter (5). Tumor-promoting phorbol esters are reported to have various effects, not only on epidermal cells and fibroblasts (3,6-8), but also on lymphoid cells (3,9-10). Some of their actions are expressed rapidly and thought to be mediated through their interactions with the cell membrane (3,11,12). We found that phorbol esters and indole alkaloids, dihydroteleocidin B and teleocidin, induce aggregation of human lymphoblastoid cells rapidly in a dose-dependent manner. This cell aggregation is easy to be measured quantitatively and so the assay system described in this paper should be useful in screening compounds with tumor-promoting activity.

Abbreviations : EB, Epstein-Barr : ODC, ornithine decarboxylase, : PBS, phosphate buffered saline (135mM NaCl, 2.7mM KCl, 5.3mM Na_2HPO_4, and 1.45mM KH_2PO_4) ; PDD, phorbol-12, 13-didecanoate ; 4α-PDD, 4α-phorbol-12, 13-didecanoate ; TPA, 12-O-tetradecanoyl-phorbol-13-acetate ; 4-O-Me-TPA, 4-O-methyl-TPA.

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MATERIALS AND METHODS

Cells : NL-3 and XPL-17 cells are human lymphoblastoid cell lines transformed by EB virus. They were established from peripheral lymphocytes of a normal adult and a patient with xeroderma pigmentosum, respectively, and were kindly supplied by Dr. A. Oikawa of Tohoku University (13). They were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum in a humidified incubator at 37°C under 5% CO_2. In order to decrease spontaneous aggregation, cells were seeded at a concentration of 3-4 x 10^5/ml and passaged every other day.

Chemicals : Phorbol, TPA, 4-O-Me-TPA, PDD and 4α-PDD were purchased from Consolidated Midland Corporation, Brewster, New York. They were dissolved in distilled water containing 30% acetone at a concentration of 1mg/ml and then diluted with PBS to 100 μg/ml. Dihydroteleocidin B and teleocidin (4) were gifts from Dr. M. Takashima, Fujisawa Pharmaceutical Industries, Ltd., Osaka, Japan. Dihydroteleocidin B is a hydrogenated compound of teleocidin, which is isolated from Streptomyces. Dihydroteleocidin B and teleocidin were dissolved in acetone at a concentration of 1 mg/ml and diluted with PBS to 10 μg/ml. Hydrolysed dihydroteleocidin B was prepared by heating dihydroteleocidin B in 6 N HCl containing 4% thioglycolic acid at 110°C for 20 hours. Under these conditions, the amide bond of the lactam ring was completely hydrolysed. Croton oil was obtained from Sigma Chemical Co., St. Louis, Mo. All chemicals were stored at -20°C and further diluted with PBS just before use.

Estimation of cell aggregation : Lymphoblastoid cells grew in suspension and formed aggregates of loosely associated cells (13). Since these aggregates were readily dispersed, a suspension consisting almost entirely of single cells could be obtained by pipetting the culture. Cells were collected by centrifugation at 350 x g for 5 minutes and resuspended by pipetting at a concentration of 7-9 x 10^5 single cells/ml in fresh medium containing 2 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid buffer (pH 7.2). Samples of 1 ml of cell suspension were seeded onto Falcon tissue culture plates No. 3001 (35 x 10 mm) containing 0.1 ml PBS with various amounts of chemicals. The cells were cultivated for 6 hours in a humidified incubator at 37°C under 5% CO_2. The degree of cell aggregation was estimated by counting the numbers of single cells in the dishes ; for this, the dishes were rocked back and forth several times to obtain a homogeneous cell suspension and then aliquots were removed and the numbers of single cells in them were determined in a hemocytometer. Duplicate dishes were used for each concentration of each compounds. ED_{50} was taken as the concentration of the test compound that decreased the number of single cells in the control culture by 50%.

RESULTS

Time course of cell aggregation

TPA and dihydroteleocidin B at a concentration of 100 ng/ml induced remarkable aggregation of NL-3 cells after cultivation for 6 hours (Fig. 1). The aggregates formed in the culture containing them could not readily be dissociated by pipetting and were larger and more spherical than those formed in the control culture. The time course of aggregation was examined in the presence of TPA (100 ng/ml), phorbol (1 μg/ml) dihydroteleocidin B

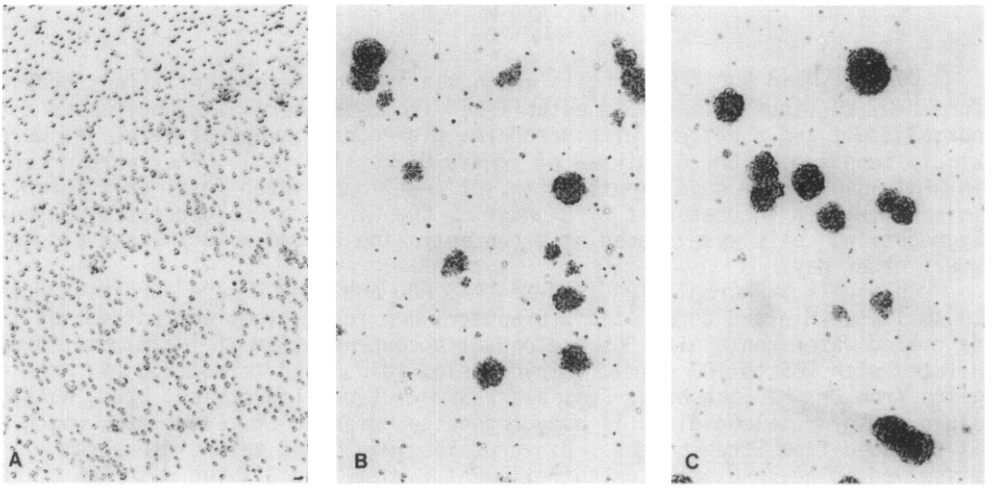


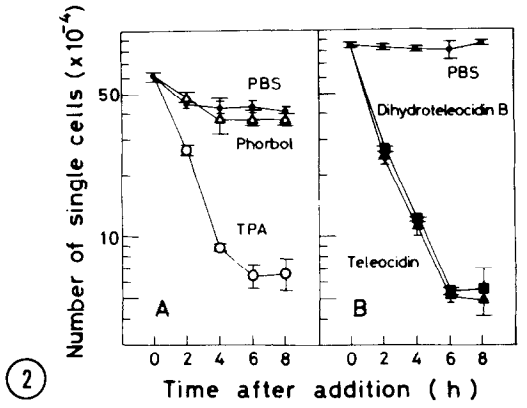
Fig. 1. Aggregation of NL-3 cells after incubation for 6 hours in the presence of (A) PBS, (B) TPA (100 ng/ml), and (C) dihydroteleocidin B (100 ng/ml) (magnification, x 28).

(100 ng/ml), and teleocidin (100 ng/ml)(Fig. 2). The effects of TPA, dihydroteleocidin B, and teleocidin on cell aggregation were apparent within 2 hours after seeding and after 6 hours, most of the cells formed aggregates, whereas 80-100% of the cell remained single in the control culture. Phorbol had no significant effects (Fig. 2). Teleocidin is not yet proven to be a tumor promoter, but ODC inducing activity of teleocidin is similar to that of dihydroteleocidin B (4,5).

XPL-17 cells showed a similar time course of aggregation in the presence of TPA and formed more aggregates spontaneously than NL-3 cells (data not shown). We also examined whether other cell lines showed increased cell aggregation after treatment with TPA and dihydroteleocidin B. The lines tested were the Raji cell line and the cell lines of murine origin, e.g., L1210, plasmacytoma (X5563), AKR lymphoma (WC-2), radiation-induced lymphoma (RLδ1) and lymphoma induced by Moloney leukemia virus (YAC). These cell lines were not aggregated remarkably.

Quantification of the effects of tumor promoters

NL-3 cells were seeded in the presence of various concentrations of TPA, dihydroteleocidin B, and teleocidin and the numbers of single cells were counted



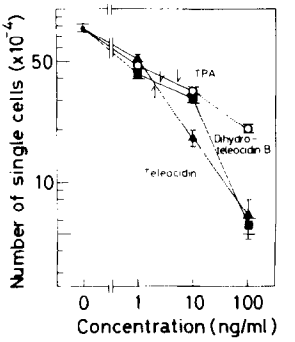


Fig. 2. Time course of cell aggregation. NL-3 cells were seeded into dishes in the presence of (A) TPA (100 ng/ml)(○), phorbol (1 μg/ml)(△), or PBS alone (●) and (B) dihydroteleocidin B (100 ng/ml)(■), teleocidin (100 ng/ml)(▲), or PBS alone (●). At various times, numbers of single cells were counted. Ranges of values are shown by bars.

Fig. 3. Dose response of cell aggregation. NL-3 cells were seeded into dishes containing indicated concentrations of TPA (○), dihydroteleocidin B (■), and teleocidin (▲). After 6 hours, numbers of single cells were counted. The arrows indicate ED_{50}'s. Ranges of values are shown by bars.

after 6 hours (Fig. 3). Aggregation was detectable with TPA, dihydroteleocidin B, and teleocidin even at a concentration as low as 1 ng/ml. The numbers of viable cells in the culture did not decrease and most cells did not adhere to the dishes (data not shown). From Fig. 3, ED_{50} values for cell aggregation were obtained as indicated by arrows. With TPA, several independent experiments using NL-3 and XPL-17 cells gave ED_{50} values of 11.2 and 3.9 ng/ml, respectively. The ED_{50} values of PDD, 4-O-Me-TPA, 4α-PDD, and croton oil were determined in the same way (Table 1). The ED_{50} of phorbol, which has no tumor-promoting activity, was more than 10 μg/ml. These ED_{50} values correlated well with the tumor-promoting activities of these compounds in vivo (1-3,10). Then abilities of indole alkaloids to induce aggregation of NL-3 cells were determined (Table 2). The ED_{50} values of dihydroteleocidin B and teleocidin were 6.5 and 3.1 ng/ml, respectively. Their abilities to induce cell aggregation were similar to or even greater than that of TPA. Hydrolysed dihydroteleocidin B, which could not induce ODC activity in mouse skin (5), was two to three hundred times less effective in inducing cell aggregation than unhydrolysed dihydroteleocidin B

Table 1. Quantification of activities of TPA and related compounds in inducing aggregation of NL-3 and XPL-17 cells

Compound	ED_{50} (ng/ml) NL-3	ED_{50} (ng/ml) XPL-17	Tumor-promoting activity
TPA	11.2 ± 6.7[a] (6)[b]	3.9 ± 0.4 (11)	+
PDD	50 ± 23 (2)	15 ± 3.8 (6)	+
4-O-Me-TPA	8,500 ± 4,600 (2)	2,700 ± 290 (3)	±
4α-PDD	>10,000 (2)	4,200 ± 1,900 (3)	-
Phorbol	>10,000 (2)	>10,000 (2)	-
Croton oil	69 ± 49 (2)	67 ± 11 (2)	+

a : Mean ED_{50} ± SEM
b : Number of independent experiments

DISCUSSION

In this work we found that EB virus-transformed human lymphoblastoid cells, NL-3 and XPL-17, aggregated rapidly and firmly in the presence of phorbol esters and indole alkaloids, dihydroteleocidin B and teleocidin. The abilities of phorbol esters to aggregate cells correlated well with their respective effects in promoting tumors in mouse skin (Table 1). Also a good correlation was observed between the abilities of indole alkaloids

Table 2. Quantification of activities of dihydroteleocidin B and related compounds in inducing aggregation of NL-3 cells

Compound	ED_{50} (ng/ml)	ODC induction
Dihydroteleocidin B	6.5 ± 2.3[a] (5)[b]	+
Teleocidin	3.1 ± 0.1 (5)	+
Hydrolysed dihydroteleocidin B[c]	460 ± 140 (2)	-
HCl/dihydroteleocidin B[d]	1.7 ± 0.8 (2)	+

a and b : as in Table 1
c : After hydrolysis in 6 N HCl containing 4% thioglycolic acid at 110°C for 20 hours, the mixture was dried up.
d : After 6 N HCl containing 4% thioglycolic acid was dried up, dihydroteleocidin B was added.

to aggregate cells and to induce ODC activity. That is, when dihydroteleocidin B was hydrolysed, it became ineffective in inducing ODC activity in mouse skin (5) and also became quite ineffective in inducing aggregation of lymphoblastoid cells (Table 2). On treatment with TPA, dihydroteleocidin B, and teleocidin, cell aggregation was detectable within 2 hours (Fig. 2). Unlike spontaneous aggregates of cells, those formed in the treated culture could not readily be dissociated by pipetting the cell suspension. Thus these tumor promoters increased the intercellular adhesiveness, possibly by changing the membrane structure of lymphoid cells. Both TPA and dihydroteleocidin B are potent tumor promoters and induce ODC activity in mouse skin, adhesion of human promyelocytic leukemia cells (HL-60), and inhibition of terminal differentiation of Friend cells(4). Thus the assay system developed in this work would detect some of non-phorbol tumor promoters that had TPA-like activities. This system is simple and rapid. So it could be used as a screening test for certain types of tumor-promoting agents.

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