3T3 FIBROBLASTS ARE STIMULATED BY 12-*O*-TETRADECANOYL-PHORBOL-13-ACETATE TO PRODUCE THYMOCYTE-ACTIVATING FACTORS

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1. Introduction

12-*O*-Tetradecanoyl-phorbol-13-acetate (TPA) is a potent tumor-promoting agent in carcinogenesis of mouse skin and also has various biological activities [1]. Recent studies concerning the activity of TPA have been extensively performed by using in vitro cultured cells. For example, TPA-treated fibroblast and epidermal cells produced specific proteins [2–4]. However, the biological activities of these proteins have not been elucidated. There are several reports that TPA stimulates lymphoid cells to produce various factors which regulate immune responses: interleukin 1 and 2 [5,6]. Thus, TPA-treated fibroblast cells may produce factors which influence immune responses. Here, we show that TPA-treated fibroblast cells produce several factors which regulate thymocyte DNA synthesis and discuss the physiological significance of these factors.

2. Materials and methods

3T3 fibroblast cells were obtained from Dr S. Kurata (Department of Radiation Biology and Health, University of Occupational and Environmental Health) and maintained in vitro culture in RPMI 1640 medium (Grand Island Biological Co.–Gibco) supplemented with 100 μg streptomycin/ml, 100 units penicillin/ml and 5% fetal calf serum (FCS, Gibco). This cell line becomes confluent usually in 3 days by 1:5 ratio split. The confluent cultured cells were washed once with RPMI 1640 medium and TPA or other phorbol esters were added at a final concentration of 100 ng/ml in RPMI 1640–FCS 5%. After incubation at 37°C for 8 h, the culture supernatant was collected and concentrated by salting out with 60% of $(NH_4)_2SO_4$ (pH 7.5). After centrifugation at 16 000 rev./min for 15 min by a Beckman JA-20 rotor, the precipitate was dissolved in 1.5 ml 10 mM phosphate-buffered saline (PBS) (pH 7.2), applied on a Sephadex G-100 column (1.8 × 24 cm), and eluted with 10 mM PBS (pH 7.2). In the case of trypsin digestion experiment, the original sample was dialyzed against PBS (pH 7.2), incubated with trypsin (25 μg/ml) at 30°C for 30 min and fractionated by the same Sephadex G-100 column.

Thymocyte proliferation assay was performed by a modification of the method in [7]: 2.5×10^5 thymocytes of C3H/He mice were cultured in vitro with 2.5 μg/ml of concanavalin A (con A) in 0.2 ml RPMI 1640–FCS 5% in wells of microtiter culture plates (Falcon no. 3042) at 37°C in 5% CO_2 and 95% air for 2 days. The culture supernatant of 3T3 fibroblast cells or each fraction from the gel filtration was added at 50% final conc. The cells were labeled with 1 μCi [^{3}H]thymidine (5 Ci/mmol, Radiochemical Center, Amersham) for 24 h and harvested by an aid of automatic cell harvester (Abe Kagaku Co.). Thymidine incorporated into thymocytes was counted by a Beckman liquid scintillation counter. The results were expressed as means and standard errors of cpm/well of triplicate assays.

3. Results and discussion

A culture supernatant of TPA-treated 3T3 fibroblast cells strongly enhanced con A-dependent [^{3}H]-thymidine incorporation of mouse thymocytes as compared with that of untreated cells (table 1). This supernatant had no effect on thymocyte response

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Table 1
Effects of various phorbol esters on the induction of lymphocyte activating factors

3T3 fibroblast stimulated with	[^{3}H]Thymidine incorporation (cpm)	
	Con A (–)	Con A (+)
–	607 ± 235	5755 ± 456
Phorbol	1425 ± 495	17 799 ± 1609
Phorbol myristate	296 ± 49	11 364 ± 1098
Phorbol-13-acetate	1520 ± 501	9994 ± 1414
TPA	396 ± 136	99 141 ± 3611

without con A. Culture supernatants of 3T3 cells treated with other phorbol esters; phorbol, phorbol-12-monomyristate, phorbol-13-monoacetate showed considerable effects on con A-dependent thymocyte response. But, TPA showed the strongest effect amongst various phorbol esters. These results suggest that TPA-treated fibroblast cells produce thymocyte-activating factors.

To investigate the nature of thymocyte activating factors, we performed Sephadex G-100 column chromatography. In the case of TPA-treated cells, the activating factors were fractionated into 2 major peaks (fig.1); one is a sharp peak at ~13 000 M_r; the other is a slightly broad peak at ~30 000 M_r (fig.1b). In contrast, these activities could not be detected in untreated cells (fig.1a). Furthermore, some inhibitory activities were detected at the positions at 100 000 M_r and 1500 M_r. When the factors were partially digested by trypsin treatment (25 μg/ml, 30 min at 30°C) before the application to the gel filtration, the activities of these factors were decreased and appeared at lower M_r levels (fig.2). The activity of the 13 000 M_r peak seems to be relatively sensitive to trypsin treatment as compared with that of the 30 000 M_r peak. We investigated the heat sensitivity of these factors. The activity at 30 000 M_r peak was lost considerably (~50% of control activity) by the treatment at 90°C for 5 min, but the peak at 13 000 M_r was relatively resistant under the same conditions. This result sug-

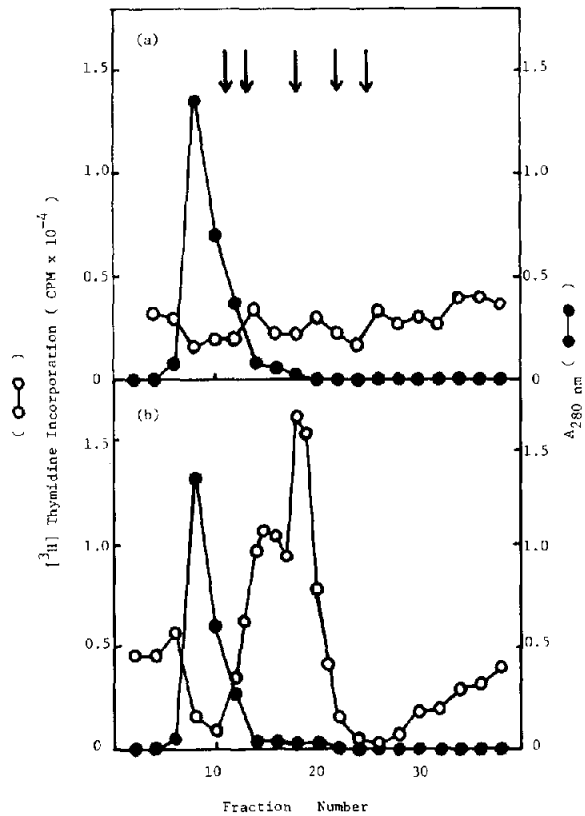

Fig.1. Sephadex G-100 column chromatography of culture supernatants from untreated (a) and treated (b) 3T3 fibroblast cells. The sample was collected from 30 ml culture supernatant as in section 2: (o——o) cpm [^{3}H]thymidine incorporation of thymocytes stimulated with con A; (•——•) absorbance at 280 nm. Arrows in the figure represent positions of bovine serum albumin (67 000 M_r), horseradish peroxidase (40 000 M_r), cytochrome *c* (13 500 M_r), insulin (5700 M_r) and bacitracin (1500 M_r) as marker proteins from the left to the right.

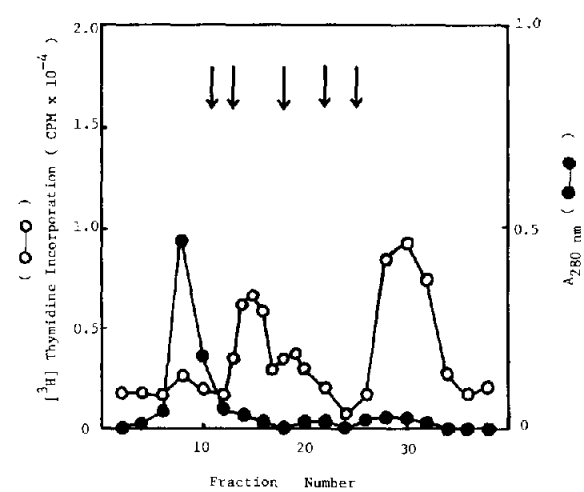

Fig.2. Sephadex G-100 column chromatography of TPA-treated 3T3 fibroblast culture supernatant after the trypsin digestion. The same amount of the sample in fig.1 was partially digested with trypsin treatment (25 μg/ml, 30 min at 30°C) and fractionated by the same procedures. The experimental conditions of other procedures and representation in the figure are the same as those of fig.1.

gests that these activating factors are proteins and that their properties are considerably different from each other.

Some researchers have reported the production of specific proteins by TPA-treated fibroblast or epidermis: in [2], 13 500 M_r and histidine-rich 27 000 M_r proteins were found at 12–48 h after TPA treatment on epidermal cells [2]; in [3] secretion of 35 000 M_r glycoprotein by TPA-treated 3T3 fibroblast cells was reported; in [4] synthesis of 41 000 M_r protein by mouse epidermis was shown. However, the physiological significance of these proteins has not been elucidated. The factors reported here may correspond to the specific proteins in [2–4]. TPA stimulates the production of interleukins by macrophages (15 000 M_r) [5] and T cells (~30 000 M_r) [6]. A similar factor (15–25 000 M_r) was found in murine keratinocyte which stimulates PHA-dependent thymocyte DNA synthesis [8].

Although the relationship between interleukins 1 and 2, keratinocyte factor and the factors derived from fibroblast remains unknown, this evidence suggests that fibroblast also regulates lymphocyte responses and may play some roles in the complex immune networks.

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