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Human Malignant Histiocytosis CD30+ DEL Cell Line Differentiates into Macrophage-like Cells When Treated with a Phorbol Diester[1]

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ABSTRACT

The histiocytic or lymphoid origin of human malignant histiocytosis is currently a subject of debate. The aim of this study was to investigate the *in vitro* effects of 12-*O*-tetradecanoylphorbol-13-acetate used as a differentiation inducer on the CD30, t(5;6) (q35;p21) DEL cell line, taken to be a reliable representative of the human malignant histiocytosis cell line.

Treatment of DEL cells with 33 nM 12-*O*-tetradecanoylphorbol-13-acetate for 6–24 h resulted in cell surface attachment (up to 80%), decrease in dividing ability, enhancement of nitro blue tetrazolium reducing capacity (from 8 to 42%), occurrence of a limited immunodependent phagocytosis, and transient increase in expression of tumor necrosis factor α gene and in production of tumor necrosis factor α protein, whereas tumor necrosis factor β remained undetectable. From these data, we can conclude that the malignant histiocytosis DEL cell line is not of lymphoid origin but stems from a myelomonocyte lineage.

INTRODUCTION

The nature of the stem cells proliferating in MH[3] is currently the subject of lively debate. By showing atypical immunohistochemical expression and frequent rearrangements for TCR-β and IgJH genes, numerous phenotypic and genotypic investigations have apparently failed to confirm the classical monocyte/macrophage histogenesis of MH, suggesting that it may be a variety of CD30+ (Ki-1+) anaplastic large cell lymphoma (1, 2). However, recent studies, performed mainly on MH cell lines, have provided results that contradict this last view and support instead the classical monocyte/macrophage origin (3–5). As was recently exemplified by the monocyte/macrophage induced differentiation observed in TPA treated HL-60, U937, and SU-DHL-1 cell lines (6–8), TPA induced differentiation represents a valuable approach in dealing with these unsettled histogenetic problems. This is what has prompted us to investigate the TPA induced differentiation of the DEL cell line, a CD30+, t(5;6) (q35;p21) MH cell line. It will be shown that the action of TPA results in cell attachment, enhancement of NBT reducing activity, appearance of an immunodependent phagocytosis, and modulation of transcription and translation of the TNF-α gene of DEL cells.

MATERIALS AND METHODS

DEL Cell Line. The DEL cell line is a MH, CD30+ cell line obtained from the pleural effusion of a child who died of MH. It bears the 5q35 breakpoint chromosomal abnormality, characteristic of MH (9, 10).

Cell Cultures. DEL cells were grown in RPMI 1640 medium containing 10% fetal calf serum, supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine at a density of 2–5 $\times 10^5$ cells/ml in a 5% CO_2 humidified atmosphere at 37°C. A T-cell leukemia cell line (CEM, originally from American Type Culture Collection), grown under the same culture conditions, was used for comparison.

Induction of Differentiation. For the differentiation experiments, DEL cells were maintained in exponential growth and treated with 33 nM TPA (Sigma Chemical Co., St. Louis, MO) in a time course experiment. Cell adhesion was determined by counting the percentage of nonadherent cells compared with the total cells. Viability was determined by trypan blue exclusion. Cytocentrifuge smears of cultured cells were examined for NBT reducing positive cells. Quantification was performed after 30 min incubation of 2×10^5 cells/ml with an equal volume of solution containing 0.2% NBT, 17 mg/ml bovine serum albumin, and 200 ng/ml freshly diluted TPA. The percentage of cells containing cytoplasmic dark formazan deposits was determined on Kernechtrot stained cytospin slide preparations by counting at least 300 cells in duplicate.

Effects of the Removal of TPA. In order to determine whether the induced differentiation was reversible, 24-h TPA treated DEL cells were washed in Hanks' solution (GIBCO, Gaithersburg, MD) and placed in fresh medium in the absence of TPA. Cell growth and viability were evaluated by trypan blue exclusion, and the ability of the cells to reduce NBT was simultaneously tested. In order to test whether TPA differentiation would affect tumorigenicity, 24-h TPA treated DEL cell suspension was injected s.c. into three nude mice (10^7 cells/mouse). Controls were conducted by injection, into three nude mice, of untreated DEL cell suspension from the same *in vitro* passage.

Phagocytosis. Functional changes induced by TPA were tested on the basis of the phagocytic capacity of DEL cells. Briefly, 0.8 μm latex particles (Difco, Detroit, MI), previously opsonized with human serum or mouse RBCs (30 min incubation of mouse RBCs in rabbit anti-mouse serum) were added to 33 nM TPA-treated DEL cells. Phagocytosis was evaluated on May-Grünwald-Giemsa stained cytospin smears or, at the ultrastructural level, on pelleted glutaraldehyde fixed cells.

RNA Extraction and Hybridization. Exponentially growing DEL cells were harvested from several different *in vitro* passages. Total cellular RNA was purified by the guanidine isothiocyanate-cesium chloride method (11), separated by electrophoresis through 1% agarose-formaldehyde gel, transferred to nitrocellulose paper, and hybridized to appropriate DNA probes. Hybridization was carried out for 16–24 h at 42°C in 50% formamide, 2× standard saline citrate, 5× Denhardt's, 0.1% sodium dodecyl sulfate, and 100 μg/ml salmon sperm DNA. Filters were washed to a stringency of 0.1× standard saline citrate at 55°C and exposed to Kodak XAR film. Autoradiograms were developed at different exposures. The following cDNA fragments were used: the *Pst*I fragment of the PE4 plasmid containing human TNF-α cDNA insert (12), the 0.8-kb *Pst*I fragment of the plasmid p16/P2 carrying the human TNF-β insert (13), and the 2.0-kb *Pst*I fragment of the chicken β-actin purified from PA1 plasmid (14). The DNA probes were labeled with [^{32}P]dCTP, using the multiprime DNA labeling system (Amersham Radiochemical Centre, Amersham, United Kingdom), described by Feinberg and Vogelstein (15). Quantitative evaluation of the specific transcripts was carried out by laser densitometric scanning of the autoradiograms (LKB 2202 Ultrascan).

L929 Bioassay. The TNF activity was measured by its cytolytic effect on the mouse fibrosarcoma cell line L929 (16). L929 cells were main-

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[3] The abbreviations used are: MH, malignant histiocytosis; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MH, malignant histiocytosis; NBT, nitro blue tetrazolium; TNF, tumor necrosis factor; cDNA, complementary DNA; kb, kilobase(s).

tained in RPMI medium with 10% fetal calf serum under standard culture conditions. For the assay, L929 cells were treated with trypsin (0.125%) (GIBCO), washed twice in culture medium, adjusted to a density of 2.5×10^5/ml, and plated (100 μl/well) in a 96-well, flat-bottomed culture plate. After incubation for 24 h at 37°C in 5% CO_2 atmosphere, the supernatant was discarded and replaced by 50 μl of the samples to be tested and 50 μl of actinomycin D (1 μg/ml) (Calbiochem, La Jolla, CA). The plate was further incubated for 18 h. Cytotoxicity was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) colorimetric assay (17) at 570 nm using a microplate reader (MR600; Dynatech, Alexandria, VA) with a reference filter at 630 nm. Samples were tested in duplicate, and results were expressed as lytic units per ml, 1 unit corresponding to a concentration of human recombinant TNF-α of 15–30 pg/ml, as defined by immunodetection. The specificity of this cytotoxic assay for TNF-α was determined by blocking DEL cell medium with serial dilutions of an anti-human recombinant TNF-α monoclonal antibody clone, 3D3 (Medgenix, Fleurus, Belgium).

RESULTS

Effects of TPA on Differentiation of DEL Cells. After 24–96-h exposure of exponentially growing DEL cells to 33 nM TPA, an almost total loss of autonomous proliferation occurred (Fig. 1), concurrently with adherence and polykaryon formation. More than 80% of the cells became firmly adherent to the flask substrate. As shown in Table 1, TPA treatment resulted in a progressive increase of the percentage of cells able to reduce NBT considerably. Whereas 5–8% of untreated cells spontaneously reduced NBT, more than 42% of the cells displayed this reducing property after 72 h of TPA exposure (Table 1). When 24-h TPA treated cells were placed in fresh medium without TPA for an additional 48 h, their proliferative capacity returned gradually. Subsequently, following 72 h of culture without an inductor, the percentage of NBT positive cells dropped back to 8%. S.c. injection of treated cell suspension in nude mice (10^7 cells/mouse) resulted in the development of a solid tumor within 5 weeks. Animals given injections of untreated cells developed tumors in a similar lapse of time.

Effects of TPA on Phagocytosis. Although untreated DEL cells were unable to spontaneously phagocytose opsonized and nonopsonized latex particles and mouse RBCs, TPA treatment induced a limited phagocytic capacity in them. The phagocytic activity of TPA treated cells was more pronounced for opsonized latex particles (4–5% of cells) than for mouse erythrocytes (3%) (Figs. 2 and 3).

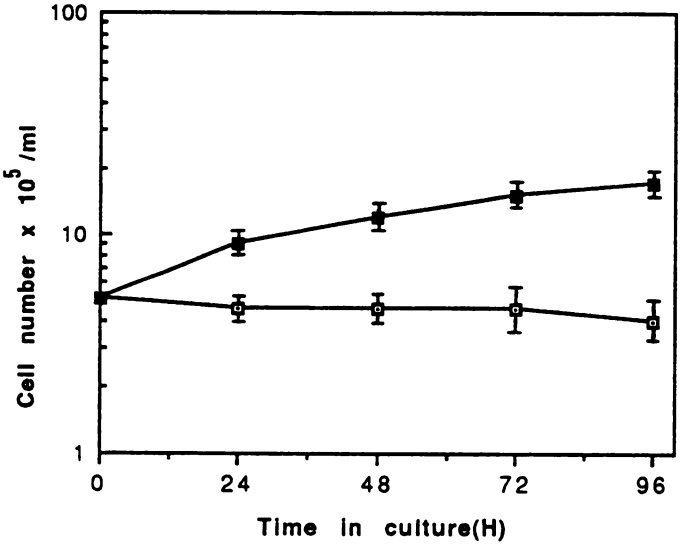

Fig. 1. Effect of TPA on DEL cell growth. DEL cells in logarithmic growth phase were seeded at 2×10^5/ml. ■, untreated DEL cells; □, 33 nM treated DEL cells. Values represent the mean ± SE for two separate experiments.

Table 1 *Effects of TPA on NBT reduction and adherence of DEL cells*

DEL cells were treated with 33 nM TPA for the indicated periods. The percentages of NBT positive and adherent cells were evaluated by counting 300 cells in duplicate. Results are expressed as mean ± SE of three experiments, each performed in duplicate.

Time of exposure (h)	NBT positive (%)	Adherence (%)
0	3.1 ± 0.5	3 ± 0.4
12	5.0 ± 0.4	32 ± 0.6
24	18 ± 0.6	82 ± 0.5
42	26 ± 0.2	72 ± 0.3
72	38 ± 0.4	46 ± 0.4

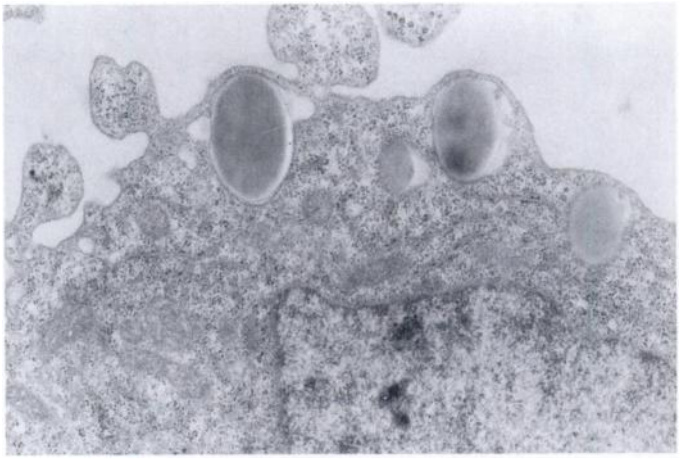

Fig. 2. Latex particles phagocytosed by 33 nM TPA treated DEL cells. Total duration of TPA exposure: 21 h; coculture with latex particles: 2 h. Electron microscopy, × 28,000.

Effects of TPA on TNF Gene Expression. Northern blot analysis of total cellular RNA from untreated DEL cells demonstrated a strong hybridization signal corresponding to the specific 1.7-kb TNF-α band. Following TPA treatment, levels of TNF-α increased from the third hour, reached a maximum by 6–12 h, and returned to normal by 24 h (Fig. 4). For comparison, there was no detectable change in actin mRNA levels (data not shown). TNF-β transcripts were not detected prior to or after induction of DEL cells, although specific TNF-β transcripts were seen in the control CEM cells (data not shown).

Effects of TPA on TNF Protein Production. Culture supernatants of untreated and TPA treated cells in a time course study, conducted over 96 h, were monitored for biological activity against TNF sensitive L929 cells. Low levels of TNF-α production, ranging between 10 and 12 units/ml, were detected in supernatants of untreated DEL cells. The level of TNF protein increased progressively throughout 24 h of TPA exposure. The maximum of 43 units/ml was attained after 6 h of exposure; the level declined thereafter to less than 10 units/ml (Fig. 5). The supernatant cytolytic activity could be inhibited by up to 83% by pretreatment of supernatants with neutralizing anti-human TNF monoclonal antibody.

DISCUSSION

The results of this study indicate that, following treatment by TPA, MH DEL cells were capable of differentiating along a more mature monocyte/macrophage lineage. Indeed, this TPA

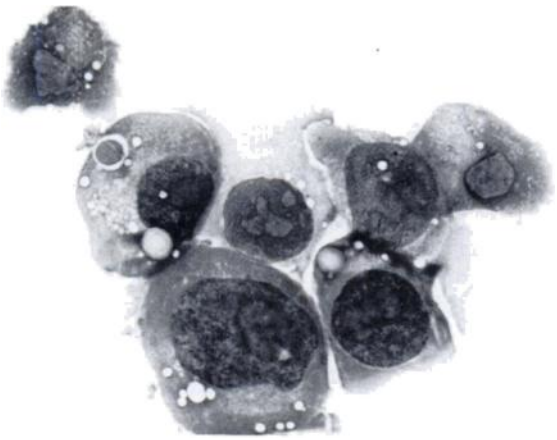

Fig. 3. Erythrophagocytic activity of 24-h, 33 nM TPA treated DEL cells. Time of coculture with mouse opsonized erthrocytes: 6 h. May-Grünwald-Giemsa, × 600.

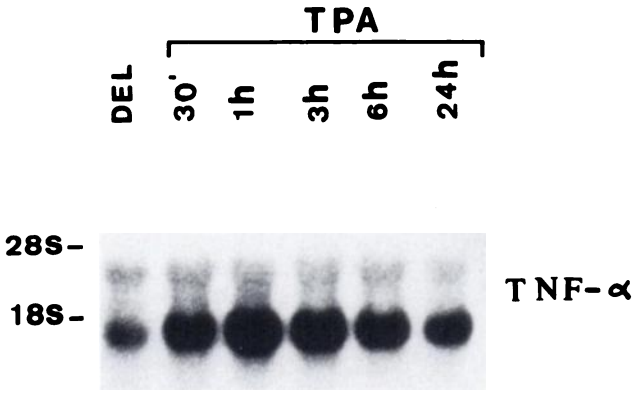

Fig. 4. Northern blot analysis of TNF-α RNA levels in DEL cells after exposure to 33 nM TPA at the indicated times. Total cellular RNA (20 μg/lane) was hybridized to a ^{32}P labeled TNF-α cDNA probe. *First lane* (*DEL*) RNA from untreated DEL cells. TPA induces approximately 4–5-fold increase of the TNF-α RNA level after 3–6-h treatment.

induced differentiation was characterized by growth inhibition, surface adherence, enhancement of NBT reducing capacity, appearance of an immunodependent phagocytic activity, and increased expression and production of TNF-α, whereas TNF-β was not expressed. Certainly, no single one of these properties, taken separately, is specific for lineage determination; however, taken as a whole, they provide convergent arguments in favor of a myelomonocytic origin.

Attachment to glass and plastic surfaces has, for a long time, been regarded as a valuable characteristic of monocyte/macrophage cells, and it is thought to be linked with the presence of some special surface receptors (18–20). As a rule, surface attachment is associated with a definite inhibition of cell growth. Indeed, 80% of 21/24-h TPA treated DEL cells became adherent and lost to a large extent their proliferative activity (Fig. 1).

The reduction of NBT into an insoluble blue formazan deposit is considered to be reliable evidence of cell oxygen consumption and production of H_2O_2 and free radicals (21, 22). The NBT reduction capacity is present in some specialized cells such as resting neutrophils, monocytes, and alveolar macrophages. This capacity increases dramatically during phagocytosis and after cell stimulation (23). In particular, Sherman and Lehrer (24) have demonstrated that TPA induces an increase in O_2^- production by adult rabbit alveolar macrophages. Since lymphoid cells have been found to be devoid of NBT reduction capacity, both spontaneously and after stimulation, a positive NBT test and especially an increased NBT level following stimulation can be used to characterize cells belonging to the granulocyte/macrophage lineage. The fact that the percentage of DEL cells able to reduce NBT increased from 5–8% to 42% following 72-h TPA exposure provides an indirect but valuable argument in favor of its myelomonocytic origin.

Immunodependent phagocytosis is generally regarded as characteristic behavior of the cells belonging to the myelocyte/macrophage lineage. Although untreated DEL cells were unable to phagocytose opsonized and nonopsonized material, the fact that TPA treatment was able to induce a phagocytic activity toward opsonized latex particles and mouse RBCs strongly suggests that DEL cells stem from a myelomonocyte lineage. However, compared with the results obtained from studies using HL-60 and U937 cells (6, 7), a percentage of 3–5% of phagocytic nonadherent cells appears rather low. Since nonadherent DEL cells, to a large extent, maintain their viability and tumorigenicity, a simple toxic effect of TPA remains unlikely.

Among the numerous regulatory peptides elaborated by activated macrophages, TNF-α is, along with interleukin 1, one of the best characterized. Although TNF-α synthesis has occasionally been reported in T- and B-cells (25, 26) and in Reed-Sternberg cells (27), its production is usually attributed to cells of the histiocyte lineage (28, 29). Although having a partial homology in the amino acid sequence and a comparable cytolytic activity, TNF-β is generally regarded as a product of T-cell activity (30). The fact that TNF-α, but not TNF-β, transcriptionally regulates the production of colony stimulating factor 1 by human monocytes accentuates the differences between these two cytokines (31). Consequently, the transcriptional activation of these cytokine genes, the production of active molecules, and their increase following drug treatment can be used as a reliable cell marker. Our results indicate that the TNF-α gene is inherently and spontaneously transcribed in DEL cells. Moreover, this TNF-α transcription is modulated by TPA treatment (Fig. 4). Conversely, TNF-β is not expressed either spontaneously or following TPA treatment. As shown by the cytolytic activity of culture supernatants on L929 responsive cells, the active transcription of TNF-α is accompanied by the production and the release of TNF-α protein, since its cytotox-

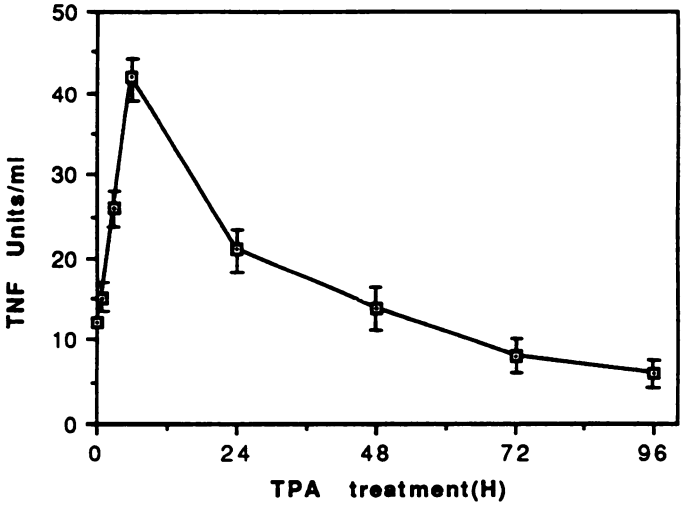

Fig. 5. Effect of TPA on production of TNF-α protein by DEL cells. The supernatants of 33 nM TPA treated DEL cells were assayed for their cytotoxic activity toward TNF sensitive L929 cells. Values represent the mean ± SE for three separate experiments performed in duplicate.

icity can be inhibited by a pretreatment with a monoclonal anti-TNF-α antibody. Again, the production and release of TNF-α protein are modulated by the inductor (Fig. 5). From these data we can conclude that DEL cells possess all of the intrinsic mechanisms for the transcription, translation, and export of TNF-α. In this regard, the production of TNF-α may account for some of the clinical and biological manifestations frequently observed in patients with MH, such as fever, wasting, areas of tissular and tumoral necrosis, and even hypertriglyceridemia.

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