12-0-Tetradecanoylphorbol-13-Acetate-induced Differentiation of a Human Rhabdomyosarcoma Cell Line

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ABSTRACT

The effect of 12-0-tetradecanoyl phorbol-13-acetate (TPA) on proliferation and differentiation of the human embryonal rhabdomyosarcoma cell line RD was investigated. The proliferation of RD cells is drastically and reversibly inhibited by 100 nM TPA. The effect is evident after 24 h of treatment and is maximal after 50–70 h. The reduction of proliferation in treated cells is followed by increased expression of differentiative characters such as a large increase in muscle myosin expression and in the binding of 125I-α-bungarotoxin. Moreover TPA induces the appearance of myotube-like structures, which contain bundles of thick and thin myofilaments along with Z bodies. The described effects are not observed if the TPA-containing medium is replaced daily, thus suggesting that these effects might be related to substances secreted by treated cells. The phosphorylation of three proteins is significantly stimulated by TPA within minutes of its administration to RD cells. Although with a different pattern, the stimulation of protein phosphorylation is clearly detectable after 6 days of incubation with TPA. These results on human rhabdomyosarcoma cells are, to our knowledge, the first evidence for a growth-inhibiting and a differentiative effect of TPA on a solid tumor of mesodermal origin.

INTRODUCTION

It is presently assumed that neoplastic transformation reflects the inability of a population of cells to couple correctly proliferative and differentiative signals, rather than the lack of differentiation potential.

Recent experimental evidence suggests that cultured tumor cells can be induced to differentiate by hormones, proteins, vitamins, or drugs which operate as suppressors of the malignant phenotype (1–3). This behavior reflects either the inability of the organism to generate an appropriate level of a differentiative factor (4) or the presence of abnormalities which do not allow transformed cells to respond properly to external differentiative stimuli. Since the discovery that proliferation of normal cells is under the control of growth factors present in the extracellular environment, suggestive evidence has accumulated that a number of transformed cells lack this form of control because they constitutively express the growth factors they are responsive to or other elements in the normal mitogenic pathway (5).

One of the elements involved in the mechanisms controlling cell proliferation and differentiation, physiologically activated by the receptor-effector system based on phosphoinositide hydrolysis, is PKC, the target of a number of tumor promoters (6–8). The phorbol ester TPA, which induces a large array of biological effects on animal cells, acts as a mitogen and induces changes including mimicry of transformation in normal cells (9–11). Cells, however, do not respond univocally to TPA: it has been reported that many cell lines, especially carcinoma or leukemia cells, are induced to differentiate when treated with TPA (12–18).

When administered to skeletal embryonic myoblasts, TPA prevents their exit from the cell cycle and reversibly inhibits their differentiation by blocking both cell fusion and accumulation of muscle-specific proteins (19–20). When TPA is administered to differentiated myotubes, preexisting striated myofibrils are dismantled and no new myofibril assembly occurs (21, 22). The rate of synthesis of acetylcholine receptor (AchR) is also decreased by TPA and its rate of degradation increased while a high level of plasminogen activator can be detected (21–26). Different myogenic cell types, however, show differential sensitivity to phorbol ester action: in "early" (embryonic) mammalian myoblasts (from somites and limbs) or adult satellite cells treated with TPA at concentrations which completely inhibit fetal myoblast differentiation, the appearance of differentiated muscle cells is not hampered (27–29). The heterogeneous response of normal myogenic cells to tumor promoters prompted us to investigate whether transformed myogenic cells, such as rhabdomyosarcoma cells, respond to TPA.

Rhabdomyosarcomas are the most common soft tissue sarcomas in childhood and they express to different extents, both in vivo and in culture, some differentiative features of the muscle phenotype. Particularly, the RD human rhabdomyosarcoma cells used in this work (30) are capable of only a limited and abortive spontaneous myogenic differentiation, probably because they secrete potent inhibitors of myogenesis such as fibroblast growth factor or transforming growth factor β (31–39). We report here that TPA inhibits RD cell growth and induces the expression of differentiative characters in a significant fraction of these cells.

MATERIALS AND METHODS

Cell Culture. RD human embryonal rhabdomyosarcoma cells, obtained from the American Type Culture Collection (Rockville, MD), were periodically clonally by limiting dilution to reduce the heterogeneity of the cell population. The clones were amplified and used between the 4th and the 18th passage. Four clones, similar to each other and to the parental cell population, were used in this study. Cells were maintained in 25-cm² tissue culture flasks in DMEM supplemented with 4 mM glutamine, 40 μg/ml gentamicin, and 10% FCS in a humidified atmosphere of 5% CO₂ in air at 37°C. Culture media were obtained from Hazelton (Denver, PA) and plastic ware from Falcon (Oxnard, CA).

Determination of Cell Growth. Triplicate 35-mm dishes were seeded with 2 x 10⁴ cells in 1.5 ml medium. After 24 h the medium was replaced with fresh medium containing 10% horse serum instead of FCS, in order to reduce the supply of proliferative factors; however, the effects of TPA reported in this study have been confirmed in FCS-grown cells (data not shown). At the same time, 100 nM TPA or non-tumor-promoting analogs were added as appropriate. Treated and control cells were labeled with 1 μCi/ml [3H]Thd (specific activity, 89 Ci/mmol; Amersham, Buckinghamshire, United Kingdom) for 4 h, then washed with cold phosphate-buffered saline (140 mM NaCl-3 mM KCl-0.5 mM MgCl₂-0.9 mM CaCl₂) in 15 mM sodium phosphate buffer,
TPA EFFECT ON RHABDOMYOSARCOMA

Fig. 1. Morphology of RD cells in the absence (A) and presence (B) of 100 nM TPA. Cells were plated in 35-mm dishes and cultured as described in “Materials and Methods” with or without TPA for 7 days. x 250.

Fig. 2. Time course of the effects of TPA (•), mezerein (○), and teleocidin (□) on [3H]Tdh incorporation of RD cells. TPA (100 nM), mezerein (100 nM), and teleocidin (50 ng/ml) were added 24 h after plating. Data represent the rate of [3H]Tdh incorporation of triplicate samples expressed as percentages of the controls in 4-α-PDD.

Fig. 3. Effect of medium replacement (in the continuous presence of TPA) on [3H]Tdh incorporation of RD cells. TPA-containing medium was replaced daily (○) or replaced only on the 5th day of treatment (•, arrow). Ordinate, rate of [3H]Tdh incorporation, expressed as percentage with respect to the controls.

Fig. 4. Effect of long-term TPA treatment on RD cell viability. Twenty-four h after plating in 35-mm dishes, cells were incubated in the presence of [3H]Tdh (1 μCi/ml) in DMEM + 10% FCS for 6 h, washed, and treated with 100 nM TPA as appropriate. At the times indicated, the radioactivity incorporated was measured as described in “Material and Methods.” Similar results were obtained by expressing the data as cpm/μg protein. Data represent the means ± SE (bars) of 3 samples. Two other experiments gave similar results. Inset, Dose-response curve of the effect of TPA on RD cell growth. Cells were plated as described in “Material and Methods” and [3H]Tdh incorporation was evaluated after 4 days of treatment.

(pH 7.4-PBS), and extracted 3 times in ice cold 5% perchloric acid for 30 min. The acid-insoluble material was then solubilized with 1 N NaOH, and an aliquot was counted after neutralization. TPA, mezerein, and the inactive phorbol ester 4-α-PDD were obtained from Sigma Chemical Co. (St Louis, MO). Teleocidin was a kind gift of Dr. M. Fujiki (National Cancer Research Institute, Tokyo, Japan).

Immunofluorescence. After washing in cold PBS, cells were fixed in cold ethanol:acetone (1:1) at -20°C for 10 min. Cells were then rinsed in PBS and treated by standard procedures for indirect immunofluorescence. The monoclonal antibody MF20 against the heavy chain of skeletal muscle myosin was a kind gift of Professor D. Fischman (Cornell University, New York); fluorescein-labeled secondary anti-mouse antibodies were obtained from Boehringer Mannheim.

Acetylcholine Receptor. The level of AchR expressed in RD cultures was measured as 125I-α-Btx-specific binding in triplicate cultures as previously described (21). Cells were incubated with 10 nM 125I-α-Btx (230 Ci/mmol; Amersham) in DMEM supplemented with 1% horse serum. The level of nonspecific binding was assessed by preincubating parallel cultures with 150 μM α-tubocurarine (Calbiochem, San Diego, CA) and was subtracted from the value obtained in the absence of d-tubocurarine.

Protein Phosphorylation and Electrophoresis. Cells were incubated for 3 h with 100 μCi/ml 32P, (2 mCi/ml; New England Nuclear, Boston, MA) in 800 μl phosphate-free culture medium with 0.1% bovine serum albumin/35-mm dish and stimulated with TPA as detailed in the legend to Fig. 7. Incubations were terminated by washing 3 times with ice-cold PBS; cells were scraped in homogenization buffer (20 mM Tris-HCl, pH 7.4-50 mM NaF-1 mM NaVO4-10 mM 2-mercaptoethanol-2 mM EDTA-10 mM ethylenebis(oxyethylenenitrilo)tetracetic acid-2 mM phenylmethylsulfonyl fluoride-0.5% Nonidet P40) vortexed, and sonicated. After centrifugation (5 min, 10,000 × g) to eliminate debris, proteins were precipitated in 12% (final concentration) trichloroacetic acid. After washing pelleted proteins were resuspended in 9.5 m urea-2% Nonidet P40-2% ampholine, pH 5-7 and 3-10 (LKB, Bromma, Sweden)-5% 2-mercaptoethanol and resolved by 2-dimensional electrophoresis as described by O’Farrel (40). Hybond MP film (Amersham)
RESULTS

Cell Morphology. Experiments were conducted with cloned cells obtained by limiting dilution from the parental cell line RD and used between the 4th and the 18th passage. Fig. 1 illustrates the striking changes in the morphology of RD cells after 7 days of TPA treatment (8th day of culture). Control cultures (Fig. 1A) displayed many different cell types, predominantly round or polygonal, but also spindle cells and some large, giant multinucleated ones, resembling those frequently occurring in several benign and malignant neoplasms and tumor cell lines (44). Moreover, myotube-like structures were occasionally found at confluence.

Conversely, TPA-treated cultures (Fig. 1B) displayed a different overall morphology, showing a dramatic increase in the percentage of elongated and multinucleated myofiber-like structures (6–10 nuclei). An accurate estimate of the percentage of nuclei present in these cells was difficult because of the irregular growth pattern. In addition, treated cultures also contained mononucleated and extremely elongated spindle cells, polygonal or star-shaped cells, and small, round cells. This last cell type rapidly proliferates when cultures are fed fresh medium (either with or without TPA) and gives rise to clones which eventually reach confluence if the medium is replaced daily. The patterns described above are not due to stable variants of the RD cell line since cloned cells showed the same heterogeneity and the same behavior as parental cells.

Cell Growth. TPA (100 nM) and other tumor promoters such as teleocidin or mezerein, known to be PKC activators, inhibited the growth of RD cells, while non-tumor-promoting analogs did not. Fig. 2 shows the time course of the effect of these agents on RD cell growth expressed as percentages with respect to the inactive phorbol ester 4α-PDD. The response of RD cells began only after 24 h of treatment and was maximal (85–95%) after 50–70 h. In order to observe the inhibitory effect, TPA had to be continuously present in the medium, since its removal as well as the change of medium in the continued presence of TPA was followed by a new round of cell proliferation; moreover, if TPA-containing medium was changed daily, RD cells continued to proliferate even though continuously exposed to TPA, and no differentiation could be observed (Fig. 3). The growth inhibition, induced by tumor promoters, is not due to toxicity: when [3H]Thd was administered before TPA treatment, the radioactivity remaining in TPA-exposed cells was comparable to that of the control ones for as long as 8 days (Fig. 4). Moreover, toxic effects were not observed even at 1 μM TPA for as long as 4 days of culture (Fig. 4, inset).

Myosin and Acetylcholine Receptor Expression. We studied the expression of both muscle myosin and AchR to better evaluate the effect of TPA on RD cell differentiation. Immunocytochemical analysis, carried out with MF20 monoclonal antibody against skeletal muscle myosin heavy chain, revealed that less than 1% of control cells were stained and therefore contained detectable amounts of sarcomeric myosin. In contrast, after 5–6 days of TPA treatment, several myosin-positive cells appeared in RD cultures. The fraction of positive cells increased progressively to reach 70–80% of the total population after 8 days of treatment (Fig. 5). Electron microscopic analysis showed in the latter samples features of initial sarcomerogenesis (Fig. 6). In addition to myotube-like structures, many of the mononucleated cells in TPA-treated cultures became positive to MF20 antibody. However, both kinds of positive cells showed uniform fluorescence with no apparent cross-striations.

Increased expression of sarcomeric myosin was paralleled by an increased expression of AchR in TPA-treated cultures: after 10 days of treatment, the amount of bound 125I-α-Btx in treated cells was more than double that of control cells (Table 1). The values of Btx binding obtained in TPA-treated cultures are comparable with those reported by other authors for human muscle cells differentiating in vitro (45).

Protein Phosphorylation. We have investigated the effect of TPA treatment on protein phosphorylation in RD cells by 2-dimensional gel electrophoresis. TPA treatment of RD cells was used for autoradiographs of dried gels.

Electron Microscopy. Cells were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed in 1% osmium tetroxide, and dehydrated in ethanol. Cells were detached from the culture vessel by rapid treatment in propylene oxide and embedded in Epon. Thin sections were contrasted with uranyl acetate and lead citrate and analyzed in a Hitachi H 7000 transmission electron microscope.

Northern Blot Analysis. After TPA treatment as described in the figure legends, total RNA was extracted as described in Ref. 41, and polyadenylated RNA was isolated on a oligo(dT) column (42). RNA was electrophoresed in formaldehyde-agarose gels and transferred on Hybond membrane (Amersham). Hybridization conditions and PKC probes were as described by Sposi et al. (43).
specifically and reproducibly increased the phosphorylation of a protein of $M_r$ 130,000 and $pI$ 6.9 and of two proteins of $M_r$ 33,000 and $pI$, respectively, 5.8 and 5.7, with the same pattern between 15 min and 3 h (Fig. 7).

The $^{32}$P-labeling increase caused by TPA was observed even after an incubation as prolonged as 6 days, although with a different general pattern with respect to the short-term incubations (Fig. 7); in fact, the phosphorylation of the protein of $M_r$ 130,000 stimulated in the short-term incubations is not observed after 6 days of TPA treatment, while the phosphorylation of another protein of the same molecular weight and $pI$ 6.8 is stimulated.

**PKC Isoform Analysis.** The inhibition of proliferation obtained with the tumor promoters teleocidin and mezerein, both PKC activators structurally unrelated to TPA, is indicative of the regulative role of this enzyme in RD cells. We sought the presence of the PKC mRNA in TPA-treated RD cells. Comparable amounts of the mRNA for the $\alpha$-isoform of PKC were found in TPA-treated and control cells at the 5th day of culture (Fig. 8), while no hybridization for $\beta$- and $\gamma$-isoforms was detected. We were unable to determine PKC activity in RD cells or to detect the protein in immunoblots using monoclonal antibodies against the $\alpha$- and $\beta$-isoforms of the enzyme.

**DISCUSSION**

The data reported in this paper demonstrate that tumor promoters which are known to activate PKC may induce growth inhibition and partial differentiation in RD cells. Because TPA usually inhibits myogenic cell differentiation, the finding that this agent induces differentiation of a rhabdomyosarcoma cell line is of interest in elucidating the regulatory mechanisms acting in both normal and transformed cells. The differentiative effect becomes evident after 5–6 days of TPA treatment and is displayed by the appearance of plurinucleated myotube-like structures with features of initial sarcomerogenesis and by a large increase in the number of skeletal myosin-positive cells. Moreover, the expression of AchR, which is expressed at a low level in control cells (46), is at least doubled in treated ones. Our data indicate that TPA induces the expression of muscle-specific products in RD cells and suggest that TPA treatment also stimulates the fusion of mononucleated myogenic cells into multinucleated myotube-like structures. However, the possibility that the giant multinucleated cells present in control cultures are converted by TPA into the myotube-like structures cannot be completely ruled out.

Since it is well known that PKC is stimulated by TPA, we have analyzed the phosphoprotein pattern of TPA-exposed and control cells: this shows that the phosphorylation of specific proteins is stimulated by TPA treatment. It is of interest to note that the TPA-induced stimulation of phosphorylation occurs, although with a different pattern, both after short-term treatment (15 min–3 h) and after long-term treatment (6 days).

Because prolonged TPA exposure is known to induce down-regulation of PKC, the differentiative effect of this agent might depend on PKC desensitization: the proliferative signals of exogenous growth factors might not be transduced any more and the consequent withdrawal from the cell cycle might increase the possibility of activating the muscle differentiative program. This hypothesis is supported by the reported decline of PKC activity in normal embryonal myoblasts undergoing fusion to form myotubes (7).

However, this hypothesis does not explain the data obtained when the TPA-containing medium is frequently renewed. Our data indicate that a striking recovery of cell proliferation and arrest of differentiation occur if the medium is changed after the cells have withdrawn from the cell cycle; moreover, if medium is replaced daily, the cells show neither inhibition of proliferation nor differentiation, despite the continuous presence of TPA (Fig. 3). These results appear unrelated to the action of growth factors added with the fresh medium, because the simple addition of serum to the conditioned medium does not induce recovery of proliferation (not shown). It is tempting to speculate that the TPA-induced inhibition of proliferation might be mediated by proliferation-inhibiting factors released.

**Table 1 Effect of TPA on $^{125}$I-$\alpha$-bungarotoxin binding in RD cells**

<table>
<thead>
<tr>
<th>Treatment time</th>
<th>24 h</th>
<th>10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10,240 ± 585</td>
<td>10,650 ± 205</td>
</tr>
<tr>
<td>TPA</td>
<td>11,640 ± 273</td>
<td>25,610 ± 493</td>
</tr>
</tbody>
</table>

* $^{125}$I-$\alpha$-bungarotoxin-specific binding (cpm/mg protein). Values are means of triplicate samples ± SE.
in the medium and removed when the medium is changed (Fig. 3). Alternatively, TPA might affect the secretion or the activity of factors such as fibroblast growth factor or transforming growth factor β, potent inhibitors of myogenic differentiation, spontaneously secreted by RD cells (31–33). The data reported demonstrate that TPA still stimulates the phosphorylation of specific proteins even after 6 days of continuous treatment. A prolonged effect of TPA on PKC has been reported in MCF 7 cells which can still maximally phosphorylate a protein of M, 28,000 after long-term exposure to TPA (47). The results support the hypothesis that PKC is not down-regulated even after long exposure to TPA. This conclusion is further supported by Northern blot analysis showing a persistent presence of mRNA for PKC α-isoform.

The impossibility of inducing differentiation in 100% of the cell population in rhabdomyosarcomas is not surprising. Even after cloning these cells show the same pattern of differentiation as parental cells. A similar behavior is described in the ratclonal rhabdomyosarcoma cell line BA-HAN-IC, which is induced to differentiate by retinoic acid (48–50). Therefore, the genes controlling the initial differentiation process may not be lost following transformation and the heterogeneity of the response may be due to the intrinsic phenotypic complexity of myogenic clones (51). Moreover, the finding that the TPA-induced differentiation process in RD cells is incomplete could be due to inadequate culture medium: differentiation of human muscle cells in culture is not as complete as that of myoblasts of other species, probably because culture media contain unsuitable components for human cells (38). Alternatively, it cannot be excluded that RD cells do not achieve full differen-

Fig. 7. Comparison of the pattern of endogenous protein phosphorylation in TPA-treated and control (4-α-PDD-treated) RD cells after short- (15 min) and long-term (6 days) treatment. For long-term TPA treatment, 100 nM TPA was added to the medium 24 h after plating and maintained for 6 days. 32P, was added for the last 3 h as detailed in “Material and Methods.” For short-term treatments, TPA was added to subconfluent cells in the last 15 min, 1 h, or 3 h of the 32P, incubation. Phosphorylated proteins were extracted as detailed in “Material and Methods” and were subjected to isoelectric focusing (IEF) followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 10% polyacrylamide gels. Arrows, proteins whose phosphorylation is affected by TPA.

Fig. 8. Northern blot analysis of polyadenylate-selected RNA showing the presence of comparable amounts of α-PKC mRNA in treated (4 days of exposure to TPA) (A) and control cells (B).
tiation because they have lost genes essential to complete this process, due to the prototypic instability of tumor cells, or because, despite the presence of TPA, they still express some transforming functions which inhibit the expression of genes of the differentiated state (34-36, 52).

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REFERENCES

12-O-Tetradecanoylphorbol-13-Acetate-induced Differentiation of a Human Rhabdomyosarcoma Cell Line

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