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COMMENTARY

ANTIPROLIFERATIVE PROPERTIES OF PHORBOL ESTER TUMOUR PROMOTERS

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12-*O*-Tetradecanoylphorbol-13-acetate (TPA*, see Fig. 1), the most active derivative among the phorbol diester tumour promotors, affects a large number of biological systems [1]. Most of its effects can be conveniently grouped into three categories, all of which begin with the letter "M" [2]. They are: (1) mimicry of cell transformation in normal cells and enhancement of expression of markers of transformation in cells transformed by chemical carcinogens or oncogenic viruses, (2) membrane changes, most likely the primary site of action of TPA, and (3) modulation of patterns of cell differentiation. Whereas in several cell lines differentiation is inhibited by TPA, in others TPA is a potent inducer of differentiation (for review see Ref. 3), a property which renders TPA growth inhibitory. Furthermore, the interaction of TPA with certain cells can cause the release of cytotoxic species [4–6]. It has been pointed out recently that there are a number of remarkable similarities in the effects of the broad spectrum antitumour antibiotic adriamycin and TPA at the cell surface [7]. Both agents alter the morphology of cell membranes, increase the uptake of ions, increase membrane fluidity, increase arachidonic acid turnover and prostaglandin synthesis, and modulate the surface receptors for epidermal growth factor (EGF) (reviewed in Ref. 7). The observations that TPA can arrest cell growth and stimulate the production of cytotoxic species, and that some of its biochemical properties are similar to those of the antitumour drug adriamycin, make TPA an intriguing agent for the experimental chemotherapist. It is clearly impossible to envisage how TPA could be exploited therapeutically as a cell growth inhibitor without inducing a multitude of deleterious cell changes, particularly those associated with tumour promotion. But insight into the nature of the growth arresting interactions between TPA and cells could conceivably lead to the identification of new biochemical targets in cancer treatment or spark a search for novel chemicals which mimic the growth inhibitory properties of TPA. This is particularly relevant in view of the lack of specificity of current therapeutic strategies to stop the growth of malignant cells. To stimulate thoughts and work which might lead to new therapeutic avenues, this review describes the systems in which TPA is directly cytostatic or exerts cytotoxicity indirectly, and outlines the mechanisms operative.

* Abbreviations: EGF, epidermal growth factor; GSH, glutathione; MPO, myeloperoxidase; PDB, phorbol dibutyrate; PMN, polymorphonuclear leukocyte; SOD, superoxide dismutase; and TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

The direct antiproliferative action of TPA

In certain cells TPA induces terminal differentiation and consequently cells lose their growth potential [3]. Cells of the human promyelocytic leukaemia cell line HL-60, when cultured *in vitro* with TPA, differentiate into cells with macrophage characteristics [8–11]. These differentiated cells, but not their undifferentiated counterparts, are cytotoxic towards leukaemia cells, including untreated HL-60 cells and HeLa cells, whereas they express little or no cytotoxicity towards nonmalignant target cells [12]. The selective cytotoxicity caused by TPA-stimulated HL-60 cells may be due, at least in part, to the secretion of the lymphokine polypeptide "tumour necrosis factor", for which the gene was cloned recently [13]. From the DNA sequence it was inferred that this factor is made as a 157 amino acid precursor from which the amino-terminal 76 amino acid segment is cleared prior to secretion.

There is evidence which suggests that the growth-inhibitory properties of phorbol esters can be separated from their tumour promoting potential. It is based on the observation that 12-*O*-retinoylphorbol-13-acetate (Fig. 1) is a derivative of TPA apparently without tumour promoting properties [14] which is still capable of inducing HL-60 cells to differentiate terminally at concentrations similar to the concentrations at which TPA causes this effect [15]. The therapeutic potential of the induction of cancer cell differentiation caused by TPA and other agents has been discussed recently [16].

TPA is also capable of inhibiting the growth of some human cells without causing cell differentiation. In several studies in which TPA-induced inhibition of cell growth was observed, its effect on differentiation patterns was not investigated. Human fibroblasts exhibit a reduced growth rate in response to TPA [17], and there is a difference between strains as to the degree of growth inhibition depending on their origin and oncogenic potential [18]. TPA inhibits cell division in MCF-7, ZR75-1 and MDA-MB-231 breast cancer cells [19, 20], SV40 virus-transformed epidermal keratinocytes [19], A431 epidermoid carcinoma cells [21], A549 lung carcinoma cells [22], and BJAB and Ramos cells derived from Epstein–Barr virus (EBV) negative Burkitt-type

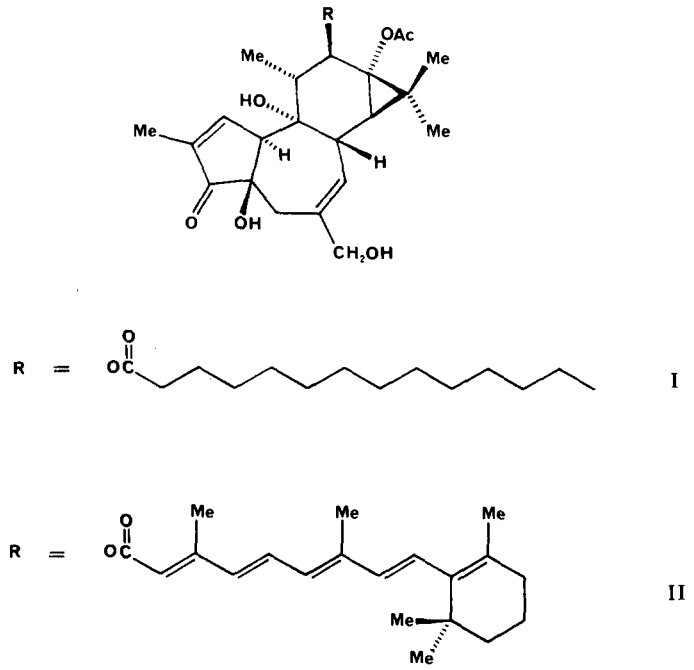

Fig. 1. Structures of 12-*O*-tetradecanoylphorbol-13-acetate (TPA, I) and 12-*O*-retinoylphorbol-13-acetate (II).

lymphomas [23]. Low density cultures of A431 cells, in which proliferation is rapid, are more sensitive towards the growth inhibitory potential of TPA than cultures approaching confluency [21]. Interestingly, BJAB and Ramos cells, converted to Epstein–Barr nuclear antigen positive lines by infection with EBV, grow in the presence of TPA at concentrations inhibitory for non-converted parental lines. Furthermore, the resistance to growth inhibition is higher in sublines converted by the transforming B95-8 strain of EBV than in those converted by the non-transforming P3-HR1 strain [23].

In most of the cell lines described above, the growth inhibition by TPA is reversible on removal of TPA from the medium. In MCF-7 cells this reversal does not occur in serum-free medium but requires the presence of serum components [20].

Mechanisms of TPA-mediated growth inhibition

The mechanisms by which TPA inhibits cell growth are not well understood. There is now considerable evidence for the contention that the complex array of responses elicited by TPA in cells is mediated via TPA–receptor interactions analogous to the way in which hormones trigger cellular responses. Driedger and Blumberg [24] were the first investigators who demonstrated the existence of specific high-affinity binding sites for TPA and related phorbol esters. Several human cell lines, the growth of which is inhibited by TPA, have been shown to possess these receptors, among them the HL-60 [25], A431 [21], SVK14 [19] and A549 [22] cells. In HL-60 cells, changes in cellular phospholipid composition change phorbol ester receptor binding [26]. The apparent affinities (K_d) observed for the specific binding of phorbol dibutyrate (PDB), a less lipophilic analogue of TPA which is most frequently used in binding studies, and the approximate number of PDB binding sites per cell are summarized in Table 1. The apparent affinity values are similar to those reported for human lymphocytes [28] or rodent fibroblast cells [29] in which TPA elicits mitogenesis. Thus, there are apparently no marked quantitative differences between phorbol ester receptors in cells in which TPA causes arrest of growth and those of cells which proliferate under the influence of TPA.

Table 1. Dissociation constants (K_d) for specific binding of [^{3}H]-PDB to receptors in human cell lines, and approximate numbers of binding sites

Cell type	K_d (nM)	Binding sites per cell	References
HL-60	51	7.0×10^4	25
	110	7.4×10^5	27
SVK40	21	1.8×10^6	19
A431	23	3.0×10^5	21
A549	12	5.6×10^5	22

The specific cellular receptor for TPA may well be the enzyme protein kinase C (for reviews see Refs. 30 and 31). TPA binds to this phospholipid and Ca^{2+} dependent enzyme and activates it *in vitro* [32, 33]. Since both TPA binding to cells and protein kinase C show the same wide tissue distribution [34–36], and as both are not separable by a variety of physical techniques [33, 37, 38], they may be closely associated or even identical. The endogenous activators of protein kinase C are diacylglycerols which are produced *in vivo* by hydrolysis of phosphatidylinositols [39]. The fact that TPA can activate protein kinase C has been suggested to reflect its structural similarity with diacylglycerols [31]. Treatment

of HL-60 cells with TPA induced rapid and specific phosphorylation of two cytosolic proteins at serine residues [40]. The assumption that this phosphorylation was catalyzed by protein kinase C is corroborated by the recent finding that protein kinase C is activated by phorbol esters also in this cell type, with a specificity paralleling the biological potencies of various TPA analogues [38]. Consequently, it is conceivable that the diverse cellular responses to phorbol esters are initiated by phosphorylations catalyzed by protein kinase C and that the nature of the substrate which is phosphorylated determines which kind of cellular response occurs. It has been speculated that, in the cells in which TPA inhibits growth rather than stimulating it, TPA may activate an already stimulated receptor-linked protein kinase system to indiscriminately phosphorylate proteins which are involved in regulating cell proliferation [21]. It appears feasible that the kind of response TPA elicits in different cells depends on the nature of the substrates which are phosphorylated by protein kinase C. Even though the ability of activated protein kinase C to phosphorylate proteins has been demonstrated unambiguously, the only functionally important substrate of protein kinase C phosphorylation shown so far has been a threonyl residue in the EGF receptor located close to the cytoplasmic face of the A431 cell membrane [41]. Cell type specific differences in substrates for protein kinase C have been found in a study using human polymorphonuclear leukocytes (PMN) and various types of human leukaemic cells [42]. It is to be expected that at least some of these substrates are involved in growth inhibitory responses to TPA.

TPA shares a number of biological properties with EGF [2, 43, 44] and both are thought to produce their effects by interactir.g with specific receptors. In A431 cells, both TPA and EGF retard cell growth [21]. Moreover, TPA enhanced the growth inhibitory effect of EGF in these cells. Concomitantly, it attenuated the EGF-induced increase of diacylglycerol production and the EGF-stimulated synthesis of phosphatidylinositol from inositol. TPA alone stimulated the production of diacylglycerol but unlike EGF had little effect on phosphatidylinositol synthesis, thus, the effect of TPA in A431 cells was concluded to be dependent on transient changes in diacylglycerol production [21]. The binding of EFG to A431 cells is unaltered by TPA [21], however, specific EGF receptor peptides in these cells are phosphorylated when they are exposed to TPA [45]. Thus, the interaction of TPA with EGF in these cells may be mediated by alterations in EGF receptor composition caused by the binding of TPA to phorbol ester receptors.

Cells in which TPA has induced terminal differentiation, such as the HL-60 leukaemia cells, ultimately die. Similarly, in the EBV-negative BJAB and Ramos cells, growth inhibition by TPA is followed by cell death [23]. In MCF-7 cells, TPA-induced blockage of cell replication is accompanied by stimulation of protein synthesis resulting in hypertrophied cells, and after 10–12 days of exposure to TPA cells detach from the culture dishes [20]. In contrast, TPA is completely without toxicity in SVK14 [19], A431 [21] and A549 cells [22]. In fact, in SVK14 and A549 cells the inhibition of cell proliferation by TPA is transient and the cells become insensitive towards TPA after several days' exposure to the agent. One explanation is that these cells undergo down-regulation of their receptors, i.e. loss of binding capacity without alteration in receptor affinity. The down-regulation of phorbol ester receptors was first observed in rat pituitary cells [46]. It remains to be elucidated whether the desensitization of cells to the growth inhibition by TPA is a direct consequence of decreased TPA receptor binding. Alternatively, receptor down-regulation might be involved in a more complicated fashion. A cell variant with acquired resistance to the differentiation-inducing properties of TPA by culturing HL-60 cells for 35 subcultures in the presence of TPA possessed the same number of specific binding sites for PDB as the parent HL-60 cells. But unlike the parental cell line, this variant lacked the ability to undergo receptor down-regulation subsequent to exposure to PDB [25]. Receptor down-regulation is presumably due to conformational changes in the phorbol ester binding sites or internalization of the sites followed by their intracellular degradation. In the case of the HL-60 cells, receptor down-regulation may be part of the events leading to the induction of cell differentiation by TPA. However, it has to be noted that reports concerning TPA effects other than growth inhibition indicate that phorbol ester resistant variants are indistinguishable from sensitive variants on the basis of down-regulatability. There is no strong evidence for a mediator role of down-regulation in any biological activity of phorbol esters.

The indirect cytotoxic properties of TPA

Human phagocytic immune cells possess a powerful cytotoxic armamentarium [47, 48]. They are capable of destroying target cells including erythroid, lymphoid and tumour cells. This destructive event can be triggered by antibodies and by pharmacological agents such as lectins [49] and the phorbol ester tumour promotors [5, 6]. The mechanisms utilized by TPA-stimulated cells to destroy their targets are not well understood, but since de Chatelet *et al.* [4] reported that TPA induces the oxidative metabolism of polymorphonuclear leukocytes (PMNs) in the same manner as phagocytosis does, attention has focused on the ability of PMNs to generate a host of potentially cytotoxic oxygen metabolites [47, 50, 51]. This ability has been associated with TPA-induced genotoxicity in leukocytes [52] and lymphocytes [53].

Following specific receptor-mediated membrane perturbation, the stimulated cell exhibits a burst in oxygen consumption associated with the generation of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and possibly hydroxyl radical ($\cdot OH$), hypochlorous acid (HOCl) and singlet oxygen ($^1O_2$) [47, 51, 54]. The enzyme responsible for the production of these oxygen metabolites in phagocytic cells is a membrane-associated pyridine nucleotide oxidase [55]. Whereas the cytotoxicity of the oxygen metabolites when generated by cell-free model systems has been well characterized [47, 51, 56–58], much less is known about the destructive potential of these agents produced in cells [6, 59–64].

Several different mechanisms have been proposed by which TPA-stimulated cells can exert cytotoxicity, and it appears that the source and type of effector and target cells, assay conditions and the method of assessing cell injury all influence which mechanism predominates under the chosen experimental conditions. Human and murine PMNs, monocytes, macrophages and also monomorphonuclear leukocytes have been effectively used as effector cells after stimulation with TPA.

The following agents and enzyme systems have been proposed as mediators of cytotoxicity induced by TPA in phagocytic cells: (a) the H_2O_2-halide-myeloperoxide system, (b) H_2O_2 alone or H_2O_2 and O_2^- together, and (c) the H_2O_2-methaemoglobin system.

(a) TPA-stimulated PMNs can release cytoxic quantities of H_2O_2 and azurophile granule enzyme myeloperoxidase (MPO), which, in concert with a halide, are able to exert a powerful toxic effect [54, 65–67]. Using a model system consisting of purified MPO, a source of H_2O_2 and various halides, Klebanoff *et al.* demonstrated the ability of this system to destroy a wide spectrum of target cells [48, 68, 69]. This system is characterized by the inhibition of cytotoxicity by addition of catalase, which degrades H_2O_2:

$$H_2O_2 \xrightarrow{\text{catalase}} \tfrac{1}{2}O_2 + H_2O$$

Superoxide dismutase (SOD) has no effect. SOD is an enzyme which catalyzes the dismutation of O_2^- according to the following scheme:

$$2\,O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

The haem enzyme inhibitors cyanide and azide also inhibit the expression of cytotoxicity in this system [65, 66]. PMNs obtained from patients suffering from hereditary MPO deficiency show markedly impaired cytotoxic activity on stimulation of the H_2O_2-halide-MPO system [61, 65].

The final mediator of toxicity in the H_2O_2-halide-MPO system is under debate. Harrison and Schultz [70] showed that the system is capable of catalyzing the oxidation of chloride to the powerful oxidant hypochlorous acid, HOCl:

$$H_2O_2 + Cl^- \xrightarrow{\text{MPO}} H_2O + OCl^-$$

HOCl reacts avidly with biological nucleophiles [71, 72], and the addition of certain amino acids to the H_2O_2-halide-MPO system has been demonstrated to inhibit cytolysis, presumably by scavenging HOCl [66]. Thus, HOCl would not be expected to accumulate in a cell system. Other candidates for the role of ultimate oxidant in this system are N-chloramines and N-chloramides. TPA-stimulated PMNs can utilize the H_2O_2-halide-MPO system to chlorinate an exogenous amine to form an N-chloro derivative [73]:

$$HOCl + H_2N - CH_2R \longrightarrow Cl - NH - CH_2R + H_2O$$

N-Chloramines are semistable compounds but have sufficient oxidizing potential to oxidize sulfhydryl groups to disulfides or thioethers to sulfoxides [71–74], and long lived oxidants with characteristics similar to those of chloramines have been detected in suspensions of TPA-stimulated PMNs [75].

Rosen and Klebanoff [76] suggested that the H_2O_2-halide-MPO system may exert cytotoxicity by generating the highly reactive singlet oxygen $^1O_2$:

$$H_2O_2 + OCl^- \longrightarrow H_2O - {}^1O_2 + Cl^-$$

There are no reagents which react selectively with $^1O_2$ and all the $^1O_2$ traps reported in the literature also react rapidly with HOCl [77, 78]. Therefore, hitherto it has been impossible to show unambiguously the generation of $^1O_2$ by an MPO system. $^1O_2$, or a similar species in an electronically excited state, is considered to be responsible for the chemiluminescence observed in TPA-stimulated human PMNs [79]. This chemiluminescence can be inhibited by retinoic acid [79] which also inhibits O_2^- formation in PMNs exposed to TPA [80]. As retinoic acid has been shown to induce protein kinase C activity in some cells, the interactions between retinoids and TPA are thought to be mediated by mutual effects on protein kinase C [81].

(b) Neutrophils are toxic towards themselves when exposed to TPA, and this autotoxicity is attributed to the H_2O_2 generated [82]. H_2O_2 has also been shown to be responsible for lysis of red blood cells and murine tumour cells by TPA-stimulated murine granulocytes and macrophages [5]. Likewise, H_2O_2 is considered to mediate toxicity of TPA-stimulated human neutrophils towards human endothelial cells [83]. In these exerpiments, catalase prevented cell damage but SOD or azide did not.

The simultaneous generation of O_2^- and H_2O_2 was considered to be responsible for the destruction of autologous erythrocyte targets by TPA-stimulated human monocytes, as both SOD and catalase inhibited cytolysis, whereas cyanide stimulated it [84].

(c) TPA-stimulated neutrophils were shown to lyse human erythrocyte targets via O_2^-, as haemolysis was inhibited by exogenous SOD and not by catalase or a glutathione (GSH)-GSH peroxidase system, which like catalase detoxifies H_2O_2 [85]. Conversion of haemoglobin to methaemoglobin by nitrite did not impair haemolysis, but the nitrite-treated targets were not protected against neutrophil toxicity by SOD. However, protection was afforded by exogenous catalase. The author concluded [85] that in this system O_2^- oxidizes oxyhaemoglobin to methaemoglobin and is itself reduced to H_2O_2 which within the cell interacts with methaemoglobin to form a cytotoxic complex. The reaction between H_2O_2 and methaemoglobin is known to lead to a powerful oxidant [86].

In addition to the two enzymes, catalase and SOD, cells might use defense systems involving GSH in order to protect themselves from the deleterious effects of TPA-induced oxygen metabolites. The protective role of GSH has been analyzed by Nathan *et al.* [87] in a number of murine tumour models. They showed that, by inhibiting GSH reductase and GSH peroxidase, the catalysts of the GSH redox cycle, tumour cells could be lysed by TPA-stimulated macrophages or granulocytes in numbers that were

ineffective in cells with uninhibited enzymes. Several authors have shown that processes associated with phagocytosis decrease the non-protein thiol concentration in human granulocytes and thus impair intracellular defense mechanisms [88, 89]. However, the thiol status of leukocytes or macrophages after stimulation with TPA has not been reported.

Conclusions

The complex responses of cells to TPA are undoubtedly triggered by TPA-receptor interactions. To be able to exploit the growth inhibitory properties of TPA, it will be necessary to understand which subsequent intracellular processes lead to growth inhibition as compared to those causing mitogenesis. Future work might show whether the pattern of phosphorylation catalyzed by TPA-activated kinases exhibits qualitative or quantitative differences between cells in which TPA causes mitogenesis and cells in which it inhibits growth. The identification of a specific substrate which upon protein kinase C mediated phosphorylation triggers growth arrest might offer a new chemotherapeutic target.

This review exemplifies multiple conditions under which TPA induces the formation of cytotoxic oxygen metabolites in leukocytes and macrophages. However, it is virtually unknown whether in other mammalian cells TPA mediates the generation of such species, or causes subtle changes in the metabolism of normally occurring oxygen metabolites. This aspect warrants investigation particularly in view of the observation of Weinberg that HL-60 cells which have undergone differentiation by exposure to TPA have acquired cytocidal and cytostatic properties [12]. It is also not clear what the role of toxic oxygen species is compared with that of toxic peptides such as tumour necrosis factor in the mediation of the cytotoxicity caused by stimulated leukocytes and HL-60 cells.

Different compounds which share the property of scavenging ·OH can inhibit TPA-induced mitogenesis in lymphocytes, which suggests that beyond its ability to induce cellular damage ·OH can function as a mediator for the transduction of mitogenic signals [90]. It is conceivable that other biochemical events, such as those associated with TPA-induced growth inhibition, are mediated by oxygen metabolites. In this context it is worth noting that ·OH can activate enzymes and thus cause alterations of metabolic pathways. For example, the enzyme guanylate cyclase is activated by ·OH [91] and cyclooxygenase and peroxidases involved in arachidonic acid metabolism are inactivated by ·OH [92]. H_2O_2 has been suggested to act in some cells as a second messenger for insulin, which can stimulate cell division *in vitro* [93].

A link between the activation of protein kinase C, the putative TPA receptor, and the TPA-induced generation of toxic metabolites has still to be established. The stimulation of the production of oxygen metabolites by TPA in phagocytic cells is considered to be the consequence of the activation of the membrane bound pyridine nucleotide oxidase. It is conceivable that the activation of this enzyme is a result of changes in the state of phosphorylation of an activator protein caused by protein kinase C [56]. A better knowledge of this link might help to exploit TPA-induced cytotoxicity therapeutically.

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