Active Oxygen Species as Factors in Multistage Carcinogenesis (43306)

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Abstract. **Oxygen, a necessary element for the life of a cell, is also the source of active states of oxygen including radicals, which can disrupt cell structure and alter cell function. Increasing evidence indicates that active oxygen species are formed in response to tumor promoters and that the cellular consequences of their actions may play a role in the process of tumor promotion. This report summarizes work from our laboratory that implicates active oxygen species derived in part from phagocytic cells in the tumor promotion process by phorbol esters and other promoters in mouse skin. Work from other laboratories indicates that phorbol ester promoters stimulate the production of active states of oxygen in mouse skin epidermal cells *in vivo* and *in vitro*. Oxidative DNA damage in epidermal cells from mice treated topically with the potent promoter phorbol myristate acetate has also been reported. The production of active states of oxygen including free radicals is discussed in relation to the mode of action of complete, first, and second stage promoters in the multistage carcinogenesis model in mouse skin.** [P.S.E.B.M. 1991, Vol 198]

During the last decade, a substantial amount of evidence has been reported which suggests that active oxygen species including free radicals may play a role in the tumor promotion process of multistage carcinogenesis. Active oxygen species, such as superoxide anion radical and hydrogen peroxide, produced in response to tumor promoters can form hydroxyl radicals. The latter can initiate peroxidative processes, leading to the production of reactive oxygen species such as protein and lipid hydroperoxides. These, in turn, form other secondary reactive oxygen species such as alkoxy and peroxy free radicals. Reactive oxygen radical species can interact with lipids, proteins, and DNA and alter their structure and function. This report will briefly review work from our laboratory which implicates active oxygen species, derived in part from phagocytic cells, in the process of tumor promotion by phorbol esters. It is not intended to be an exhaustive review on active oxygen species in tumor promotion, and is restricted to work from our laboratory and to oxidant generation and its consequences in response to phorbol ester tumor promoters in mouse skin and epidermal cells.

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Multistage Carcinogenesis Models in Mouse Skin

The multistage carcinogenesis model is an experimental system for the induction of tumors by sequential treatment with chemicals (Fig. 1). The central features of this model, initiation and promotion, are derived from studies in the 1940s by Mottram (1), Berenblum and Shubik (2), and others, who showed that tumor induction by chemical carcinogens in the skin of mice or rabbits can be accelerated by repeated wounding or treatment with croton oil, a chemical irritant. These studies developed into two-stage carcinogenesis, a model in which tumors are induced by initiation and promotion. In this model, a subcarcinogenic dose of a whole carcinogen (the initiator) followed by repeated treatment with a promoter results in tumor induction. Either treatment alone is ineffective. This model for tumor induction has been used extensively in mouse skin with initiators such as dimethylbenz(*a*)anthracene and benzo(*a*)pyrene.

The early studies in chemical carcinogenesis used croton oil, a strong irritant derived from the seeds of *Croton tiglium L.*, as a promoter of tumor development. Isolation and characterization of the active principles of croton oil in the late 1960s (3, 4) revealed that the promoting activity is due to phorbol esters, the most potent being phorbol myristate acetate (PMA). This compound and structural analogs were subsequently used to characterize the biochemical changes resulting

Multistage Induction of Tumors in Mouse Skin

Initiation	Promotion		Progression
•Permanent alteration of cell genotype	CONVERSION ⟶ Stage 1	PROPAGATION Stage 2	•Additional genetic alterations?
•Somatic gene mutation	•Partially reversible	•Reversible	

Figure 1. Experimental stages of the multistage carcinogenesis model in mouse skin.

from treatment with phorbol ester tumor promoters. The studies indicated that promoters, unlike initiators, do not bind to DNA, and that the metabolic changes caused by promotion treatment are not the result of a direct interaction of promoters with genetic material (5). The process of initiation is believed to involve covalent binding of ultimate carcinogens to DNA. Replication before repair results in a mutation that is irreversible and heritable. Studies from several laboratories suggest that the Ha-*ras* oncogene is activated by a single-base substitution and that this activation is central to initiation in mouse skin (6, 7). Promotion, on the other hand, has been suggested to involve epigenetic processes that stimulate the expression of the altered genotype within the initiated cells.

The promotion phase of two-stage carcinogenesis was shown by Slaga *et al.* (8) in the SENCAR mouse to be accomplished by a two-stage protocol consisting of a short-term, 2-week treatment with PMA (twice a week) followed by a long-term treatment (18 weeks) with mezerein (MEZ), a plant product with a structure similar to that of PMA. In this protocol, short-term treatment of initiated mice with PMA did not lead to tumor induction, whereas short-term treatment with PMA followed by long-term treatment with MEZ resulted in a high tumor yield. Two-stage promotion in mice was also demonstrated in initiated NMRI mice by using short-term treatment with PMA (1–4 times) followed by long-term treatment with the semisynthetic phorbol ester retinoylphorbol acetate (9). Based on this protocol, tumor promoters have been classified as Stage 1 or Stage 2 promoters, depending on their activity in the two stages. Promoters that are active in only one of the two stages are termed "incomplete" promoters, while promoters like PMA, which are active in the classical initiation promotion experiment (in which Stage 1 and Stage 2 promotions consist of treatment with the same chemical), are termed "complete" promoters. Using these criteria, MEZ, which is a weak complete promoter, has been shown to be a strong Stage 2 promoter, whereas the PMA analog 4-O-methyl PMA (4-O-Me-PMA) and the calcium ionophore A23187 were demonstrated to be effective first-stage promoters (8, 10). Slaga *et al.* (11) further demonstrated that inhibitors of complete promotion can be stage specific, suggesting that Stage 1 and Stage 2 promoters have different mechanisms of action.

Compared with initiation, which is irreversible since tumors can be induced even when promoter treatment is delayed for several months, promotion is believed to be reversible, since it is almost abolished by extending the time interval between promoter applications (12, 13). Using the two-stage promotion protocol, with PMA as first-stage promoter and RPA as second-stage promoter, Furstenberger *et al.* (14) demonstrated that the effects of PMA are irreversible for at least 8 weeks, implying that a "memory effect" is involved in the mode of action of first-stage promoters. The reversibility of tumor promotion was related by Furstenberger *et al.* (14) to the second stage of promotion and thought to reflect the reversibility of PMA-induced epidermal hyperplasia. This group further demonstrated that the tumor response is independent of whether stage one of promotion occurs before or after initiation (15), indicating that initiated cells in the skin are not required for the induction of cellular alterations necessary for stage one of the promotion phase. These findings support the concept that genetic modifications are involved in the mode of action of Stage 1 promoters.

In Vitro Stimulation of Superoxide Anion Radical Production in Human Neutrophils by Tumor Promoters

Phorbol myristate acetate is a potent inflammatory agent in mouse skin. The inflammatory response to PMA is characterized by an influx of neutrophils followed by macrophages (12). Based on a correlation between promoting activity and the inflammatory activity of phorbol esters, early studies suggested a role for inflammation in tumor promotion (4, 16). Much later, studies indicated that stimulated phagocytic inflammatory cells produce large amounts of active oxygen species including free radicals during the oxygen burst. The oxygen burst, which is triggered in phago-

cytic cells 30–40 sec after exposure to an oxygen burst initiator, is characterized by increased oxygen consumption, increased hexose monophosphate shunt activity, and the production of large quantities of superoxide anion radicals (O_2^-) and other radicals active in the killing of bacteria (17).

Superoxide anion radicals are metabolized by superoxide dismutase (SOD) to hydrogen peroxide (H_2O_2). In the presence of iron and other metals, superoxide anion radicals and hydrogen peroxide can undergo a Haber-Weiss reaction, which generates hydroxyl radicals ($OH^\bullet$) and singlet oxygen ($^1O_2$). Hydroxyl radicals can also be generated from H_2O_2 by metal ion-catalized Fenton reactions. Myeloperoxidase in white cells can metabolize H_2O_2 to the oxidant hypochlorous acid. Neutrophils and macrophages, once stimulated, thus produce a variety of active oxygen species and oxygen radicals that can react with cellular components to form secondary radicals and active oxygen species (18). Among the active oxygen species formed, the hydroxyl radical is by far the most reactive. It reacts indiscriminantly with cellular components and is not expected to reach nuclear material or diffuse through cell membranes if generated in the cytoplasm. Hydrogen peroxide, on the other hand, is longer lived and can diffuse through membranes out of cells as well as into the nucleus, where it can oxidize DNA.

In studies on the activation of human neutrophils *in vitro* by PMA and other promoters, we noted a correlation between tumor-promoting activity and the rate of superoxide anion radical production (Table I; 19, 20). Superoxide anion radical was measured by the reduction of ferricytochrome C to ferrocytochrome C, using the increase in the optical density at 550 nm as a measure of O_2^- formation. Using this assay, PMA, the most potent phorbol ester tumor promoter, was also the most potent phorbol ester with respect to the stimulation of O_2^- production. Phorbolol myristate acetate, a metabolite of PMA in mouse skin equipotent to PMA in causing inflammation, but only one fifth as active as PMA in a promotion bioassay (21), was a weak stimulator of O_2^- production. Acetic acid and phenol, two compounds with potent inflammatory activity in mouse skin and very weak promoting activity, were inactive in stimulating O_2^- production. Mezerein, on the other hand, a potent irritant and very weak complete promoter, was as active as PMA in this system. In other studies, we showed that inhibitors of promotion, such as protease inhibitors and retinoids, are also inhibitors of the oxygen burst stimulated by PMA (22, 23). Based on these results, as well as the known inhibition of promotion by protease inhibitors (24) and antioxidants (25), including DMSO, a hydroxyl radical scavenger (26), we suggested that free radicals and active states of oxygen derived in part from phagocytic cells play a role in the mechanism of action of tumor promoters (27).

Table I. Stimulation of Polymorphonuclear Leukocyte Superoxide Anion Radical (O_2^-) Production by Tumor Promoters and Related Compounds[a]

Compound (5.8×10^{-8} *M*)	O_2^- produced (nmol/min/7.1 $\times 10^5$ PMN/ml)
Phorbol myristate acetate	11.3
Phorbol dibutyrate	5.7
Phorbolol myristate acetate	1.3
4-O-Methyl phorbol myristate acetate	0.0
Phorbol diacetate	0.0
Mezerein	12.9
Acetic acid (1.72×10^{-7}–1.72×10^{-4} *M*)	0.0

[a] From Witz *et al.* (19).

The Peritoneal Macrophage System for *In Vivo* Stimulation of Oxyradical Production by Promoters

In order to demonstrate that phagocytic cells can be stimulated *in vivo* by phorbol ester tumor promoters, we developed a murine peritoneal model system (28). In this system, the test compounds are intraperitoneally injected into unmanipulated mice or mice treated intraperitoneally 6 days earlier with the inflammatory agent thyoglycollate. Peritoneal exudate cells (PEC) are harvested 2 hr after treatment with the test compounds. Macrophages isolated by adherence or whole PEC (consisting of 70–80% macrophages, 20–30% lymphocytes, and less than 1% neutrophils, eosinophils, and basophils) are subsequently incubated with nitroblue tetrazolium. Nitroblue tetrazolium is reduced by superoxide anion radical to an insoluble blue formazan precipitate, which accumulates in the cytoplasm of phagocytic cells producing O_2^-. Incubation of cells with nitroblue tetrazolium in the presence of SOD allows assessment of O_2^--specific reduction of nitroblue tetrazolium.

Using the system described above, intraperitoneal administration of 0.1 μg PMA to unmanipulated CD-1 mice caused an increase in formazan-positive PEC, indicating that PMA stimulates O_2^- production *in vivo* in resident peritoneal phagocytic cells. At the same dose (0.1 μg), neither the incomplete promoters mezerein and 4-O-Me-PMA and the calcium ionophore A23187, nor phorbol, the nonpromoting parent compound of PMA, were able to stimulate O_2^- production *in vivo*. Dose-response studies demonstrated that 4-O-Me-PMA and MEZ are able to stimulate O_2^- production at higher doses (about 50 times that of PMA), but no *in vivo* O_2^- stimulation was observed for phorbol dibutyrate (PDB), phorbol diacetate (PDA), phorbol or A23187. When the same compounds were administered at the 0.1-μg dose to mice pretreated with thioglycollate, PMA, PDB, 4-O-Me-PMA, MEZ, and A23187, all caused an increase in the number of formazan-positive

cells in elicited PEC 2 hr after treatment. The PEC that reduced nitroblue tetrazolium in response to tumor promoters were predominantly adherent and esterase-positive cells, suggesting that they were macrophages (29). The differences in response of resident PEC to PMA and MEZ after *in vivo* administration are notable since, with few exceptions, PMA and MEZ cause similar biochemical responses, including O_2^- production by phagocytic cells *in vitro*. The finding that PMA and MEZ are equipotent in stimulating O_2^- production *in vivo* in thioglycollate-elicited PEC, along with the differences in response of resident PEC to PMA and MEZ, indicates that the physiological state of the macrophage plays a role in the response to various types of tumor promoters (29).

An inhibition of PMA promotion in SENCAR mice has been reported for 4-O-Me-PMA and MEZ (8), as well as for PDB in NMRI mice (30), when these compounds were co-administered with PMA. It was, therefore, of interest to determine whether these compounds could also inhibit the *in vivo* stimulation of oxyradical production by PMA (31). Co-administration of PMA (0.1 μg) with 4-O-Me-PMA (1 ng–1 μg) and PDB (1 ng–1 μg) resulted in a dose-dependent decrease in formazan-positive cells in resident PEC. At equimolar doses of PMA and 4-O-Me-PMA or PDB, the decrease was about 40–50%. Phorbol diacetate, an inhibitor of PMA promotion in NMRI mice (30) but not in SENCAR mice (8), was ineffective in inhibiting PMA-stimulated O_2^- production in PEC *in vivo*, as was MEZ, an inhibitor of PMA promotion in SENCAR mice (8). However, production of O_2^- by thioglycollate-elicited PEC stimulated *in vivo* with MEZ (0.1 μg) was inhibited in a dose-dependent manner by co-administration with PDA (1 ng–1 μg) (31). These results suggested that modulation of oxyradical production may play a role in the inhibition of PMA promotion by phorbol esters. They also suggested that differences may exist in the mode of action of PMA and MEZ with respect to the stimulation of O_2^- production by PEC.

Inhibition of Second-Stage Promotion and Oxyradical Production by Phorbol Diacetate

In order to examine whether inhibition of O_2^- production in mezerein-stimulated PEC by PDA is relevant to second-stage promotion by MEZ, a bioassay was carried out in female SENCAR mice using dimethylbenz(*a*)anthracene initiation (25.6 μg), followed by first-stage promotion with PMA (2 μg 4 times) for 2 weeks and then second-stage promotion with MEZ (2 μg, twice a week, 14 weeks) with and without PDA (2 and 20 μg per dose just prior to MEZ treatment) (32). Co-administration of MEZ with 2 or 20 μg of PDA reduced the number of papillomas after 14 weeks of treatment by 38% and 44%, respectively, compared with MEZ alone (Table II). Phorbol diacetate did not significantly alter the percentage of mice with tumors when administered with MEZ during second-stage promotion, and did not cause an increase in the number of tumors formed compared with the acetone control group when administered without MEZ. In addition, PDA also did not inhibit second-stage promotion by PMA when co-administered at a dose of 20 μg (33).

The inhibition by PDA of MEZ-stimulated superoxide anion radical production *in vivo* by murine PEC and of second-stage promotion by MEZ suggests that oxyradicals play a role in second-stage promotion by MEZ. The observed order of potency of *in vivo* stimulation of oxyradical production in resident PEC decreased in the following order: PMA (complete promoter) > 4-O-Me-PMA (first-stage promoter) > MEZ (second-stage promoter). These limited data suggest that the mode of action of complete, as well as first- and second-stage, promoters may involve active states of oxygen, including free radicals. Since neither second-stage promotion by PMA nor the stimulation of oxyradical production in murine PEC by PMA *in vivo* were found to be inhibited by PDA, the results also suggest that second-stage promotion by MEZ differs from second-stage promotion by PMA.

In Vivo Stimulation of Active Oxygen Production in Mouse Skin and Peripheral Blood Cells

An immediate response to PMA in mouse skin is evidenced by increased phospholipid metabolism (34). A great many studies, summarized by Fischer (35), suggest that the lipoxygenase pathway of arachidonic acid metabolism is important, if not essential, to tumor promotion. Strong support for this concept comes from studies which have shown that lipoxygenase inhibitors and inhibitors of both cyclooxygenase and lipoxygenase inhibit promotion, whereas cyclooxygenase inhibitors enhance promotion.

Arachidonic acid metabolism via the arachidonic acid cascade results in the formation of endoperoxides and hydroperoxides, i.e., secondary active oxygen species whose formation and/or decomposition may be accompanied by the emission of light. Low levels of chemiluminescence have been demonstrated in a number of biological systems during the production and metabolism of active states of oxygen (36). In some *in vitro* systems, the spectral properties of the emitted light are identical to those emitted by the decay of singlet oxygen (37). Light emission, however, may also occur from excited state species such as excited carbonyls and cleavage products of dioxetanes formed during oxidations in biological systems (38, 39). Using a luminol-enhanced chemiluminescence assay, Fischer and Adams (40) demonstrated the production of active oxygen species in SENCAR mouse epidermal cells *in vitro* in response to tumor promoters, indicating that inflammatory cells are not the only cells that may respond to

Table II. Phorbol Diacetate Inhibition of Second-Stage Promotion by Mezerein in SENCAR Mice[a]

Group[b]	Treatment	Average no. of papillomas per mouse (% inhibition)	Percentage of mice with papillomas	No. of mice with carcinomas
1	DMBA, PMA, acetone	3.0	33	0
2	DMBA, PMA, mezerein	22.3	100	0
3	DMBA, PMA, mezerein/PDA 2 μg	13.8 (38)[c]	93	0
4	DMBA, PMA, mezerein/PDA 20 μg	12.4 (44)[c]	97	1
5	DMBA, PMA, PDA 20 μg	1.4	33	2
6	DMBA, PMA, PMA/PDA 20 μg	26.3	100	0

[a] Adapted from Czerniecki *et al.* (32). DMBA, dimethylbenz(*a*)anthracene.
[b] Groups 1–5 consisted of 30 mice per group and Group 6 consisted of 20 mice. Promotion was terminated at 14 weeks.
[c] Significantly different from Group 2, as determined by chi-square analysis, $P < 0.05$.

PMA by producing active oxygen species including free radicals. In these studies, PMA, the most potent phorbol ester tumor promoter, was most effective in stimulating chemiluminescence, and MEZ was as potent as PMA. The chemiluminescence response to other phorbol esters was found to correlate with their promoting activity. The chemiluminescence response to PMA was inhibited by SOD and the SOD-mimetic compound CuDIPS (Cu^{+2} [3,5-diisopropylsalicylic acid]$_2$), suggesting involvement of superoxide anion radicals. Negligible inhibitory effects were observed when catalase, which metabolizes H_2O_2, or mannitol, a hydroxyl radical scavenger, was added to the assay system. The chemiluminescence response to PMA was inhibited by retinoic acid, an inhibitor of promotion by PMA. In addition, inhibitors of arachidonic acid metabolism, which are effective in inhibiting PMA promotion, were also inhibitors of the PMA-induced chemiluminescence response. Specifically, lipoxygenase inhibitors such as nordihydroguaiaretic acid and agents effective against both lipoxygenase and cyclooxygenase were active in diminishing the PMA-induced chemiluminescence response, compared with the cyclooxygenase inhibitors indomethacin and flurbiprofen, which exhibited no or a slight enhancing effect. Based on these results, the authors suggested that arachidonic acid metabolism, most likely by lipoxygenases, may account in large part for the PMA-induced chemiluminescence in mouse epidermal cells. This conclusion is supported by other studies by Fischer *et al.* (41) which showed that phospholipase C mimics the PMA-induced chemiluminescence response in SENCAR epidermal cells. Since the chemiluminescence response was specific to phospholipase C from *Clostridium perfringens* and not from *Bacillus cereus* or phospholipase A_2, the authors suggested that specific lipids may be involved in the chemiluminescence response occurring during arachidonic acid metabolism. Additional studies by this group showed that antioxidants, protease inhibitors, and other agents, such as trifluoperazine, which suppress PMA activity in other cell systems are also active in suppressing the PMA-induced chemiluminescence response in SENCAR epidermal cells.

A natural balance of active oxygen species including free radicals is maintained in the cell by antioxidant enzymes and nonenzymatic mechanisms (18). Among these, the glutathione peroxidase system, which metabolizes hydrogen peroxide and organic hydroperoxides, plays a prominent role. Glutathione peroxidase reduces hydrogen peroxide and organic hydroperoxides to H_2O and ROH compounds, respectively, at the expense of glutathione, which is oxidized to oxidized glutathione (GSSG). Glutathione reductase subsequently reduces GSSG to reduced glutathione (GSH), and NADPH is regenerated by glucose-6-phosphate dehydrogenase, thus restoring cellular GSH and reductant equivalents. A decrease in antioxidant enzymic defenses was originally observed by Solanki *et al.* (42) who found a decrease in SOD and catalase activity in mouse skin treated with PMA. Subsequently, an inhibition of tumor promotion by PMA and of ornithine decarboxylase induction, a biochemical response to promoters in mouse skin, was shown for CuDIPS, a copper coordination complex with superoxide dismutase activity, suggesting that protection against $O_2^{\cdot-}$ attenuates the tumor response (43). Studies by Perchellet *et al.* (44, 45) examining glutathione peroxidase activity and the GSH to GSSG intracellular ratio in isolated mouse epidermal cells suggest that the GSH-dependent peroxidase system is initially stimulated by PMA followed by a rapid inhibition. The rapid decrease in the intracellular GSH to GSSG ratio, the prolonged decrease in glutathione peroxidase activity, and the induction of ornithine decarboxylase activity caused by PMA were all inhibited by free radical scavengers, GSH level-raising agents, and Na_2SeO_3-containing compounds (46, 47). Levels of hydroperoxides of mouse skin homogenates treated with PMA *in vitro* and in skin homogenates prepared from mice treated *in vivo* with PMA were increased relative to control homogenates (48). The levels of hydroperoxides increased with the increasing number of PMA applications and reflected

almost entirely increased hydroperoxide production in the epidermis. The hydroperoxide response was also demonstrated for phorbol esters with complete and/or Stage 2 promoting activity, but not for agents with only inflammatory, hyperplastic, or Stage 1 promoting activity, except for the Ca^{2+} ionophores A23187 (a Stage 1 promoter) and ionomycin. Based on biochemical characterization studies of the promoter-stimulated hydroperoxide response in terms of DNA synthesis and ornithine decarboxylase induction, the authors suggested that PMA- and ionophore-induced DNA synthesis may be linked to hydroperoxide production, rather than to ornithine decarboxylase induction (48, 49).

Oxidation of nonfluorescent 2′,7′-dichlorofluorescin (DCFH) has been used to measure intracellular hydroperoxides. In this assay, cells are incubated with 2′,7′-dichlorofluorescin diacetate, which is hydrolyzed by intracellular esterases to DCFH. In the presence of H_2O_2 or other hydroperoxides, DCFH is oxidized to DCF, which is highly fluorescent. The oxidation of DCFH to DCF by epidermal cells from mouse skin treated with PMA *in vivo* was investigated by Robertson *et al.* (50) in order to determine the ability of PMA to produce hydroperoxides in mouse skin epidermal cells *in vivo*. Epidermal cells from CD-1 mice 24 hr after acetone (vehicle) treatment were relatively homogeneous in cell size and density and oxidized low levels of DCFH. At 24 hr after treatment with 10 μg of PMA, two keratinocyte cell populations oxidized 2 and 10 times more DCFH, compared with dermal cells from acetone-treated mice. Inclusion of catalase in the cell suspension prior to loading with DCFH diacetate suppressed DCFH oxidation to control levels, suggesting that intracellular H_2O_2 is responsible for the enhanced DCFH oxidation in cells derived from PMA-treated mice. The authors concluded that specific subpopulations of keratinocytes produce elevated levels of intracellular peroxides following treatment of mouse skin with PMA *in vivo*. This group also demonstrated the production of reactive oxygen species by peripheral blood leukocytes in mice following exposure to PMA (51). Their results indicate that PMA (0.2–20 μg) induces a dose-dependent increase in polymorphonuclear leukocytes, while simultaneously decreasing the number of peripheral blood monocytes. Hydroperoxide production by polymorphonuclear leukocytes as measured by DCFH oxidation was enhanced 2-fold 4 hr after treatment of mice with PMA (10 μg). The authors suggest that polymorphonuclear leukocytes are activated prior to their infiltration into the epidermis and that they may serve as a primed cell population that significantly contributes to the cutaneous alterations observed during acute and chronic inflammation following PMA treatment.

Oxidation of Epidermal Cell DNA *In Vivo* by Phorbol Myristate Acetate

The memory effect of first stage promotion in the two-stage promotion experiments, as well as the finding that tumor response is independent of whether Stage 1 of promotion occurs before or after initiation, suggests that heritable genetic modifications may also be involved in the mode of action of tumor promoters. It is well known that radiation damage of cellular DNA resulting in strand breaks and oxidized bases is mediated in part by hydroxyl radicals (52). Frenkel *et al.* (53, 54) recently demonstrated oxidative damage in DNA incubated in the presence of PMA-stimulated neutrophils (53), as well as in DNA of HeLa cells exposed to PMA with and without neutrophils (54). 5-Hydroxymethyl uracil and thymine glycol derived from the oxidation of thymine were consistently present in these oxidized DNA. These oxidized DNA bases, as well as 8-hydroxyguanine, were recently reported by Wei and Frenkel (55) to be present in epidermal DNA of SENCAR mice treated topically with PMA. These findings demonstrate that PMA has the ability to alter DNA *in vivo*, most likely via the intermediacy of active oxygen species. Additional studies by this group showed that sarcophytol A, an inhibitor of PMA promotion and H_2O_2 production in stimulated neutrophils, also inhibits the formation of oxidized DNA bases in the epidermis of SENCAR mice treated with PMA for 16 weeks. The formation of oxidized bases correlated with skin hyperplasia and infiltration by inflammatory phagocytic cells (56). Based on these and other findings, the authors suggest that oxidative DNA damage in target cells by active oxygen species derived from epidermal and phagocytic cells play a role in tumor promotion and progression.

Conclusion

Studies from our laboratory suggest that active oxygen species derived in part from inflammatory phagocytic cells are involved in tumor promotion. Limited but sufficient evidence indicates that active oxygen species are also formed *in vitro* and *in vivo* in epidermal cells in response to tumor promoters. However, the cellular processes of promoter-stimulated production of active states of oxygen in epidermal cells remain unclear and need to be determined. It is likely that active oxygen species formed during the oxygen burst by stimulated inflammatory phagocytic cells, as well as active oxygen species formed in epidermal cells, participate in cellular processes leading to promotion. Since promoters are believed to alter cellular metabolism and gene expression by activation of protein kinase C, the intrinsic effects of active oxygen species on protein kinase C activation, as well as their role, if any, on promoter-stimulated protein kinase C activation, need to be elucidated. Germane to this topic are the findings by

Gopalakrishna and Anderson (57), which showed that protein kinase C can initially be activated by mild oxidative modification and subsequently be inactivated by further oxidation. Although tumor promoters do not act by directly binding to DNA, studies indicate that they can oxidatively modify DNA *in vivo* via the intermediacy of active oxygen species. Whether oxidative genetic damage during first stage promotion plays a role in the memory effect or gene expression of initiated cells is a central question in delineating events during two-stage promotion. Numerous phorbol esters and other promoters with complete Stage 1 or Stage 2 activity are currently available. Their use as probes for elucidating mechanisms of active oxygen production and the chemical nature of the active oxygen species formed, coupled with biochemical and molecular studies of promoter action, should provide a clearer picture of the role of active oxygen species in multistage carcinogenesis.

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