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THE ROLE OF OXYGEN RADICALS AS A POSSIBLE MECHANISM OF TUMOR PROMOTION

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INTRODUCTION

In recent years, considerable progress has been made in our understanding of the basic concepts of carcinogenesis. Epidemiological and experimental animal studies indicate that cancer can develop in tissues in a multistep process. The concept of two-stage carcinogenesis was first described by Berenblum (1) in mouse skin and this description has been the primary model for investigating the mechanism of tumor promotion. Tumor induction can be divided into two distinct treatment stages: initiation and promotion. The initiation phase requires a single application of either a direct or an indirect carcinogen at a subthreshold dose and is essentially irreversible; the promotion stage requires repeated treatments *after* initiation and is initially reversible, later becoming irreversible (2). The initiation step in this process probably involves a heritable modification in the genetic material of the cells, as evidenced by the good correlation between the mutagenicity and the carcinogenicity of many chemical agents (3). Most tumor-initiating agents are electrophilic reactants or must be converted metabolically into a chemically reactive electrophilic form that then binds covalently to cellular DNA and other macromolecules (4). Initiation is produced after a single exposure to an initiating carcinogen that presumably converts a small proportion of the target cell population to cells that are competent to be transformed into tumors (5). On the other hand, promoters are not mutagenic and do not bind covalently to DNA. Many promoters, like phorbol esters, are almost exclusively membrane-active agents. The cell mem-

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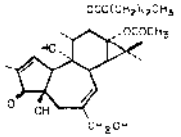
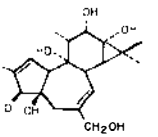
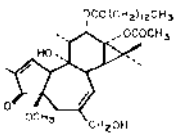


PMA



Phorbol



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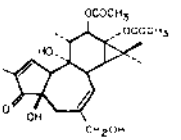
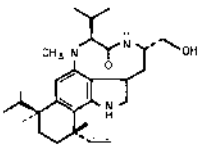
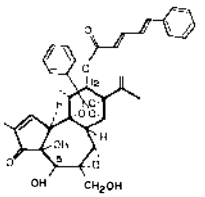


Phorbol 12,13-Diacetate



Teleocidin



Mezerein

Figure 1 Structures of PMA, phorbol, 4-O-methyl PMA, phorbol 12,13-diacetate, teleocidin, and mezerein.

branes of many tissues contain high-affinity, specific receptors that interact with biologically active phorbol esters in a reversible and saturable manner (6, 7). Promotion requires multiple or prolonged exposure to the promoting agent before tumor growth becomes inevitable. Promotion has been further divided into at least two phases (8, 9). An additional step different from promotion has also been described, i.e. the progression of a benign lesion to a malignant neoplasm (10).

Evidence for the existence of two-stage carcinogenesis has been shown in a number of other species and tissues and with other promoting agents (11).

The most potent tumor promoter is phorbol-myristate-acetate (PMA), which has been isolated from croton oil (12, 13). In addition to the phorbol esters, a wide variety of other compounds [teleocidin (14), mezerein (15), and various organic peroxides (16) (Figure 1)] have been shown to have skin tumor–promoting activity. Although tumor promoters cause many cellular and biochemical changes in mouse skin, it is difficult to determine which of the many effects associated with tumor promotion are in fact essential components of the promotion process.

PROTEASE ACTION IN TUMOR PROMOTION

The observation that the inflammatory tumor promoter PMA induces proteases in mouse skin led to the suggestion that proteases play a role in tumor promotion. Further studies have shown that the action of proteases in tumor

promotion and neoplastic transformation can be modified by protease inhibitors when these inhibitors are applied directly to tissues or when fed to animals or man.

The first indication that proteases affect tumor promotion was the observation that protease inhibitors suppress tumor induction by PMA in mouse skin. Low doses (1–10 μg) of tosyl-L-lysine chloromethyl ketone (TLCK), tosyl-phenylalanine-chloromethyl ketone (TPCK), and tosyl-L-arginine methyl ester (TAME) counteract the effect of PMA on mouse skin in two-stage carcinogenesis (17). The number and incidence of tumors are decreased and the latent period is increased. The most effective agent is TPCK. This inhibition by protease inhibitors has been confirmed by Hozumi et al, who used the protease inhibitor leupeptin (18), one of the small inhibitors isolated from streptomycetes (19), to suppress tumorigenesis in the mouse-skin model. The importance of proteases in the tumorigenic process was further kindled by the discovery of Reich and his associates that tumor cells contain more plasminogen activator than their normal counterparts (20). Plasminogen activators are trypsin-like serine proteases. Furthermore, the level of plasminogen activator is increased when fibroblasts are transformed by viruses or promoters (21). Intracellular plasminogen activator appears to be associated with the membrane fraction (22).

In the early studies, the inhibitors were applied percutaneously. Later experiments have shown that feeding a raw soybean diet rich in protease inhibitors suppresses the appearance of tumors in mouse skin treated with nitroquinoline oxide and PMA (23), of breast tumors induced by X-ray irradiation in Sprague-Dawley rats (24), and of spontaneous liver cancer in C_3H mice (25). Similarly, feeding leupeptin (26) or N,N-dimethylamino-[p-p'-(guanidinobenzyloxy)]-benzilcarbonyloxyglycolate, an effective trypsin inhibitor, also delays and suppresses rat mammary tumors induced by 7,12-dimethyl benz(a)anthracene (DMBA) (27).

Additional evidence for the reduction of tumor formation by protease inhibitors has been obtained with the oral administration of ε-aminocaproic acid, which inhibits 1,2,dimethyl-hydrazine-induced mouse colorectal tumors (28). Furthermore, the feeding of a synthetic protease inhibitor [N,N-dimethylcarbamoylmethyl 4-(4 guanidinobenzoyloxy)-phenylacetate]methanesulfate (FOY-305) suppresses the incidence of carcinomas in mouse skin induced by repeated applications of the carcinogen 3-methylcholanthrene, which presumably has both initiating and promoting capabilities (29). FOY-305 is a strong inhibitor of trypsin, thrombin, and kallikrein.

Ingested protease inhibitors may play a direct role in blocking tumor promotion in mouse skin, or they may act in an indirect manner by limiting the digestion of proteins (30).

Two major inhibitors purified from soybeans are the Kunitz inhibitor and the Bowman-Birk inhibitor (31). When the Bowman-Birk inhibitor is fed to rodents, a large part of the inhibitor is excreted in the feces as a protease: protease inhibitor complex that retains its antiprotease activity. We have proposed that the anticarcinogenic activity of Bowman–Birk like inhibitors may be an indirect one resulting from the consequent inhibition of protein digestion (32).

In addition to protease inhibitors, other agents that block the induction of tumors by PMA in mouse skin are the anti-inflammatory steroids, dexamethasone (33) and fluocinolone acetonide (34); certain retinoids (35); a combination of retinoids and anti-inflammatory agents (36); and certain antioxidants (16).

The finding that these agents block tumor promotion has raised new questions about how tumor promotion is derailed by these agents.

OXYGEN RADICALS IN TUMOR PROMOTION

Recent developments have stimulated a growing interest in the possible role of free oxygen radicals in tumor promotion. More and more, the data support the view that these radicals are important in promotion in vivo as well as in vitro.

Tumor induction by PMA in mouse skin is accompanied by an inflammatory response; within 24 hours after promotion treatment, polymorphonuclear leukocytes (PMNs) infiltrate the dermis (37). Protease inhibitors applied directly to mouse skin block this response (17). When phagocytes (PMNs and macrophages) are exposed to appropriate stimuli, a series of metabolic events takes place known as the respiratory burst (38, 39). Many agents, both particulate and soluble, are able to evoke this response, which is accompanied by the production of superoxide anions ($O_2^-\cdot$), hydrogen peroxide (H_2O_2), hydroxy radical ($\cdot OH$), and singlet oxygen. The production of reactive oxygen species by phagocytic cells contributes to the antimicrobial and antitumor activity of these cells (38–40).

Evidence from our laboratory suggests that tumor promoters such as PMA may enhance tumor formation by stimulating superoxide anion production through the action of an NADPH-dependent oxidase. Very small amounts of PMA (5–10 ng) stimulate the formation of superoxide anions, H_2O_2 (41), and chemiluminescence (42) in PMNs. Mezerein, a diterpene related to the phorbol esters, also stimulates $O_2^-\cdot$ production (43) as well as that of chemiluminescence (42). Indole alkaloids such as teleocidin, lyngbyatoxin, debromoaplysiatoxin, and aplysiatoxin (14), which are chemically distinct from the phorbol esters, are another class of tumor promoters that stimulates $O_2^-\cdot$ production in human PMNs (44). The degree of stimulation of PMNs by various phorbol esters correlates with their promoting activity in mouse skin. PMA is greater than phorbol dibutyrate, which is greater than phorbolol myristate acetate (45).

On the other hand, phorbol, phorbol diacetate, and 4-0-methyl phorbol myristate acetate, which are inactive in tumor promotion, are also inactive in stimulating $O_2^-\cdot$ production (42, 45). PMA, but not 4-0-methyl-PMA, also stimulates the release of $O_2^-\cdot$ and H_2O_2 in guinea pig macrophages (46). No superoxide anion production has been observed in either human or Chinese hamster fibroblast cultures (47). These oxygen radicals induced by the promoter may contribute to tumor promotion by being released at an inappropriate time.

One might expect protease inhibitors capable of inhibiting tumor promotion in mouse skin to be able to antagonize the stimulation by PMA in human leukocytes. Such is indeed the case; a number of protease inhibitors (soybean trypsin inhibitor, lima bean trypsin inhibitor, benzamidine, and antipain) suppress the production of $O_2^-\cdot$ in PMNs stimulated by PMA. Soybean trypsin inhibitor antagonizes the stimulatory effect of a variety of other stimulating agents and inhibits $O_2^-\cdot$ production in rat PMNs as well as in alveolar macrophages (41). TLCK and TPCK suppress oxygen consumption and superoxide production in PMA-stimulated rat pulmonary macrophages (48). Crude extracts of canned legumes, as well as protease inhibitors purified from these legumes, have been effective in blocking the effect of PMA on the $O_2^-\cdot$ response of PMNs (49).

Derivatives of Vitamin A have also been shown to inhibit tumor promotion (35). Thus, it is not surprising that retinol and Vitamin A analogs (retinyl acetate and retinoic acid) effectively inhibit $O_2^-\cdot$ production in PMA-stimulated PMNs in a dose-dependent manner (42, 50). Similar to PMNs, rat alveolar macrophages also show a decrease in $O_2^-\cdot$ formation when stimulated by PMA in the presence of retinol. Stimulation of $O_2^-\cdot$ production by mezerein and teleocidin B is also inhibited by retinol. The mechanism of action of retinol in this system could be explained by its alteration of cell membrane fluidity. Retinoic acid has been shown to alter membrane fluidity in red cell membranes (51). Thus, the inhibition of PMA stimulation of PMNs observed with retinoids may involve a general effect such as an alteration in the dynamic properties of plasma membranes (50).

The anti-inflammatory corticosteroid dexamethasone, which can strongly inhibit tumor promotion (33), also inhibits the PMA-stimulated PMNs. The anti-inflammatory property of the steroids may in part be due to their antioxidant properties (52). An alternative explanation is that the inhibitory action of dexamethasone is due to the elaboration of a protease inhibitor. Dexamethasone has been shown to induce an inhibitor of plasminogen activator (53, 54).

Superoxide production probably is initiated on the cell surface by the interaction of the cell surface with appropriate stimuli. Surface-active agents such as cytochalasin E and Concanavalin A generate $O_2^-\cdot$ formation in PMNs and monocytes (55). In addition, anti-IgE induces production of $O_2^-\cdot$ in human

basophils (56). The $O_2^-\cdot$ production in all of these cells is inhibited by potent inactivators of serine proteases in a dose-dependent fashion. The inhibitory effect of inactivators of chymotrypsin-like proteases is more effective than that of inhibitors of trypsin-like proteases, suggesting that a chymotrypsin-like protease is involved in $O_2^-\cdot$ production by PMNs and basophils. $O_2^-\cdot$ produced by the xanthine-xanthine oxidase superoxide anion–generating system is not impaired by protease inhibitors, indicating that these inhibitors do not themselves react with superoxide.

More direct evidence for the involvement of free radicals in tumor promotion comes from the work of Slaga et al (2), who have shown that a number of free radical–generating compounds (benzoyl peroxide, chloroperbenzoic acid, lauroyl peroxide) are effective skin tumor promoters in Sencar mice after initiation with DMBA. A significant number of papillomas and squamous cell carcinomas are produced by benzoyl peroxide, which is not effective as a complete carcinogen or as an initiator.

One expected effect of PMA stimulation is an increase in lipid peroxidation. However, Logani et al observed that lipid peroxidation in mouse skin is decreased by PMA treatment and that TPCK has no effect on this response (57). In contrast, Belman & Garte (58) found that mouse epidermal lipid peroxidation is unchanged after one treatment with PMA but is markedly suppressed after six treatments.

Stimulation of superoxide release by PMA has been observed only in phagocytes. Recently, Fisher & Adams (59; S. M. Fischer, unpublished data) presented evidence that treatment of isolated mouse epidermal cells with PMA results in increased production of chemiluminescence, an index of the generation of superoxide anions and singlet oxygen. The PMA-mediated chemiluminescence is dose-dependent and peaks within 15 minutes. The extent of the response correlates with the tumor-promoting ability of the phorbol esters tested. This response can be suppressed by superoxide dismutase (SOD) and Cu(II)-3,5,diisopropylsalicylate (CuDIPS) as well as by retinoic acid. Inhibitors of the arachidonic acid cascade, such as benoxaprofen and phenidone, also suppress the response. The data suggest that the metabolism of arachidonic acid is responsible for the PMA-stimulated response in mouse epidermal cells. Thus, free radicals may be generated by tumor promoters without the intervention of PMNs.

DEFENSE MECHANISMS AGAINST FREE OXYGEN RADICALS

The reduction of molecular oxygen in all aerobic eukaryotic cells results in intermediates ($O_2^-\cdot$, H_2O_2, and $\cdot OH$) that are highly toxic. As a result, organisms have developed an elaborate system of defenses against these intermedi-

ates (60). The primary defense is provided by enzymes that catalytically scavenge the intermediates of oxygen reduction. Superoxide anions are eliminated by SOD, which catalyzes a dismutation reaction leading to the formation of $O_2^-\cdot$ plus H_2O_2. The latter can be destroyed by the action of catalases and glutathione peroxidase (61). Although $O_2^-\cdot$ and H_2O_2 individually may not be particularly damaging in aqueous solution, it has been postulated that their combined action leads to the formation of a highly reactive product, the hydroxy radical via the Haber-Weiss reaction (62), according to the following equation:

$$O_2^-\cdot + H_2O_2 \rightarrow \cdot OH + OH^- + O_2$$

This reaction is very slow; however, there is substantial evidence that, when catalyzed by metal ions present in all biological systems, this reaction can proceed at a pace that would make it biologically relevant (63). Hydroxy radicals will attack and destroy most molecules in living cells (39, 40); hence, efficient methods of removing $O_2^-\cdot$ and H_2O_2 are necessary to prevent the damage that can result from these radicals.

Treatment of adult mouse skin with 2 μg PMA results in decreased levels of both SOD and catalase activities in the epidermis. A fairly good correlation has been found between the tumor-promoting ability of various promoters and their ability to decrease the activity of these enzymes. The effect of PMA is selective for SOD and catalase, since general protein synthesis is not inhibited by PMA (64). Mezerein, a resiniferonal derivative with weak promoting activity that nevertheless acts as a strong stage II promoter, is more potent than PMA in lowering the levels of SOD and catalase. Therefore, lowered SOD and catalase levels may be characteristic of stage II promotion.

Reduced levels of SOD have been found in human lymphoblast and fibroblast cultures after PMA treatment (47). Such treatment also lower levels of both SOD and catalase in $C_3H/10T½$ cells (J. Yavelow, personal communication).

Two types of superoxide dismutases are found in all eukaryotic cells: a copper and zinc-containing SOD (CuZnSOD) and a manganese-containing SOD (MnSOD) (61). Lowered levels of CuZnSOD and especially of MnSOD are characteristic of many of the tumor systems studied so far (65). CuZnSOD activity is significantly decreased in the liver of rabbits bearing the VX-2 carcinoma in the maxillary sinus, whereas MnSOD is not affected. However, in the VX-2 carcinoma itself no MnSOD activity and only low levels of CuZnSOD have been found (66). In a separate study, solid tumors were induced in CBA/J mice by implantation of Ehrlich carcinoma cells. Depressed levels of SOD activity were seen in a number of the organs of these tumor-bearing mice. In liver, spleen, and kidneys, the MnSOD activity was lowered some time after implantation, even though there was no evidence of metastases in these organs (67). An earlier study showed that Ehrlich carcinomas in inbred

CBA mice contain reduced amounts of SOD activity when compared to the activity of control mice. Furthermore, when CuDIPS was administered at various doses, reduction in tumor size, delay of metastases, and a significant increase in survival of the hosts were observed (68). CuDIPS is a lipid-soluble, low molecular–weight compound that exhibits superoxide dismutase activity and therefore can act as an intracellular $O_2^-\cdot$ scavenger. CuDIPS also reduces the frequency of PMA-induced mouse papillomas and inhibits the induction of ornithine decarboxylase (ODC) (69), the enzyme shown by Boutwell to be characteristic of tumor promotion (70).

An excess of free radicals, in conjunction with a deficiency in protective enzymes such as SOD and catalase, could lead to adverse effects that may contribute to the cancer phenotype.

THE ROLE OF TUMOR PROMOTERS IN MUTAGENESIS

Free oxygen radicals generated during phagocytosis can be harmful or beneficial with respect to neoplasia. Products of phagocytosis such as $O_2^-\cdot$, H_2O_2, and $\cdot OH$ are part of the killing mechanism of neutrophils and macrophages (40). On the other hand, these products can peroxidize lipids and form cytotoxic metabolites such as malondialdehyde, which is capable of interacting with DNA and producing mutations (71). Additional evidence for the genotoxic effects of the products of phagocytosis has been furnished by the observation that mutagenic activity could be associated with human PMNs stimulated by phagocytosis. When histidine-requiring mutants of *Salmonella typhimurium* strain TA 100 are incubated with PMNs, these bacteria revert to histidine independence (72). The mutagenicity of PMNs from patients with chronic granulomatous disease (CGD) is markedly diminished compared to that of normal cells. Neutrophils from a patient with CGD have a defect in the NADPH oxidase-superoxide generating system and are thus unable to generate $O_2^-\cdot$ and H_2O_2. Further support for the idea that phagocytosis induces mutations comes from work with luminous bacteria. In this case, dark mutants of luminous bacteria incubated with human neutrophils activated by opsonized zymosan revert to hereditary stable luminescent forms. Heat-killed phagocytes are not mutagenic. Scavengers of oxygen radicals, such as β carotene, mannitol, and benzoate, as well as SOD, prevent these mutations (73). Moreover, $O_2^-\cdot$ generated from potassium superoxide induces mutagenic and cytotoxic effects in Chinese hamster ovary (CHO) cells. These effects are reversed by SOD (74).

PMA-stimulated PMNs cause other types of cytotoxic changes in CHO cells, resulting in a concentration-dependent increase in sister-chromatid exchanges. PMA alone and PMNs from a patient with CGD did not increase sister-chromatid exchanges. A significant increase in sister-chromatid exchanges is

induced when the cells are exposed to a cell-free superoxide-generating system (75).

The production of reactive oxygen species could explain how leukocytes induce mutation and generate sister-chromatid exchanges and suggests that PMA may participate in tumor promotion in vivo by stimulating toxic oxygen radical production in PMNs.

IN VITRO TUMOR PROMOTION SYSTEMS

PMA can enhance the level of transformation induced by chemical carcinogens (76), ultraviolet light (77), and X-ray in $C_3H/10T½$ cells (78) just as it enhances promotion in vivo. Protease inhibitors have been shown to prevent tumor promotion in vivo and they also have the ability to inhibit malignant transformation in vitro. Protease inhibitors that have the ability to suppress X-ray transformation in $C_3H/10T½$ cells include antipain, leupeptin, and the Bowman-Birk inhibitor, whereas antipain and soybean trypsin inhibitor suppress PMA enhancement of transformation (79). Similar results have been obtained by Borek et al (80). Antipain, elastatinal, chymostatin, and leupeptin also block chemical transformation of $C_3H/10T½$ cells (81).

Mouse erythroleukemia cells respond to PMA by an increase in cell adhesiveness. This response is associated with activation of protease activity. TLCK prevents cell adhesiveness. Pentamide isethionate, a trypsin-like inhibitor, decreases cell adhesiveness as well as PMA-induced proteolytic activity (82).

The enhancement of X–ray induced sister-chromatid exchanges by PMA in $C_3H/10T½$ cells is also suppressed by antipain and leupeptin (83).

Induction of skin tumors and ornithine decarboxylase by PMA is inhibited by retinoids (35). Thus, the ability of retinoids (β-all trans retinoic acid and the trimethyl methoxyphenyl analog of N-ethyl retinamide) to inhibit not only X–ray induced transformation but also the enhancement of this transformation by PMA in both $10T½$ and hamster embryo cells is to be expected. The effectiveness of the retinoids in preventing promotion takes place a short time after their addition to the media and their action is irreversible (84).

Zimmerman & Cerutti (85) have shown that active oxygen species act directly as a promoter in $C_3H/10T½$ cells. Extracellular superoxide produced by xanthine-xanthine oxidase has the capacity to promote $C_3H/10T½$ fibroblasts. Cell cultures initiated with ^{137}Cs γ-rays or benzo[a]pyrene diol epoxide I transform three to 30 times more effectively when subsequently treated with xanthine-xanthine oxidase for three weeks. SOD or SOD together with catalase reduces the number of transformed foci.

The effect of SOD and catalase on bleomycin and X–ray induced transformation has been examined in hamster embryo cells. SOD, but not catalase,

inhibits the transformation induced by X-rays and bleomycin and the enhancement by PMA of X–ray induced transformation (86). These results support the idea that oncogenic transformation is mediated in part by free radicals and that SOD is most effective in inhibiting events associated with promotion.

Mouse epidermal cells (JB6) have been shown to respond to late-stage tumor promoters with an irreversible induction of anchorage-independent growth. Mezerein, a strong stage-II promoter, stimulates promotion of anchorage-independent growth of JB6 cells without accompanying induction of DNA double- or single-strand scissions. On the other hand, both benzoyl peroxide, a complete tumor promoter, and H_2O_2, an efficient stage-I tumor promoter, produce single-strand DNA scission under conditions that do not induce anchorage-independent growth. These data lead one to believe that strand scission in DNA may not be involved in late-stage promotion in JB6 cell lines (87).

However, PMA and mezerein induce DNA single-strand breaks in primary mouse epidermal cells when incubated in the presence of macrophages. PMA or macrophages alone do not induce strand breaks. One concludes from this data that active phorbol ester tumor promoters cause the release of a clastogenic factor from macrophages that can then exert its effect on a known target cell, the epidermal cell (88). Induction of anchorage independence by PMA in JB6 mouse epidermal cell lines is inhibited by antioxidants, CuZnSOD, and CuDIPS, but not by catalase. Scavengers of ·OH (benzoate, mannitol) are moderately active, suggesting that $O_2^{\cdot-}$ · and possibly ·OH, but not H_2O_2, play a role as mediators of promotion of neoplastic transformation by PMA in JB6 mouse epidermal cells (89).

The addition of fresh medium to mouse mammary tumor cells (Mm 5 mt/C1) induces ornithine decarboxylase. Further, PMA enhances this induction. SOD, catalase, SOD plus catalase, and mannitol (a scavenger of hydroxy radicals) partially inhibit ODC induction, supporting the notion that active oxygen species $O_2^{\cdot-}$·, H_2O_2 and ·OH participate in the induction of ODC (90).

DNA DAMAGE BY FREE RADICALS

At the biochemical level, the major difference between initiators and promoters is their site of action. Whereas initiators (or their metabolites) bind covalently to DNA, the primary site of action of the phorbol ester tumor promoters appears to be the cell membrane through their interaction with specific cell receptors (6, 7). It usually has been assumed that tumor promoters (in particular PMA) exert their pleotropic effects entirely by interaction with epigenetic targets. However, recent observations have provided evidence that the phorbol ester tumor promoters may act directly or indirectly to produce DNA damage and chromosomal aberrations via activated forms of oxygen.

Very low levels of PMA induce extensive DNA strand break damage (DSBD) in human PMNs. Just as in the case of mouse skin, nonpromoting analogues are inactive. Damage is decreased by the addition of either catalase or SOD, providing evidence that the damage is caused by products of the respiratory burst (91). The damage to DNA involves both superoxide anion and H_2O_2, but the precise radical has not been determined. A likely candidate is the highly reactive ·OH radical, which can be formed from $O_2^-\cdot$ and H_2O_2 in the presence of certain trace metals (63). However, the use of ·OH scavengers does not provide any definite proof of ·OH involvement (92). The following observations provide strong evidence that PMA-induced DNA damage is related to the respiratory burst: (*a*) DSBD is prevented by the inhibitor 2-deoxyglucose, which blocks superoxide production by interfering with the hexose monophosphate shunt responsible for generating NADPH. (*b*) The PMNs from patients with CGD have a defect in the NADPH-oxidase superoxide-generating system and are thus unable to generate $O_2^-\cdot$ and H_2O_2. Exposure of PMNs from patients with CGD to PMA induces no detectable DSBD. (*c*) PMA-stimulated PMNs induce DSBD in mouse erythroleukemia cells. DSBD is blocked by catalase but not be SOD. Complete inhibition by catalase but not by SOD is also obtained when DSBD is induced in PMNs by xanthine-xanthine oxidase, which enzymatically generates superoxide anions. Other promoters that produce DSBD in leukocytes are benzoyl peroxide and teleocidin B (93).

The action of oxygen radicals may also exert its effect through the formation of a clastogenic factor, a diffusible cellular product that induces chromosomal damage. PMA, but not its weakly or nonpromoting derivatives, induces chromosomal aberrations with high efficiency in phytohaemogglutinin-stimulated lymphocytes (94). The clastogenic activity of the compounds tested (PMA, 4-O-methyl PMA, and phorbol) parallels their effectiveness as promoters in the mouse skin system. This clastogenic activity of PMA is indirect because it can be suppressed by SOD, which catalyzes the breakdown of $O_2^-\cdot$. It is mediated by secondary products formed by the cell in response to the interaction with PMA. Active oxygen species, as well as metabolites of arachidonic acid, are intermediates in the formation and action of the clastogenic factor because CuZnSOD, certain antioxidants, and inhibitors of the cyclooxygenase and lipooxygenase pathways are anticlastogenic (95, 96). PMA also induces the formation of a diffusable clastogenic factor in human leukocytes (97).

Clastogenic activity also can be detected in concentrated ultrafiltrates of media from cultures of fibroblasts of patients with Bloom's syndrome (98). No activity has been found in the media of normal fibroblasts. Because bovine SOD strongly suppresses the clastogenic potency of the ultrafiltrates, the authors speculate that the primary genetic defect in Bloom's syndrome may be a deficiency in the detoxification of active oxygen species that cause the formation of a clastogenic factor. Preliminary evidence suggests that the

clastogenic factor consists of free arachidonic acid plus lipid hydroperoxides and aldehydic compounds. Cerutti et al have proposed a model for "membrane-mediated chromosomal damage," i.e. membrane-active agents such as PMA stimulate the arachidonic acid cascade, elicit an oxidative burst, and perturb membrane integrity so that phospholipids become more susceptible to auto-oxidation and induce chromosomal damage (99).

Radiation causes strand breaks (100) and modification of bases in DNA (101, 102). It is well established that hydroxy radicals are responsible for this damage (62). Modification of thymine in cellular DNA through the action of γ-irradiation results in formation of 5-hydroxymethyl-2'-deoxyuridine (HMdU) (102). If PMA is similar to ionizing radiation in generating ·OH radicals, one would expect the same type of modification of the thymine moiety in DNA as has been shown to occur with γ-irradiation. Preliminary results indeed show that PMA-stimulated neutrophils cause formation of HMdU in DNA coincubated with PMNs in the presence of ferrous ions or autologous serum (103). H_2O_2 also modifies thymine, forming ring-saturated products such as thymine glycol (104). Therefore, it appears that at least some of the properties of tumor promoters, expressed through the PMN-mediated generation of oxygen radicals, are similar to initiating carcinogens in causing modification of DNA constituents.

TUMOR PROMOTION IN OTHER SYSTEMS

The significance of the concept of tumor promotion has been questioned because initially mouse skin was the only vehicle in which induction of tumors by PMA could be demonstrated. Recent developments, however, show that two-stage carcinogenesis is not limited to mouse skin, and the importance of promotion in the development of several other cancers has been confirmed.

In addition to the phorbol esters, several other substances exhibit promoting activity. Phenobarbital appears to act as a tumor promoter by increasing the incidence of tumors in the liver when its administration is preceded by initiation with 2-acetylaminofluorene (105). Similarly, the incidence of bladder cancer in animals has been enhanced after a single dose of a carcinogen followed by consumption of diets containing saccharin or sodium cyclamate (106, 107). Bile acids promote colon cancer initiated by N-methyl-N-nitro-N-nitrosoguanidine (108).

Although tobacco smoke contains a number of known carcinogens, the incidence of cancer associated with smoking could just as well be related to the tumor promoters in smoke. The risk of cancer occurrence in heavy smokers who have given up the habit for 15 years is only slightly higher than that of individuals who have never smoked. This reduction in risk is more consistent

with the view that promoters rather than initiators are the major agents of cigarette smoke that cause cancer (109).

Prolonged inhalation of asbestos produces two types of lung cancer. Asbestos has a promoting effect on tumor development in rodent trachael grafts previously exposed to subcarcinogenic doses of 7,12-dimethylbenz(a)-anthracene. Increased DNA synthesis and the induction of ornithine decarboxylase accompany the morphological changes induced by asbestos in tracheal epithelium cells. The addition of exogenous SOD, but not catalase, to these cultures prevents the cytotoxicity induced by asbestos, suggesting that $O_2^-\cdot$ may be important in mediating asbestos cytotoxicity (110).

Production of duodenal adenocarcinomas in mice initiated by methyl-azoxymethanol acetate is enhanced when hydrogen peroxide is given in the drinking water (111). This effect, which is larger in mice with low levels of catalase (112), has been ascribed to the promoting action of oxygen radicals, probably $\cdot OH$ radicals generated from H_2O_2.

The decisive action of an initiating carcinogen is to cause formation of a cancer-initiated cell that differs from normal cells in having an increased risk of tumor formation on subsequent exposure to carcinogens or promoting agents. A possible model for a cancer-initiated cell is one that has lowered repair capacity, as do cells of individuals with genetic diseases such as *Xeroderma pigmentosum* or *Ataxia telangectasia*. For example, an initial insult to cells by viral infection or chemical carcinogens (e.g. N-acetyl-N-amino-fluorene) results in the loss of their ability to repair carcinogen-induced damage (113). This is also true of lung explants exposed to cigarette smoke (114).

The effect of one agent (promoter) increasing the effect of another (initiator) has been noted in some epidemiological studies (115, 116). Ionizing radiation, which experimentally is used as an initiator in cell culture (78) or to induce breast cancer in rats (23), may act as an initiator in man. Asbestos workers who are smokers have a ten-fold higher lung cancer mortality than asbestos workers who do not smoke (11). Cigarette-smoking uranium miners occupationally exposed to radon have radiation-induced lung cancer rates about five times higher than those of miners who do not smoke (115). Here, we have a choice of whether to assign the role of initiator to the radiation or to the tobacco smoke.

A clearer human case for ionizing radiation acting as an initiator is the description of a 15-fold increase in risk for cutaneous cancer in psoriatic patients undergoing PUVA treatment (8-methoxypsoralen plus long-wave ultraviolet light) if they had been previously treated with ionizing radiation (116). A possible mechanism suggested for these findings is the lowering of DNA repair capacity by the ionizing radiation (116). The phenomenon described here has some relevance to multistage carcinogenesis, where a single dose of one agent (X-rays) predisposes cells to the action of subsequent

exposure to a second agent (PUVA). A single X-ray treatment of V79 cells acts in a manner expected from an initiator. PUVA-induced mutability of the hypoxanthine guanine-phosphoribosyl locus of V79 cells increases for at least 108 days after the initial insult by X-ray treatment (117).

A second possible mechanism for increased vulnerability to promoter-induced damage after the primary damage by the initiating agent has occurred is a diminution in the capacity of the cells to detoxify active oxygen species. This might lead to increased amounts of DNA damage and the release of clastogenic factors. It has been postulated that clastogenic factors contribute to the chromosomal fragility of Bloom's syndrome and *Ataxia telangectasia* (96). The agents that detoxify active oxygen species include protective enzymes such as SOD that inhibit transformation when added to tissue culture systems (86).

POINTS TO PONDER

A number of problems concerning the role of free oxygen radicals in tumor promotion remain to be solved. How does the tumor promoter recognize the initiated cell and convert it to a tumor? What are the ultimate reactive species producing promotional or clastogenic changes in cellular DNA? What is the relationship between DNA damage induced by active oxygen and tumor promotion? How does one relate tumor induction in mouse skin with the phenomenon of oxygen radical production in neutrophils? In what way do protease inhibitors, retinoids, and antioxidants work to inhibit tumorigenesis? These and other questions still remain to be answered before the underlying mechanism of tumor promotion can be fully understood.

SUMMARY

In this chapter we have reviewed the evidence that free oxygen radicals play a role in tumor promotion. We have emphasized the fact that protease as well as other inhibitors exert their action by modulating the oxygen radical response. The finding that chemical agents are capable of blocking the carcinogenic process offers some hope that cancer may be prevented by diets rich in these agents.

Nutrition is an important modifying factor in chemical carcinogenesis. A relationship between diet and cancer incidence has been demonstrated by studies that show a wide variation in cancer incidence from country to country and even within the same country, depending on the dietary habits of the populations (118). Even more convincing is the fact that in migrant populations the incidence of cancer changes from the level characteristic of the mother country to that of the new country as the immigrants adapt to the dietary preferences of their new home (119, 120).

The Western diet, which is rich in fat content but low in vegetables, appears to contribute to a higher occurrence of breast, colon, and prostatic cancers (121, 122). The incidence of these cancers is lower in populations eating vegetarian diets (123–125).

We have shown that protease inhibitors interfere with tumor promotion by modulating the oxygen radical response. Protease inhibitors are widely distributed in plants, especially soybeans, which are a major source of protein in vegetarian diets. Therefore, the finding that protease inhibitors interfere with tumor promotion, taken together with the lower incidence of cancer in populations eating vegetarian diets, offers hope for the future that protease inhibitors may play an important role in preventing cancer.

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