Induction of 5,6-Ring-saturated Thymine Bases in NIH-3T3 Cells by Phorbol Ester-stimulated Macrophages: Role of Reactive Oxygen Intermediates

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ABSTRACT

Because oxygen intermediates secreted by inflammatory leukocytes are postulated to play a role in potentiating carcinogenesis, we investigated the ability of macrophages to induce oxidative DNA damage in eukaryotic cells. Murine macrophages, obtained from sites of inflammation and stimulated with 12-O-tetradecanoylphorbol-15-acetate, induced the formation of 5,6-ring-saturated thymine bases in the DNA of cocultured NIH-3T3 cells; macrophages or 12-O-tetradecanoylphorbol-15-acetate alone did not induce such alterations. Reagent H2O2, at concentrations produced by macrophages in the ambient medium (i.e., ~10^-5 M), induced saturated thymines in the target cells in a dose-dependent manner. The reaction between reagent H2O2 and cellular DNA was rapid, reaching maximum levels in 30 min, and similar amounts of saturated thymines were induced at 4° or 37°. The 3T3 targets were able to repair the saturated thymines rapidly (i.e., over 70% of the lesion was removed in 2 hr). Catalase completely inhibited macrophage-mediated induction of saturated thymines, although superoxide dismutase enhanced induction. Taken together, the data indicate that macrophages exposed to phorbol diesters can induce a specific, quantifiable lesion in the DNA of bystander eukaryotic cells and that reactive oxygen species from the macrophages participate in producing the lesion.

INTRODUCTION

Evidence from many investigators has indicated that ROI can play a major role in carcinogenesis (5, 13, 45, 47). Studies of mutagenesis, transformation, chromosome damage, DNA breakage, and tumor promotion have shown that these processes can be inhibited by scavengers of H2O2, O2-, or OH or by inhibitors of ROI secretion, inflammation, and arachidonic acid metabolism (8, 9, 18, 19, 29, 48, 49, 51, 52). Direct evidence for the participation of ROI in tumor promotion has come from studies demonstrating that benzoil peroxide acts as a potent tumor promoter in mouse skin (45). In addition, H2O2 itself has been shown to be a weak promoter (30). The major sources of ROI in the body and critical cellular targets of ROI, however, have not been fully established. One potentially abundant source of ROI is the inflammatory leukocytes-neutrophils and macrophages. When these cells are stimulated in vitro by active tumor promoters such as TPA, they secrete sufficient ROI to produce relatively high concentrations of H2O2 and O2- in the culture medium (i.e., up to 10^-4 M) (23, 39). Recent studies have shown that blood leukocytes exposed to TPA suffer single-strand breaks in their DNA and that eukaryotic cells cocultured with TPA-stimulated leukocytes have increased rates of sister chromatid exchanges (9, 48).

In 2-step models of carcinogenesis, tumor promotion in response to irritants, such as phorbol esters, generally requires months, a period of time consistent with the presence of chronic, rather than acute, inflammation. This suggests that macrophages might well be the major inflammatory cell in the skin during TPA exposure in the promotion step of carcinogenesis. Macrophages have been suspected of contributing to the development of neoplasia through several mechanisms. Macrophages can metabolically activate carcinogens (27). Kupffer cells have been shown to participate directly in DNA damage, repair, and replication in the liver during carcinogen exposure (33, 34). The coadministration of particulates and carcinogens to the lung can significantly increase tumor yield (14), and in humans, smokers often have higher rates of lung cancer if there is a concurrent exposure to particulates (43). It is important to stress that macrophages, unlike polymorphonuclear leukocytes, are not end cells, but long-lived cells of varying functions and capacities depending on the state of maturation or activation achieved after leaving the bone marrow (4). Of particular importance is the observation that macrophages in different stages of activation have significantly different capacities for ROI secretion, which can be further augmented by environmental pollutants (15).

Previous studies of the chemical alterations induced in DNA by ROI have mainly measured the induction of single-strand breaks. While these methods are sensitive at detecting ROI-DNA interactions, they provide little information about the actual chemical structure of the alterations induced in DNA. Single-strand breaks can represent strand scissions directly caused by ROI or discontinuities induced by the repair of many different forms of DNA damage. One of the major types of base alterations induced in DNA by ionizing radiation, thought to be mediated by OH radicals, is saturation of the 5,6-double bond in thymine (25). In fact, T' have been shown to be formed in isolated DNA reacted with reagent H2O2 at concentrations of ~10^-1 M (17).

For these reasons, we investigated the induction of this class of chemically defined lesions in the DNA of NIH-3T3 cells by macrophages. We here report that T' is induced in 3T3 cells by TPA-stimulated inflammatory macrophages or by physiologically relevant concentrations of reagent H2O2 in a quantitatively similar manner. We further report that the induction of T' in target cells by macrophages is fully inhibited by catalase and enhanced by SOD.

MATERIALS AND METHODS

Cell Culture. NIH-3T3 cells, generously provided by Dr. Darell Bigner, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin, and streptomycin. Casein-elicited murine...
macrophages were obtained 3 days after i.p. injection of 120 mg of casein in 1 ml of water into C57BL/6J mice. They were cultured and purified by adherence as described previously to result in monolayers of >98% macrophages (3).

Treatment of DNA with OsO₄. OsO₄ selectively induces 5,6-dihydroxythymine in DNA (11). [³H]Thymidine-labeled DNA was isolated from prelabeled NIH-3T3 cells by hydroxyapatite chromatography as described previously (7). The labeled DNA was denatured by heating to 90°C followed by rapid cooling in an ice bath. Following denaturation, the DNA was exposed to 1% OsO₄ at 4°C for 15 min. Excess OsO₄ was removed by ether extraction, and the DNA was desalted by passing it through 1 × 15-cm columns containing Sephadex 25 (Pharmacia) eluted with deionized water.

Quantitation of Saturated Thymines (T'). The number of saturated thymine bases (T') was measured by quantifying the acetol fragment, resulting from selective fragmentation of the saturated pyrimidine ring by successive treatments with dilute base, followed by acid as described by Harinaran and Cerutti (26). If the thymine base is labeled with ³H in the methyl position, then the label will be contained in the acetol fragment which can be separated by ion-exchange chromatography (26). Columns measured 1.0 × 30 cm and contained a 6-cm layer of AG 50W-X8 (H⁺) 200 to 400 mesh on the bottom, an 8-cm layer of AG 1-X8 (OH⁻) 200 to 400 mesh in the middle (Bio-Rad), and a 6-cm layer of DEAE-Sephadex A25 (Pharmacia) on top. The columns were eluted with degassed, deionized water. The assay for T' was performed as described (26), except that after centrifugation and neutralization the samples were centrifuged in microconcentrators (Amicon) which contained YM filters with a molecular weight cutoff of 10,000. The filtrate containing the low-molecular-weight material was then passed over the ion-exchange columns. Target cells were labeled with [³H]thymidine to a level of 11 to 35 × 10⁶ cpm per sample as described below. Following precipitation and centrifugation through the microconcentrators, the amount of radioactivity applied to the columns was reduced by 50 to 70%. Counts recovered from the columns were normalized with respect to applied counts, background was subtracted, and the net cpm were divided by the total radioactivity to calculate the number of T' per 10⁶ thymine bases. Backgrounds were consistently in the range of 100 to 500 cpm. Only samples which were at least 100 cpm over background were used for quantitation. All samples were chilled in the dark and counted for 10 min in a Packard Model 3385 scintillation counter. Counting efficiency for ³H was 40%. Specificity of the assay results from the fact that only the thymine bases are labeled, and only saturated thymines are labile to ring fragmentation when treated with mild base and then acid (11). Chart 1 is a radiochromatogram of base-acid-treated control DNA and DNA treated with OsO₄ passed over the ion-exchange columns. Only counts from OsO₄-treated DNA containing saturated thymines pass through the columns. Seven % of the thymine bases were saturated in this example.

Exposure of Mice to TPA. The backs of BALB/c mice were clipped of hair with laboratory clippers. Five days after clipping, TPA (Sigma) dissolved in acetone was applied at a dose of 10 μg in 20 μl of acetone. Control animals received acetone alone. Animals were sacrificed 2 days after a single exposure and 3 days after 2 exposures spaced 3 days apart. Skin was taken and fixed in 10% formalin, processed for histology, and stained with hematoxylin and eosin according to standard procedures.

Exposure of Target Cells to H₂O₂. NIH-3T3 cells were labeled with [methyl-³H]thymidine (specific activity, 20 Ci/mmol; New England Nuclear) for 16 to 24 hr in complete medium with a concentration of 6 to 8 μCi/ml. Nearly confluent, labeled monolayers of NIH-3T3 cells in 75-cm² flasks were washed 3 times with Hank's balanced salt solution, and the medium was replaced with 20 ml of RPMI 1640 without serum, which was buffered with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Reagent H₂O₂ was diluted in deionized H₂O from a stock 30% solution (Mallinckrodt) and added in the appropriate concentrations, and the flasks were incubated for varying amounts of time and at different temperatures. The concentration of H₂O₂ was determined by the horse-radish peroxidase-catalyzed oxidation of phenol red (41). Following incubation, the medium was removed, and the cells were collected by trypsinization and centrifugation. In repair experiments, the plates were washed free of H₂O₂, and RPMI 1640 plus 10% fetal bovine serum was added, and the flasks were incubated at 37°C for various times before the cells were collected. The macromolecules were precipitated by the addition of 7% trichloroacetic acid. T' was then quantified as described above.

Exposure of Target Cells to Macrophages. Casein-elicited peritoneal exudates were obtained as described previously (3) and added to T-75 flasks containing near-confluent monolayers of prelabeled NIH-3T3 cells in RPMI-1640 containing 10% fetal bovine serum. The flasks were incubated for 60 min at 37°C. Following incubation, the nonadherent lymphocytes and polymorphonuclear leukocytes were removed by washing the plate 3 times with Hanks' balanced salt solution. This resulted in monolayers of macrophages of >98% purity (3). After adherence purification, the medium was changed to serumless RPMI 1640 with and without TPA (100 ng/ml). The flask was incubated for a further 60 min at 37°C, the medium was removed, and the cells were collected by trypsinization and centrifugation. The amount of T' in the 3T3 cells was quantified as described above. Controls were target cells incubated at 37°C without macrophages; the same number of macrophages was added at 4°C immediately before the precipitation of DNA. An additional control was targets exposed to TPA alone. In experiments in which catalase and SOD were used, the targets and macrophages were cocultured in suspension in order to avoid previously reported difficulties of accessibility of these large-molecular-weight scavengers to critical sites (29). Polymorphonuclear leukocytes and RBC were removed from the casein-elicited exudates by density gradient centrifugation over Lymphoprep (Litton Bionetics), resulting in suspensions of >90% macrophages as determined by differential counts of Wright-stained smears. Then, 5 × 10⁶ prelabeled targets were added to 20 × 10⁶ macrophages in 20 ml of RPMI 1640 which contained no serum, 10 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, TPA (100 ng/ml), catalase (100 units/ml; Calbiochem), or SOD (100 units/ml; Sigma). Activity of catalase was confirmed by the method of Beers and Sizer (6), and SOD activity was confirmed by the inhibition of O₂⁻ reduction of ferricytochrome c (38). The samples were incubated for 60 min at 37°C and agitated every 5 min. Following incubation, the cells were collected by centrifugation, and the amount of T' was measured as described above. Controls were the same as described for the adherent exposures.
RESULTS

Morphology of TPA-induced Inflammation in Mouse Skin. Because i.d. inflammatory leukocytes may serve as a source of genotoxic ROI during treatment of skin with phorbol esters, we first determined what leukocytes were present in mouse skin during exposure to TPA. One day after a single application of TPA, a large acute inflammatory response comprised neutrophils almost exclusively (Fig. 1A). Three days after 2 applications of TPA given 3 days apart, the reaction had changed to chronic inflammation comprising mononuclear cells, most of which were small monocytes and large macrophages (Fig. 1B).

Induction of T' in Target Cells by Inflammatory Macrophages. We next determined if macrophages taken from sites of chronic inflammation could induce saturated thymines (T') in surrounding mammalian cells. When 3T3 cells were cocultured with peritoneal inflammatory macrophages plus TPA, a significant number of saturated bases (i.e., >20 T'/10⁶ molecules of thymine) were produced (Chart 2). T' were not detected in cells exposed to TPA alone, while very low concentrations were induced by macrophages alone (Chart 2).

Fig. 1. Photomicrographs of inflammation in mouse skin induced by TPA. The backs of BALB/c mice were clipped free of hair 5 days before treatment. A, the skin of a mouse 2 days after a single application of 10 μg in 20 μl of acetone; B, the skin of a mouse 3 days after 2 applications of TPA spaced 3 days apart. H & E, x 350.

Chart 2. Induction of T' in the DNA of NIH-3T3 cells cocultured with inflammatory macrophages (Mφ) stimulated with TPA. Casein-elicited peritoneal exudate cells were obtained as described previously (3) and added to monolayers of 3T3 cells which had been prelabeled with [methyl-³H]thymidine. The flasks were incubated for 60 min, and the nonadherent cells were washed off the target cells. Following adherence purification of the macrophages, serumless medium was added with and without TPA (100 ng/ml). The flasks were incubated for a further 60 min and T' was quantified as described in "Materials and Methods." Controls and samples cultured with TPA alone received the same number of macrophages at 4° immediately before precipitation of the macromolecules (mean of duplicate samples).

Chart 3. Relationship between the density of macrophages (Mφ), the concentration of H₂O₂ in the culture medium, and the amount of T' induced in target cells. Conditions under which these experiments were conducted were the same as those described in the legend to Chart 2, except that the density of the macrophages in the cultures was varied as indicated. The curves connect the means at duplicate determinations. Points, individual determinations.

Relationship of the Induction of T' by Macrophages and the Amount of H₂O₂ Secreted. To determine if the amount of T' induced in target cells was related to the amount of H₂O₂ released into the ambient medium, target cells were cocultured with varying densities of TPA-stimulated macrophages. The amount of T' induced in the targets and the concentration of H₂O₂ in the medium rose proportionately (Chart 3).
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Chart 4. Induction of T' in the DNA of NIH-3T3 cells of reagent H2O2. Prelabeled monolayers of target cells were exposed to varying concentrations of H2O2 for 60 min at 4° or 37°. Following exposure, the cells were collected by trypsinization and centrifugation, and T' was quantified as described in "Materials and Methods" (mean of duplicate samples).

Chart 5. Kinetics of T' formation in target cells exposed to H2O2. Monolayers of prelabeled target cells were exposed to 100 µM reagent H2O2 for varying periods of time, and the amount of T' was quantified as described in "Materials and Methods." The curve connects the means of duplicate samples. Points, individual determinations.

Induction of T' in NIH-3T3 Cells by Reagent H2O2. Because the induction of T' increased in proportion to the concentration of H2O2 released into the culture medium by macrophages and because T' has been shown to be formed in isolated DNA reacted with reagent H2O2 (17), we next determined if reagent H2O2 itself, at concentrations actually produced by macrophage, could induce T' in cells. When target cells were exposed to reagent H2O2 at 10 to 250 µM, T' was formed in a dose-dependent manner (Chart 4). The amount of T' induced and the concentration of H2O2 were linear over the range of 10 to 100 µM H2O2 (Chart 4). Similar amounts were induced at 4° or 37°, except that no T' was detected at the lowest concentration of H2O2 when the cells were exposed at 37°. The reaction was rapid and almost complete by 30 min (Chart 5). Following exposure to 75 µM H2O2 for 45 min at 4°, 3T3 cells were able to repair T' rapidly (Chart 6). After an initial lag period of 30 min, the target cells removed over 70% of the initial T' from their DNA by 2 hr (Chart 6). This loss was not due to the loss of cells, because the total amount of radioactivity recovered did not change over the time allowed for repair (legend to Chart 6).

DISCUSSION

Several lines of evidence thus indicate macrophages can induce genotoxic injury via release of ROI. 5,6-Ring saturated...
thymine bases were induced in the DNA of NIH-3T3 cells cocultured with inflammatory macrophages that were stimulated with TPA. Reagent H$_2$O$_2$, at concentrations which macrophages actually produced in the ambient medium, also induced formation of saturated thymines in 3T3 cells. Catalase, a specific scavenger of H$_2$O$_2$, completely inhibited the induction of saturated thymines by phorbol-stimulated macrophages. Taken together, the data demonstrate that inflammatory macrophages stimulated with the tumor promoter TPA can induce oxidative alterations of known chemical structures in the DNA of surrounding eukaryotic cells. The studies further suggest that secreted ROI from the macrophages, H$_2$O$_2$ in particular, are important mediators of this damage. The amount of T' induced by macrophages with respect to the amount of H$_2$O$_2$ secreted is quantitatively similar to the amount induced by the same concentrations of reagent H$_2$O$_2$ (compare Chart 3 with Chart 4). For example, macrophages cultured at $10^5$ cells/ml produced a 49.8 $\mu$M solution of H$_2$O$_2$ and induced 39 T'/10$^6$ (Chart 3). The amount of T' induced by that concentration of reagent H$_2$O$_2$, as predicted from the data in Chart 4, is 36 T'/10$^6$.

The enhancement of T' induction by SOD observed in these studies is not surprising. SOD converts O$_2^-$ to H$_2$O$_2$ (38), and H$_2$O$_2$ alone can induce T' in the DNA of cells (Chart 4) and in isolated DNA (17). The amounts of H$_2$O$_2$ secreted by macrophages fall on the linear portion of the dose curve for H$_2$O$_2$ (Charts 3 and 4). Thus, increases in the concentration of H$_2$O$_2$ due to the action of SOD on O$_2^-$ would be expected to produce proportional increases in H$_2$O$_2$ and hence in T' (Chart 4). Recently, it has been shown that SOD enhances radiation-induced transformation in vitro (28). Leukocyte-induced genotoxicity in other studies has been blocked by many inhibitors and scavengers including SOD, catalase, mannitol, benzoate, indomethacin, and imidazol (8, 9, 18, 19, 29, 44, 48, 49, 52), implicating O$_2^-$, H$_2$O$_2$, OH$,^-$, and oxidized lipid products. One potential explanation of the apparently different action of SOD in the present studies is that, under conditions of chronic exposure, O$_2^-$ might be required to reduce Fe$^{3+}$ as postulated by the metal-catalyzed Haber-Weiss reaction (24, 31, 36, 37, 50). Reduction of Fe$^{3+}$ would generate more Fe$^{2+}$, which would react with H$_2$O$_2$ to produce more OH$^-$ to react with DNA. Under conditions where the amount of Fe$^{2+}$ was rate limiting, SOD would therefore remove O$_2^-$ and inhibit regeneration of Fe$^{2+}$ from Fe$^{3+}$. Our studies with reagent H$_2$O$_2$ do suggest that there was a limiting factor(s) in the reaction, since little increase in the amount of T' produced was observed at concentrations of H$_2$O$_2$ over 100 $\mu$M (Chart 5). For example, a limited number of reactive sites in the DNA could account for this observation.

Saturated thymine ('T') has been reported to be induced in isolated DNA by concentrations of H$_2$O$_2$ in the range of 10$^{-1}$ M (17). By contrast, the effective concentrations of H$_2$O$_2$ used in this study with intact cells ranged from 10$^{-5}$ to 10$^{-4}$ M. Several obvious differences exist between these 2 studies, including the presence or absence of metal ions (Fe$^{2+}$ in particular), reducing agents such as ascorbate, and cell defenses against oxidative damage. Numerous potential sources of Fe$^{2+}$ exist in the intact cell. H$_2$O$_2$ is stable in solutions containing no metal ions, and the reaction of DNA with H$_2$O$_2$ is greatly enhanced by the addition of Cu$^{2+}$, Fe$^{2+}$, or chelates of both (10, 20, 31, 35, 42). On the other hand, cellular defenses such as the glutathione-glutathione reductase system would be expected to protect the cell from the effects of ROI. The observation that no T' was induced in targets exposed to the lowest concentration of H$_2$O$_2$ (10 $\mu$M) at 37°C (Chart 4) suggests that some protection may have occurred. The data in Chart 6 demonstrate that these target cells did possess the ability to repair saturated thymines rapidly after they are formed. This is in accord with other studies which have shown that mammalian cells rapidly repair saturated thymines induced by ionizing radiation (36). The role and importance of these various factors in promoting or ameliorating DNA damage need to be assessed precisely.

Although there is a large body of evidence demonstrating a strong connection between tumor production and ROI, and ROI and DNA damage, the importance of ROI-mediated DNA damage in tumor promotion is still undetermined. Some in vitro studies have shown that C3H/10T$^{1/2}$ cells can be transformed following carcinogen exposure by TPA in the absence of inflammatory cells (28). Other studies have shown that JB6 epidermal cells can be promoted to the transformed state by TPA without measurable single-strand DNA breaks (22). These studies suggest that inflammatory cells are not an absolute requirement for TPA promotion and that DNA may not be the crucial target of TPA action. Conversely, Zimmerman and Cerutti (52) have shown that C3H/10T$^{1/2}$ cells can be directly transformed or promoted to transformation following initiation with $\gamma$-radiation or benzo(a)pyrene diol-epoxide I by xanthine-xanthine oxidase. Both the direct transformation and promotion were inhibited by SOD and catalase. Kennedy et al. (28) have shown that TPA promotion of irradiated C3H/10T$^{1/2}$ cells was inhibited by catalase. These studies indicate that ROI from exogenous or endogenous sources are important in transformation in the C3H/10T$^{1/2}$ cells.

The relative contributions of ROI-mediated DNA damage, alterations in gene expression via signal transduction, and selective stimulation of cell replication to the process of tumor promotion remain to be established. Therefore, the importance of thymine glycol and hydroperoxy compounds in mutagenesis and carcinogenesis is uncertain. Some alterations in DNA bases, such as the O-alkyl products induced in thymine and guanine bases by methylating and ethylating agents, have been shown to be promutagenic (1, 2) and correlate closely with tumor initiation (33, 34, 46). The precise chemical structures involved in oxidative DNA damage which could potentially be responsible for the potentiation of carcinogenesis are not known. Formation of saturated thymines may be a critical class of DNA damage for 4 reasons: (a) T' represents a major class of DNA base damage induced by ionizing radiation (25, 26); (b) this modification of DNA disrupts the planar nature of pyrimidines and thus potentially of base stacking (12); (c) mutagenicity studies have shown that bacterial test strains which require mutations at A-T loca-

REFERENCES

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