Modification of DNA Bases in Chromatin of Intact Target Human Cells by Activated Human Polymorphonuclear Leukocytes

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ABSTRACT

We investigated whether phorbol-12-acetate-13-myristate (PMA)-activated human polymorphonuclear leukocytes (PMNs) induce modifications in target cell DNA in vivo. Human PMNs produced 9.4 ± 0.8 (SD) nmol of H₂O₂/10⁶ cells during 50 min of exposure to 2 µg/ml PMA and 13.7 ± 2.8 nmol/10⁶ cells during exposure to PMA plus 5 µM NaN₃. Neither nonstimulated PMNs, nor PMA alone, nor NaN₃ alone induced base modifications in chromatin-associated DNA of human Ad293 cells above control levels, when assayed by gas chromatography/mass spectrometry with selected-ion monitoring. However, a 60-min exposure to 1.7 ± 0.4 × 10⁶ PMNs/ml in the presence of 2 µg/ml PMA induced a 2-3-fold increase in the level of all modified bases detected by gas chromatography/mass spectrometry with selected-ion monitoring. The guanine-derived products 8-hydroxyguanine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine, and the adenine-derived product 4,6-diamino-5-formamidopyrimidine were induced to the highest levels among those bases detected. These data demonstrate that exposure to activated PMNs causes DNA base modifications in target cells in vivo typical of those induced by hydroxyl radical attack. The induction of potentially promutagenic modified bases may contribute to the mutagenicity of activated PMNs.

INTRODUCTION

Activated leukocytes are among the principal physiological sources of fluxes of oxygen-derived species. They are known to produce superoxide anion, nitric oxide, and H₂O₂, as well as other strong oxidizing species, such as hypohalous acids (for reviews, see Refs. 1 and 2). Activated leukocytes cause DNA strand breaks in target cells (3) and the formation of a variety of base modifications in isolated DNA (4, 5). The induction of certain DNA base modifications in human leukocytes and mouse skin cells in vivo by exposure to PMA has also been demonstrated (6–8).

Weitzman and Stossel (9, 10) first demonstrated that activated leukocytes are mutagenic. They are also carcinogenic in vitro (11), an effect with a well-documented clinical correlate, in that sites of chronic inflammation and chronic woundng are predisposed to developing cancer (12). These events can also be elicited by reactive oxygen species generated enzymatically in the absence of cells (11, 13) and can be inhibited by free radical scavengers and inhibitors of lipid peroxidation (10). Also, the tumor-promoting capability of first-stage tumor promoters for the mouse skin system correlates well with their ability to stimulate H₂O₂ release by leukocytes (8).

Although it is clear that activated leukocytes are mutagenic, little is known about the mechanism(s) of mutagenesis of mammalian DNA other than it is probably oxidant mediated. Hydroxyl radical, produced by reduction of H₂O₂ by intracellular Fenton chemistry or by decomposition of peroxynitrite radical formed by the coupling of superoxide anion and nitric oxide, has been proposed as the mediator of leukocyte-induced strand breaks in target cells (3) and of DNA base modifications in vitro (5). Several of the DNA base modifications, the content of which is increased in purified DNA exposed to activated leukocytes, have been shown to be weak mutagens in site-specific mutagenesis assays, principally causing point mutations. These include thymine glycol (14) and 8-0H-Gua (15–17). Abasic sites resulting from enzymatic removal of modified bases may also serve as templates for misincorporation (18, 19). The purpose of this study was to determine whether potentially promutagenic DNA base modifications were induced in vivo in target cells exposed to activated leukocytes. We show that a variety of DNA base modifications were induced in vivo by activated leukocytes and that the pattern of base modifications is consistent with attack by hydroxyl radical or a species with similar reactivity.

MATERIALS AND METHODS

Target Cells. Human Ad293 cells were grown as a monolayer in Dulbecco’s modified Eagle’s medium (Gibco, Grand Island, NY) containing 5% heat-inactivated fetal bovine serum in a humidified 5% CO₂ atmosphere at 37°C. Monolayer cultures were 85–90% of confluency at the time of PMN exposure.

PMN Preparation. Fresh human PMNs were isolated from 10 ml of normal volunteer peripheral blood obtained by venipuncture in tubes anticoagulated with EDTA by the method of Boyum (20). Briefly, whole blood was diluted 1:3 (v/v) with 154 m.MNaCl, layered over 45% (v/v) Percoll (Pharmacia), and centrifuged at 400 × g for 40 min. The PMN-containing interface was isolated and mixed with 1 ml plasma and 1.3% (w/v) dextran sulfate; PMNs were sedimented at 1 × g for 40 min at 4°C. Residual erythrocytes were lysed by the addition of two volumes of ice-cold distilled water for 1 min. PMNs were recovered by centrifugation at 300 × g for 8 min and resuspended in serum-free RPMI 1640 (Gibco) for immediate use. PMNs obtained by this procedure were routinely 96–97% viable as assayed by exclusion of trypan blue dye.

PMN Exposure and Chromatin Isolation. Target Ad293 cells in 30-ml tissue culture flasks were overlayed with 5 ml of serum-free RPMI 1640 containing, where appropriate, 1.7 ± 0.4 × 10⁶ (SD) PMNs/ml ± 2 µg/ml PMA (Sigma Chemical Co., St. Louis, MO) ± 5 µM NaN₃ for 60 min at 37°C. After exposure, PMNs, nonviable target cells, and media were poured off, and target cells were rinsed in ice-cold phosphate-buffered saline and recovered by scraping, followed by centrifugation for 5 min at 300 × g at 4°C. Chromatin was isolated from the recovered cells by the procedure of Gajewski et al. (21). Chromatin, which was obtained in 1 ml Tris-Cl (pH 7.4), was dialyzed extensively against 1 µM phosphate buffer (pH 7.4), which had been treated with Chelex resin. The entire isolation procedure was carried out at 4°C. After dialysis, chromatin was homogenized with 4–5 strokes in a glass homogenizer.

The absorption spectra of chromatin obtained by this procedure resembled absorption spectra characteristic of mammalian chromatin (21) with the following characteristics: A₂₆₀/A₃₀₀ ranged from 1.50 to 1.66 and A₂₃₅/A₃₂₀ from 1.269.
Hydrolysis, Derivatization, and Gas Chromatography/Mass Spectrometry. Chromatin samples were hydrolyzed with 60% (v/v) formic acid (Mallincrodt, St. Louis, MO) in evacuated and sealed tubes at 140°C for 30 min (22). Samples were lyophilized and trimethylsilylated with 0.1 ml of a mixture of N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trichloromethylsilane (Pierce Chemical Co., Rockford, IL) and acetonitrile (4:1, v/v; Pierce Chemical Co.) at 130°C for 30 min. Gas chromatography/mass spectrometry with selected-ion monitoring analyses of derivatized samples were performed as described previously (21).

Hydrogen Peroxide Production. H₂O₂ production by stimulated PMNs was measured by the procedure of Pick and Keisari (23). Briefly, 1 × 10⁶ PMNs were incubated in 1 ml of 140 mM NaCl, 10 mM potassium phosphate (pH 7.0), 5.5 mM glucose, 0.28 mM phenol red, and 8.5 units/ml horseradish peroxidase (Sigma Chemical Co.). At t = 0, 2 µg/ml PMA ± 5 mM NaN₃ were added to the appropriate dishes. At various times thereafter, cell-free supernatants were harvested, NaOH was added to 100 mM, and A₅₅₀ was determined. The H₂O₂ content of each sample was determined by comparison to a linear standard curve.

RESULTS

The objective of this work was to examine whether PMA-activated human PMNs cause modification of purines and pyrimidines of DNA in the chromatin of cultured human cells. First, we measured the generation of H₂O₂ by PMNs in both the presence and absence of PMA. Without PMA, no generation of H₂O₂ by PMNs was detected. However, addition of PMA (2 µg/ml of culture medium) stimulated PMNs to generate 9.4 ± 0.8 nmol of H₂O₂/10⁶ cells in 50 min (mean ± SD; n = 3). The addition of PMA and 5 mM NaN₃ caused PMNs to generate 13.7 ± 2.8 nmol of H₂O₂/10⁶ cells under the same conditions.

Chromatin samples were isolated from cultured Ad293 human cells which were exposed either to PMA-activated PMNs or to nonactivated PMNs or to PMA alone. Chromatin was also isolated from untreated cells as a control. The Ad293 cell line grows as a monolayer attached to the incubation flask. Thus, Ad293 cells which remained attached to the flask were readily separated from nonviable cells and PMNs after treatment. Neither PMA, nonactivated PMNs, nor PMA-activated PMNs diminished recovery of flask-attached Ad293 cells as compared to untreated controls. Trimethylsilylated hydrolysates of chromatin samples were analyzed by gas chromatography/mass spectrometry with selected-ion monitoring for modified purine and pyrimidine bases. Modified bases and their quantities are given in Table I. 5-Hydroxycytosine and 5-hydroxycytidine result from acid-induced modification of cytosine glycol, the former by deamination and dehydration and the latter by dehydration (24). All of the modified bases listed in Table I were observed in chromatin from control Ad293 cells. Exposure of Ad293 cells to PMA (2 µg/ml of culture medium) had no significant effect on the background levels of any base modifications measured in this study. Neither did exposure to nonstimulated PMNs. However, exposure of target cells to PMNs in the presence of PMA (2 µg/ml of culture medium) induced significant increases (ranging from 200% to 500% of untreated control values; P values ranged from 0.018 to <0.0001 by t test) in the amounts of modified bases above background levels. The highest increases were observed in the amounts of FapyGua and cytosine glycol (taken as the sum of the amounts of 5-hydroxycytosine and 5-hydroxycytidine). The amounts of hydantoin (5-hydroxy-5-methylhydantoin and 5-hydroxyhydantoin) had the least increases. Among the modified bases measured, guanine-derived bases FapyGua and 8-OH-Gua had the highest net amounts produced by PMA-activated PMNs (8 molecules/10⁵ DNA bases), followed by adenine-derived bases FapyAde and 2-hydroxyadenine (4 and 3 molecules/10⁵ DNA bases, respectively).

Coaddition of 5 mM NaN₃, an inhibitor of PMN myeloperoxidase, to PMA-activated PMNs did not cause a significant further induction of some of the modified bases in viable Ad293 cells (data not shown). However, approximately two-thirds of the exposed target Ad293 cells were lost as nonviable detached cells after coaddition of NaN₃.

DISCUSSION

Our results demonstrate that PMA-activated PMNs induce DNA base modifications in intact target cells. The pattern of modified bases...
is typical of DNA base modifications induced by well-known -OH-producing systems (reviewed in Ref. 25). This suggests that the reactive intermediate was -OH or a similarly reactive species. Production of H₂O₂ by leukocytes, with subsequent generation of -OH via a Fenton-type reaction leading to DNA base modification, has been suggested previously (4–7). Our data are consistent with this view. In vivo generation of -OH may occur in a site-specific manner in reactions of H₂O₂ with chromatin-bound metal ions (26, 27). Furthermore, evidence indicates that -OH plays a role in biological effects of H₂O₂ in vivo involving metal ions (27, 28).

Recently, DNA base modification caused by the exposure of target cells to H₂O₂ has been identified and quantified (29). The pattern of base modification caused by 2–20 mM H₂O₂ was similar to that observed in Ad293 cells after exposure to PMA-activated PMNs, which generate 10–15 nmol of H₂O₂/ml of culture medium. The similar pattern of damage supports the hypothesis that generation of H₂O₂ is an important part of DNA base modification in cells by activated PMNs. There may be at least two reasons for the approximately 1000-fold greater effect of activated PMN-generated H₂O₂. First, the measurements of H₂O₂-induced DNA damage in cells were taken after exposure of the target cells to H₂O₂ in medium containing fetal bovine serum (29). Fetal bovine serum contains sufficient catalase to inactivate much of the H₂O₂ in the medium prior to entry into cells. Second, the H₂O₂ generated by activated PMNs is produced at or near the cell surface and must diffuse into the medium for measurement. It is likely that the concentration of H₂O₂ encountered by the target cells which directly abut the activated PMNs is considerably higher than the measured concentration.

Wei and Frenkel (7, 8) observed the induction of 8-OH-Gua, cis-thymine glycol, and 5-(hydroxymethyl)uracil in the epidermal cellular DNA of mice after local application of tumor promoters, including PMA. Induction of these modified bases was attributed to local infiltration of the epidermis by activated inflammatory cells in response to application of the tumor promoter (8). Our results are consistent with their hypothesis and indicate that PMA-activated PMNs may generate oxidants at a rate sufficient to overcome Ad293 cellular antioxidant and DNA repair defenses, resulting in the detectable induction of modified bases. Although PMA may stimulate H₂O₂ production in some noninflammatory cells, e.g., mouse epidermal cells (30), the production rate may be insufficient to induce detectable DNA base modification. Similarly, Lewis and Adams did not detect the induction of ring-saturated products of thymine in target NIH-3T3 fibroblasts by PMA alone, but did so with PMA-activated mouse macrophages (31). On the other hand, PMA may potentiate activated leukocyte-induced damage by causing a diminution of antioxidant enzymes in target cells (30).

There were some interesting differences in the pattern of DNA base modification caused by PMA-activated PMNs in Ad293 cells as compared to the pattern in isolated DNA in vitro (5). In the latter instance, the yields of formamidopyrimidines FapyAde and FapyGua were substantially less than those of 8-hydroxyadenine and 8-OH-Gua. In the present study, however, production of substantial amounts of formamidopyrimidines was observed. The net yield of FapyGua was similar to that of 8-OH-Gua, and the net yield of FapyAdo was higher than that of 8-hydroxyadenine. The reduction and oxidation of C-8-OH-adenosine radicals of purines lead to formation of formamidopyrimidines and 8-hydropurines, respectively (reviewed in Ref. 32). Thus, the high yields of FapyAde and FapyGua in cells may suggest that the intracellular environment provides reducing equivalents which are not available to isolated DNA in solution. Modification of guanine in Ad293 cells exposed to PMA-activated PMNs was predominant in terms of the measured products of all four DNA bases. This is in agreement with previous results obtained with H₂O₂-treated or γ-irradiated mammalian cells (22, 29) and may indicate the high reactivity with -OH (or a related reactive species) of guanine residues in chromatin in cells. Reid and Loeb (33) have studied the mutagenesis of phage M13 mp2 DNA by in vitro exposure to PMA-activated PMNs. They observed primarily base substitutions; G to T transversions and G to C transversions were the most prominent alterations. This implies a role for guanine-derived modified bases as promutagenic intermediaries of PMN-induced mutagenesis. A number of modified DNA bases have been investigated for their biological consequences (14–17, 34–36). Some of them, including 8-OH-Gua, have been found to possess mutagenic properties (14–17, 35–36). Induction of promutagenic base modifications in critical target genes may be one mechanism whereby inflammatory cells stimulate neoplastic transformation. The variety of modified DNA bases formed in cells hampers the assessment of their role in biological end points such as mutagenesis and carcinogenesis. The contribution of specific modified DNA bases, which have been identified in the present study, to PMN-induced mutagenesis in biological systems remains to be defined.

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