Immunoperoxidase Detection of 8-Hydroxydeoxyguanosine in Aflatoxin B₁-treated Rat Liver and Human Oral Mucosal Cells

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Abstract

An immunoperoxidase method using a monoclonal antiserum that recognizes 8-hydroxydeoxyguanosine has been developed for detection and quantitation of oxidative damage in single cells. The method was initially applied to cultured cells treated with H₂O₂ or aflatoxin B₁ and then to cryostat liver sections of rats treated with aflatoxin B₁. To demonstrate that the method has sufficient sensitivity for detection of damage in human samples, oral mucosal cells from a total of 12 pairs of smokers and nonsmokers were analyzed. Mean staining intensity of oral cells of smokers was 1.6-fold higher than in nonsmokers. The immunoperoxidase method, requiring a small number of cells and eliminating the need for isolation of DNA, will be useful for evaluation of oxidative damage in a wide range of biological samples.

Introduction

8-Hydroxy- or 8-oxo-deoxyguanosine is recognized as a useful marker for the estimation of DNA damage produced by oxygen radicals generated endogenously or exogenously. Although numerous oxidative lesions occur in DNA, oxidation of the C8 of guanine is one of the more abundant types (1); it is a major mutagenic lesion producing predominately G→T transversion mutations (2, 3). 8-OHdG accumulates in DNA exposed to mutagenic and carcinogenic agents, such as reducing agents, X-rays, asbestos, and polynuclear hydrocarbons that generate oxygen-free radicals (4). Increased levels of 8-OHdG are also observed in DNA from γ-irradiated mouse liver, X-irradiated HeLa cells, and H₂O₂-treated Salmonella typhimurium (5).

A number of methods have been developed for quantitation of femtomol levels of 8-OHdG in cellular DNA, including HPLC-EC (6), GC/MS (7), ³²P postlabeling (8), and immunoassays (9, 10). The HPLC-EC and GC/MS methods are used most frequently, but both have some limitations that may be responsible for discrepancies in quantitative values. Two- to 11-fold greater amounts of 8-OHdG in DNA were reported with the GC/MS method than with the HPLC method (11). Inaccurate estimates of 8-OHdG by the GC/MS method may be due to incomplete derivatization, formation of by-products, and possible destruction or formation of oxidized bases during acid hydrolysis, whereas complete enzymatic hydrolysis and coelution of interfering substances may be potential problems in the HPLC-EC method. Monitoring of human tumor and non-tumor tissue has documented the occurrence of 8-OHdG in DNA. Higher levels of 8-OHdG were found in WBC of smokers than nonsmokers (12) and in tumor tissue compared to non-tumor tissues in colon, stomach, ovary, brain, breast, and lung (13, 14).

Recently, we reported development of monoclonal antisera and an immunoassay for quantification of 8-OHdG in human samples (15). Antiserum 1F7 was used for immunoaffinity isolation of 8-OHdG from DNA hydrolysates, followed by ELISA quantitation with antiserum 1F11. To validate the assay, DNA extracted from human placental tissue was assayed by ELISA and HPLC-EC. Values by both methods correlated well, but the levels determined by ELISA were approximately 6-fold higher than those determined by HPLC. This may be due to oligonucleotides detected by ELISA, but not by the HPLC method, or cross-reaction of the antisera with other damaged bases present in the immunoaffinity-purified material.

In this study, we determined that the more selective antibody 1F7, can be used for detection of 8-OHdG in single cells by an immunoperoxidase procedure. Initial studies were performed with cells treated in culture with two inducers of oxidative damage, H₂O₂ and AFB₁. Then a small pilot study was carried out on human subjects to test the applicability of the method for detection of oxidative damage caused by smoking as a model environmental exposure. We also adapted antisera 6A10, used previously for immunofluorescence detection of AFB₁-DNA adducts (16), to the immunoperoxidase technique to allow side-by-side comparisons of AFB₁-DNA and 8-OHdG in the same tissue.

Materials and Methods

Chemicals. AFB₁, DNase, RNase, proteinase K, H₂O₂, polyethylene glycol, and 3-amino propyltriethoxysilane were purchased from Sigma Chemical Co. (St. Louis, MO). FCS was obtained from Sterile Systems (Logan, UT). RPMI 1640 was obtained from ICN Pharmaceutical, Inc. (Costa Mesa, CA). DMEM was purchased from Gibco-BRL Life Technologies (Gaithersburg, MD). Permount was purchased from Fisher Scientific (Pittsburgh, PA). Eight chamber slides used here were from Miles Laboratories (Naperville, IL). Woodchuck hepatitis (WC3) cells were obtained from Dr. C. Rogler (Albert Einstein Medical Center, New York, NY). Mouse ABC and 3,3′-dimethylaminobenzene kits were obtained from Vector Laboratories (Burlingame, CA).

H₂O₂ Treatment of 10T½ Cells. The 10T½ cells were maintained in DMEM medium containing 10% FCS. Exponentially growing cells in 8-chamber slides were treated with 10–200 μM H₂O₂ in complete culture medium for 15 min at 37°C. Exposure was stopped by removing the media and rinsing the cells twice with 120 mM NaCl, 2.7 mM KCl, 1 mM EDTA, and 10 mM K₂HPO₄ (pH 7.4). The cells were fixed with 75% ethanol at —20°C.

AFB₁, Treatment of Woodchuck Hepatocytes. WC3 cells were cultured in RPMI 1640 containing 10% FCS. Cells were treated with 0.4–10 μM AFB₁, in DMPO or DMSO alone for 6 h, washed with PBS twice, and fixed with 75% ethanol.

AFB₁, Treatment of Rats. Frozen tissues of 1-year-old male Sprague-Dawley rats treated previously with 2.5 mg/kg AFB₁, and sacrificed at 2, 4, 8, 24, and 48 h after treatment were available (16). Tissue samples were isolated 5 mm from the surface, sectioned (5 μm) on a cryostat (Leica, Deerfield, IL), and placed on glass microscope slides coated with 3-amino propyltriethoxysilane and fixed in 75% ethanol for 10 min at —20°C.
Immunoperoxidase Staining for 8-OHdG. Slides were washed with 1X PBS twice, treated with RNase (100 μg/ml) in Tris buffer (pH 7.5; 10 mM Trizma Base, 1 mM EDTA, and 0.4 mM NaCl) at 37°C for 1 h. After washing with PBS, cells were treated with proteinase K (10 μg/ml) at room temperature for 7 min. After rinsing with PBS, DNA was denatured by treatment with 4 M HCl for 7 min at room temperature. The pH was adjusted with 50 mM Trizma base for 5 min at room temperature. After washing with PBS, the cells were treated with 10% normal horse serum in 10 mM Tris (pH 7.5) for 1 h to block non-specific binding sites and then incubated with primary antibody IF7 (1:30 dilution of hybridoma supernatant) at 4°C overnight (15). After washing with PBS, cells were treated with goat antimouse IgG conjugated to biotin at room temperature for 30 min. Endogenous peroxidase was blocked by treating the cells with 3% H2O2 in methanol for 30 min at room temperature. After rinsing with PBS, cells were treated with 10% normal horse serum in 10 mM Tris (pH 7.5) for 1 h to block non-specific binding sites and then incubated with primary antibody IF7 (1:30 dilution of hybridoma supernatant) at 4°C overnight (15). After washing with PBS, cells were treated with goat antimouse IgG conjugated to biotin at room temperature for 30 min. Endogenous peroxidase was blocked by treating the cells with 3% H2O2 in methanol for 30 min at room temperature. After washing with PBS, ABC reagent, avidin conjugated to horseradish peroxidase was added, and the slides were incubated for 30 min at 37°C, followed by PBS and 1% Triton X-100 PBS washes. To localize peroxidase, cells were treated with diaminobenzidine for 10 min at room temperature. After rinsing with PBS, the cells were treated with 10% normal horse serum in 10 mM Tris (pH 7.5) for 1 h to block non-specific binding sites and then incubated with primary antibody IF7 (1:30 dilution of hybridoma supernatant) at 4°C overnight (15). After washing with PBS, cells were treated with goat antimouse IgG conjugated to biotin at room temperature for 30 min. Endogenous peroxidase was blocked by treating the cells with 3% H2O2 in methanol for 30 min at room temperature. After washing with PBS, ABC reagent, avidin conjugated to horseradish peroxidase was added, and the slides were incubated for 30 min at 37°C, followed by PBS and 1% Triton X-100 PBS washes. To localize peroxidase, cells were treated with diaminobenzidine for 10 min at room temperature. Finally, slides were washed with H2O2, dehydrated by a series of 95 and 100% ethanol and xylene washes, mounted with cover glass using Permount, and followed by quantitation with a Cell Analysis System CAS 200 microscope (Becton Dickinson, San Jose, CA). The relative intensity of nuclear staining of 30–50 randomly selected cells was measured using the Cell Measurement Program software package. Data presented are the object average absorbance multiplied by 1000.

To demonstrate specificity, cells and tissues were pretreated with DNase (100 μg/μl for 1 h at 37°C) before staining and stained with a nonspecific antiserum 8Gl (1:10 dilution) recognizing DNA damage produced by the photoactivated drug 8-MOP (17) or with antiserum IF7 preabsorbed with 8-OHdG (1 μg/μl) for 20 min at room temperature before use.

Immunohistochemical Analysis of AFB1-DNA Adducts in Tissue. Slides were treated with 15 mM Na2CO3 and 30 mM NaHCO3 (pH 9.6) for 2 h at room temperature to ring open the guanine adducts and then rinsed with PBS. Degradation of RNA and protein was performed as described above. For denaturation of DNA, 50 mM NaOH in 40% ethanol was used (15 s at room temperature). After a quick rinse with 40% ethanol, the pH was adjusted by treatment with 5% acetic acid in 40% ethanol for 1 min at room temperature. After blocking of nonspecific binding sites with 10% normal horse serum, primary antibody 6A10, recognizing imidazole ring-opened AFB1-DNA adduct (18), in 1% goat serum (1:50 dilution) was applied overnight at 4°C. The remaining steps were performed as described above.

Human Subjects. Volunteers were recruited from the local area around Columbia Presbyterian Medical Center for the smokers' study. After informed consent was obtained, oral mucosal cells were collected by gently scraping the inside of the cheek with a wooden tongue depressor or by rinsing the mouth with saline. Cells collected by scraping were smeared on slides. Cells collected by rinsing were centrifuged at 1000 rpm for 10 min. The cell pellet was resuspended in sucrose buffer [0.25 M sucrose, 1.8 mM CaCl2, 25 mM KCl, and 50 mM Trizma Base (pH 7.5)] to a final volume of 1 ml. About 30–50 μl of cell suspension were added to 300 μl carbowax-ethanol buffer (0.8% polyethylene glycol in 70% ethanol). After briefly vortexing, one-half of the suspension was cytopsied at 300 rpm for 5 min onto each of two slides precoated with 0.2% poly-D-lysine. Slides were air dried, fixed in 95% ethanol, and stored at −20°C until staining. For analysis of the human samples, paired samples of oral cells of smokers and nonsmokers, matched by age, race, and sex, were assayed together.
Results

Detection of 8-OHdG in Single Cells. The immunoperoxidase method was used to detect oxidative DNA damage in WC3 cells treated with 0—10 μM AFB₁. Representative staining for 8-OHdG demonstrating specific nuclear antibody binding in cells treated with 2 μM AFB₁ is illustrated in Fig. 1a, while weaker background staining in untreated control cells is seen in Fig. 1b. Duplicate cell preparations were stained for AFB₁-DNA adducts using antiserum 6A10 and also demonstrated specific nuclear staining in treated but not control cells (data not shown). Quantitation of staining in 50 randomly selected cells treated with 0, 0.4, 2, and 10 μM AFB₁ indicated a dose-related increase in mean nuclear staining in AFB₁-treated cells with both antisera recognizing AFB₁-DNA and 8-OHdG. Mean nuclear staining for 8-OHdG was 212 ± 43, 416 ± 109, 720 ± 159, and 848 ± 107, respectively. Staining intensity for AFB₁-DNA for the same doses was 193 ± 47, 323 ± 86, 563 ± 90, and 686 ± 84, respectively. Preabsorption of primary antibody 1F7 with 8-OHdG before use decreased staining from 717 ± 102 to 179 ± 22 for cells treated with 2 μM AFB₁ (data not shown). Staining of these same cells with a nonspecific antiserum recognizing DNA damage produced by 8-methoxypsoralen resulted in a staining intensity of 212 ± 66 (Fig. 1c), while pretreatment with DNase decreased relative staining (161 ± 32; Fig. 1d).

To demonstrate that the method was also applicable to detection of oxidative damage induced by another agent in an alternate cell line, 10T½ cells were treated with 0–200 μM H₂O₂. Again, specific nuclear staining was observed in treated but not control cells (data not shown). Quantitation of staining on a total of 30 randomly selected cells indicated a 2.5-fold increase in cells treated with 100 μM H₂O₂ (205 ± 29) compared to control cells (82 ± 6). No further increase in staining intensity was observed in cells treated with 200 μM H₂O₂ (185 ± 19).

Detection of 8-OHdG and AFB₁-DNA in Aflatoxin B₁-treated Rats. The immunoperoxidase method was then used to detect the presence of 8-OHdG and AFB₁-DNA in cryostat liver sections of rats treated with AFB₁. Fig. 2. a and b, show representative specific nuclear staining for 8-OHdG of liver tissue of a control untreated rat and a rat treated with 2.5 mg AFB₁/kg and sacrificed 2 h later, respectively. Mean relative staining intensity of 50 cells for these samples was 265 ± 55 and 850 ± 120, respectively. Fig. 2. c and d, show decreased nuclear staining when animals were sacrificed at 8 h (455 ± 62) and 24 h (313 ± 56) after AFB₁ treatment, respectively. To demonstrate specificity of staining, liver tissue from an animal sacrificed 2 h after 2.5 mg/kg AFB₁ treatment was stained with antiserum 1F7 preabsorbed with 8-OHdG (1 μg/μl), and low nuclear staining was observed (274 ± 71; data not shown). Staining with a nonspecific antiserum recognizing 8-MOP-DNA damage was also decreased (270 ± 42; data not shown), compared to use of specific antiserum. Pretreatment of this same tissue with DNase decreased relative staining to 180 ± 30 (data not shown). Tissue sections were also stained for AFB₁-DNA, and the time course comparison of both

![Fig. 2. Immunoperoxidase staining for 8-OHdG in rat liver tissues. Rats were treated with 0 or 2.5 mg/kg AFB₁ and sacrificed at various time intervals. a, control, untreated rat. Rats were treated with 2.5 mg/kg and sacrificed 2 h (b), 8 h (c), and 24 h (d) after treatment.](cancerres.aacrjournals.org)
from a total of 12 pairs of smokers and nonsmokers were analyzed. Representative staining for 8-OHdG in a smoker (subject no. 7) and a nonsmoker (subject no. 36) are illustrated in Fig. 4, a and b, respectively. Preabsorption of antibody 1F7 with 8-OHdG before use decreased staining in cells of smoker no. 7 from 431 ± 67 to 61 ± 17 (Fig. 4c), while staining with a nonspecific antiserum recognizing 8-MOP-DNA gave a value of 118 ± 35 (Fig. 4d). Pretreatment of slides with DNase decreased relative staining to background levels (58 ± 24; data not shown). Quantitative staining intensity data as well as demographic data and number of cigarettes smoked for each subject are given in Table 1. Higher levels of specific nuclear staining were observed in every smoker compared to their matched nonsmoker. Mean level of relative staining was elevated 1.6-fold in smokers (mean 381 ± 70; P < 0.001) compared to nonsmokers (mean 244 ± 43). There was no association between staining intensity and the number of cigarettes smoked/day.

Discussion

This study describes the development of a new method for monitoring oxidative damage. Monoclonal antibody 1F7, used previously for ELISA quantitation of 8-OHdG in DNA isolated from tissues (15), is also applicable to immunohistochemical analysis of 8-OHdG in single cells or tissue sections. Both H2O2 and AFB1 were used as inducers of 8-OHdG in cultured cells. The maximum increase in oxidative damage induced by AFB1 (4-fold) was higher than that produced by H2O2 (2.5-fold). AFB1 was reported to induce 8-OHdG
formation in rat hepatic DNA in a time- and dose-dependent manner (19). The 1 mg/kg dose used in this study increased the level of 8-OHdG from 1.5 ± 0.6 pg/g DNA to 4.5 ± 1.0 pg/g DNA 1 day after treatment (a 3-fold increase). The immunoperoxidase technique reported here detected a 3.2-fold increase in the 8-OHdG level in rats treated with 2.5 mg AFBl/kg, compared with control untreated rats. In animals sacrificed at different time points after treatment, a decrease in 8-OHdG and AFBl levels was observed, and both types of damage had similar half-lives of approximately 24 h (Fig. 3). This is probably due to a combination of DNA repair and cell turnover. The data on AFBl-DNA are similar to those published previously using immunofluorescence or spectrofluorescence methods on the same tissue samples (16). A good correlation (r = 0.95, P < 0.01) was found between relative staining intensity for AFBl-DNA adducts by the immunoperoxidase and immunofluorescence methods.

The immunohistochemical assay can also detect oxidative damage in oral cells. A 1.6-fold increase in the 8-OHdG level was observed in oral cells of smokers versus nonsmokers. We have observed previously a 2-fold increase in polycyclic aromatic hydrocarbon-DNA adducts in oral cells of smokers versus nonsmokers using an immunoperoxidase technique (20). A 1.5-fold increase in the level of 8-OHdG in WBC of smokers over nonsmokers has been reported using the HPLC-EC method (12). However, another study was unable to confirm these results (21). In our study, no relationship was found between the number of cigarettes smoked per day and oxidative damage. In our prior studies on polycyclic aromatic hydrocarbon-DNA adducts in mononuclear cells by ELISA (22) or oral cells by immunoperoxidase (20), we were also unable to detect a relationship between numbers of cigarettes smoked per day and DNA damage levels. This may be due to differences in smoking habit, brand of cigarette, as well as genetic factors which influence carcinogen metabolism and DNA repair. It may also be the result of other environmental exposures or differences in diet or life-style of study subjects.

Limitations of the immunoperoxidase method are that: (a) it is semiquantitative and only provides information on relative levels of 8-OHdG; and (b) there is the potential for cross-reactivity with other DNA damage. Although the competitive ELISA demonstrated that antisera 1F7 is relatively specific for 8-OHdG, some low cross-reactivity was observed for several competitors (15). These limitations are, however, far outweighed by the advantages of the immunohistochemical method. The requirement for small numbers of cells allows its application to tissue biopsies. Although the method works well on frozen sections, we are currently determining its applicability to stored paraffin blocks. If successful, this would greatly expand the types of samples that can be assayed. In addition, since there is no need for isolation and processing of DNA, the potential for artifactual generation of oxidative damage is eliminated. Thus, the immunoperoxidase method developed here should be useful for evaluation of oxidative damage in a wide range of in vitro, animal, and human studies and will help in the determination of its relationship to human cancer.

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References


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