A Highly Sensitive and Specific Method for Quantitation of O-Alkylated DNA Adducts and Its Application to the Analysis of Human Tissue DNA

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Introduction

O\textsuperscript{6}-Alkylguanine and O\textsuperscript{4}-alkylthymine have been demonstrated to be the most relevant lesions among DNA modifications for induction of cancer by N-nitroso compounds in diverse experimental systems (1-3). Detection of such DNA lesions in human tissues, therefore, is one of the best markers for the biologically relevant exposure to alkylating agents and may eventually lead to a better risk assessment (4-6). Because DNA modifications in human tissues have been previously demonstrated to be at extremely low levels, the use of an assay with high sensitivity as well as specificity is needed for molecular epidemiological studies on cancer in man. To address this problem, we have established a quantitation method for O-alkyl DNA adducts and examined its validity for application to human samples in a pilot experiment.

Materials and Methods

DNA was enzymatically hydrolyzed to nucleoside-3'-monophosphate (3'-MP) using micrococcal nuclease and spleen phosphodiesterase (7). Each alkylated 3'-MP was fractionated by a reverse-phase HPLC system under essentially the same conditions as previously described (5). The alkylated 3'-MP was then phosphorylated by polynucleotide kinase and [\textsuperscript{32}P]ATP according to Reddy et al. (7), followed by 3'-dephosphorylation by decreasing the pH to 6.0 (8) for better recognition by antibodies. After purification by HPLC, alkylated 5'-[\textsuperscript{32}P]MP was immunoprecipitated with corresponding monoclonal antibodies [ER-6 for O\textsuperscript{6}-methylguanine, EM-3-33 for O\textsuperscript{4}-methylthymine, and ER-01 for O\textsuperscript{4}-ethylthymine (9,10)], and the radioactivity was determined by liquid scintillation counting. Human DNA samples were isolated from peripheral leukocytes of 11 healthy volunteers who had no apparent history of exposure to alkylating agents.

Results and Discussion

Procedure of PREPI

Conditions of each reaction step were optimized using standard DNA treated with N-methyl-N-nitrosourea or N-ethyl-N-nitrosourea. The content of O\textsuperscript{6}-methylguanine (O\textsuperscript{6}-MethG), O\textsuperscript{4}-methylthymine (O\textsuperscript{4}-MethT), and O\textsuperscript{4}-ethylthymine (O\textsuperscript{4}-EtT) in the standard DNA was determined by competitive radioimmunoassay using titrated tracers and antibodies as described (5). Completion of DNA hydrolysis was monitored by injecting aliquots onto HPLC. O\textsuperscript{6}-Methylguanosine-3'-monophosphate, O\textsuperscript{4}-methylthymidine-3'-monophosphate, O\textsuperscript{4}-ethylguanosine-3'-monophosphate, and O\textsuperscript{4}-ethylthymidine-3'-monophosphate...
could be separated from each other and also from normal 3'-MP under the present conditions, which facilitated independent analysis of each adduct from a given amount of DNA and reduced the amount of [32P]ATP necessary in comparison with the original postlabeling method. After postlabeling, 3'-phosphate was removed to enhance the recognition by antibodies because the monoclonal antibodies used were originally raised against alkylated nucleosides (9,10). The second HPLC fractionation was used to remove [32P]ATP and nonspecific radioactive byproducts before final immunoprecipitation. The precise amount of alkylated DNA adducts was determined by comparing a curve obtained by serial dilution of samples with a standard curve.

**Sensitivity of the Method**

A representative standard curve for O⁴-EtT is shown in Figure 1. Standard curves for O⁴-MetG and O⁴-MetT were essentially similar to that for O⁴-EtT (data not shown). One femtomole of each alkylation product could be precisely determined. Therefore, the relative sensitivity (II), i.e., the lowest relative modification level to be determined is about 1 × 10⁻⁷ as a molar ratio to normal counterpart when 100 μg of DNA is available for analysis (Table I).

**Specificity**

Combining several analytical methods with different physicochemical bases is preferable for attaining the high specificity needed, particularly in the case of human samples. The present method is highly specific because each step, i.e., reverse-phase HPLC fractionation, enzymatic reactions including postlabeling, and immunoprecipitation, contribute to increasing the specificity through different physicochemical or molecular mechanisms.

**Relevance versus Feasibility**

The validity of any analytical methods for use in molecular epidemiological studies with respect to cancer may be assessed by two independent parameters: relevance to cancer development and feasibility or ease of use for mass screening. Quantitation of O-alkylated DNA adducts is considered to have higher relevance to carcinogenesis than hemoglobin adducts or N7-alkylguanine, for example. In our previous studies, to detect O⁴-EtT in human liver DNA by radioimmunoassay using a tritiated tracer, we had to use more than 20 mg of DNA (corresponding to 20–30 g liver tissue) because of the limited sensitivity. We attempted to improve the feasibility by increasing the sensitivity of the quantitation method without sacrificing the relevance. In the present assay, we need only 100 μg DNA (corresponding to 10 mL of peripheral blood or several hundred milligrams of biopsy tissue specimens) for the analysis of similar relative modification levels.

**Analysis of Human Leukocyte DNA**

To examine the validity of the present method, we quantitated O⁴-MetG, O⁴-MetT, and O⁴-EtT in peripheral leukocyte DNA obtained from 11 healthy volunteers. O⁴-MetG was detected in all 11 cases, with a mean value of 2.0 ± 1.3 × 10⁻⁸ (range 0.78–4.6 × 10⁻⁸) as a molar ratio to guanine. O⁴-MetT and O⁴-EtT were below the detection limit of 0.8 × 10⁻⁸ as a ratio to thymine. The result indicates that humans are exogenously and/or endogenously exposed to methylating agents, and the present method can be practically applied to the analysis of human samples. We are now undertaking studies on a possible correlation between levels of alkylated DNA adducts and the incidence of cancer using the method described above.

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