Detection of 8-Hydroxy-2'-deoxyguanosine in Deoxyribonucleic Acid by the $^{32}$P-Postlabeling Method

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Using synthesized 8-hydroxy-2'-deoxyguanosine 3'-monophosphate as a marker, the $^{32}$P-postlabeling method was adapted with minimum modifications for the analysis of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) content in deoxyribonucleic acid (DNA). This method allows the analysis of one 8-OH-dG per $10^8$ DNA nucleotides with only 10 pmol of nucleotides required. The amounts of 8-OH-dG in DNA detected by the postlabeling method correlated well with the electrochemical detection method but were consistently lower.

Keywords: $^{32}$P-postlabeling; 8-hydroxydeoxyguanosine; DNA; oxidative damage

Introduction

8-Hydroxy-2'-deoxyguanosine (8-OH-dG) represents one specific type of deoxyribonucleic acid (DNA) damage product induced by oxygen radical producing agents. 3) Site specific mutagenesis analysis shows that 8-OH-dG is a mutagenic lesion. 3,23) Mechanistic studies concerning the formation of 8-OH-dG indicates the involvement of a single oxygen species. 34) An enzyme that specifically removes 8-OH-dG has been purified from E. coli implying the formation of this adduct in vivo. 35) This adduct was first detected in in vivo samples 36) through the combined use of high performance liquid chromatography (HPLC) and electrochemical detection developed by Floyd et al. 37) A fluorescence postlabeling method was also recently described for the analysis of this adduct. 38) Povey et al. 39) purified 8-hydroxy-2'-deoxyguanosine 3'-monophosphate (8-OH-dGp) from DNA by HPLC and quantitated this nucleotide using the postlabeling method. 40) The present report describes a simple modification of the $^{32}$P-postlabeling method that allows the detection of one 8-OH-dG per $10^5$ nucleotides in DNA without the prepurification of this nucleotide by HPLC. The HPLC purification step can enhance sensitivity but requires substantial amounts of DNA for analysis.

Experimental

8-Hydroxy-2'-deoxyguanosine 3'-monophosphate (dGp), calf thymus DNA, horseradish peroxidase, and hydrogen peroxide were purchased from Sigma Chem. Co. Sources of all reagents used for the $^{32}$P-postlabeling method were described in detail. 3,10) $[{\gamma}^{32}$P]adenosine triphosphate (ATP) was synthesized and activities determined in our laboratory as described. 10)

Synthesis of 8-OH-dGp

8-OH-dGp was synthesized as described 10) with the following modifications. dGp (10 mg) was dissolved in 10 ml of 0.1 M phosphate buffer, pH 6.8 containing 14 mM ascorbic acid, 6.5 mM ethylenediaminetetraacetic acid and 1.3 mM FeSO$_4$. The mixture was bubbled with oxygen at 37°C for 3.5 h. The water was removed by lyophilization. The residue was solubilized with a minimum amount of water, applied to a Sephadex LH 20 column (2 × 38 cm), eluted with water, and detected by an electrochemical detector. Fractions containing 8-OH-dGp were pooled for further purification by HPLC. The pooled fractions were applied to a LiChrospher 100 RP-18(e) column (4.0 × 125 mm, Merck) and eluted isocratically with 10 mM Na$_2$HPO$_4$ containing 2% methanol. Salt was removed by HPLC using a TSKgel-ODS 80Tm column (4.6 × 100 mm, Tosho). The solvent used for elution was water containing 10% methanol. Ultraviolet (UV) spectra (absorption maximum, nm) of 8-OH-dGp were 247 and 293 at pH 1 and pH 8.6, and 248 and 280 at pH 11. This spectra is identical to that of 8-OH-dG. The present paper describes a simple modification of the $^{32}$P-postlabeling method that allows the detection of one 8-OH-dG per $10^5$ nucleotides in DNA without the prepurification of this nucleotide by HPLC. The HPLC purification step can enhance sensitivity but requires substantial amounts of DNA for analysis.

Results and Discussion

Figure 1 shows a suitable separation of 8-OH-dpGp from dpGp after two dimensional TLC (thin layer chromatography) on a PEI-cellulose plate. Figure 1A shows the pattern of control calf thymus DNA which has a background level of seven 8-OH-dG per $10^5$ nucleotides as determined by the electrochemical detection method. Figure 1B shows the pattern of calf thymus DNA with 1493 8-OH-dG per $10^5$ nucleotides. Figure 1C shows the pattern of a mixture consisting of a DNA sample (same as Fig. 1B) and an aliquot of [5'-$^{32}$P]-OH-dpGp. Figure 1D represents authentic dpGp and 8-OH-dpGp. The result shown in Fig. 1C indicates that the 8-OH-dG moiety in DNA does co-chromatograph with an authentic marker.

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The 1.25 M ammonium formate, pH 3.5 and overnight development were chosen, since this condition retains only dpGp and 8-OH-dpGp on the TLC plate. The overnight development removed the remaining $^{32}$P-labeled normal nucleotides and $^{32}$Pi (from excess $[\gamma-^{32}$P]ATP after potato apyrase treatment), thus, reducing >90% of the radioactivity to which personnel would otherwise be exposed. Occasionally, 3',5'-bisphosphate of 2'-deoxyadenosine was also retained at the upper right hand corner of the chromatogram. The overnight development can be performed for 15—17 h. 8-OH-dGp was found to be sensitive to the digestion of nuclease P1. Therefore, nuclease P1 cannot be used to enrich 8-OH-dGp. The amount of $[\gamma-^{32}$P]ATP used was greater than the amount of nucleotides used for this adaptation of the postlabeling method.\(^{10}\)

Figure 2 compares the levels of 8-OH-dG contained in DNA samples analyzed by the postlabeling method and electrochemical detection. In general, 8-OH-dG contents were consistently lower by ca. 4-fold if analyzed by the postlabeling method compared to electrochemical detection. The reason for this difference is unknown. Whether this is due to suboptimal enzymatic digestion or reaction conditions is not known. We did not observe any significant differences in the 8-OH-dG content when the amount of ATP used was deficient (data not shown). More studies may be needed to explain the difference. However, in the postlabeling method, the same samples were simultaneously analyzed for both dG and 8-OH-dG. Since in this instance dG served as an internal standard, data obtained from the postlabeling method cannot be attributed to a variation in the amounts of nucleotides used for analysis. A 20% label-
ing efficiency was reported for thymine glycol when the postlabeling method was used for the quantification of this type of oxidative DNA base damage.\textsuperscript{12} The reduced labeling efficiency observed for both thymine glycol and 8-OH-dGp may result from the loss of their respective parent base ring structures.

In spite of the differences in the actual levels of 8-OH-dG determined using the postlabeling method and electrochemical detection, Fig. 3 shows that data obtained by the two methods do correlate well. The equation for linear regression analysis is \( y = 0.054 + 0.204x \), with the multiple correlation coefficient being 0.92 and the coefficient of determination being 0.85. From this equation, the actual background counts in the 8-OH-dG spot area is calculated to be 0.054\% of dG (e.g., \( x = 0 \) based on electrochemical detection data) over the surrogate background area. From these parameters, assuming a sample size of 5, using a power of detection at 0.8, and a level of statistical significance equivalent to 0.05, a theoretical limit of the minimal value for detection above the background was calculated to be approximately 0.061\% of dG (ca. 0.012\% of total nucleotides).

One advantage of using the postlabeling method is that it requires substantially decreased amounts of DNA (1/300 \( \mu \)g DNA) compared to either electrochemical detection (100—500 \( \mu \)g DNA pending on the extent of modifications) or the fluorescence postlabeling method (100 \( \mu \)g DNA). The latter two procedures, however, do not expose personnel to radiation. Since the background levels of 8-OH-dG content in control DNA are ca. 1 modification per 10\(^5\) nucleotides, this procedure is sufficient for the analysis of in vitro samples. DNA from cells or tissues contains 0.6—1.4 molecules of 8-OH-dG per 10\(^5\) dG.\textsuperscript{11} Improving the labeling efficiency will enable us to analyze the formation of 8-OH-dG in cells or tissues.

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\textbf{References}