Ionizing-radiation-induced damage in the DNA of cultured human cells

Identification of 8,5'-cyclo-2'-deoxyguanosine

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Epstein–Barr-virus-transformed peripheral-blood B-lymphocytes were γ -irradiated at 0 °C at doses from 10 to 100 Gy. The cells were immediately lysed and the DNA was isolated. Subsequently, the DNA was hydrolysed to 2'-deoxyribonucleosides with a mixture of DNAase I, venom and spleen exonucleases and alkaline phosphatase. The hydrolysate was dried, trimethylsilylated and analysed by capillary gas chromatography–mass spectrometry with selected-ion monitoring. The (5'R)- and (5'S)-diastereomers of 8,5'-cyclo-2'-deoxyguanosine were observed in a ratio of 1:3, and their formation was dose-dependent. It was possible to detect and characterize one such lesion in approx. 4×10^4 guanine nucleotide subunits of DNA.

INTRODUCTION

DNA of living cells is considered to be the principal target for radiation-induced damage, which might result in mutation, chromosomal aberrations, carcinogenesis and cell death (for reviews see Painter, 1980; Ward, 1981). Knowledge of the chemical nature of this damage is required for an understanding of its biological consequences and enzymic repair.

The capillary g.c.–m.s./s.i.m. technique has been shown to be a useful analytical tool for detection and characterization of the radiation-induced damage to DNA in aqueous solution (Dizdaroglu, 1985, 1986; Dizdaroglu & Bergtold, 1986). We now report the first use of g.c.–m.s./s.i.m. in the identification of DNA damage induced by ionizing radiation in living cells. The lesion identified is 8,5'-cyclo-dGuo, which has not previously been known to occur in irradiated cells.

MATERIALS AND METHODS

Cell lines and culture medium

Epstein–Barr-virus-transformed human peripheral-blood B-lymphocytes were from the Institute for Medical Research, Camden, NJ, U.S.A. The two cell lines used (GM 130 and RB 4580) were derived from normal volunteers. Medium RPMI 1640, foetal bovine serum and phosphate-buffered saline were from GIBCO, Grand Island, NY, U.S.A.

Materials for DNA isolation

Unless otherwise stated, all chemicals were of analytical-reagent grade and were used without purification. SDS was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Proteinase K and RNAase A were from Sigma Chemical Co., St. Louis, MO, U.S.A. The RNAase stock solution (10 mg/ml) was heated for

10 min in a boiling-water bath. Tris and phenol (redistilled) were from Bethesda Research Laboratories, Gaithersburg, MD, U.S.A. Phenol was obtained frozen and stored at -20 °C. A 10 ml volume of water was added per 100 g bottle of phenol, which was then heated in a 60 °C water bath until the solid dissolved, and the phenol was used within a few hours. Buffers A, B and C were prepared in accordance with Gross-Bellard *et al.* (1973). Solvent P is buffer B/phenol (1:10, v/v). Solvent P was added to the nucleic acid preparation in combination with solvent CI [chloroform/3-methylbutan-1-ol (chloroform/isoamyl alcohol), 24:1, v/v]. TE buffer is 10 mM-Tris/HCl buffer, pH 8, containing 1 mM-EDTA.

Culture and irradiation of cells

Lymphoblasts were grown to 10^6 cells/ml in medium RPMI 1640 supplemented with foetal bovine serum (20%, v/v), chilled and centrifuged at 500 g in the cold. The pellets were washed once by resuspension in cold phosphate-buffered saline followed by centrifugation. The washed cells were then resuspended in cold phosphate-buffered saline at a concentration of 10^8 cells/ml, stored on ice and transported to the radiation source. The cells were γ -irradiated with a ^{60}Co source (82 Gy/min) while their container was submerged in an ice/water bath.

Isolation of DNA

Immediately after irradiation, the cells were centrifuged, resuspended at 10^8 cells/ml in cold TE buffer and lysed in 10 vol. of buffer A, and incubated for 3 h at 55 °C with proteinase K (100 $\mu\text{g}/\text{ml}$). After three extractions with equal volumes of solvent P/solvent CI (1:1, v/v) followed by extraction with an equal volume of solvent CI, the aqueous phase was chilled and

Abbreviations used: Ado, adenosine; dGuo, 2'-deoxyguanosine; dAdo, 2'-deoxyadenosine; g.c.–m.s./s.i.m., gas chromatography–mass spectrometry with selected-ion monitoring; Me₃Si, trimethylsilyl.

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Fig. 1. G.c.-m.s./s.i.m. plots of some characteristic ions of 8,5'-cyclo-dGuo(Me₃Si)₄ obtained from trimethylsilylated enzymic hydrolysates of DNA samples isolated from (a) γ -irradiated (100 Gy) cells and (b) non-irradiated cells

The column was a fused silica capillary coated with SE-54 (25 m $\times$ 0.31 mm internal diam.), programmed from 150 to 250 $^{\circ}$ C at 10 $^{\circ}$ C/min. Arrows indicate the elution positions of (5'R)- and (5'S)-diastereomers of 8,5'-cyclo-dGuo(Me₃Si)₄. The full scales of the *m/z* 448 and 538 ions are twice those of the other ions plotted here.

adjusted to 0.2 M-sodium acetate with 3 M-sodium acetate buffer, pH 5. Nucleic acid fibres were spooled on a glass rod after addition of 2 vol. of ethanol prechilled to -20° C. The fibres were washed with 70% (v/v) ethanol, then dissolved in buffer C, and the resulting solution was incubated at 37 $^{\circ}$ C with RNAase A (50 μ g/ml) for 2.5 h followed by a 30 min incubation with proteinase K (100 μ g/ml). The three extractions with solvent P/solvent CI and one extraction with solvent CI were then repeated. DNA fibres were again collected by ethanol precipitation, washed with 70% ethanol and dissolved in water. After exhaustive dialysis

(72 h) against water in the cold, the preparation was freeze-dried.

Treatment and analysis of DNA

Enzymic hydrolysis, trimethylsilylation and g.c.-m.s./s.i.m. were performed as previously described (Dizdaroglu, 1986).

RESULTS

Trimethylsilylated enzymic hydrolysates of DNA isolated from γ -irradiated or non-irradiated cells were

Fig. 2. Mass spectrum generated on the basis of the ions and their area counts at the elution position of (5'*R*)-8,5'-cyclo-dGuo(Me₃Si)₄ indicated with the arrow in Fig. 1(a)

analysed by g.c.-m.s./s.i.m. Characteristic ions from the known mass spectrum (Dizdaroglu, 1986) of the trimethylsilyl derivative of 8,5'-cyclo-dGuo [8,5'-cyclo-dGuo(Me₃Si)₄] were monitored simultaneously. Fig. 1(a) illustrates selected-ion-current profiles obtained from a DNA sample from cells irradiated at a dose of 100 Gy. Here, the readily discernible peaks of the monitored ions are seen at the expected retention times of the (5'*R*)- and (5'*S*)-diastereomers of 8,5'-cyclo-dGuo(Me₃Si)₄ (indicated with the two arrows in Fig. 1a). Since the intensities of the recorded ions relative to one another also have to match those of the same ions in the mass spectrum of the authentic compound for an unequivocal identification, the area counts of the ions in Fig. 1(a) were calculated by the integrator of the instrument. The acquired data were used subsequently to generate a mass spectrum by setting the area count of the most abundant ion, i.e. *m/z* 397, equal to 100% of relative intensity. The partial mass spectrum obtained in this way from the signals at the g.c. retention time of (5'*R*)-8,5'-cyclo-dGuo(Me₃Si)₄ in Fig. 1(a) is illustrated in Fig. 2. This spectrum was directly correlatable to that of authentic (5'*R*)-8,5'-cyclo-dGuo(Me₃Si)₄ published previously (Dizdaroglu, 1986). Similarly, the partial mass spectrum obtained from the signals at the g.c. retention time of (5'*S*)-8,5'-cyclo-dGuo(Me₃Si)₄ in Fig. 1(a) was also directly correlated to that of authentic (5'*S*)-8,5'-cyclo-dGuo(Me₃Si)₄ (results not shown here). The spectrum in Fig. 2 contains four more ions than those plotted in Fig. 1(a). Since the instrument used permits the monitoring of up to six ions simultaneously, of necessity the data shown in Fig. 2 were obtained from two runs. The most abundant ion *m/z* 397 was also recorded in the second run in order to obtain the relative intensities of the additional ions. The (5'*S*)-diastereomer was more abundant than the (5'*R*)-diastereomer (Fig. 1a). A ratio of approx. 3:1 (5'*S*/5'*R*) was calculated on the basis of the area counts of the most intense ion *m/z* 397.

As Fig. 1(b) indicates, small but detectable amounts of 8,5'-cyclo-dGuo were also observed in the DNA samples isolated from non-irradiated cells. The origin of

8,5'-cyclo-dGuo in the DNA of non-irradiated cells is not known.

With the use of (5'*S*)-8,5'-cyclo-Ado as the internal standard, g.c.-m.s./s.i.m. was used to measure the amounts of 8,5'-cyclo-dGuo in the DNA from γ -

Fig. 3. G.c.-m.s./s.i.m. plot of the *m/z* 553 ion obtained from the trimethylsilylated enzymic hydrolysate of a DNA sample from γ -irradiated cells

A known amount of the internal standard (5'*S*)-8,5'-cyclo-Ado was added to the DNA sample before enzymic hydrolysis. Peak assignment: 1, (5'*R*)-8,5'-cyclo-dGuo(Me₃Si)₄; 2, (5'*S*)-8,5'-cyclo-Ado(Me₃Si)₄; 3, (5'*S*)-8,5'-cyclo-dGuo(Me₃Si)₄. It should be noted that the time scale here is different from that in Fig. 1.

irradiated and non-irradiated cells. DNA samples containing a known amount of the internal standard were subjected to enzymic hydrolysis followed by trimethylsilylation and g.c.-m.s./s.i.m. (5'S)-8,5'-Cyclo-Ado(Me₃Si)₄ has the same fragmentation patterns and the same molecular mass as 8,5'-cyclo-dGuo(Me₃Si)₄. In addition, the mass spectra of these compounds show molecular ions (*m/z* 553) with nearly equal relative intensities (Dizdaroglu, 1986). For this reason, the *m/z* 553 ion was plotted for quantitative analysis, and the relative molar response factors of molecular ions for both 8,5'-cyclo-Ado(Me₃Si)₄ and 8,5'-cyclo-dGuo(Me₃Si)₄ were assumed to be equal. Fig. 3 illustrates a representative ion-current profile of *m/z* 553 from a DNA sample from γ -irradiated cells. An excellent separation of (5'S)-8,5'-cyclo-Ado(Me₃Si)₄ from both (5'R)- and (5'S)-diastereomers of 8,5'-cyclo-dGuo(Me₃Si)₄ was obtained (Fig. 3). The amount of 8,5'-cyclo-dGuo found in the DNA of non-irradiated cells corresponded to one lesion in 4×10^4 – 4.5×10^4 guanine nucleotides. One 8,5'-cyclo-dGuo lesion per 4×10^4 guanine nucleotides was induced by 10 Gy, and 7 times as many were induced by 100 Gy.

DISCUSSION

The mechanism suggested for the formation of 8,5'-cyclo-nucleosides (or -nucleotides) from nucleosides (or nucleotides) in aqueous solution (Keck, 1968; Cadet & Berger, 1985; Raleigh & Fuciarelli, 1985) can be assumed for the formation of 8,5'-cyclo-dGuo in cellular DNA with the initial attack at C-5' of the dGuo moiety by hydroxyl radicals generated by radiation from cellular water. The stereoselectivity of the following cyclization process appears to depend much on the nature of the substrate. For DNA irradiated in aqueous solution, the ratio of (5'R)-8,5'-cyclo-dGuo to (5'S)-8,5'-cyclo-dGuo was 3:1 (Dizdaroglu, 1986). In contrast, for the DNA of irradiated cells reported here the ratio is 0.33:1. Variations have also been reported for the ratios of (5'R)- and (5'S)-diastereomers of 8,5'-cyclo-Ado formed from adenosine and from 5'-AMP and in poly(A) upon γ -irradiation in aqueous solution (Fuciarelli *et al.*, 1986).

The induction by radiation of (5'R)- and (5'S)-8,5'-cyclo-dGuo in cellular DNA may pose a threat to the survival of a living cell. The contribution of these lesions to the detrimental effects of ionizing radiation on living

cells is not yet known. The covalent cyclic bonding of a sugar to the base in the same nucleotide residue may cause a conformation distortion of the DNA structure. Although it is unlikely that such a cyclic lesion could be repaired via the base-excision pathway, it might well be subject to repair by the nucleotide-excision pathway (Friedberg, 1985). Of some interest also is the possibly different action of DNA repair enzymes on (5'R)- and (5'S)-diastereomers of 8,5'-cyclo-dGuo.

In conclusion, the results presented here show the induction by ionizing radiation of (5'R)- and (5'S)-8,5'-cyclo-dGuo in the DNA of living cells. Our methodology should permit studies of the repair of such lesions in normal cells as well as in radiosensitive cells from patients with neurodegenerations such as ataxia telangiectasia, Alzheimer's disease, Parkinson's disease and Huntington's disease (Robbins *et al.*, 1985).

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