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Reduction in the amount of 8-hydroxy-2′-deoxyguanosine in the DNA of SV40-transformed human fibroblasts as compared with normal cells in culture

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DNA damage due to oxidative free radicals is considered to be a major cause of ageing and age-related diseases including cancer. Of more than 20 modifications formed in DNA by the action of hydroxyl radicals, 8-hydroxy-2′-deoxyguanosine (oh^{8}dG) is potentially highly mutagenic and is known to occur most frequently. Using HPLC combined with electrochemical (HPLC/EC) detection of oh^{8}dG, fivefold higher levels of oh^{8}dG are detected in the DNA of cultured normal human skin fibroblasts as compared with SV40-transformed human fibroblasts MRC-5V2. In comparison, the levels of oh^{8}dG were similar in the growth medium of both types of cells. Applications of this method range from studies on the genomic stability and instability of normal and cancerous cells to the clinical and laboratory testing of toxic substances and drugs.

Oxidative DNA damage; 8-Hydroxydeoxyguanosine; Transformed cell; HPLC/EC

1. INTRODUCTION

Free radical-induced oxidative damage to DNA and other macromolecules has been linked with ageing and several diseases including ischemia, chronic inflammation, autoimmune diseases and cancer [1,2]. The oxidative DNA damage in vivo arises most commonly by the initial reaction of hyroxyl radicals generated by metal ions bound to DNA and cellular oxidants [3]. The same radicals are also the principal reactive species in the mutagenic and carcinogenic effects of ionizing radiations. Of more than 20 different modifications supposed to be formed in DNA by the action of hydroxyl radicals, 8-hydroxy-2′-deoxyguanosine (oh^{8}dG) [4,5] has been reported to occur in the DNA of various organs of ageing rats [6], and is detected in rodent and human urine [7]. Furthermore, the presence of oh^{8}dG has also been reported in rat liver nuclear and mitochondrial DNAs [8].

Considering the strong mutagenic capacity of such a modified base [9,10], it is very important to measure the levels of oh^{8}dG in both the DNA of cells, tissues and organisms, and in body fluids such as plasma and urine in order to monitor the extent of oxidative damage and repair during normal ageing and pathological conditions. We have compared the levels of oh^{8}dG both in cellular DNA and released in the culture medium, by using HPLC combined with electrochemical (EC) detection of oh^{8}dG. Due to the well known advantages of cultured cells for studies on cell cycle regulation, transformation, immortalization, cellular ageing and toxicity testing [11,12], having a method of determining the extent of oxidative damage and repair in cultured cells and in growth media is potentially highly useful. Using this method, we have observed that transformed human cells contain lower amounts of oh^{8}dG in DNA as compared with normal cells.

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2. MATERIALS AND METHODS

Nonradiolabeled standard samples of oh^{8}dG were synthesized from deoxyguanosine (dG) with Udenfriend system as described by Kasai and Nishimura [13]. Herring sperm DNA and dG were purchased from Boehringer Mannheim, Germany. All other materials were of the highest grade available.

Rapidly growing normal adult human skin fibroblasts of passage level 8 and SV40-transformed immortal fibroblasts designated MRC-5V2 were used in this study. The methods for the maintenance and growth of normal and transformed cells has been described previously [14]. DNA from about 2×10^6 cells was extracted by following a modified method of Miller et al. [15]. Cells were collected by scrapping of the growth surface of the culture flask. After washing once with buffered saline, the cells were resuspended in 0.5 ml lysis buffer (10 mM Tris-HCl pH 8.2, 400 mM NaCl and 2 mM EDTA). The cell lysate was then digested overnight at 37°C with 1 mg protease K in 1% SDS and 2 mM Na_2EDTA in order to remove proteins. The lysate was treated with 1 ml saturated NaCl (6 M), and DNA was precipitated with two volumes of ethanol. DNA was dissolved in 0.5 ml 50 mM Tris-HCl pH 8.1 buffer and digested with 400 units of pancreatic ribonuclease for 1 h at 37°C in order to remove associated RNA which can also contain oxidized products DNA was precipitated again with ethanol.

Hydrolysis of DNA to nucleosides was done in 0.5 ml of 20 mM

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sodium acetate pH 4.8 buffer using 20 μg P1 nuclease for 2 h at 37°C followed by treatment with 2 units of bacterial alkaline phosphatase adding 0.5 ml of 0.1 M Tris-HCl pH 7.5 buffer at 37°C for 1 h. For HPLC analysis, 20–50 μl sample of DNA or culture medium was analyzed on a Gilson HPLC system with a Separon SGX C18 column (150 × 3.3 mm; Tessek, Denmark) with precolumn connected to a Gilson UV detector and a Coulochem II multielectrode detector with a potential of 400 mV (ESA Inc., USA). The buffer used was according to [4] except for methanol which was 5%. The flow rate was 0.6 ml/min. Pure sample of oh^{8}dG as obtained by purification on HPLC on the C18 column and a buffer of 10 mM ammonium acetate pH 5.3 and a gradient of 2.5 to 25% of methanol over 30 min was used to establish a caliberation curve. Similarly, a caliberation curve was established for dG. Nelson software was utilized for the quantitation of oh^{8}dG and dG.

3. RESULTS AND DISCUSSION

Oxidized DNA adduct oh^{8}dG can be quantitated with a high degree of sensitivity and selectivity by EC detection after separation by HPLC. The oh^{8}dG synthesized by us showed the same spectral and electrochemical characteristics as those described previously [13]. Fig. 1a shows the UV- and EC-spectra for dG and oh^{8}dG, respectively. We have applied this method for the detection of oh^{8}dG in the DNA of normal and transformed human cells and have observed that by using this detector equipped with a high sensitivity analytical cell and a high pressure guard cell, oh^{8}dG can be detected both in cellular DNA and in the growth medium of cultured cells. Fig. 1b and c show representative UV- and EC-spectra of cellular DNA and culture medium, respectively. Retention time of oh^{8}dG in the analysed samples was confirmed each time by coinjection of synthetic oh^{8}dG. It is clear from Fig. 1b that extremely small levels of oh^{8}dG can be detected by EC detector and the ratio of oh^{8}dG to dG in the UV spectrum is calculated to be 2.1×10^{-5}. Under these conditions, amounts of oh^{8}dG as low as 3×10^{-15} M could be detected in a DNA sample whose concentration was in the range of 0.034 A_{260} to 1.0 A_{260} units. Furthermore, Fig. 1c shows that oh^{8}dG could also be detected in the culture medium from human cells in which no peak for dG could be detected in UV spectrum. No oh^{8}dG could be detected in the unused stock culture medium.

The amounts of oh^{8}dG in various samples are given in Table I. Quantitative analysis of HPLC/EC data shows that normal human cell genome contains about 1.05 oh^{8}dG per 10^4 dG in their DNA. In comparison, SV40-transformed MRC-5V2 cells had 2.1 oh^{8}dG per 10^5 dG in their DNA, which is almost 5 times less than in the normal fibroblasts. These differences in the amounts of oh^{8}dG in the DNA of normal and transformed cells may be a reflection of their differences in terms of oxygen consumption, cell division rates and macromolecular turnover rates. For example, it is well known that transformed and cancerous cells generally have a low oxygen utilization as compared with normal cells [16]. Therefore, the extent of oxidative damage is

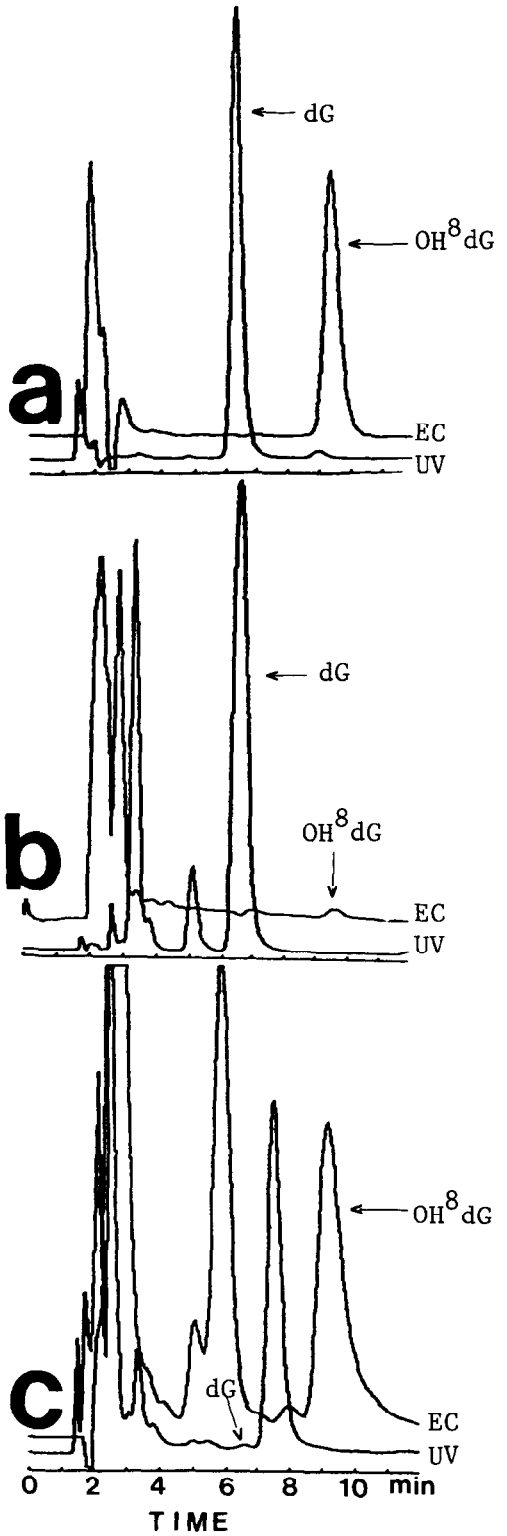

Fig. 1. Ultraviolet (UV) and electrochemical (EC) spectra for dG and oh^{8}dG in various samples analysed on a Gilson HPLC system connected to a UV detector and a Coulochem II multielectrode detector. (a) synthetic samples of dG and oh^{8}dG; (b) total cellular DNA from SV40-transformed MRC-5V2 cells; (c) culture medium from normal human skin fibroblasts.

Table I

Amounts of oh^{8}dG in DNA and growth medium of cultured cells as detected by HPLC-electrochemical analysis

Sample	Amount of oh^{8}dG	Ratio of oh^{8}dG/dG
DNA (human skin fibroblasts)	N.D.*	1.05×10^{-4}
DNA (SV40-transformed MRC-5V2 cells)	0.45×10^{-14} M/1 A_{260}	2.10×10^{-5}
DNA (herring sperm; commercially purchased)	$5.24 \times 10^{-14} \times$ M/1 A_{260}	1.20×10^{-4}
Growth medium from normal cells	7.02×10^{-11} M/ml	–
Growth medium from transformed cells	7.20×10^{-11} M/ml	–

*N.D., not determined.

expected to be low in transformed cells. Furthermore, rapid cell cycle rates of transformed cells will also increase the rate of DNA replication and, hence, removal of damage from the DNA.

Differences in the rates of occurrence of oxidative DNA damage and its repair in normal and transformed cells is also evident from the amounts of oh^{8}dG present in the culture medium. Table I shows that although the amount of oh^{8}dG in the DNA of normal and transformed cells differed by 5-fold, there was no significant difference in the amount of oh^{8}dG per ml of the culture media collected from these cells. This is a reflection of higher repair efficiency of transformed cells as compared with the normal human cells.

We have also analysed a sample of commercially purchased herring sperm DNA and found that it contained 1.2×10^{-4} oh^{8}dG, a level that is about 15% higher than that in the DNA extracted from normal skin fibroblasts in culture. A reason for relatively higher levels of oh^{8}dG in the commercial preparation of DNA may lie in the extraction procedure in which the DNA is extracted using phenol and hence several new modifications may be introduced during this process [17].

Finally, the significance of HPLC/EC method of estimating the levels of oxidative damage product oh^{8}dG lies in its applicability in both basic research and clinical diagnostic purposes. For example, determining the levels of oh^{8}dG in body fluids such as plasma and urine can be a marker of the extent of DNA damage and repair under various pathological and nutritional conditions, including normal ageing. Inflammatory diseases in which high oxygen free radicals are considered to be a causative factor can be monitored by this method both for the origin and progression of disease and for testing the efficiency of various treatments. Furthermore, the effects of UV and other oxidative damage-causing agents particularly tobacco smoking can also be monitored by this method. Thus if a sensitive and reproducible data base can be created for the extent of various oxidative DNA damages in cells, tissues and body fluids, this could develop into a very important and useful marker of diseases such as autoimmune diseases and cancer, and also provides means to testing the effectiveness of various therapies with respect to the cure of those diseases.

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