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Effect of Antioxidants on UV-Induced DNA Breakage in Human Peripheral Lymphocytes

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It has long been recognized that the most frequent Ultraviolet (UV) -induced DNA damage resulting from the direct absorption of UV radiation is the formation of cyclobutane pyrimidine dimers and the pyrimidine (6-4) pyrimidone photoproduct. Initial UV-induced damage triggers a variety of events that sometimes lead to the occurrence of skin cancer (WHO 1994). Exposure of skin to UV can generate reactive oxygen species (ROS) and reduce epidermal enzymatic and non-enzymatic antioxidants (Fuchs et al. 1989; Pence and Naylor 1990). ROS are thought to be mainly related to cutaneous photoaging (Cerimele 1990), and to cutaneous immunosuppression (Clement 1996). Ames (1983) and Pence et al. (1994) reported that selenium or vitamin-E in the diet can reduce UV-induced skin cancer, suggesting that ROS might be involved in UV-induced DNA damage. But direct evidence for ROS involvement in DNA breakage is lacking. To define further the role of ROS in UV-induced DNA breakage, we performed experiments in which human peripheral lymphocytes were incubated with antioxidants and DNA breakage measured by the COMET assay (Anderson et al. 1994). Our results suggest a role for oxidative mechanisms in UV-induced DNA damage.

MATERIALS AND METHODS

Before evaluating the role of ROS, the relationship of UV dose to DNA breakage was tested. Lymphocytes were isolated from peripheral blood specimens of six individuals. Each specimen was divided into four samples and exposed to 0, 1, 3, or 5 minimal erythemal dose units (MED) of UV radiation. COMET assays were performed to measure the DNA breakage. The length of DNA migration, which is the length of 'comet' tail composed of DNA fragments, served as an index of the amount of breakage. DNA migration (μm) was divided into 5 categories: 0-5, 6-15, 16-25, 26-35, ≥36. For each sample 50 lymphocytes were measured, and the percentage of lymphocytes in each of the migration categories was obtained. Statistical comparisons among various

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doses of UV exposure were made by means of the chi -square test. After finding a UV dose capable of inducing significant DNA breakage, the effect of antioxidants on UV-induced DNA breakage was analyzed. Antioxidant (dissolved in PBS) was added to lymphocytes in Dulbecco's modified minimum essential medium (DMEM) and incubated at 37° C for 1h and exposed to 3 MED of UV radiation. The concentrations (µg/mL) of antioxidants used in the assay were: superoxide dismutase (SOD)- 25, 50, or 100; β-carotene- 20, 40, or 100; catalase (CAT)- 100, 300, or 500; or glutathione (GSH)- 100, 300, or 500. These dose levels were established in accordance with levels in the dermis and from reports in the literature (Anderson et al. 1994). Cultures without the addition of antioxidant served as positive controls. COMET assays were performed immediately following UV exposure and the resulting distribution of DNA migration for each treatment was compared with the positive control by chi-square test. The mean and standard deviations of DNA migration were also calculated and compared between various antioxidants by Student-Newman-Keuls (SNK) test.

Heparinized human peripheral blood specimens were collected from 12 non-smoking healthy adult donors, aged 25-45, including 6 males and 6 females. Of these, 6 were used for testing UV-dose dependence of DNA breakage, and the other 6 were used for testing the effect of antioxidants on UV-induced DNA breakage. Lymphocytes were separated with lymphocyte separation medium which has a density of 1.077, washed with phosphate-buffered saline (PBS) and resuspended in small aliquots of PBS (3×10^6 cells/ml). For each sample, 10 µL of lymphocyte-containing medium were mixed in 75µL 5% low melting point agarose. The mixture was pipetted onto a microscope slide, then exposed to UV. A mercury/argon lamp which emitted a broad spectrum of radiation including UV-B and UV-A was used as the UV source. The major wavelengths of the radiation were 300, 315, and 365 nm. The MED was measured on the medial side of the forearm of five adults. The average MED was equivalent to 2 minutes of exposure at a distance of 50 cm from the lamp. After the UV-exposure, single-cell gel electrophoresis assays (the COMET assay) were performed as described by Anderson et al. (1994). The slides were immersed in freshly prepared cold lysing solution (2.5M NaCl, 100 mM Na_2EDTA, 10mM Tris; pH 10, with 1% Triton X- 100 and 10% DMSO added just before use) for 1 h at 4°C. The slides were then removed from the lysing solution, drained, and placed in a horizontal gel electrophoresis tank side by side. The tank was filled with fresh electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH) to a level approximately 0.25 cm above the slides. The slides were left in the high pH buffer for 20 min to allow unwinding of the DNA before electrophoresis. Electrophoresis was conducted at room temperature for 20 min at 5 V/cm. After electrophoresis slides were washed gently to remove alkali and detergents with 3 changes of 0.4 M Tris, pH 7.5, each for 5 min. After neutralization, slides were stained with 50-100 µl of 2 µg/ml ethidium bromide (EtBr) in distilled water and

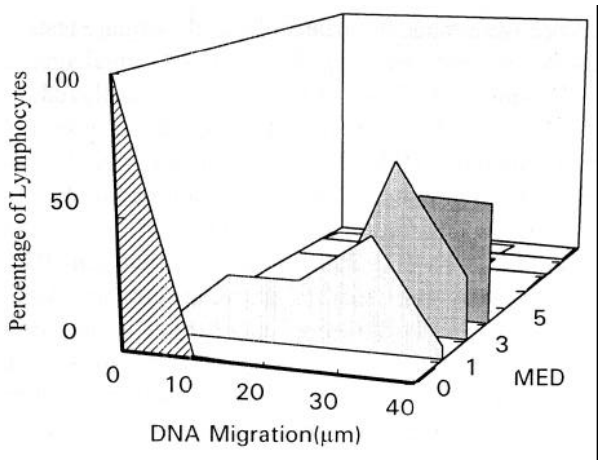


Figure 1. The distribution of DNA migration distances for human peripheral blood lymphocytes exposed to 0-5 MED UV. Data represent an average of 6 samples, consisting of 50 cells per sample.

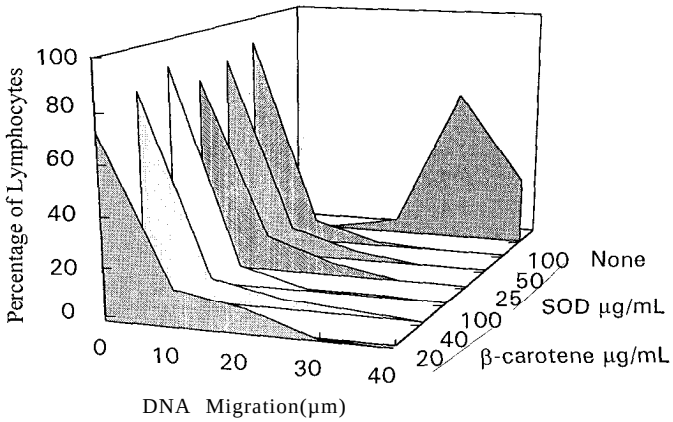
covered with a coverslip. The DNA migration lengths were calibrated with a scanning MRC- 600 laser confocal microscope.

To test the effects of various antioxidants on UV-induced DNA breakage, another 6 lymphocyte samples were collected. Lymphocytes were incubated with different antioxidant concentrations in DMEM in microcentrifuge tubes at 37°C for 1 h. Cell suspensions were mixed in low melting point agarose, exposed to 3 MED of UV radiation, and a COMET assay was performed.

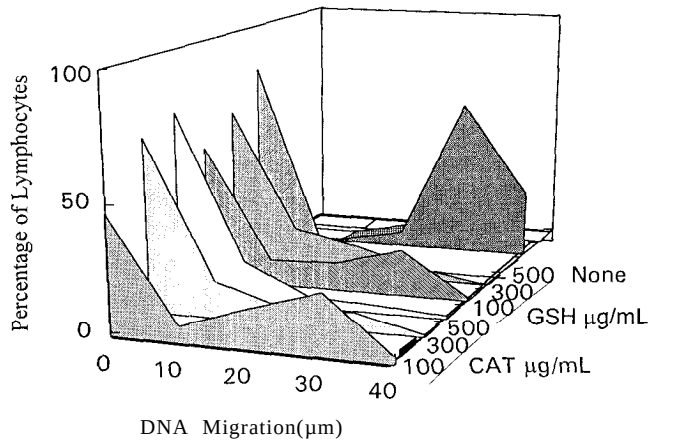
Chemicals used were purchased from the following suppliers: β-carotene, E. Merck Darmstadt, Germany: Cu,Zn-SOD, Sigma Chemical Company, St. Louis, MO, U.S.A.: lymphocyte separation medium, Shanghai 2nd Chemical Industry, Shanghai, China: low melting point agarose, Sino-American Biotec, Peijing, China: L-glutathione reduced, catalase (CAT), EtBr, Trizma base (Tris) and Triton X-100, Shanghai Chemical Purchase & Supply Station, Shanghai, China. DMEM Life Technologies Inc, Grand Island, N.Y. U.S.A.

RESULTS AND DISCUSSION

Human lymphocytes irradiated with UV in the dose range of 1-5 MED exhibited significant DNA single-strand breakage (Fig. 1). Lymphocytes exposed to 1 MED of UV resulted in 50% of cells with a migration length of ≥ 30 μm in comparison to 90% for 3 MED exposures. By contrast, unexposed lymphocytes (negative controls) showed very little migration (Fig. 1); only a few cells had a migration length <5μm. The difference among various UV doses was



(A)



(B)

Figure 2. The effect of various antioxidants on UV-induced DNA breakage in human blood lymphocytes after exposure to 3 MED UV, evaluated by the COMET assay. Data are based on an average of 6 samples, consisting of 50 cells per sample. (A) Lymphocytes incubated with 20, 40, or 100 μg/mL β-carotene or 25, 50, or 100 μg/mL of SOD. (B) Lymphocytes incubated with 100, 300, or 500 μg/mL of CAT or 100, 300, or 500 μg/mL of GSH.

significant ($p<0.01$), based on the chi-square test, and indicate that the UV - induced DNA strand breaks were dose-dependent.

The UV-induced single-strand breaks in lymphocytes were inhibited significantly by various antioxidants. After incubation with β-carotene or SOD at concentrations of 20-100 μg/ml, 74-90% of the lymphocyte DNA migrated

Table 1. Effects of varying concentrations of antioxidants on average length of DNA migration.*

Antioxidant	Antioxidant concentration			
	none	low	middle	high
CAT	31.8± 0.4	12.7± 0.6	5.4 ± 3.7	3.8 ± 2.5
GSH	31.8± 0.4	9.6 ± 3.9	5.3 ± 1.5	1.6± 0.5
β-carotene	31.8± 0.4	3.8± 0.8	2.5 ± 1.1	1.3± 0.6
SOD	31.8± 0.4	2.6 ± 0.5	1.9± 0.3	1.2 ± 0.1

*mean±standard deviation (μm).

within the range of normal cells, while the positive control group (the same dose of UV radiation without antioxidant) exhibited more than 90% with a DNA migration of 30 μm or more (Fig. 2A). CAT and GSH exhibited similar but lesser effects on UV-induced DNA breakage (Fig. 2B). Based on the chi-square test, the DNA breakages of any antioxidant even at the low dose levels used in the present study were significantly lower than the positive controls ($P<0.01$). However, DNA breakage differences among the various dose levels for β-carotene, and SOD were not significantly different based on the same test ($P>0.05$). The likely reason for this finding is that even the lowest doses of antioxidants in our study strongly inhibited DNA breakage, so the higher doses were unable to increase the protection. Exceptions were CAT and GSH. The effect of CAT at the lowest dose was only about 50% protective, therefore a dose-dependent change in DNA breakage was observed at the higher doses.

In order to compare the protective efficiency of the four antioxidants, means and standard deviations of DNA migration distances for each treatment were calculated (Table l), and statistically compared by using the SNK test. The results show that protective effectiveness was not the same for the various antioxidants. The rank order of protective effectiveness for the various antioxidants was SOD, β-carotene, CAT and GSH respectively. The differences between SOD or β-carotene versus GSH or CAT were significant ($P<0.05$) based on the SNK test.

The differences in effectiveness among the antioxidants may be explained in part by the non-enzymatic antioxidant β-carotene can quench singlet oxygen, the major form of ROS generated by iron-free porphyrins under exposure to UV radiation (Tyrell 1992); SOD catalyzes the conversion of the superoxide anion, the major source of ROS to hydrogen peroxide, and blocks the chain reaction of oxygen radicals initiated by superoxide; glutathione is involved in a number of antioxidant reactions, including reduction of hydrogen peroxide (as a cofactor of GSH peroxidase), detoxification of free radicals, reduction of protein disulfides, and competition with protein thiols for oxidizing species. It has clearly been shown in cell culture models that GSH depletion leads to an increased sensitivity

to UV-A and UV-B radiation (Tyrell and Pidous 1988). Therefore, both glutathione and CAT contribute to the defense against ROS. The relative absence of single-strand breaks in UV-irradiated cells exposed to antioxidants in the present study indicated that oxidative lesions are the main cause of single-strand breaks, although we cannot rule out the possibility that other minor pathways lead to DNA strand breakage.

The present studies provide support for the idea that ROS plays an important role in mediating UV-induced DNA damage and may partly explain how antioxidants in the diet protect skin from UV-induced skin damage and reduce the tumor response to UV radiation.

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