Free Rad. Res., Vol. 29, pp. 307–313
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Protective Effects of Antioxidants against UVA-Induced DNA Damage in Human Skin Fibroblasts in Culture

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Accepted by Prof. H. Sies

(Received 5 January 1998; In revised form 15 May 1998)

Ultraviolet A radiation (UVA, 320–400 nm) is mutagenic and induces genomic damage to skin cells. *N*-acetyl-cysteine (NAC), selenium and zinc have been shown to have antioxidant properties and to exhibit protective effects against UVA cytotoxicity. The present work attempts to delineate the effect of these compounds on genomic integrity of human skin fibroblasts exposed to UVA radiation using the single cell gel electrophoresis (SCGE) or Comet assay. The cells were incubated with NAC (5 mM), sodium selenite (0.6 μM) or zinc chloride (100 μM). Then cells were embedded in low melting point agarose, and immediately submitted to UVA fluences ranging from 1 to 6 J/cm^2. In the Comet assay, the tail moment increased by 45% (1 J/cm^2) to 89% (6 J/cm^2) in non-supplemented cells ($p < 0.01$). DNA damage was significantly prevented by NAC, Se and Zn, with a similar efficiency from 1 to 4 J/cm^2 ($p < 0.05$). For the highest UVA dose (6 J/cm^2), Se and Zn were more effective than NAC ($p < 0.01$).

Keywords: Strand breaks, Comet assay, *N*-acetyl-cysteine, trace elements, human skin fibroblasts, UVA radiation

Abbreviations: FCS, fetal calf serum; GSH, glutathione; MT, metallothionein; NAC, *N*-acetyl-cysteine; PBS, phosphate-buffered saline; ROS, reactive oxygen species; SCGE, single cell gel electrophoresis; Se-GPX, selenium-dependent GSH peroxidase; UVA, ultraviolet radiation in the UVA region (320–400 nm)

INTRODUCTION

Cumulative exposure of human skin to solar radiation results in premature aging and increased risk of skin cancers. Ultraviolet A radiation (UVA, 320–400 nm) exerts chemical effects on cellular DNA, mediated predominantly by endogenous photosensitizers.[1] Photoexcited sensitizers are likely to generate reactive oxygen species (ROS), including superoxide ($O_2^{\bullet -}$) and singlet oxygen ($^1O_2$) through the type I and type II mechanism respectively. $O_2^{\bullet -}$ is not reactive

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towards DNA, at least in aqueous solution.[2] Hydrogen peroxide (H_2O_2) is the precursor of the reactive hydroxyl radical ($^{\bullet}OH$) in the Fenton reaction. The contribution of the two reactive species $^{\bullet}OH$ and $^1O_2$ towards photooxidation of DNA has not yet been established.[1,3] Oxidation reactions including the formation of 8-oxo-7,8-dihydroguanine occur upon UVA irradiation of cellular DNA.[4–8]

A variety of enzymatic and nonenzymatic antioxidants protect cells against ultraviolet (UV) related adverse effects. However, if the dose of UV is great enough, the inherent antioxidants are altered and the antioxidant defense systems are overcome. This may induce damage to cellular components (proteins, lipids, DNA), and subsequent cell death may occur.

In previous studies from this laboratory, *N*-acetyl-cysteine (NAC), selenium and zinc have been identified as cytoprotectors of human skin fibroblasts exposed to UVA radiation. NAC, a cysteine donor, was able to significantly counteract UVA deleterious effects, mainly through glutathione (GSH) metabolism.[9] Se affords cytoprotection via the inhibition of lipid peroxidation through selenium-dependent glutathione peroxidases EC 1.11.1.9. (Se-GPX) activity.[10] Several nuclear selenoproteins e.g. thioredoxin reductase[11] exist in mammalian cells, so, Se could be implied in the maintenance of genomic integrity. Zn inhibits free radical production, stabilizes cell membranes[12] and is involved in the conformational control of many proteins, particularly nuclear enzymes and transcription factors.[13] Zn also stimulates the transcription rate of the gene encoding for metallothionein which significantly increases the survival rate of cells exposed to UVA[14] and protects DNA from oxidative damage.[15] Finally, we have demonstrated that Zn inhibited UVB[16] and UVA (unpublished data) induced DNA strand breaks determined by the method described by Birnboim and Jevcak.[17]

Recently, several studies on genotoxicity of oxidative stress have been reported using the Comet assay, also called the single cell gel electrophoresis assay (SCGE).[18–21] Firstly introduced by Östling and Johanson,[22] the technique was modified by Singh *et al.*[23] who added an alkaline treatment allowing the unwinding of the DNA. Using the Comet assay, we examined whether NAC, Se and Zn could protect against genomic damage induced by physiological UVA doses in human skin fibroblasts.

MATERIALS AND METHODS

Cells and Culture

Primary culture of human normal skin fibroblasts was established from excess tissues of breast plastic surgery specimens from adults aged 20–40 years. Cells were tested between population doubling 7 and 14. Fibroblasts were trypsinized each week (ratio 1:2) and further propagated in RPMI 1640 supplemented with 10% FCS (ATGC Biotechnologie, Noisy-le-Grand, France), 300 µg/ml L-glutamine (Boehringer, Mannheim, Germany), 0.5 µg/ml fungizone (Boehringer), 0.17 µg/ml penicillin and streptomycin (Polylabo, Paul Block & Cie, Strasbourg, France), 54 µg/ml kanamycin (Gibco, Grand Island, NY). Fibroblasts were grown to near confluency in 75 cm^2 culture flasks (NUNC, Roskilde, Denmark) at 37°C in a 5% CO_2 atmosphere. Experiments were performed at near-confluency. Antioxidant treatments were performed as previously described.[9,10] Briefly, the pretreatments with Se (0.6 µM, as sodium selenite; Sigma Chemical Co, St Louis, MO) or Zn (100 µM, as zinc chloride; Merck, Darmstadt, Germany) were performed for 3 and 1 wk respectively. NAC (5 mM; Sigma Co) was added for 4 h before UVA irradiation because this resulted in a maximum of intracellular GSH content as determined by pilot experiments using NAC concentrations between 1 and 20 mM.

UVA Irradiation

The UVA source was a high pressure Tecimex apparatus (Dixwell, St Symphorien d'Ozon,

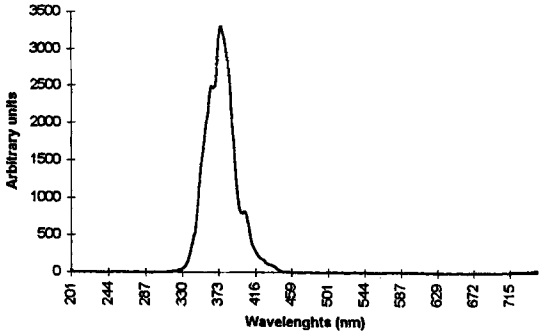

FIGURE 1 Relative spectral distribution of the high pressure Tecimex apparatus.

France). The typical spectral distribution of this lamp (Figure 1) was kindly determined by D. Masserot (Laboratoire de Métrologie des UV, EUDIL-USTL, Villeneuve d'Ascq, France). The dose-rate measured by a radiometer (Dixwell, France) was 33 mW/cm^2, and the maximal exposure time was 3 min.

Single-Cell Gel Electrophoresis

The procedure used was a modification of the protocol described by Singh *et al.*[23] Typically, 150 µl of 0.6% normal melting agarose (Gibco) in phosphate-buffer-saline (PBS), without calcium and magnesium (Eurobio, Les Ullis, France), were dropped on frosted microscope slides (Touzart et Matignon, Les Ulis, France), covered with a coverslip, and kept at room temperature until their subsequent use. After trypsination, fibroblasts were washed twice with PBS, and the cell pellet was suspended in PBS. Around 2×10^4 cells were mixed with 75 µl of 0.6% low melting point agarose (FMC Bioproducts, Rockland, ME) in PBS, held at 37°C. Embedded cells were immediately irradiated using low UVA fluences ranging from 1 to 6 J/cm^2. Then, the slides were kept in the dark at 4°C, in order to limit non-specific DNA damage and repair process. A final layer of agarose (75 µl of 0.6% low melting point agarose) was applied as previously described. Then, the slides were immersed in ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, NaOH to pH 10, 1% sodium sarcosinate, 1% Triton X-100 and 10% DMSO were added just before use) and kept at 4°C overnight. Slides were then transferred to an electrophoresis tank containing a fresh alkaline buffer (300 mM NaOH, 1 mM EDTA), at room temperature, for 40 min to allow the unwinding of the DNA. Electrophoresis was performed for 30 min at 25 V and 300 mA. Then, the slides were rinsed three times for 5 min with 0.4 M Tris, at pH 7.5, stained with 50 µl ethidium bromide (20 µg/ml) (Sigma), and covered with a coverslip. The slides were placed in a humidified air-tight container to prevent drying of the gel, until the analysis was performed. Each slide was processed in duplicate.

Slide Analysis

Slides were examined using an epifluorescence microscope, Zeiss Axioskop 20 (Carl Zeiss, Microscope Division, Oberkochen, Germany), equipped with a short arc mercury lamp HBO (50 W, 516–560 nm, Zeiss), and filters 5 and 15 (Zeiss) at 20× magnification. Fifty randomly selected comets on each duplicated slide were scored with a Pulmix TM 765 camera (Kinetic Imaging, Liverpool, UK), linked to an image analysis system Komet 3.0 (Kinetic Imaging, UK). This software defines head and tail regions, measures the fluorescence intensity of these parameters and determines their length. In our test, quantification of DNA damage was performed using the tail moment, the product of the tail distance (i.e., the distance between the centre position of the head and the centre of gravity of the tail) and the % of DNA in the tail (relative to the total amount of DNA in the entire comet (head + tail)).[24]

Statistics

Each experiment was repeated three times. One-way analysis of variance (ANOVA) and a Newman–Keuls test were performed to determine whether differences in tail moment values were statistically significant over the range of UVA doses investigated and among the different cell treatments.

RESULTS

DNA Damage Induced by UVA Radiation

DNA from non-irradiated cells appeared as a bright circular area (Figure 2) and measured tail moments were very low (5.46 ± 2.44), indicating that trypsination and the experimental procedure were well tolerated by the cells (Figure 3). UVA irradiation over the range of doses investigated resulted in comet images, with distinct head and tail, as shown in Figure 2. This distinction allows us to discriminate a clear increase ($p < 0.01$) in tail moment from 24.6 ± 3.5 for the lowest fluence ($1\,J/cm^2$) up to 48.4 ± 2.8 for $6\,J/cm^2$ (Figure 3).

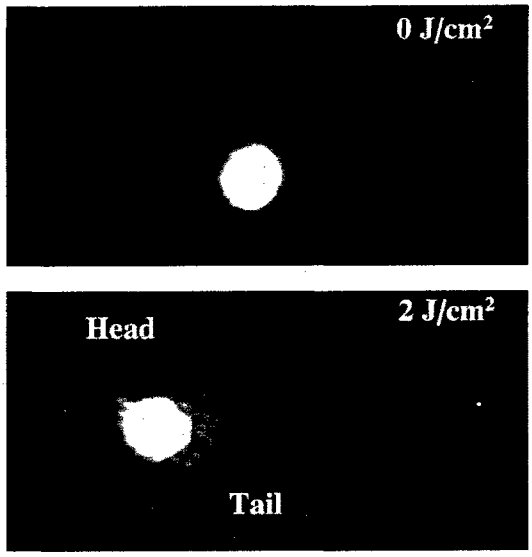

FIGURE 2 UVA-induced DNA damage in non-supplemented human skin fibroblasts. Photographs of representative cells were taken after electrophoresis.

Effect of Antioxidants

NAC, Se, Zn, and the combination NAC–Se did not modify the tail moment of non-irradiated

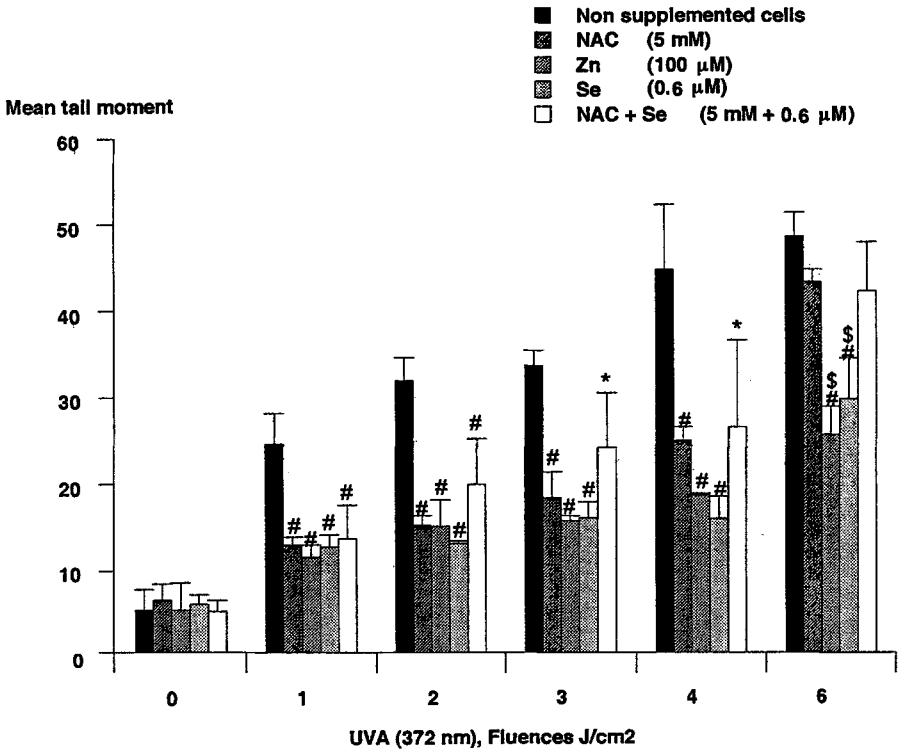

FIGURE 3 Effect of NAC, Zn, Se and NAC + Se on UVA-induced DNA damage in human skin fibroblasts. Yield of damage is represented by the mean tail moment of 50 cells per slide. $n = 3$, $^{*}p < 0.05$, $^{\#}p < 0.01$ supplemented cells vs. non-supplemented cells and $^{\$}p < 0.01$ Zn and Se-treated cells vs. NAC-treated cells.

cells. NAC had a statistically significant ($p < 0.01$) protective effect on UVA-induced DNA damage (Figure 2). With the exception of the highest dose ($6\,J/cm^2$), mean values of the tail moment of each point were decreased by a factor of 2, compared to non-treated cells.

Alkali-labile sites and strand breaks were significantly lowered after treatments by Se and Zn (Figure 2). These trace elements afforded a reduction of 50% ($p < 0.01$) of the tail moment in irradiated-treated cells (UVA doses varying from 1 to $4\,J/cm^2$). For $6\,J/cm^2$, the protection was less marked but Se and Zn were significantly ($p < 0.01$) more effective than NAC.

In a previous study, Se was found to significantly enhance the NAC protective effect on human skin fibroblasts exposed to UVA radiation.[9] The tail moment was not significantly different in cells treated with either NAC or NAC–Se (Figure 2). Moreover, for UVA doses comprised between 3 and $6\,J/cm^2$, NAC–Se combination did not provide any protection compared to non-treated cells.

DISCUSSION

Monitoring the formation of specific radiation-induced DNA damage within cells is still considered as a challenging goal. The Comet assay or SCGE is a rapid method for the measurement of alkali-labile sites and DNA strand breaks in individual cells. Although rather unspecific, SCGE has been reported to be about 10-fold more sensitive than high performance liquid chromatography associated with electrochemical detection in determining ionizing radiation DNA damage.[25]

UVA-induced DNA damage involved ROS and *in vitro* studies suggested that the $^{\bullet}OH$ radical may play a key role.[26] GSH depletion using buthionine-S,R-sulfoximine (BSO) as a specific inhibitor of γ-glutamyl-cysteine synthetase has been found to sensitize either human skin fibroblasts or epidermal keratinocytes to the damaging effects of UVB and UVA.[27] In addition, the treatment was found to enhance the frequency of UVA radiation-induced mutants in human cells.[28] UVA radiation induced the depletion of GSH in human skin fibroblasts.[9] Applegate *et al.*[29] have extended this work in distinct differentiation stages of human skin fibroblasts and keratinocytes. We have also shown that increased levels of GSH by NAC treatment led to a protection against UVA lethal effect.[9] In the present work, we reported a significant decrease of DNA damage in human skin fibroblasts grown in medium supplied with NAC.

Using the single-cell gel electrophoresis method, we have also demonstrated that Zn protects DNA from UVA radiation effects, in agreement with our previous findings which showed that Zn inhibited apoptosis.[16] This protective effect could be related to the induction of metallothionein (MT). MT selectively binds metal ions such as zinc, copper or cadmium.[30] Increased levels of MT by Zn prevent the cytotoxic effect of UV,[16,31] and provide protection against DNA damage.[15,32] Moreover, MT was suggested to act as a scavenger of active oxygen species by its free SH groups.[33,34] The binding of Zn to the thiol groups of proteins is another potential mechanism of Zn protective effect.[35] This hypothesis is in agreement with the observation that depletion of Zn by the metal chelator *N-N-N′-N′*-tetrakis 2-(pyridylmethyl)-ethylene diamine (TPEN) led to a depletion of intracellular GSH.[36]

Recentely, Leist *et al.*[37] have demonstrated that an adequate Se supply almost completely inhibited single-strand breaks induced by H_2O_2, with subsequent reduced cell death. In this context, our data (Figure 3) reinforce the hypothesis that Se–GPX activity protects against UV genotoxicity.

The combination of NAC with Se was found previously to improve the protective factor of the thiol molecule against the cytotoxic effects of UVA radiation.[9] However, no further decrease in DNA damage levels was observed in fibroblasts treated with both NAC and Se compared to

NAC alone. Under these conditions, NAC and Se protection against UVA-induced DNA damage involved different pathways.

In conclusion, the main aim of the present study was to evaluate the potentialities for NAC, Se and Zn to decrease UVA-induced DNA damage in human skin fibroblasts, using the single-cell electrophoresis approach. We observed a five-fold increase of the tail moment at our lowest UVA dose ($1\,J/cm^2$), whereas the finding of a two-fold increase in 8-oxo-7,8-dihydro-2′-deoxyguanosine levels, using HPLC-EC, required $20\,J/cm^2$.[5] Similar results (1.5-fold increase; $40\,J/cm^2$) were obtained by Zhang *et al.*[8] on HeLa cells. Our results demonstrated that the use of the Comet assay can be an interesting approach to evaluate the efficiency of antioxidants on DNA damage induced by physiological UVA doses. Hence, these results are supportive of the hypothesis that GSH plays an important role in improving genetic stability of human skin fibroblasts exposed to UVA radiation.

Acknowledgements

This study was supported by Pierre Fabre Dermocosmétique (France).

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