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Journal of Photochemistry and Photobiology B: Biology 37 (1997) 101–106

Journal of PHOTOCHEMISTRY AND PHOTOBIOLOGY B:BIOLOGY

Zinc and DNA fragmentation in keratinocyte apoptosis: its inhibitory effect in UVB irradiated cells

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Received 15 December 1995; accepted 1 March 1996

Abstract

Zinc has been shown to have antioxidant properties and to exhibit inhibitory effects on apoptosis. In this work we investigated the effect of zinc on DNA integrity and on apoptosis of HaCaT keratinocytes. Cells were submitted to zinc deprivation by a diffusible zinc chelator, (*N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine) (TPEN) or supplied with zinc chloride and submitted to UVB radiation.

After cell exposure to TPEN for 2 h, strand breaks significantly impaired DNA resistance to alkaline denaturation. DNA strand breaks induced by a 6 h TPEN application were significantly prevented if zinc chloride was supplied together with the chelator. TPEN also generated, after 4–6 h of application, cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes), features of cell death by apoptosis.

Moreover, UVB irradiation led to early DNA strand breaks and to an increase in cytoplasmic nucleosomes which was maximum 10 h after irradiation. These effects were prevented by the supply of zinc chloride (0.1 mM) in the culture medium.

These results suggest that zinc ions interfere with the apoptosis process at an early stage, by decreasing DNA damage able to trigger apoptosis.

Keywords: TPEN; Zinc; Keratinocyte; DNA; Apoptosis; DNA strand breaks; Antioxidant; UVB

1. Introduction

Apoptosis is the morphological aspect of the active process of cellular self-destruction occurring in various physiological or pathological conditions. This process allows the homeostatic control of cell number in multicellular organisms (programmed cell death), and the suicide of cells to avoid cancer or senescence if their DNA is irreparably damaged after a genotoxic stress. Apoptosis plays an important role in epidermal biology. Indeed genotoxic radiations such as UVB are known to induce apoptosis. Several studies have recently shown an antiapoptotic effect of Zn(II) ions in various leukocyte cell types submitted to different apoptotic stimuli [1–3]. It is thus relevant to wonder whether zinc is implicated in keratinocyte apoptosis.

The most commonly accepted hypothesis to explain this effect of zinc is the inhibition of an endonuclease activated in apoptosis, originally described by Wyllie [4]. However, this inhibition of DNA fragmentation by zinc does not always prevent subsequent cell death [5,6]. Zinc ions exhibit other properties which can explain their antiapoptotic effect; zinc is involved in several enzymes of DNA metabolism [7], and in transcription factors potentially activated in apoptosis [8]. Finally, zinc has antioxidant properties which might be involved in its interference with apoptosis pathways, for reactive oxygen species are described as apoptosis mediators [9,10]. Now reactive oxygen species (ROSs) are produced in the skin irradiated by UV light [11], and zinc supplied to cultured skin cells protect them towards UVA and UVB cytotoxic effects [12].

The aim of this work was to study the effect of zinc on DNA integrity and on apoptosis of HaCaT keratinocytes. The effect of zinc deprivation was investigated using a diffusible zinc chelator, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethylenediamine) (TPEN). To assess the effect of zinc supply in these cells, UVB irradiation was used as an apoptotic stimulus. Indeed, UVB is a physiologically relevant stimulus of keratinocytes apoptosis for it generates in vivo sunburn cells

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PII S1011-1344(96)07334-4

in the epidermis, which are considered as good markers of UV-induced acute effects on skin.

2. Materials and methods

2.1. Chemicals

L-glutamine was purchased from Gibco (Grand Island, USA). RPMI medium, fetal calf serum (FCS), penicillin, streptomycin, trypsin–ethylenediamine tetraacetic acid (EDTA) were purchased from Boehringer (Mannheim, Germany). TPEN and Dulbecco's phosphate-buffered saline without calcium chloride and magnesium chloride were purchased from Sigma Chemical Co. (Saint-Louis, MO, USA) and zinc chloride from Prolabo (Paris, France).

2.2. Cell culture

Spontaneously immortalized human keratinocyte cell line HaCaT was characterized and developed in the Division of Differentiation and Carcinogenesis in Vitro of the German Cancer Research Center (Heidelberg, Germany) and kindly supplied to us [13]. Culture medium RPMI 1640 with $NaHCO_3$, penicillin (50000 UI l^{-1}), streptomycin (50 mg l^{-1}) and L-glutamine (4 mM) was added with 10% FCS. Cells were incubated at 37 °C in a 5% CO_2-enriched atmosphere (Forma Scientific incubator). Culture flasks were from Nunc (Grand Island, USA), Petri dishes from Beckton Dickinson (Plymouth, UK) and 96 wells strip plates from Costar (Cambridge, MA, USA).

2.3. N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine application

TPEN stock solution (5 mM) was prepared by dilution in deionized water and filtered through a 0.2 μm pore membrane. Dilutions in culture medium were realized before use and applied on confluent cells for varying times.

2.4. Zinc chloride supply

The RPMI medium with 10% FCS was added with $ZnCl_2$ (stock solution (100 mM) prepared in deionized water and filtered through a 0.2 μm pore membrane). The final concentrations of zinc-enriched media were checked by electrothermal atomic absorption. Cells were supplied for 36 h before UVB irradiation with fresh medium added or not with zinc.

2.5. UVB irradiation

Confluent cells were irradiated using a Biotronic apparatus equipped with three 40 W Vilbert–Lourmat T-40M fluorescent tubes (spectrum distribution, 280–320 nm; $\lambda_{max} = 312$ nm). Radiation fluences were measured using an international light radiometer (RX Vilbert–Lourmat). The apparatus is controlled by microprocessor monitoring the time of irradiation according to the suitable energy. Control keratinocytes were sham irradiated. Cells were irradiated in PBS without calcium–magnesium (10 ml per flask or 1 ml per dish). Dishes were irradiated without lid. For apoptosis determination, cells were replaced in their respective medium after irradiation for up to 24 h.

2.6. DNA strand breaks determination

Strand break determination was carried out using the method described by Birnboim and Jevcak [14]. This assay measures degree of double-strandedness of DNA through binding of fluorescent dye. Immediately after cell irradiation or TPEN application, cells in 75 cm^2 flasks were rinsed twice with 10 ml of PBS without calcium chloride and magnesium chloride, trypsinized and centrifuged at 1200 rev min^{-1} for 4 min. The pellet from two flasks per assay was then resuspended and assayed as described in the original method. Strand breaks were detected by exposing cell lysates to alkaline solutions and monitoring after denaturation for 40 and 80 min the rate of strand unwinding.

2.7. Apoptosis detection and measurement

After incubation with TPEN for up to 6 h or up to 24 h after UVB irradiation, apoptosis was evaluated using the cell death detection ELISA from Boehringer (Mannheim, Germany) after the following sample processing: culture medium was removed from the wells (TPEN application) or the dishes (exposure to UVB) and cells were rinsed with PBS without calcium chloride and magnesium chloride. Lysis was then realized by adding 320 μl per well or 1 ml per dish of incubation buffer and incubating for 30 min at 4 °C. The lysate was then centrifuged at 5000 rev min^{-1} for 40 min and the supernatant carefully removed and stored at −20 °C until testing. Results are expressed as relative absorbance at 405 nm, which increases as the amount of cytoplasmic nucleosomes rises. For UVB-irradiation-induced apoptosis, the enrichment factor was calculated in order to compare the effect of UVB on zinc supplied and control cells. The enrichment factor is given by the absorbance at 405 nm of irradiated cells divided by the absorbance at 405 nm of the corresponding unirradiated cells.

2.8. Statistics

The data have been analysed by a Mann–Whitney *U*-test.

3. Results

3.1. Effect of N,N,N',N'-tetrakis(2-pyridylmethyl)ethylenediamine application

3.1.1. On DNA integrity

Fig. 1(a) shows the results of strand breaks determination after treatment of the cells by TPEN for 6 h (mean ± standard deviation, $n = 3$). After alkaline denaturation for 80 min, the

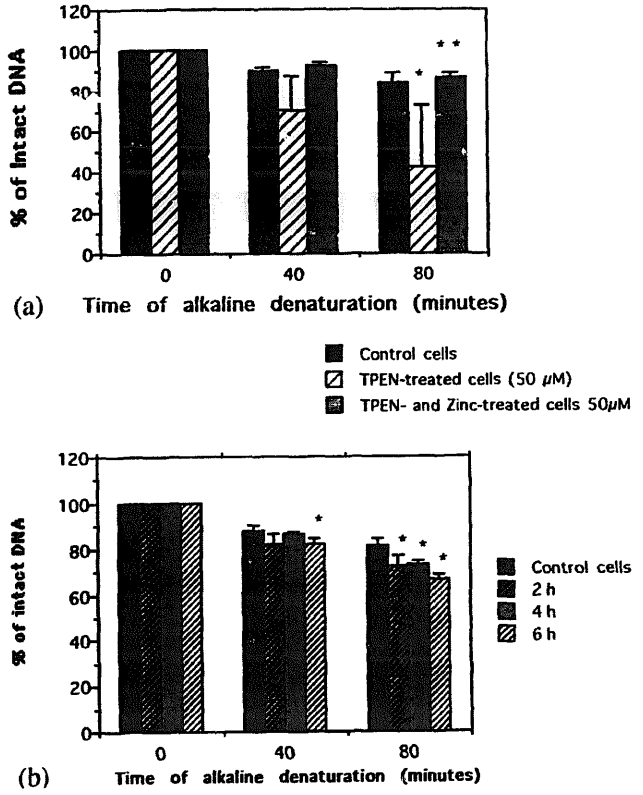

Fig. 1. (a) TPEN-induced DNA strand breaks. DNA strand breaks were evaluated immediately after exposure of the cells to TPEN or to TPEN + zinc in equimolar concentration (50 μM) by DNA resistance to alkaline denaturation (values represent mean ± standard deviation of three separate experiments): *, $p = 0.05$, TPEN-treated cells vs. control cells; **, $p = 0.05$, zinc- and TPEN-treated cells vs. TPEN-treated cells. (b) Effect of duration of TPEN application on DNA strand breaks. DNA resistance to alkaline denaturation was evaluated immediately after exposure to TPEN (50 μM) for 2, 4 or 6 h (values represent mean ± standard deviation of three separate experiments): *, $p = 0.05$, TPEN-treated cells vs. control cells.

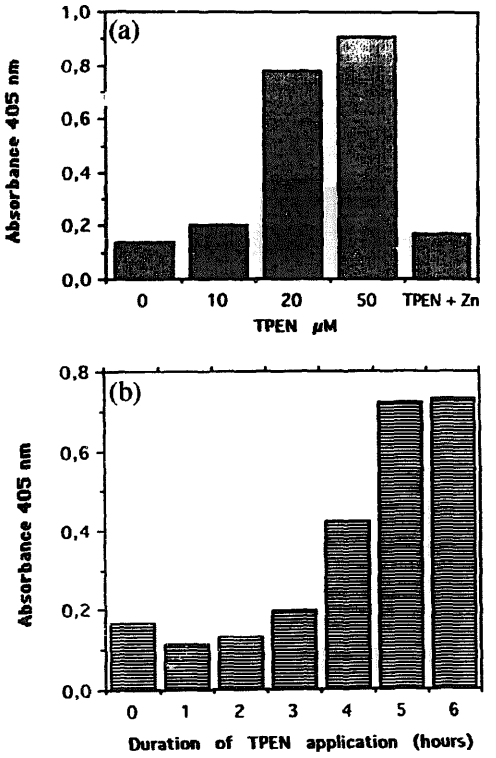

Fig. 2. (a) Apoptosis of HaCaT cells induced by TPEN. Cells were submitted to TPEN at various concentration or to TPEN + zinc in equimolar concentration for 6 h and apoptosis immediately measured using ELISA detection of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes). Values are expressed as relative absorbances. (b) Time course of apoptosis appearance under continuous exposure to TPEN (50 μM). Cytoplasmic nucleosomes are detected by ELISA method. Values are expressed as relative absorbances.

percentage of intact DNA is significantly reduced for TPEN-treated cells and this effect is prevented if the equimolar association of the chelator plus zinc is applied instead of the chelator alone.

The appearance of DNA strand breaks as a function of the time of TPEN application at the concentration 50 μM is shown in Fig. 1(b). After incubation with TPEN for 2 h, DNA strand breaks are already present, impairing DNA resistance to alkaline denaturation. The percentage of intact DNA is significantly reduced after 40 minutes of alkaline denaturation for 6 h of exposure to TPEN, as well as after 80 minutes of alkaline denaturation for 2, 4 and 6 h of exposure to TPEN.

3.1.2. On cell apoptosis

The effect of TPEN applied for 6 h, at various concentrations, on the level of cytoplasmic histone-associated DNA fragments is presented in Fig. 2(a). The absorbance at 405 nm increases with increasing TPEN concentration and this effect is also avoided if the equimolar association of the chelator plus zinc is applied instead of the chelator alone. The effect of duration of TPEN application is shown in Fig. 2(b). The TPEN concentration used is 50 μM. No increase in the level of cytoplasmic histone-associated DNA fragments is detected before 4 h of exposure to TPEN.

3.2. Effect of zinc supply on cells submitted to a genotoxic stress

3.2.1. UVB-induced DNA strand breaks

DNA strand breaks are detected immediately after UVB irradiation of the cells, as shown in Fig. 3. Zinc chloride treatment of the cells before irradiation provides a statistically significant protection.

3.2.2. UVB-induced cell apoptosis

Fig. 4(a) presents the appearance of nucleosomes in the cytoplasm induced at different time points by a single dose of UVB (40 mJ cm^{-2}). The enrichment factor of the cytoplasm in oligonucleosomes and mononucleosomes due to UVB irradiation increases 4 h after irradiation and this increase is maximum after 10 h irradiation.

Fig. 4(b) shows an increase in the enrichment factor of the cytoplasm in histone-associated DNA fragments at different doses of UVB 10 h after irradiation. This effect is partially prevented by zinc chloride supply in the culture media.

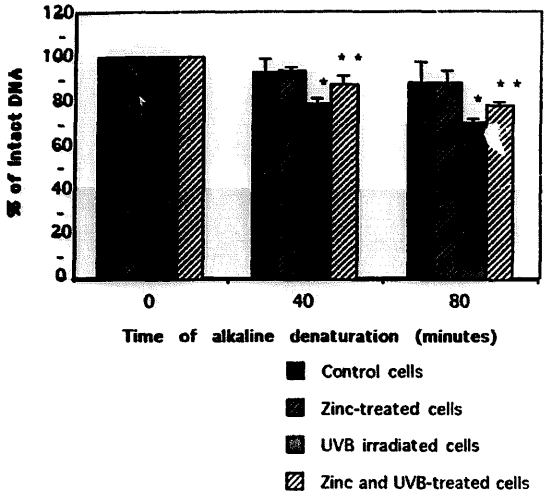

Fig. 3. Effect of zinc supply (0.1 mM) on the UVB-induced DNA strand breaks on HaCaT. DNA strand breaks were evaluated immediately after UVB irradiation (400 mJ cm^{-2}) by DNA resistance to alkaline denaturation (values represent mean ± standard deviation of three separate experiments): *, $p = 0.05$, irradiated cells vs. control cells; **, $p = 0.05$, zinc- and UVB-treated cells vs. UVB-irradiated cells.

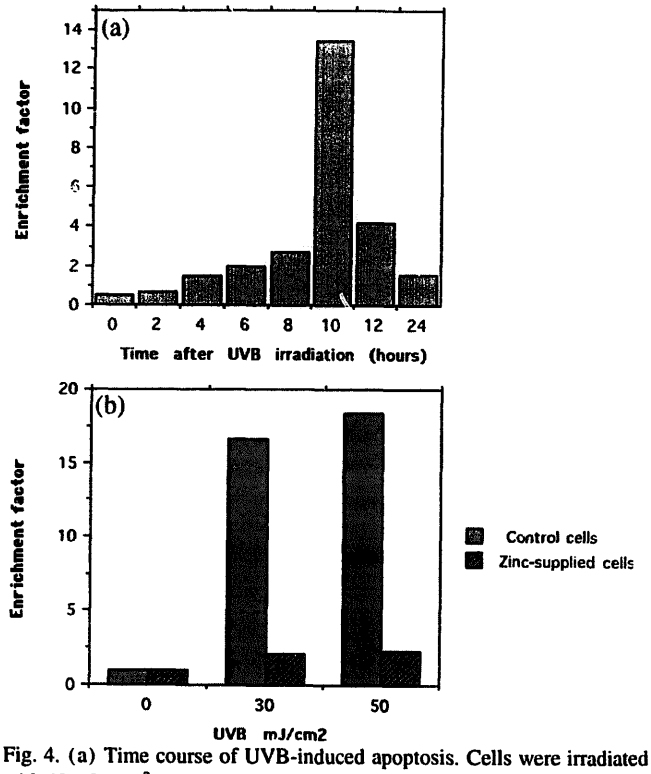

Fig. 4. (a) Time course of UVB-induced apoptosis. Cells were irradiated with 40 mJ cm^{-2} UVB or sham irradiated and lysed at different time points after UVB irradiation to detect cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes). Values are expressed as the enrichment factor of cytoplasm in nucleosomes. (b) The effect of zinc supply on UVB-induced apoptosis. Cells were treated with zinc-chloride-enriched medium (0.1 mM) for 36 h before UVB-irradiation and replaced in their respective medium for 10 h. Apoptosis was then measured using ELISA detection of cytoplasmic mononucleosomes and oligonucleosomes. Values are expressed as the enrichment factor of cytoplasm in nucleosomes.

4. Discussion

The ROSs generated in the skin by UV irradiation [11] are involved in the deleterious effects caused by sun exposure. These include acute effects such as erythema or photosensitization, and long-term effects such as photocarcinogenesis or photoaging [15]. UVB irradiation induces in keratinocytes damage characteristic of ROSs, such as lipid peroxidation [16]. ROSs also act as apoptosis mediators [9,10]. There is growing evidence that the sunburn cells formed in epidermis following by UV irradiation in response to DNA damage are actual apoptotic cells [17,18].

Previous studies have shown that zinc supplied to cultured skin fibroblasts is able to prevent some damaging features of UVA radiations [19] and UVB cytotoxicity [12]. Moreover, zinc has an important role in epidermal biology. This trace element is also known to inhibit apoptosis in several leucocyte models [2,20]. The zinc effect on keratinocyte apoptosis thus appears to be important to assess, and to our knowledge no work on this matter has been published so far.

To investigate the role of zinc in keratinocyte apoptosis, chelation of intracellular zinc was realized by applying TPEN on the cells. Our findings show that TPEN is itself a genotoxic stress, for DNA strand breaks appear when 50 μM of this chelator are added in the culture medium. This is an early event for, after TPEN application for 2 h, DNA strand breaks are already detected. The involvement of zinc in the observed effect is shown by the prevention of TPEN effect when it is applied in equimolar concentration with zinc. Because the alkaline unwinding method for assaying strand breaks involves alkaline denaturation of the DNA, single-strand breaks as well as frank double-strand breaks or any alkali-labile site are detected [21].

Apoptosis is best characterized biochemically by cleavage of chromatin at internucleosomal regions into fragments of 180–200 base pairs, yielding a classical DNA ladder pattern on gel electrophoresis. In our experiments, histone-associated DNA fragments were assayed in the cytoplasm of cells using ELISA detection. TPEN application led to mononucleosome and oligonucleosome enrichment of the cell cytoplasm, indicating that HaCaT keratinocytes apoptosis was triggered. In our experiments this effect appears after TPEN application for 4 h, is dose dependent and is prevented by zinc chloride addition during TPEN application. This observation confirms what was observed on leucocyte models, which have been extensively used to study the effect of zinc on apoptosis [1–3].

Our results also show an increase in cytoplasmic nucleosomes after UVB irradiation. The enrichment factor increases when the energy of irradiation increases. Moreover, this apoptotic feature is seen from 2–24 h after UVB irradiation. These results are in accordance with the finding in mice skin of apoptotic keratinocytes using fluorescent in situ end labelling of DNA nicked strands 24 h after UVB irradiation [17]. However, in our experiment, UVB-induced apoptosis is best detected at the tenth hour after UVB irradiation. To explain

the decrease in cytoplasmic nucleosome measurement after 10 h in our system, we hypothesize that dead cells detached from the dish bottom are eliminated with the culture medium and are thus not submitted to the lysis buffer.

In our study, HaCaT cells submitted to UVB (400 mJ cm^{-2}) also exhibit an increase in DNA strand breaks as detected by DNA resistance to alkaline denaturation, measured immediately after irradiation. Both UVB and UVA wavelengths are known to induce DNA strand breaks [21]. In the apoptosis occuring after a genotoxic stress, nickel DNA can be either the signal initiating apoptosis or the result of the activity of the activated calcium- and magnesium-dependent endonuclease responsible for internucleosomal DNA fragmentation. In our experiments, DNA strand breaks are assayed immediately after UVB irradiation. They thus reflect the initial damage to DNA.

To assess the effect of zinc on HaCaT keratinocytes apoptosis, we pre-treated the cells with zinc chloride (0.1 mM) for 36 h before UVB irradiation. This provides significant protection against UVB-induced DNA strand breaks. These results are in accordance with the inhibition by zinc of DNA fragmentation shown on chicken embryo cells by viscometry of alkaline cell lysates after exposure to various genotoxic agents, including UV light [22]. Our findings show the protective effect of zinc supply on DNA integrity following UVB exposure. Moreover, when zinc chloride is supplied in the culture medium, the UVB-dependent appearance of nucleosomes in the cytoplasme of HaCaT keratinocytes is strongly reduced. This result is in favour of an antiapoptotic effect of zinc supplied to keratinocytes before UVB irradiation.

The hypothesis usually advanced to explain the antiapoptotic effect of zinc is an 18 kDa calcium-dependent endonuclease responsible for internucleosomal degradation and able to be inhibited by zinc. However, some of these studies show that zinc inhibits DNA fragmentation but has no effect on subsequent cell death [5,6]. In fact, it has been recently demonstrated that nuclear fragmentation during apoptosis occurs in two stages, involving two distinct endonucleolytic activities preexisting in the cell, with only the first activity being essential for cell death and only the second undergoing inhibition by zinc [23].

It is thus relevant to consider other potential action levels for zinc in the apoptosis pathway. It has been proposed that zinc inhibits the intracellular acidification associated with apoptosis, consequently inhibiting deoxyribonuclease II activation and hence DNA digestion [24]. Reactive oxygen species, generated by exogenous stress exposure or during normal developmental processes, are believed to be strongly implicated in apoptotic cell death. Various morphological features of apoptosis, such as DNA strand breaks or membrane damage, are typical deleterious effects of ROSs. Moreover, many of the chemical or physical stimuli capable of inducing apoptosis enhance cellular oxidative metabolism, decrease cellular defences against ROSs or are oxidants themselves (hydrogen peroxide, radiation, buthionine sulfoximine and tumour necrosis factor α), whereas treatments able to inhibit apoptosis have antioxidant properties or stimulate antioxidant cellular defences (catalase, *N*-acetyl cystein, glutathione, vitamin E and Bcl-2) [9,10]. Now zinc exhibits antioxidant properties; this trace element prevents the production of ROSs and is involved in cell membrane stabilization, in metallothionein transcription and in the copper, zinc superoxide dismutase structure [25]. Moreover, when supplied to cultured cells, zinc increases their resistance to oxidative stress [12].

Another explanation for the effects of zinc comes from its strong involvement in DNA metabolism, through numerous enzymes containing or activated by zinc [7] or through transcription factors [8]. Our results confirm that zinc ions are of prime importance to DNA integrity, for zinc deprivation by TPEN induces DNA strand breaks which are prevented is zinc is added in equimolar concentration. DNA strand breaks may thus be one mechanism involved in TPEN-induced apoptosis.

In HaCaT keratinocytes treated by either TPEN or UVB, DNA strand breaks are present at an early time when apoptosis cannot yet be detected. In both cases, zinc is able to prevent partially this early damage as well as the subsequent apoptosis. This observation involves zinc upstream in the inhibition of TPEN- or UVB-induced apoptosis.

Our findings show that zinc deprivation using TPEN as a chelator is a genotoxic stress and leads to cell apoptosis as detected by increased nucleosomes in the cytoplasm. We also demonstrate that the zinc supply is able to decrease the appearance of nucleosomes in the cytoplasm of a keratinocyte cell line submitted to UVB, a genotoxic stress occurring in vivo when skin is exposed to solar radiations. Furthermore, our study suggests that zinc ions have at least one role, at an early stage, by decreasing the DNA damage able to trigger apoptosis. Further experiments are required to elucidate other possible levels of action for zinc in apoptosis. These results further implicate zinc in the control of processes of prime importance for epidermal cells, such as carcinogenesis and senescence.

Acknowledgments

We thank Dr. T. Mammone and Dr. P. Hainaut for stimulating discussions, and gratefully acknowledge the technical assistance of J. Meo and A.M. Monjo. This work was partially supported by a grant from the Volvic Centre for Research on Trace Elements.

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