Acta Derm Venereol 2002; 82: 325–328

Taylor & Francis
healthsciences

INVESTIGATIVE REPORT

Relevance of Reactive Oxygen Species in the Induction of 8-Oxo-2′-deoxyguanosine in HaCaT Keratinocytes

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There is growing evidence that solar radiation-induced oxidative DNA damage may play an important role in carcinogenesis of the skin. One substantial modification in this context is the oxidation of the guanine base to 8-oxo-2′-deoxyguanosine. Using HaCaT keratinocytes, measurement of the 8-oxo-2′-deoxyguanosine content in this study was performed by flow cytometry on whole cells. Hydrogen peroxide and hydroxyl radicals seem not to be involved in the process of this DNA alteration. However, our results demonstrate that ultraviolet A can cause DNA damage at guanine sites primarily via photosensitized reactions. Although singlet oxygen can also lead to 8-oxo-2′-deoxyguanosine, the major mechanism seems to be based on formation of the guanylcation radical through excited riboflavin and can therefore proceed without the involvement of reactive oxygen species. *Key words: flow cytometry; free radicals; guanylcation radical; riboflavin; UV-irradiation.*

(Accepted 27 May, 2002.)

Acta Derm Venereol 2002; 82: 325–328.

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Increased exposure to ultraviolet A (UVA) radiation as a result of lifestyle changes enhances the risk of development of skin tumours (1). It is estimated that between 20% and 50% of solar UVA is capable of penetrating deeply into the skin structures, as far as the papillary and reticular dermis, whereas only about 9–15% of solar UVB reaches this depth (2). Between 90% and 95% of the solar UV radiation energy that reaches the surface of the earth is UVA. UVA therefore has to be attributed an essential effect on the formation of skin cancer. For example, although UVB is more strongly absorbed by DNA than UVA, intracellular melanin strongly absorbs UVA too, and therefore might affect DNA by direct energy transfer from melanin to DNA (3). Investigations on pigmented fishes of the genus *Xiphophorus* have shown melanoma-inducing effects at wavelengths >320 nm (4), thus confirming a role for UVA in this context.

Oxidative damage to DNA is considered to be an important contributor to degenerative diseases, including cancer. One form of oxidative DNA damage after UV radiation is the induction of 8-oxo-2′-deoxyguanosine (8-oxodG) (5). This base modification is approximately 5% of the total oxidized bases known to occur in DNA (6). 8-oxodG promotes the misincorporation of deoxynucleotide triphosphates by DNA polymerase resulting in the formation of guanosine to thymidine transversion mutations during replication *in vitro* and *in vivo* (7). An additional aspect for the mutagenic relevance of 8-oxodG is the fact that the repair through DNA proof-reading enzymes is poor (8). It seems consistent to attribute this DNA alteration to the induction of reactive oxygen species (ROS). However, the type of ROS involved is controversial (5, 9–11).

Our aim was to investigate the role of certain ROS and UVA in the process of 8-oxodG induction in HaCaT keratinocytes. We employed flow cytometry as a method that can be carried out without a hydrolysis step and which is easy to perform.

MATERIALS AND METHODS

Cell culture

HaCaT keratinocytes (kindly provided by N. E. Fusenig, DKFZ Heidelberg, Germany) (12) were cultivated in 25-cm^2 culture flasks in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS in a humidified atmosphere containing 10% CO_2 and 90% air at 37°C. Cells were used for the experiments at a confluence of 80–90%.

Chemical treatments

Cells in the flasks were incubated for 3 h with vitamin C at a concentration of 500 μM and riboflavin at concentrations from 2.5 to 20 μM. Before irradiation, cells were trypsinized, washed and resuspended in PBS. In each case, a volume of 1 ml suspension containing 1 million cells was transferred to 35-mm culture dishes and then irradiated at about 1°C by placing the dishes in a bath of ice-water.

The following treatments were performed on cells trypsinized and resuspended in PBS as described above. Hydrogen peroxide was added at final concentrations from 1 to 4 mM and sodium azide prior to irradiation at 5 mM only. Hydroxyl radicals were generated through 8-hydroxyquinoline/iron(II)-sulphate in combination with hydrogen peroxide (13) at final concentrations from 0.1 to 0.4 mM. To this, 8-hydroxyquinoline

 ISSN 0001-5555

and iron(II)-sulphate were mixed to bind iron to the cell permeable chelator and only then added to the cells.

UVA irradiation

UVA was applied using an irradiation chamber equipped with TLK40W-10R tubes (Philips, Hamburg, Germany) and a UVA sensor for controlling the doses indicated. These lamps emit predominantly from 350 to 390 nm, with an UVB/UVA ratio of less than 0.1%. The distance between lamps and 35-mm dishes was 20 cm. The dishes were left open for the duration of irradiation.

Detection of 8-oxo-2′deoxoquanosine

After irradiation, cells were washed with PBS and resuspended in 500 μl of PBS. A volume of 500 μl of 2% paraformaldehyde was added and the samples were then incubated on ice for 15 min. After washing twice with PBS, cells were fixed in ice-cold 70% ethanol for 30 min. Washing was repeated once with PBS and once with a special wash solution (OxyDNA Assay Kit Fluorogenic; Calbiochem, Bad Soden, Germany). Non-specific binding sites were blocked with 50 μl blocking solution (OxyDNA Assay Kit, Fluorogenic) for 1 h at 37°C followed by washing twice with the wash solution. The cells were then incubated for 1 h with 100 μl of a FITC-labelled protein with an affinity to 8-oxodG (OxyDNA Assay Kit, Fluorogenic). After washing twice with the washing solution, the cells were finally resuspended in PBS. All measurements were performed on a flow cytometer FACScan (Becton Dickinson, San Diego, USA) using a 515 nm bandpass filter for FITC detection.

Determination of intracellular ROS generation

The content of intracellular peroxides was measured with dihydrorhodamine 123 (DHR; purchased from Sigma, Deisenhofen, Germany) (14). In this way the non-fluorescent DHR is irreversibly oxidized to the green fluorescent rhodamin 123. Cells were seeded in 96-well microtitre plates and cultivated and incubated as described above. Prior to UVA irradiation, DHR was added at a final concentration of 5 μM and incubated for 45 min at 37°C. Irradiation was carried out after washing with PBS. Fluorescence intensity was measured using the microtitre plate reader Fluoroskan Ascent (Labsystems, Helsinki, Finland) with filters for excitation at 485 nm and emission at 538 nm.

RESULTS

As expected, UVA induced a dose-dependent increase in peroxides in the HaCaT cells. Preincubation with vitamin C led to a clearly lower peroxide level (Fig. 1). Compared to the unirradiated control, exposure to UVA at doses of 30, 60 and 90 $J \times cm^{-2}$ significantly induced 8-oxodG dose-dependently and resulted in an increase of up to 138% at 90 $J \times cm^{-2}$. In contrast to the reduction in intracellular peroxide level (Fig. 1), vitamin C could not lower the UVA-induced increase in 8-oxodG content (data not shown).

The influence of specific ROS was tested by adding them directly to the cells or by making use of the Fenton reaction. The application of hydrogen peroxide alone failed to induce 8-oxodG supporting the results presented above. By contrast, artificial hydroxyl radical

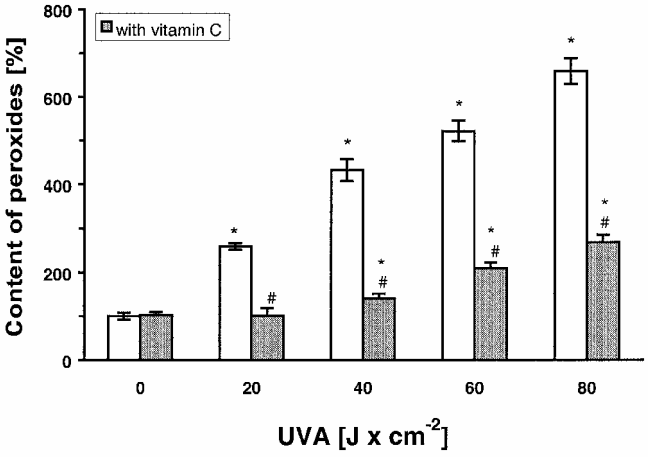

Fig. 1. Effect of vitamin C on the content of peroxides in UVA irradiated HaCaT keratinocytes. Unirradiated cells without vitamin C treatment were considered a control and set to 100%. $*p < 0.05$ vs. control, $\#p < 0.05$ vs. the same UVA dose (two-tailed *t*-test). The results are given as mean ± SD ($n = 6$ observations).

generation led to an enhanced 8-oxodG content (Fig. 2).

The following results were obtained applying a UVA dose of 60 $J \times cm^{-2}$. Preincubation of the cells with riboflavin was performed in order to study the contribution of photosensitizing effects to the 8-oxodG formation. Riboflavin treatment resulted in the highest increase in the UVA-induced 8-oxodG content, which reached a level 4.36-fold (20 μM riboflavin) of the irradiated control (0 μM riboflavin) (Fig. 3). The effect of singlet oxygen on the 8-oxodG induction was investigated using the specific singlet oxygen quencher sodium azide. As shown in Fig. 4, sodium azide was partially able to abolish the increase in 8-oxodG content in UVA-irradiated and riboflavin-treated cells. Nevertheless, this increase remained about two-thirds of that which occurred without adding sodium azide. When riboflavin was omitted, the UVA-induced

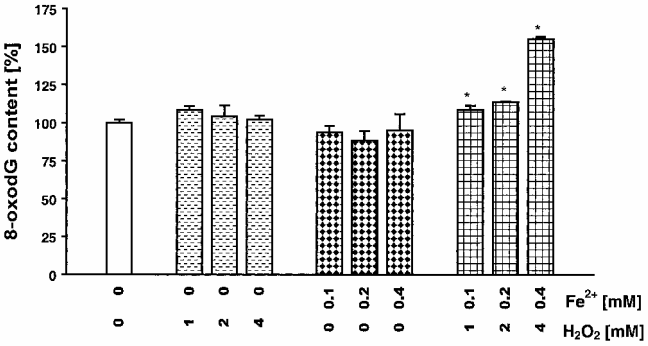

Fig. 2. Effect of hydrogen peroxide and the hydroxyl radical (formed from H_2O_2 and Fe^{2+} according to the Fenton reaction) on the 8-oxo-2′-deoxyguanosine (8-oxodG) formation in HaCaT keratinocytes. Untreated cells were considered a control and set to 100%. $*p < 0.05$ vs. control (two-tailed *t*-test, $p < 0.05$). The results are given as mean ± SD ($n = 3$ observations).

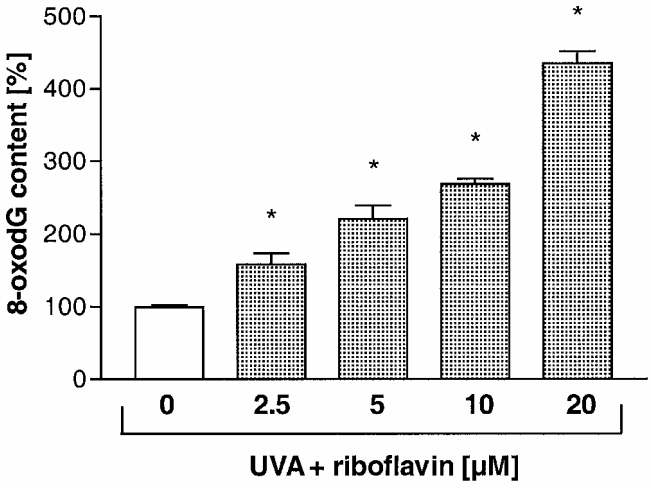

Fig. 3. Detection of the 8-oxo-2′-deoxyguanosine (8-oxodG) induction in HaCaT keratinocytes treated with riboflavin prior to UVA (60 J × cm^{-2}). Irradiated cells without riboflavin treatment were considered a control and set to 100%. *$p < 0.05$ vs. control (two-tailed *t*-test). The results are given as mean ± SD ($n = 3$ observations).

increase in the 8-oxodG content could not significantly be affected by sodium azide (Fig. 4).

DISCUSSION

Several studies have demonstrated the involvement of ROS in the process of 8-oxodG generation after UV irradiation. Using UVA and selective ROS quenchers, Zhang et al. (5) found a significant influence of singlet oxygen, hydrogen peroxide and, to a smaller extent, hydroxyl radical, whereas superoxide anion had no impact on the alteration of the guanine base. Similar results in respect of singlet oxygen have been provided

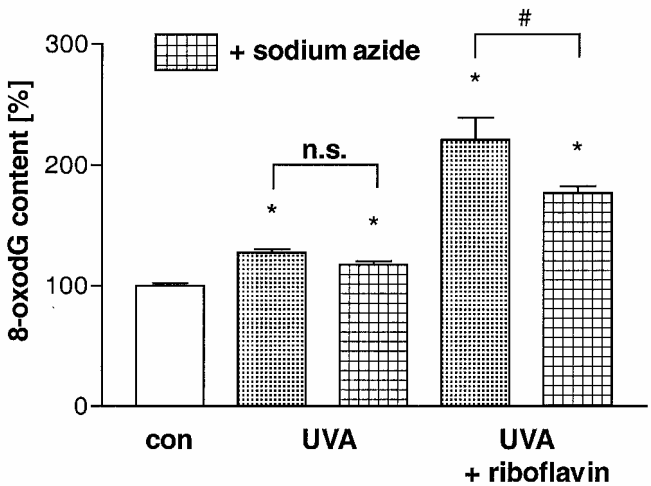

Fig. 4. Effect of sodium azide [5 mM] on the 8-oxo-2′-deoxyguanosine formation after UVA irradiation alone and after UVA irradiation (60 J × cm^{-2}) in combination with riboflavin pretreatment [5 μM]. Unirradiated cells without sodium azide treatment were considered a control and set to 100%. *$p < 0.05$ vs. control; #$p < 0.05$ vs. without sodium azide; n.s. not significant (two-tailed *t*-test). The results are given as mean ± SD ($n = 3$ observations).

by Devasagayam et al. (15) and Kvam & Tyrrell (11), but the hydroxyl radical could not be attributed any effect. Przybyszewski et al. (16) generated ROS with selective inducers rather than UV. However, the application of 12-*O*-tetradecanoylphorbol-13-acetat e or paraquat as intracellular inducers of hydrogen peroxide or superoxide anion did not lead to an 8-oxodG induction in mouse keratinocytes. Consistent with Beehler et al. (17), we were unable to demonstrate an effect of hydrogen peroxide in HaCaT cells. As expected, UVA alone considerably raised the intracellular peroxide level. Still, the preincubation with vitamin C as a ROS scavenger led only to a distinct decrease in peroxides (Fig. 1); it could not inhibit the increase in 8-oxodG content. Thus, peroxides seem not to be involved in the specific DNA alteration. In contrast, artificially generated hydroxyl radical as a result of the Fenton reaction after the addition of Fe^{2+} and hydrogen peroxide to the cells resulted in 8-oxodG formation, whereas the addition of Fe^{2+} alone had no effect (Fig. 2). Therefore, under natural conditions the hydroxyl radical can at best be involved only weakly.

Another approach to studying 8-oxodG generation is to excite photosensitizers with light of the UV or visible wavelength range. By ROS-quenching after application of benz[a]pyrene and UVA, Liu et al. (10) showed that superoxide anion alone was correlated to the formation of 8-oxodG. Besides artificially synthesized compounds, riboflavin as a natural cell constituent plays an essential role in such investigations. Using calf thymus DNA and UVA-excited riboflavin, Ito et al. (18) could not find any influence of singlet oxygen, and attributed the formation of 8-oxodG to hydroxylation of guanylcation radical. In the present study on HaCaT keratinocytes, the preincubation with riboflavin followed by UVA irradiation also led to a dramatic increase in 8-oxodG (Fig. 3). Contrary to Ito et al. (18), we observed an involvement of singlet oxygen at least to some extent by sodium azide quenching (Fig. 4). This effect occurred significantly only when riboflavin was present and not after UVA irradiation alone. In this case, the higher proportion of 8-oxodG content is probably a result of hydroxylation of the guanylcation radical. Mori et al. (19) found that hydroxylation of DNA bases through the influence of excited riboflavin exclusively occurs at guanine sites. As the hydroxyl radical possesses no base specificity (20), it can be suggested, in line with Kvam & Tyrrell (11), that the photoreaction of DNA with riboflavin proceeds without the generation of hydroxyl radicals. The interaction between photosensitized riboflavin and deoxyguanosine is based on intercalation and/or hydrogen binding and this may be responsible for the electron transfer from the guanine base to the excited riboflavin. Guanine has the lowest ionization potential of the four DNA bases (21). Consequently, an electron transfer of the guanine

base to excited riboflavin is highly likely. Cadet & Vigny (22) and Kasai et al. (23) have shown that the guanylcation radical actually reacts with water alone, which means that the formation of 8-oxodG can proceed without ROS involvement (24), presumably according to photodynamic reaction type I (18).

In summary, intracellular photosensitized riboflavin can cause DNA mutations at guanine sites after UVA irradiation. This process does not require the presence of ROS and may be induced primarily via hydroxylation of the guanylcation radical. ROS scavengers thus seem unable to prevent this potential source of skin cancer.

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