Induction of oxidative DNA base damage in human skin cells by UV and near visible radiation

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The premutagenic oxidative DNA base damage, 7,8-dihydro-8-oxoguanine, is induced in human skin fibroblasts by monochromatic radiation ranging from a UVB wavelength (312 nm) up to wavelengths in the near visible (434 nm). The oxidative damage is not generated by absorption of radiation in DNA but rather by activation of photosensitizers generating genotoxic singlet oxygen species. The spectrum for the yield of the oxidative damage in confluent, non-growing, primary skin fibroblasts shows that it is UVA (above 334 nm) and near visible radiations which cause almost all of this guanine oxidation by natural sunlight in the fibroblast model. We estimate that the total amount of oxidation of guanine induced by sunlight in fibroblasts in the epidermis of the skin equals or exceeds the amount of the major type of direct DNA damage, cyclobutane pyrimidine dimers. In rapidly dividing lymphoblastoid cells, no oxidative guanine damage was induced. However, in melanoma cells almost as much damage as in non-growing fibroblasts (1.1 per 10^4 guanine bases after 1200 kJ/m^2 UVA) was found. We conclude that oxidative DNA base damage can probably contribute to the induction of both non-melanoma and melanoma skin cancer by sunlight.

Introduction

Skin cancer is the most common type of cancer in Caucasian populations and its incidence is increasing steadily. The primary cause of skin cancer, particularly non-melanoma skin cancer, is evidently exposure to ultraviolet (UV*) radiation (1,2). The most energetic part of natural solar UV radiation, the middle ultraviolet (280–320 nm) (UVB) region, is most efficient in producing the direct DNA damage, cyclobutane pyrimidine dimers and (6–4) photoproducts, which are produced by absorption of radiation in DNA (3). These photoproducts are associated with mutagenesis and cancer (4,5).

Near ultraviolet radiation (320–380 nm) (UVA) is also carcinogenic (6) and is a possible cause of highly lethal malignant melanomas (7). However, in contrast to UVB, the mechanisms causing mutations and cancer after UVA are not well known. Many of the biological effects of UVA, including cell inactivation, are strictly dependent upon the presence of molecular oxygen (8). Thus, oxidations of biomolecules are prominent after UVA irradiation of cells or skin.

*Abbreviations: UV, ultraviolet; UVA, near ultraviolet (320–380 nm); UVB, middle ultraviolet (290–320 nm); 8-OHdG, 8-hydroxydeoxyguanosine or 7,8-dihydro-8-oxo-2′-deoxyguanosine; dG, 2′-deoxyguanosine; FPG, formamidopyrimidine DNA glycosylase; D_2O, deuterium oxide.

Oxidative DNA damage consists of a large group of oxidations of DNA components, notably the DNA bases (9). The spectrum of oxidation products which appear depends on the damaging agent which include hydroxyl radicals and singlet oxygen. The most frequently studied oxidation product, 7,8-dihydro-8-oxoguanine, is a premutagenic lesion in bacteria and mammalian cells (10,11). There are DNA repair enzymes both in mammalian cells and in bacteria (formamido-pyrimidine DNA glycosylase [FPG]) which can excise the damaged base (12,13).

It is known that UV and visible light can induce oxidative DNA base damage in the form of 7,8-dihydro-8-oxoguanine or lesions sensitive to the FPG enzyme (14–16). However, the wavelength dependent yield of 7,8-dihydro-8-oxoguanine or its nucleoside derivative by monochromatic UV and visible light has not been available. We have studied the spectrum for yield of 7,8-dihydro-8-oxo-2′-deoxyguanosine (8-hydroxydeoxyguanosine [8-OHdG]) in human skin fibroblasts, the mechanism of induction of damage by UVA and the yields of damage by UVA in other cell types found in the skin. The spectrum, which gives information about the physiological importance of induction of 8-OHdG, was compared to the spectrum for pyrimidine dimers.

Materials and methods

Cell cultivation

Human primary skin fibroblasts (FEK4) were cultivated using 15% fetal calf serum in Earle's Minimal Essential Medium (Gibco, Basel, Switzerland). The cells were replated twice a week and used for experiments between passages 9 and 15. Fibroblasts were either plated 2 or 5 days before light exposure. In the latter case, the cells were confluent and had stopped growing for 2 days before irradiation.

Human lymphoblastoid cells (TK6) were grown using 10% fetal calf serum in RPMI 1640 medium (Gibco, Basel, Switzerland). The cells were kept at a density of between 2×10^5 and 10^6 cells per ml by diluting three times a week.

Human melanoma cells (GLL19) (17), which were a kind gift from Dr Donata Rimoldi, were grown using the same medium as for lymphoblastoid cells.

Cell irradiation

Before irradiation with the broad-spectrum UVASUN 3000 lamp (Mutzhas, Munich, Germany) (350–450 nm), cells attached to Petri-dishes were washed with PBS. All the PBS used was supplemented with 0.01% Ca^{2+} and Mg^{2+}. Cells were irradiated at ~1°C by placing the Petri-dishes in an ice-water bath. Suspension cells (TK6) were irradiated suspended in PBS.

To study the mechanism of induction of 8-OHdG, cells were washed and incubated with PBS containing 100% deuterium oxide (D_2O) (Sigma, Buchs, Switzerland) for 30 min prior to and during irradiation. Cells were also incubated with the iron chelator desferal (0.5 mM) for 60 min before or the thiol-compound cysteamine (10 mM) 30 min before and during the irradiation. The treatment with D_2O, desferal or cysteamine were all found to be non-toxic to the cells.

Fibroblasts were irradiated with monochromatic light from a 2.5 kW Hg-Xe lamp passed through a monochromator and a cut-off filter (18). Before irradiation, cells were trypsinized, washed and resuspended in PBS. A volume of 1.5 ml cell suspension containing 1.5 million cells was irradiated in a quartz cuvette. The cells were stirred continuously during irradiation and the cuvette was kept at 0–2°C by circulating cold liquid around the cuvette. Condensation of water on the irradiated surface of the cuvette was prevented by blowing N_2 at the surface. Cells were irradiated from 90–120 min and the fluences used were 200 kJ/m^2 for 313 nm, 300 kJ/m^2 for

334 nm, 1200 kJ/m^2 for 365 nm, 600 kJ/m^2 for 405 nm and 1000 kJ/m^2 for 434 nm. Half band-widths were 8 nm for 313 nm and 17.6 nm for the other wavelengths. Radiation fluence rates were measured as previously described (18) and the fluences were corrected by the Morowitz (19) method from data for light transmission (which was >87%) through the cuvette.

Analysis of DNA damage

After irradiation, cells were kept in an ice-water bath. For cells irradiated in plates, the PBS was replaced with ice cold PBS supplemented with antioxidants 2 mM (final concentration) desferal, 10 mM histidine and 6 mM reduced glutathione, to prevent oxidation of DNA during isolation of DNA. The cells were scraped off the plates using an ice-cold rubber policeman. The scraping did not change the yield of induced 8-OHdG since we had identical yields with fibroblasts irradiated in suspension or attached to plates (data not shown). However, the background levels of 8-OHdG were doubled by the scraping. Antioxidants at the same final concentrations as above were also added to suspension cells (TK6) immediately after irradiation.

After irradiation of fibroblasts with monochromatic light, antioxidants were added into the cuvette. Cells were either immediately frozen (–70°C) or DNA was isolated. Both methods gave identical yields of DNA damage.

DNA was isolated from cells using the procedures and columns provided by Qiagen (Blood & Cell Culture DNA Kit) (Basel, Switzerland). Initially, nuclei were isolated by adding ice-cold hypotonic buffer (final concentrations 64 mM saccharose, 1 mM MgCl$_2$, 2 mM Tris, 0.2% Triton X-100, 20% PBS, pH 7.2) to the cell suspension. However, before the addition of hypotonic buffer, antioxidants (as described above) were added to the cell suspensions. The isolated DNA was dissolved in 10 mM Tris (pH 7.5) and enzymatically digested to nucleosides (20). High performance liquid chromatography (HPLC) was performed as previously described (20) except that the isocratic HPLC condition of 50 mM phosphate buffer and 8–9% methanol was used. Nucleosides were detected by optical density at 254 nm and 8-OHdG was measured by electrochemical detection (ESA, Model 5200 Coulochem II detector equipped with a 5011 analytical cell, Stagroma, Zurich). Standards for 8-OHdG were synthesized as described (20).

Results

Spectrum of yield for induction of oxidative DNA base damage

We have determined how efficiently different wavelengths of light induce the oxidative DNA base damage 8-OHdG in human primary skin fibroblasts. Cells were exposed to nearly monochromatic or broad-band UVA radiation and the temperature was kept at 0–2°C to prevent DNA repair and cell degradation. The dose-response for broad-band UVA radiation (350–450 nm) was found to be linear up to 1200 kJ/m^2 (Figure 1). For irradiation of cells with monochromatic wavelengths from 312–434 nm only one dose per wavelength was given in 90–120 min. Assuming a linear dose-response also for all monochromatic wavelengths, the spectrum for yield of 8-OHdG per quantum of incident light was determined (Figure 2).

Irradiation with the wavelengths of 365 nm produced significantly more oxidative damage than did the wavelenths of 334 and 434 nm (two-sided Student's *t*-test, $P < 0.05$) (Figure 2). Furthermore, radiation at the wavelength of 405 nm was significantly more efficient than radiation at the wavelength of 334 nm (*t*-test, $P < 0.04$). The yield of 8-OHdG at 312 nm was, due to higher uncertainty (Figure 2), not significantly different from yields at longer wavelengths. However, when compared to the yield of cyclobutane pyrimidine dimers produced in human primary skin fibroblasts or lymphocytes (Figure 2), the yields of 8-OHdG at both 312 and 334 nm were small. The yield of dimers at 365 nm was 65% of the yield of 8-OHdG. For wavelenths longer than 365 nm, the yields of dimers are generally close to or below the detection limit.

The mechanism of induction of 8-OHdG

Because it was clear that induction of 8-OHdG was significantly higher than the induction of pyrimidine dimers for wavelengths above 365 nm (Figure 2), we investigated the mechanism of induction of 8-OHdG for broad band UVA and near visible
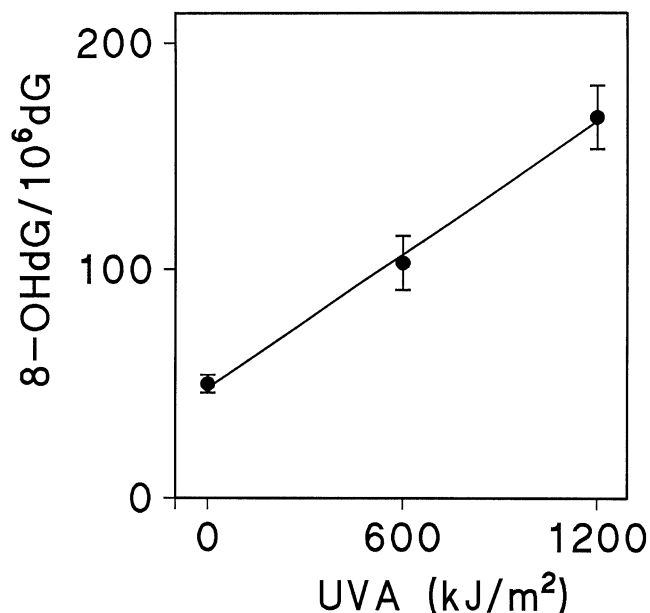
Fig. 1. Dose-response for induction of the oxidative DNA base damage 8-OHdG in human primary skin fibroblasts (FEK4) by broad-band UVA (350–450 nm) radiation. The error bars represent standard deviation for 4 samples. The cells used were confluent and non-growing.
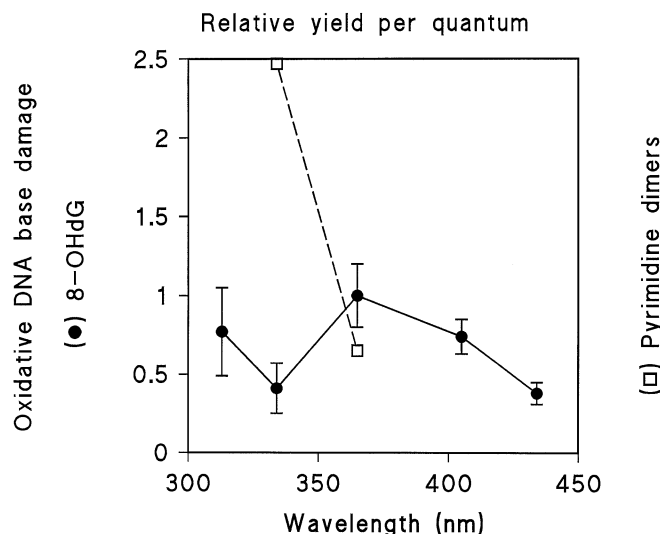

Fig. 2. Spectrum for the yield of the oxidative DNA base damage 8-OHdG (●) after irradiation of human primary skin fibroblasts (FEK4). For comparison, the yield of cyclobutane pyrimidine dimers (□), determined also in primary skin fibroblasts by Enninga *et al*. (31) or peripheral lymphocytes (334 nm) (39), is also shown. The yields represent the relative amount of damage produced per quantum of incident radiation. The yields are normalized relative to the yield for 8-OHdG at 365 nm radiation which produced 20 8-OHdG per 10^6 basepairs after 600 kJ/m^2. The level of damage in non-irradiated cells (15–26 per 10^6 basepairs) was subtracted and the error bars represent standard error of the mean for 4 or 5 samples. The relative yield of of pyrimidine dimers at 312 nm was 181 (31).

radiation. Replacing the water in the PBS used during irradiation with deuterium oxide (D$_2$O), enhanced the yield of 8-OHdG by 2.7 times (Figure 3). This is an indication that singlet oxygen is involved in the production of 8-OHdG, since the lifetime of singlet oxygen is several times longer in a D$_2$O than in a H$_2$O environment (20). Singlet oxygen may be produced by energy transfer from an excited photosensitizer to molecular oxygen or as a by-product of UVA-induced lipid
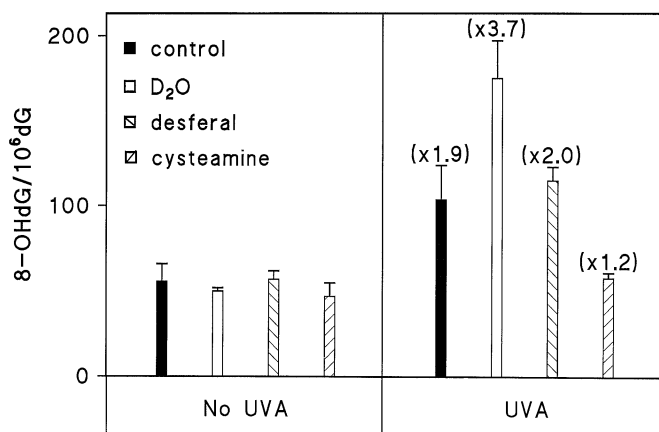
Fig. 3. Induction of 8-OHdG by 600 kJ/m^2 UVA irradiation of FEK4 fibroblasts in the presence of deuterium oxide (D$_2$O), desferal or cysteamine. The error bars represent standard deviation for 4 samples. The fold inductions of 8-OHdG for UVA irradiations are indicated in parentheses.

Fig. 4. Induction of 8-OHdG by 1200 kJ/m^2 UVA irradiation of different types of cells found in the human skin; exponentially growing and confluent, non-growing skin fibroblasts (FEK4), lymphoblastoid cells (TK6) and melanoma cells (GLL19). The error bars represent standard deviation for 4 samples.

peroxidation (21). In the latter case, one expects to be able to modulate induction of 8-OHdG by sequestration of iron by desferal treatment since desferal treatment strongly reduces UVA induced lipid peroxidation in the fibroblasts (22). However, sequestration of iron by desferal treatment, did not influence the yield of 8-OHdG (Figure 3). The lack of change with desferal treatment also suggests that iron dependent Fenton reactions producing genotoxic hydroxyl radicals are probably not a major cause of 8-OHdG production after UVA irradiation. Irradiation of cells in the presence of the thiol-compound cysteamine significantly decreased the UVA-induced yield of 8-OHdG. Cysteamine is a potent antioxidant which is able to react with singlet oxygen as well as other reactive oxygen species. However, cysteamine's reaction mechanism *in vivo* seems to be different from that *in vitro* since cysteamine enhances singlet oxygen induced DNA damage in isolated DNA (23).

The yield of UVA-induced 8-OHdG varies with the type of skin cell and with the state of growth of a particular cell type

The yield of UVA-induced 8-OHdG in confluent, non-growing fibroblasts (FEK4) was 110 per million deoxyguanosine nucleosides (dG) after 1200 kJ/m^2 UVA (Figures 1 and 4). This fluence of UVA corresponds to the UVA fluence accumulated in several hours of summer midday sunlight. The induced level of damage is 2.4 times higher than the background level of damage in the fibroblasts. However, the background level of 8-OHdG was partly caused by scraping of the cells (see Materials and methods) and the background level is probably lower in living tissue than in cells in culture.

We also determined the yields of 8-OHdG after 1200 kJ/m^2 UVA for other cell types found in the skin (Figure 4). In exponentially growing melanoma cells (GLL19), the yield (90 8-OHdG/10^{6}dG) was similar to that of confluent fibroblasts. However, in exponentially growing lymphoblastoid (TK6) cells, it was not possible to induce 8-OHdG by UVA. Previously, we have also failed to detect induced mutations after UVA irradiation of these cells (24). Interestingly, when non-confluent exponentially growing fibroblasts were irradiated with UVA, only 26 8-OHdG/10^{6}dG, compared to 110 for non-growing fibroblasts (Figures 1–3), was induced. Hence, except for melanoma cells, little or no oxidation of DNA bases could be induced in exponentially growing cells.
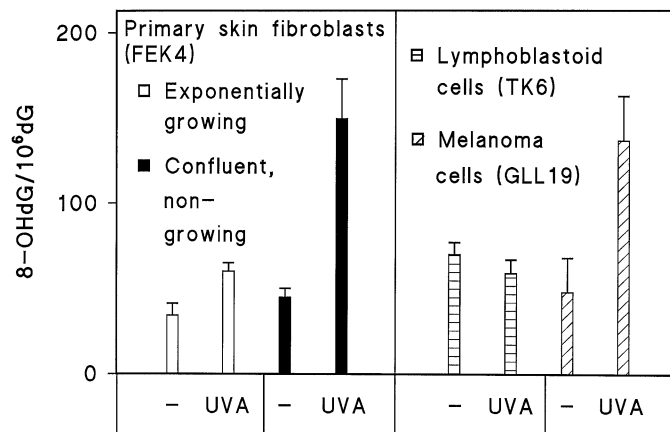
Discussion

To investigate the role of oxidative stress in UV and visible light carcinogenesis, we have determined the induction of the premutagenic oxidative DNA base damage, 8-OHdG, in human skin cells. The wavelength dependent yield of 8-OHdG in non-growing human skin fibroblasts showed a moderately sized peak at 365 nm (Figure 2). The spectrum of 8-OHdG from 312 and 365 nm is clearly very different from the action spectrum for pyrimidine dimers (Figure 2) which decreases strongly with increasing wavelength and which resembles the DNA absorption spectrum (25). Thus, 8-OHdG is clearly a type of DNA damage that is induced independently of absorption of radiation in DNA itself. It appears that production of singlet oxygen (Figure 3) by UVA is a major factor in the induction of 8-OHdG. Singlet oxygen also seems to be involved in production of the other types of oxidative DNA damage induced by sunlight — the presumably non-mutagenic DNA strand breaks (26) and protein-DNA crosslinks (27).

What role can induction of 8-OHdG have in solar carcinogenesis? Figure 5 shows the action spectrum for induction of pyrimidine dimers and the spectrum for the yield of 8-OHdG corrected for transmission of radiation to the basal layer of the epidermis where most skin cancers are believed to originate. Figure 5 also shows the action spectrum for induction of squamous cell carcinoma in human skin (extrapolated from data obtained in mice [28]). The spectrum for 8-OHdG has been arbitrarily normalized to equal that for pyrimidine dimers at 334 nm. It is clear that the direct DNA damage, mainly pyrimidine dimers, can to a large degree account for UVB (280–320 nm) carcinogenesis but most likely not for UVA carcinogenesis. The action spectrum for skin cancer shows a peak above 365 nm (Figure 5), although the spectrum for skin cancer is highly uncertain for UVA and near visible wavelengths (28). The induction of 8-OHdG is also clearly strongest for wavelengths above 334 nm (Figure 5). Thus, 8-OHdG, together with other oxidative guanine base damage induced by singlet oxygen, is likely to play an important role in UVA and visible light carcinogenesis.

How much 8-OHdG and pyrimidine dimers would be expected to be produced in quiescent fibroblasts situated near the basal layer of the epidermis during sunlight exposure? Figure 6 shows the relative production rates for 8-OHdG and
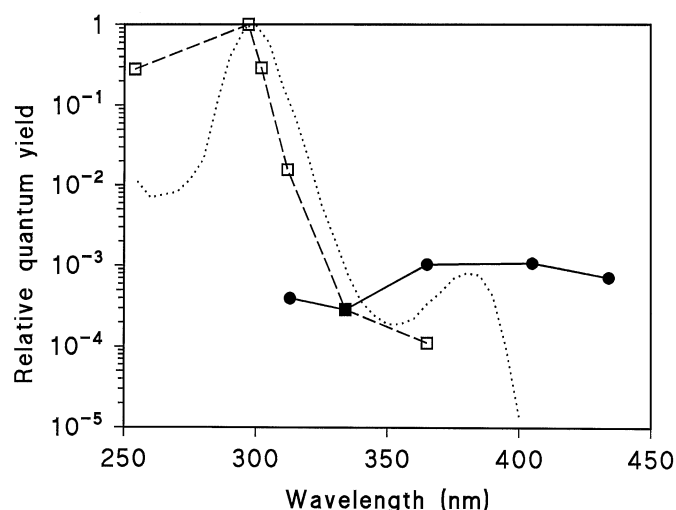
Fig. 5. Relative yields of 8-OHdG (●), pyrimidine dimers (□) and human non-melanoma skin cancer (.....) (determined from mouse data by de Gruijl *et al.* [28]) for the basal layer of the human epidermis. The yields of the DNA damage 8-OHdG (Figure 2) and pyrimidine dimers (31) in human skin fibroblasts were corrected for transmission of light (70 μm) through the skin (18). The spectra for pyrimidine dimers and skin cancer were normalized to 1 at 302 nm, and the spectrum for 8-OHdG was arbitrarily normalized so that the yield of 8-OHdG was equal to that of pyrimidine dimers at 365 nm.
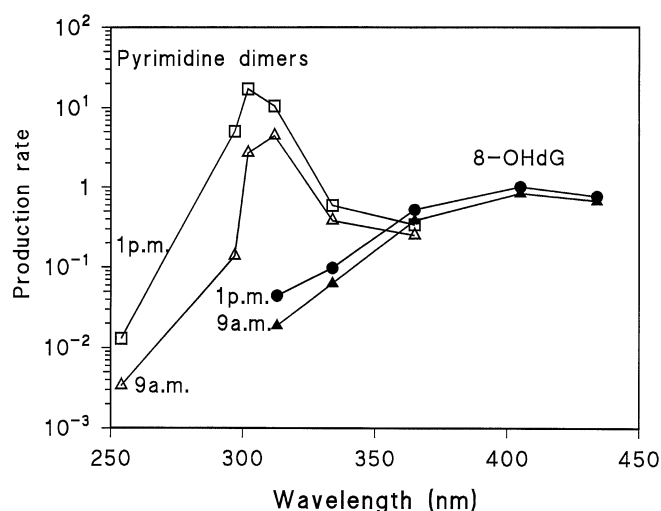

Fig. 6. Production rates of 8-OHdG (filled symbols) and pyrimidine dimers (open symbols) in fibroblasts the basal layer of the epidermis at 9 am (▲,△) and 1 pm (●,□). The data were normalized to 1 for 8-OHdG induced by 405 nm radiation at 1 pm. The production rates are the products of the yields of damage from Figure 2, corrected for transmission of light (70 μm) through the skin and multiplied with the sun irradiance spectra measured in July in Albuquerque, New Mexico (18).

pyrimidine dimers using the sun irradiance spectra for 9 am and 1 pm in mid-summer in Albuquerque (18). Pyrimidine dimers are produced much faster at 1 pm than at 9 am due to the high intensity of UVB radiation at midday. In contrast, the production rate for 8-OHdG, which is significant from 365–434 nm, does not vary much during the day. The peak for production of 8-OHdG at 405 nm at 1 pm is an order of magnitude lower than the corresponding peak for production of pyrimidine dimers at 302 nm. However, if we take into account that 8-OHdG constitutes only ~3% of the total oxidation of the guanine base caused by singlet oxygen (20), Figure 6 indicates that the rate of oxidation of the guanine base is as

large as or, especially in the morning and afternoon, larger than the rate of production of pyrimidine dimers.

Most of the sunlight induced 8-OHdG in fibroblasts is clearly induced by the UVA and near visible components of sunlight (Figure 6). It is therefore relevant to note that many commercial sunscreens used until now have reduced UVB exposure of skin and consequently induction of direct DNA damage, but have not significantly reduced UVA exposure (data not shown). Hence, use of sunscreens may have significantly increased the ratio of the oxidative DNA base damage relative to the direct DNA damage.

The wavelength dependent yield of oxidative DNA base damage was generated using non-growing primary fibroblasts (Figure 2). In theory, the spectrum should be equal to the absorption spectrum of the chromophore(s) which induces the DNA damage (29). Thus, candidates for such chromophores or photosensitizers are porphyrins, flavins and other photosensitizers which have peaks of absorption between 334 and 434 nm. The photosensitizers are either not photodegraded by UVA or their degradation products are equally good photosensitizers since the dose-response for 8-OHdG induction by UVA is linear (Figure 1). Other cell types are likely to contain a different composition of photosensitizers. Thus, Kielbassa *et al.* found a maximum yield above 400 nm for FPG sensitive sites (including 8-OHdG) induced in Chinese hamster cells (16). In the hamster cells, the DNA damaging photosensitizer seemed to be photodegraded since the yield of damage decreased sharply with a fluence of visible light above 20 kJ/m² (16).

The amount of 8-OHdG induced by UVA varies substantially between exponentially growing and confluent, non-growing fibroblasts, melanoma cells, lymphoblastoid cells (Figure 4) as well as human HeLa cells (14). It seems unlikely that these large differences can be entirely explained by variations in intracellular antioxidants that prevent the damage. Hence, it appears that the composition or concentration of photosensitizers varies much with the cell type as well as with the state of growth of the cell. It is remarkable that non-growing cells tend to suffer more DNA damage than growing cells. This suggests that DNA-damaging photosensitizers are present in non-growing cells. Thus, the heme precursor protoporphyrin IX could be one of the photosensitizers involved since its concentration was significantly higher in non-growing than in growing fibroblasts (data not shown). It is also tempting to speculate that melanin may play a role in the induction of 8-OHdG in the growing melanoma cells (Figure 4) which contained melanin (1 pg per cell). Certain forms of melanin or melanin precursors are known to have photosensitizer properties (30). We are currently investigating whether this is relevant *in vivo*.

What role does 8-OHdG have in solar mutagenesis? It is clear that mutations induced by wavelengths from 254 nm up to 320 nm in cells in culture are to a large degree caused by direct DNA damage including pyrimidine dimers (25,31). The most frequent type of point mutation induced in this wavelength region is G to A transitions located at dipyrimidine sites (32,33). These mutations are not frequently induced with 8-OHdG (34). However, UVA or visible wavelengths contribute significantly to solar mutagenesis (32). Thus, mutations at AT base pairs are much more frequent with UVA or sunlight than with UVC or UVB irradiation (32,33). The mechanisms of DNA damage causing these mutations at AT base pairs are not known. However, 8-OHdG can generate mutations at AT base pairs next to the damaged guanine base (34). On the

other hand, 8-OHdG or other singlet oxygen induced guanine damage have been found to cause mainly G to T transversions (10,11,35) which is a minor type of mutation after UV or solar irradiation of cells. Taken together, the data for point mutations indicate the complexity of mutagenesis, and the data are not conclusive for the role of 8-OHdG or other oxidative DNA base damage in solar mutagenesis.

The mechanism of generation of melanoma skin cancer appears to be different from that of non-melanoma skin cancer (7): Episodic high-dose exposure to sunlight at an early age (36) and a lack of *p53* gene mutations (37) are among the features which set melanomas apart from other types of skin cancers. Furthermore, from the experimental Xiphophorus fish model, there is evidence that the UVA component of the solar spectrum might be the major cause of melanoma (38). It is therefore important to investigate to what degree premutagenic oxidative DNA base damage, which at least in fibroblasts is most frequently generated by UVA and near visible radiation (Figure 6), contributes to the generation of melanoma skin cancer.

Acknowledgements

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