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Differences in Activation of G2/M Checkpoint in Keratinocytes after Genotoxic Stress Induced by Hydrogen Peroxide and Ultraviolet A Radiation

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Long-wave ultraviolet radiation (UVA) may cause extensive DNA damage via reactive oxygen species (ROS). In this study we examined whether UVA- and H_2O_2-mediated DNA damage have equivalent effects on the induction of G2/M phase checkpoint and cell cycle progression in a transformed keratinocyte cell line HaCaT. By employing single cell gel electrophoresis (comet assay) we determined the equipotent doses of UVA and H_2O_2 with respect to the induction of alkali-labile sites (an indicator of oxidative DNA decay). However, in contrast to H_2O_2 which caused a pronounced G2/M cell cycle arrest 24 h after treatment, UVA irradiation did not affect cell cycle progression. Increasing UVA doses up to 150 kJ/m^2 did not affect cell cycle and proliferation whereas increasing H_2O_2 concentrations caused a cell cycle block or cell death. Cytometric analysis revealed that G2/M cell cycle arrest took place beyond the cyclin B1 restriction point. We conclude that the DNA damage induced by UVA is easily repaired and does not perturb cell growth, whereas the H_2O_2-induced damage leads ultimately to cell cycle arrest or cell death.

Keywords: Ultraviolet radiation, hydrogen peroxide, keratinocytes, DNA damage, cell cycle

Abbreviations: DSB, double strand breaks; FITC, flourescein isothiocyanate; LSC, laser scanning cytometry; PI, propidium iodide; ROS, reactive oxygen species; SSB, single strand breaks; UVA, ultraviolet radiation A (320–400 nm)

INTRODUCTION

There is substantial experimental and epidemiological evidence that short-waved UV radiation (UVB) is a complete carcinogen involved in the pathogenesis of both melanoma and non-melanoma skin cancer (for review see Ref. [1]). Conversely, very little is known about the carcinogenic effect of long-waved ultraviolet radiation (UVA). This issue is of a high practical

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importance since UVA comprises about 95% of solar ultraviolet energy and is difficult to filter out with traditional sun blockers. In mouse skin only extremely high doses of UVA may induce skin cancer. Experimental evidence suggesting the relevance of UVA for melanoma induction [2] (for review see Ref. [3]) turned out to be difficult to extrapolate into humans.

Important evidence supporting the causative role of UVB in skin cancer development has been gained by the analysis of its genotoxic and mutagenic properties. The predominant type of UVB-mediated DNA damage are cyclobutane pyrimidine dimers which are repaired by nucleotide excision. Improper repair leads to the formation of a characteristic mutation pattern at dipyrimidine sites involving C→T substitutions (UVB footprint) which has been found in several oncogenes (e.g. p53) involved in skin carcinogenesis.[4–7] UVA that unlike UVB is not absorbed by nucleic acids exerts the genotoxic effect via reactive oxygen species (ROS). UV irradiation causes an immediate intracellular synthesis of singlet oxygen and H_2O_2 the latter being further decomposed into a highly reactive, but short-lived, hydroxyl radical ($HO^{\bullet}$). There is compelling evidence that ROS-species cause oxidative DNA damage.[8–11] However, the carcinogenic importance of oxidative DNA damage turned out to be much more difficult to scrutinize. Many different types of base modifications have been described, most often changes at A/T base pairs[12] but no clear-cut "UVA footprint" has been found. DNA damage exerted by UVA is extensive, but DNA is quickly repaired in most cells and mutations occur very rarely.

DNA damage often results in alterations of the cell cycle. Proliferating cells react to DNA damage by cell cycle arrest, most commonly at the transition from G1 to S phase (the G1 checkpoint) and/or from G2/M to M phase (G2/M checkpoint). The arrest at checkpoints prevents the replication of damaged DNA and is thus a mechanism that prevents mutagenesis. The G1 checkpoint is mostly regulated by p53 which is induced directly by DNA damage and activates the cell cycle inhibitor $p21^{Cip1/WAF}$ [13–15] Evidence for the pivotal role of p53 in G1 arrest has been specifically found in keratinocytes.[16,17]

A different molecular machinery operates at G2/M checkpoint. The targeted protein is Cdc2 that after complexing with cyclin B allows G2-M progression.[18–20] One of the pathways leading to Cdc2 inactivation is that mediated by a protein kinase Chk1 that phosporylates and inhibits the function of the protein phosphatase Cdc25C which in turn removes inhibitory phosphates from Cdc2.[21–23] A closely related pathway is mediated by proteins CdS1,WEE1 and MIK1.[24–27] Activation of p53-$p21^{Cip1/WAF}$ axis is not required for induction of G2 checkpoint, but seems to sustain the block allowing a more efficient DNA repair.[28] A cell cycle inhibitor $p16^{CDKN2A}$ has also been implicated in G2 arrest after UV irradiation.[29]

An immortalized keratinocyte cell line HaCaT [30] has a defective G1 checkpoint because of mutation in the p53 gene and therefore responds to genotoxic stress solely by G2/M cell cycle arrest. (Ref. [31], unpublished data) In order to further elucidate the role of UVA as a mutagen we determined here whether it is able to induce DNA damage of the magnitude requiring the checkpoint activation and whether UVA irradiation affects cell proliferation and survival. Since UVA may cause DNA damage via ROS generated from H_2O_2 the effect of UVA was compared with those observed after H_2O_2 treatment at equal genotoxic doses.

MATERIALS AND METHODS

Cell Culture

HaCaT keratinocytes is a commercially available cell line is derived from spontaneously transformed human keratinocyte [30] and was obtained from Dr Mark Pittelkow (Department of Dermatology, Mayo Clinic, Rochester, Minnesota, USA).

HaCaT cells were cultured in Dulbecco's minimal essential medium (DMEM; Gibco BRL, Life Technologies, Rockville, MD) supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco). Cultures were maintained at 37 °C in an atmosphere of 100% humidity and 5% CO_2. Cells were seeded in culture bottles at a density of 7×10^3 cells/cm^2 and experiments were performed on subconfluent cells. The cells were trypsinized with a 0.05% trypsin and EDTA solution (Biological Industries, Israel). Trypan blue exclusion assay was used to determine cell viability.

UVA Irradiation

The UV irradiation system used in this study consisted of a horizontal planar array of 7 Phillips TL 10R tubes (Phillips, Eindhoven, The Netherlands) with an intensity of 37 W/m^2. The TL 10R tubes emit 99.8% of the total UV radiation in the spectral region between 340–400 nm (UVA1) with a peak emission at 367 nm.[32] The emitted intensities were measured using an International Light (IL) 1700 research radiometer with a SED 400 detector as described previously.[33] The cells were irradiated from above in 60 cm^2 Petri dishes or 25 cm^2 tissue culture bottles through a glass window (3 mm thickness) and a 0.5 cm layer of phosphate-buffered saline (PBS) at doses of 0–150 kJ/m^2.

Laser Scanning Cytometry (LSC)

A slide-based laser scanning cytometer (CompuCyte Corp., Cambridge, MA) equipped with a 488 nm line argon laser excitation source, a BX50 Olympus microscope with a 20 × dry objective and a XC-75 Sony CCD camera was used. The LSC provides data equivalent to flow cytometry and enables quantitative measurement of fluorescence from single cells.[34] HaCaT cells were analyzed on multichamber slides prepared from cover-slipped standard microscope slides divided into chambers with double-adhesive Scotch 3M (St. Paul, MN) 467MP tape, as previously described.[35] Cell suspension was pipetted into a 5 × 20 mm (~40 µl) chamber and the chamber was sealed with nail polish. The propidium iodide (PI) and flourescein isothiocyanate (FITC) fluorescence was detected by photomultipliers equipped with 530 ± 30 nm and 625 ± 28 nm band pass filters, respectively. The LSC™ software (WinCyte™, CompuCyte) was used for evaluation of the data.

Cell Cycle Analysis

For a simultaneous analysis of DNA content and cyclin B1 expression the cells were harvested by trypsinization, washed with PBS and fixed in ice-cold ethanol (62%) at −20 °C overnight. Prior to staining ethanol was removed by centrifugation and the pellet was washed with PBS. The cells were permeabilized with 0.5% Triton X-100 for 5 min on ice, washed with 1% heat-inactivated bovine serum albumin (BSA) in PBS, and incubated for 30 min with 1:5 FITC-conjugated mouse anti-human cyclin B1 (Pharmingen, San Diego, CA) at room temperature in darkness. To determine the background staining, the cells were incubated under the same conditions with 1:5 FITC-conjugated mouse IgG1 monoclonal isotype control serum (Pharmingen). The cells were washed with PBS/BSA, and stained with 50 µg/ml PI in PBS with 200 µg/ml RNase for 0.5–2 h at 4 °C. The cells were subjected to LSC using the same hardware as described above. PI was used as a gating parameter and flourescence was collected in green and red channels.

Comet Assay

The cell preparation and gel electrophoresis were performed as previously described.[36–39]

In brief, non-confluent HaCaT keratinocytes were harvested 3–5 days after seeding, trypsinized and resuspended in PBS at a density of 3×10^5 cells/ml. The cells were treated with H_2O_2 at final concentrations up to 200 µM for 5 min and then cells (1.5×10^4 cells/slide) were

cast in low melting agarose (SeaPlaque, FMC, Rockland, ME) on custom frosted microscope slides. For experiments involving UVA irradiation the cells had to be irradiated directly in the agarose gels, which were later used in the comet assay, due to the very fast DNA repair kinetics in studies involving UVA irradiation. It should be noted that HaCaT cells need surface

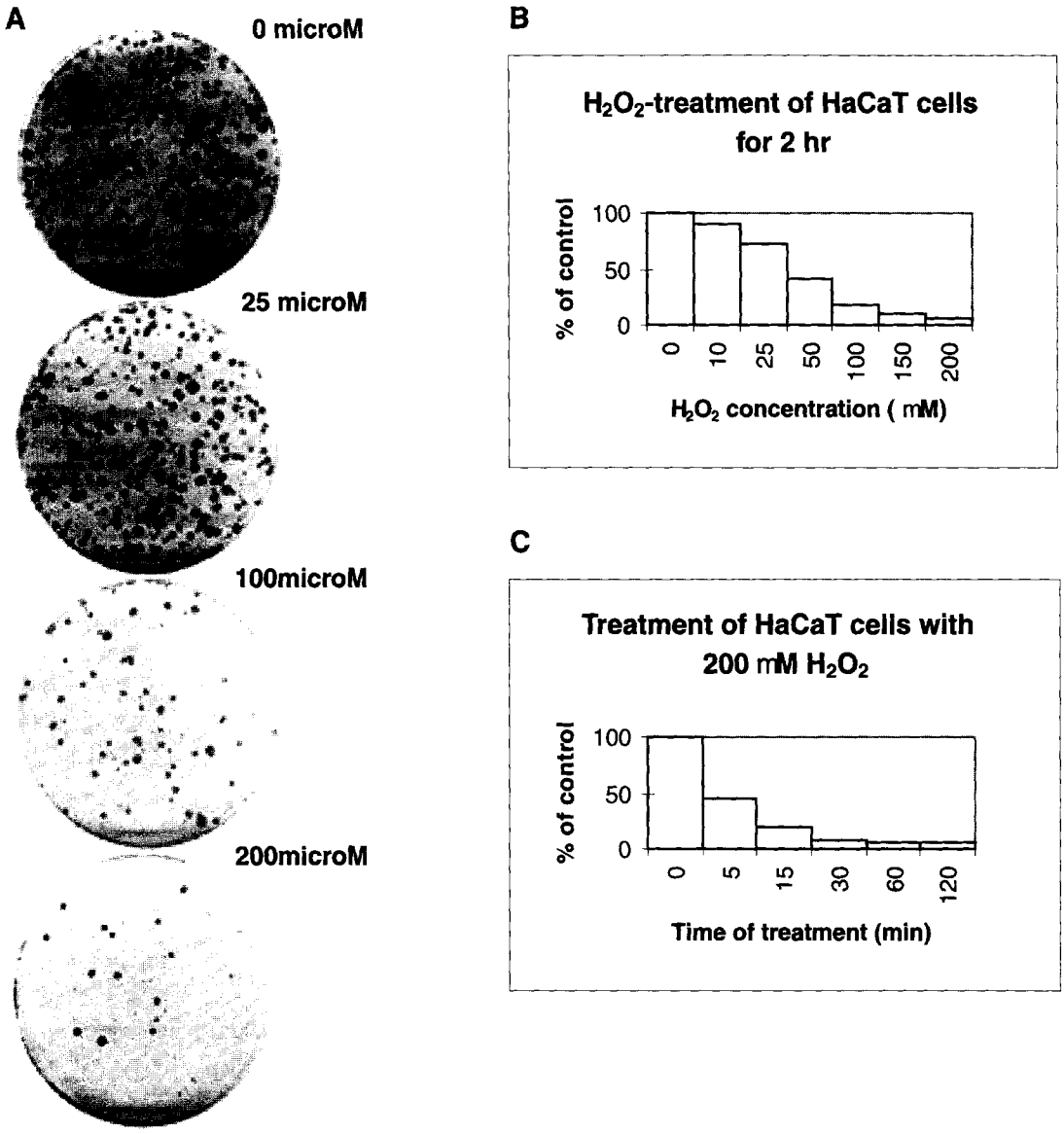


FIGURE 1 Influence of H_2O_2 on clonal growth. HaCaT cells were seeded at a density of 10 cells/cm^2, allowed to attach for 5 h and treated with different concentrations of H_2O_2 (A, B) or with 200 μM H_2O_2 for different periods of time (C). After H_2O_2 treatment, the cells were refed with fresh growth medium and were further incubated for two weeks. The cells were then fixed with 2% paraformaldehyde and stained with 0.5% crystal violet in distilled water. Number of colonies is expressed as a percentage of the control, PBS-treated cultures. Bars represent mean values of two independent experiments.

attachment for cell growth and thus cannot proliferate in agarose gel suspension. The agarose-embedded cells were placed on an ice-cold plate and irradiated with UVA (0–76 kJ/m^2). Immediately after the treatment the slides were immersed in a cold lysis solution (2.5 M NaCl, 100 mM EDTA, 1% N-laurylsarcocine, 10 mM Tris-base, pH10 with 10% DMSO and 1% Triton X-100) at 4 °C for at least 1 h to remove cytoplasm, membranes and most nuclear proteins. DNA was unwound for 20 min in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13.2) at 4 °C. Electrophoresis was conducted at 20 V, 300 mA for 20 min at 4 °C. After neutralization in 400 mM Tris-HCL, pH 7.5 the slides were stained with 50 μg/ml PI in PBS with 200 μg/ml RNase at 4 °C overnight. Tail moment of the comets corresponding to the degree of DNA damage, was calculated using the Euclid Comet Analysis software (Euclid Analysis, St. Louis, MO, USA).

RESULTS

Effect Of H_2O_2 and UVA on Cell Viability and Clonal Growth

HaCaT cells were tested for viability after a 2 h treatment with various concentrations of H_2O_2 by the trypan blue exclusion assay. $H_2O_2 \leq$ 200 μM had no effect on cell viability (>95% surviving cells) whereas concentrations >200 μM were cytotoxic to the cells (<50% surviving cells). These data are in agreement with previous results on fibroblasts.[40] By the trypan blue exclusion assay we found that UVA doses up to 150 kJ/m^2 did not have any effect on cell viability (>95% surviving cells).

H_2O_2 interfered with clonal growth in a time and dose-dependent manner (Figure 1A–C). Treatment with 10–200 μM H_2O_2 for 2 h caused a dose-dependent decrease in the number of colonies. The effect of H_2O_2 was rapid, since a pulse treatment for 5 min resulted in a decrease

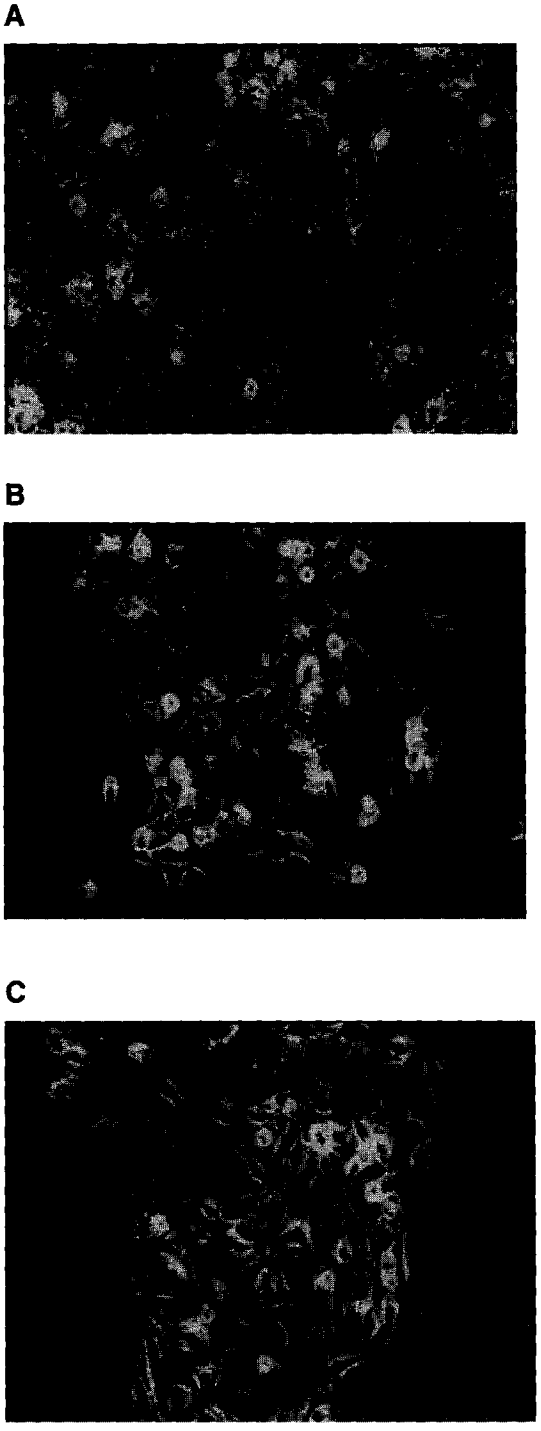
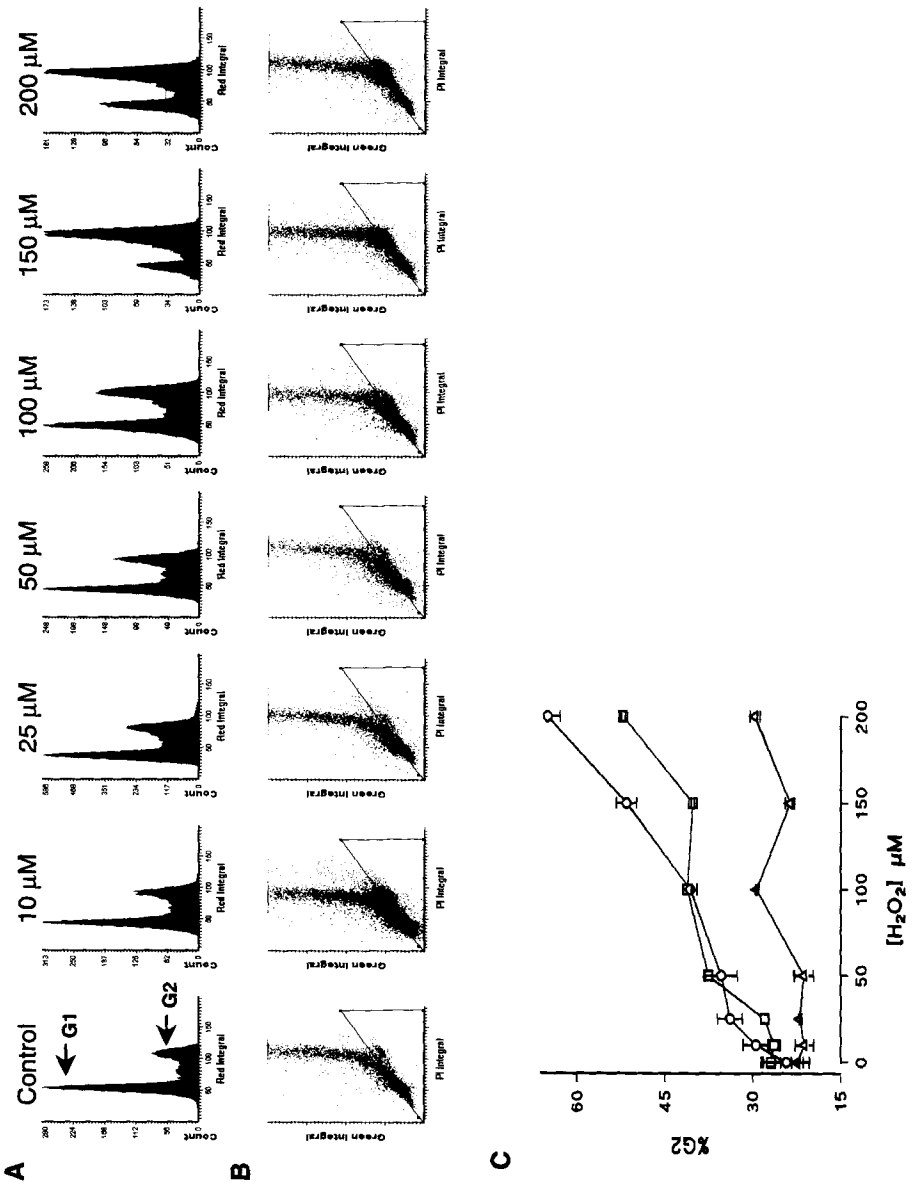


FIGURE 2 Morphology of H_2O_2-treated HaCaT colonies. Exponentially growing cells were treated with PBS (control cells, A) or 100 μM H_2O_2 for 2 h (B, C) and photographed after 48 h. Note an increased number of detaching cells in B and C.



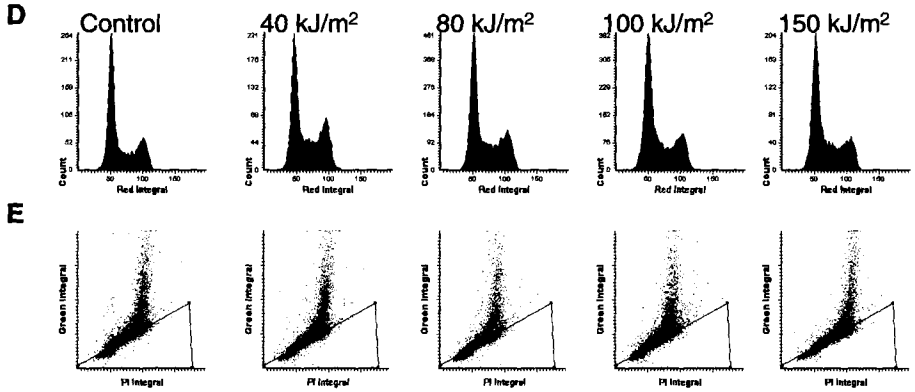


FIGURE 3 Cell cycle analysis of HaCaT cells treated with UVA or various concentrations of H_2O_2. Cells were seeded at a density of 7×10^3 cells/cm^2, grown to ~60% confluence and treated with UVA (A, B) or H_2O_2 (D, E) as indicated. The cells were grown overnight in culture medium, harvested by trypsinization and stained with FITC-conjugated anti-cyclin B1 antibody and PI, as described in Materials and Methods. DNA histograms (A, D) and cyclin B1 staining vs DNA content (B, E) are shown. The black lines in B, E demarcate the background staining determined by a non-specific IgG2b FITC-conjugated antibody. The position of G1 and G2 peaks are indicated in A. The experiment was reproduced three times with similar results. C – changes in the number of cells in the G2 phase after treatment with various concentrations of H_2O_2. The cells were analyzed 1 day (circles), 2 days (squares) or 5 days (triangles) after H_2O_2 treatment. Symbols represent means with SD.

in the number of colonies. There was a decrease in the number of colonies with increased duration of H_2O_2 treatment until ~30 min exposure after which the effect stabilized and was comparable to that seen after 2 h exposure.

In an attempt to determine the cause of the decrease in clonogenic potential the fate of H_2O_2-treated cells was followed daily. Forty-eight hour after treatment with 100–200 μM H_2O_2 a detachment of cells was noted (Figure 2). The cells had a characteristic morphology with rounded shape and condensed nuclei suggesting apoptosis. During clonal growth we detected cells that remained attached to the dish but did not proliferate. Thus both cell death and growth-arrest contributed to the decreased clonogenic potential after H_2O_2-treatment. However, the surviving cells proliferated at a normal rate since H_2O_2 did not significantly affect the number of population doublings (11.6 vs 10.1 population doublings in the controls and H_2O_2 (200 μM, 2 h) treated cells). The size of the colonies derived from H_2O_2-treated cells did not differ from the control.

UVA treatment (0–150 kJ/m^2) had no adverse effect on HaCaT clonal growth. The capacity to form colonies was the same in both UVA-treated and untreated HaCaT cells.

Effect of H_2O_2 and UVA on Cell Cycle of HaCaT Keratinocytes

Cell cycle distribution was assessed at various times after H_2O_2 or UVA-treatment. When non-confluent HaCaT cells were treated with various concentrations of H_2O_2 for 2 h, a dose-dependent G2/M block occurred (Figures 3A and B). This block was reversible since >2 days after the H_2O_2 treatment the cells began to re-enter the cell cycle showing an almost complete recovery 5 days after the H_2O_2-treatment (Figure 3C).

To map the observed G2/M block we examined expression of cyclin B1. The H_2O_2 (200 μM, 2 h) treated cells had a similar expression of cyclin B1 in the G2/M phase as the control cells (Figure 3B). This indicated that the cells were arrested at a very late G2/M phase after the cdc2/cyclin B1 restriction point.

The cell cycle distribution and cyclin B1 expression was not affected by UVA in doses up to 150 kJ/m^2 (Figures 3D and E).

Capability of H_2O_2 and UVA to Induce DNA Damage

To investigate whether the differences in the biological effects of UVA and H_2O_2 were caused by different abilities to induce DNA damage, we performed comet assay to determine the amount of single strand breaks (SSB), double strand breaks (DSB) and alkali-labile sites introduced during oxidative DNA damage by these agents. By calculating tail moment of comets from H_2O_2 or UVA-treated HaCaT cells we found that 100 μM H_2O_2 induced the same amount of DNA damage as 76 kJ/m^2 UVA irradiation, while 25 μM H_2O_2 had a genotoxic activity of 47 kJ/m^2 UVA irradiation (Figure 4).

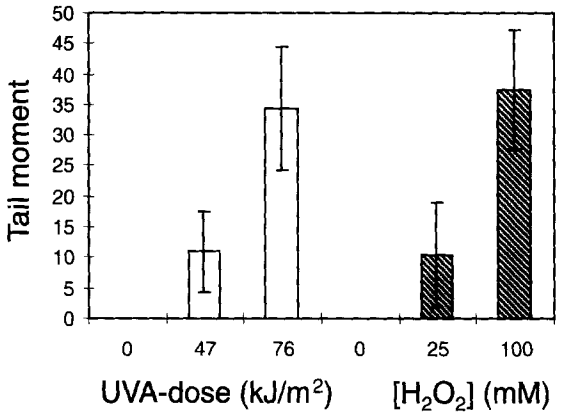


FIGURE 4 Comparison of the DNA damage induced by H_2O_2 and UVA. The cells were embedded in agarose for comet assay and treated with H_2O_2 (0–200 μM) for 5 min or irradiated with UVA (0–76 kJ/m^2). Only equal genotoxic doses of UVA and H_2O_2 with respect to the induction of alkali-labile sites are shown. The tail moment is correlated with the amount of alkali-labile sites per nucleus. Intervals on the bars are standard deviations (SD) of three repeated experiments. SD is based on all images scored.

DISCUSSION

Hydrogen peroxide is known to induce G2/M block in prokaryotic and eucaryotic cells, such as *Saccharomyces cerevisiae*,[41] fibroblasts[42] and hamster ovary cells.[43] Hydrogen peroxide does not interact directly with DNA,[44] but it gives rise to hydroxyl radical that produces single and double strand breaks and abasic sites.[45–47] As shown here by alkaline comet assay, H_2O_2 produced extensive DNA damage in HaCaT cells and induced G2/M block after the mitotic cyclin B1 restriction point. Despite the presence of p53 gene mutation in HaCaT cells and taking into consideration that ROS-induced damage is repaired within 1–2 h,[38] the G2/M block was relatively long-lived (24 h). The release from the block was associated with a massive cell death. Besides cell death, a permanent arrest in G2/M phase was probably also caused by H_2O_2 since microscopic inspection of the dishes seeded for clonogenic assay revealed the presence of single, non-proliferating cells. The cells that resumed proliferation after H_2O_2-induced oxidative stress grew at a normal rate forming colonies of the size comparable to those of the control cells.

Irradiation of the cells with UVA led to extensive DNA damage as detected by comet assay. There is evidence suggesting that this genotoxic effect is largely mediated by H_2O_2. First, UVA irradiation of HaCaT cells causes an intracellular synthesis of H_2O_2 from superoxide.[48] Second, the genotoxic effect of UVA is blocked by diethyldithiocarbamate (inhibitor of superoxide dismutase) and desferrioxamine (iron chelator) but not the singlet oxygen scavenger NaN_3, indicating that H_2O_2 is, at least partially, converted into $^{\bullet}OH$ which attacks DNA.[49] Therefore, we found it surprising that unlikely H_2O_2, UVA was unable to affect cell cycle progression at the comparable genotoxic doses. UVA irradiation was neither able to activate the G2/M checkpoint nor to affect cell survival and clonal growth of HaCaT cells. However, the genotoxic stress of comparable magnitude induced by H_2O_2 resulted in G2/M cell cycle arrest.

The present results reveal a fundamental difference in the nature of the oxidative stress induced by UVA and H_2O_2. A similar phenomenon has recently been observed in *Saccharomyces cerevisiae* where H_2O_2 was a potent inducer of G2 block whereas the exposure of the cells to superoxide caused a G1 arrest.[41] It has been noted that *RAD9* protein is involved in cell cycle arrest after exogenous H_2O_2 but not in response to superoxide, which means that the cells activate different defense mechanisms depending on the source of ROS. Although the mechanism(s) of this phenomenon are unclear at present, one should mention several possibilities.

The alkaline comet assay used in this study is not specific for any particular type of oxidative DNA lesion but reflects the amount of single- and double strand breaks and alkali-labile sites, such as abasic sites of sugar backbone lesions. We chose this assay because all types of DNA damage detected by comet assay are known to be recognized by cell cycle checkpoint systems and induce cell cycle arrest. However, since comet assay does not allow distinguishing between different types of DNA lesions, we cannot exclude the possibility that UVA irradiation produces a different type of DNA damage detectable in comet assay yet easily repaired and not causing any cell cycle arrest.

Second, there are differences in time scale of oxidative stress between H_2O_2 treatment and UVA irradiation. H_2O_2 causes and immediate effect whereas the formation of ROS in response to UVA is more slow as requiring enzymatic reactions converting several ROS species into the DNA-attacking hydroxyl radical. It is possible that this factor plays a role in the cellular effect of oxidative stress.

In contrast to H_2O_2 that induces hydroxyl radical as the main ROS species, UV induces different types of ROS, including superoxide and singlet oxygen. It is possible that the latter radicals induce additional defense mechanisms,

such as the recently demonstrated SOS-like response in eucaryotic cells,[50] leading to a more proficient repair of oxidatively damaged DNA. If true, this may reflect an evolutionary adaptation by natural selection favoring the development of UVA-protective mechanisms in sunlight-exposed keratinocytes ensuring efficient DNA repair and preservation of epidermal integrity. This hypothesis is corroborated by the fact that ultraviolet radiation, both UVA and UVB more readily causes cell cycle delay and cell death in the cell types that are normally shielded from UV radiation (lymphocytes, fibroblasts, kidney cells) than in keratinocytes.[12,51,52] It will be fascinating to inquiry into the potential role of UV defense mechanisms in the processes of mutagenesis and carcinogenesis in the skin.

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