UVA-induced apoptosis studied by the new apo/necro-Comet-assay which distinguishes viable, apoptotic and necrotic cells


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Introduction

The genotoxic and cytotoxic effects of solar radiation on cells have been extensively studied (1–3). In particular, the ultraviolet component of solar radiation (UVR) causes DNA damage in individual cells and thus increases the workload for DNA repair enzymes. Because UVB (280–320 nm) radiation can cause the formation of DNA lesions by direct interaction (1) UVB has been considered to be more damaging to cellular DNA than UVA (320–400 nm). However, it has become clear that exposure to UVA can also cause DNA base lesions and adducts such as 8-oxo-guanine and abasic sites via reactive oxygen species (ROS) reactions (4), and its effects can lead to persistent genetic instabilities such as micronuclei (5).

To counter DNA damage induced by UVR, mammalian cells have evolved primary defense strategies that include enzymatic and non-enzymatic antioxidants to quench or neutralize ROS (6), as well as secondary defenses such as DNA repair and apoptosis (7). Nevertheless, exposure to UVR can result in persistent DNA damage, mutations and necrosis (8).

In the study reported here a variety of methods including single cell gel electrophoresis (SCGE) (otherwise known as the Comet-assay) (9,10) was used to plot a time course for necrosis and apoptosis and to examine the repair kinetics in human keratinocytes (HaCaT) following exposure to UVA radiation.

Both necrosis and apoptosis contribute to decreased cell survival and reduced clonogenic colony forming ability. Necrosis is an unregulated event that comprises the release of cellular components that can result in localized inflammation. In contrast, apoptosis leads to an organized elimination of cellular components (11) and is defined by some key events which are controlled by pro- and anti-apoptotic regulators (11). Common events in apoptosis include chromatin condensation (12), translocation of phosphatidyl serine (PS) to the outer cell membrane (12), proteolytic activation of caspases (11), cytochrome c release from mitochondria (13), DNA fragmentation (14) and membrane blebbing. All of these events can be used as markers in the detection of apoptosis. Apoptosis is tightly regulated by a number of distinct pathways and can be induced after a cell has experienced DNA damage (15–19).

The Comet-assay is especially sensitive to the presence of cells undergoing apoptosis and necrosis. This is because apoptotic DNA fragmentation can lead to an overestimation of the DNA damage induced by genotoxins (including UVA), and necrosis may result in an underestimation of DNA repair capacity. Also with the Comet-assay technique that is widely in use today it is not possible to determine comets formed from cells undergoing apoptosis or necrosis; therefore, a large portion of this paper is devoted to an adaptation of the Comet-assay that enables such differentiation. This adaptation is based on the staining of individual cells for specific biochemical changes associated with apoptosis and necrosis or otherwise normal viability, which could then be compared with the comets formed subsequently by the same individual cells. This was made possible because the cells were stained while embedded in the agarose gel, after they had been exposed to UVR and before they had been placed in lysis solutions, i.e. before they had been processed in the Comet-assay (20).

During this stage the cells could be immuno- or cyto-stained as appropriate. Hence, by examining the viability status of individual cells with the comets subsequently formed from the
same cells it was possible to correlate the pattern of DNA fragmentation with normal cells or cells undergoing early, mid- or late apoptosis and/or necrosis.

While facilitating the investigation into the time course of UVA-induced apoptosis this adaptation to the Comet-assay also enabled the question to be answered of whether DNA fragmentation induced by apoptotic events interferes with the detection of strand break repair using the Comet-assay.

**Materials and methods**

All chemicals were purchased from Sigma Aldrich, unless stated otherwise.

**Cell culture**

The human keratinocyte cell line HaCaT was kindly provided by P. Boukamp and N. Fusenig, DKFZ Heidelberg, Germany (21) and was maintained in Roswell Park Memorial Institute 1640 medium (RPMI), 10% fetal calf serum (FCS), 5% CO2 and 95% humidity at 37°C. Cells were harvested by trypsinisation. Cell viability was routinely controlled before the experiments using the trypan blue exclusion test of cell viability. Briefly, cells were incubated in 0.4% trypan solution (1:1 vol/vol) for 5 min and scored as membrane damaged if the cells were stained blue. Cell cultures were also routinely controlled for mycoplasma contamination by DAPI staining.

**Irradiation**

Cells were irradiated in an adherent state maintained in pre-warmed phosphate-buffered saline (PBS) using a 300 W Osram Ultraviolet® bulb. The emitted light was filtered by the two colour glass filters Ug-11 and Kg-1 to select the UVA spectrum (peak at 365 nm, no detectable emission below 320 nm). Fluency rate was measured at 0.5 J/m² and spectral intensity was characterized using a Solarcube UV-spectroRadiometer (Solaselt, Croydon, UK). Temperature was controlled and kept constant (36.6 ± 1°C) during irradiation by a two-chamber air vent system. The doses in the experiments described ranged from 160 (5 min exposure) to 1280 kJ/m² (40 min exposure).

**Hyperthermic induction of apoptosis**

To test the newly developed technique, apoptosis was induced in HaCaT cells by a hyperthermic (heat) treatment and necrosis was simulated by vortexing another set of cells. These cells were embedded in agarose on a microscope glass slide and exposed to the three-colour staining solution described below to analyse the viability status of the cells before proceeding with the Comet-assay.

**Etoposide treatment to induce apoptosis**

HaCaT cells were grown in 6 cm Petri dishes to a confluence of ~70%. Etoposide treatment was performed in full medium containing 5 μg/ml Etoposide final concentration. Cell exposure to Etoposide was performed for 1 h. The cells were then incubated in fresh (untreated) medium as described above for the Annexin-V staining with the exception that a 20 μM Annexin-V-FLUOS/ml, 6 μM calcein-blue–AM and 1 μM Ethidum–Homodimer were added to the staining solution. After 20 min, cells were lysed in alkaline lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% SDS, 10% dimethyl sulfoxide, 1% Triton X-100, pH 10.0) for 1 h at 4°C. Unwinding was also conducted at 4°C for 1 h in electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13.1). Electrophoresis was performed in a pre-cooled electrophoresis tank at 4°C with 1 V/cm resulting in a current of 475 mA. After electrophoresis, slides were neutralized in 400 mM Tris–HCl (pH 7.4) for 10 min. Total DNA was stained with 50% Sybr Green® (Molecular Probes), diluted 1:500, 50% antifade.

The ‘neutral’ Comet-assay, to detect DNA double strand breaks (DSBs), was conducted according to the protocol of Olive et al. (22). Embedding was performed as described above: lysis was conducted in neutral lysis buffer (EDTA 50 mM, SDS 0.5%, pH 8.0) for 4 h at 4°C. Slides were then equilibrated in neutral electrophoresis buffer (1× TBE), which was replaced before the slides were transferred to the pre-cooled electrophoresis tank. Electrophoresis was performed at 4°C for 30 min at 1 V/cm resulting in a current of 80 mA. After electrophoresis, the slides were counterstained as described above.

**Apoptotic detection**

Apoptotic cells were detected using two different fluorescent-based staining techniques:

(i) Three-colour viability/apoptosis/necrosis staining: The ‘Annexin-V-FLUOS’ (Roche) was used for the identification of cells with PS translocated to the outer membrane. This technique uses a FLUOS conjugated Annexin-V that binds the PS on the outer cell surface emitting a green fluorescence. The Annexin-V was combined with the viability stain calcein-blue–AM and the membrane integrity dye Ethidum–Homodimere (E–HD) (Molecular Probes Leiden), to identify necrotic cells with compromised membrane integrity. The staining solution containing 20 μL Annexin-V-FLUOS/ml, 6 μM calcein-blue–AM and 1 μM E–HD in 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl and 5 mM CaCl₂, Aliquots containing 60 μl of the staining solution were added to each slide which were then covered with a plastic coverslip and incubated at 37°C for 25 min. A total of 180 cells were scored from three independent, replicated experiments (60 × 3).

(ii) TUNEL staining: TUNEL staining was conducted using the ‘In Situ Cell Death Detection Kit, Fluorescin’ (Roche, Mannheim Germany), following the manufacturer’s instructions. A total of 400 cells were scored from two independent experiments (2 × 200).

**Combining the Comet-assay with viability staining**

The method described is based on cytological staining of living cells embedded in agarose prior to the lysis stage of the Comet-assay. The study was performed using ‘window slides’, which have a clear area to facilitate microscopy and an etched edge to enable the agarose to adhere. Cells were embedded as described above (agarose, culture). The agarose was briefly left at room temperature to solidify before the cells were incubated with the three-colour viability staining solution described above (apoptosis detection). The cells were analysed at five locations on the slide marked by a cross using a diamond-tip glass cutter on the underside of the clear slide area. After washing the cells twice in PBS the slides were further processed in the Comet-assay as described above (Comet-assay).

**Trypan blue dye exclusion test**

To determine the viability of the cells embedded in agarose and verify the efficacy of the three-colour staining solution, the cells were exposed to a trypan blue exclusion test. Briefly, cells were incubated in 0.4% trypan solution (1:1 vol/vol) for 5 min and scored as membrane damaged if the cells were stained
blue. As a positive control for the trypan blue exclusion test, cells were incubated in PBS containing 10 mg/ml saponin to perforate cell membranes.

Results

Development of the apo/necro-Comet-assay staining method

To establish a staining procedure for the discrimination of apoptotic and necrotic cells in the comet assay, we controlled the results of cytological staining (Annexin-V, membrane integrity and cytotoxic esterase activity) of agarose embedded cells exposed to various treatments. This enabled a comparison with the results obtained from cells grown on microscope glass slides and stained in suspension as well as morphological images of the cells embedded in agarose and stained with trypan blue to test for membrane integrity.

Figure 1 shows agarose embedded cells and exposed to the three-colour viability/apoptosis/necrosis staining solution before and after the Comet-assay. Each figure represents a different treatment regime, i.e. untreated cells [Figure 1, A(1–6)], vortexed cells [Figure 1, B(1–6)] and heat-treated cells [Figure 1, C(1–6)]. The individual colour images (1–3) represent the different stains that were used: calcine-blue–AM (DAPI-filter: Figure 1, A-1, B-1, C-1); Annexin-V-Fluos (FITC-filter: Figure 1, A-2, B-2, C-2); E–HD (Rhodamine-filter: Figure 1, A-3, B-3, C-3); the three-colour images are overlaid (Figure 1, A-4, B-4, C-4) and displayed along with an image of the resulting comets obtained subsequently after electrophoresis and having relocated the same position on the slide (Figure 1, A-6, B-6, C-6). The white lines represent the displacement vector between first and second imaging. Together with the fluorescence images phase contrast images are shown of parallel prepared slides showing the agarose embedded cells stained with trypan blue inside the gel (Figure 1, A-5, B-5, C-5).

The untreated cells (Figure 1A) show a bright blue fluorescence indicating a high cytoplasmic esterase activity (normal viability) and an intact membrane. No red or green fluorescence is visible (Figure 1, A-2 and A-3). This indicates that no apoptotic or necrotic cells are present. Further study revealed that a total percentage 2.1 ± 1.7 of apoptotic and 1.1 necrotic cells were detected in this control (untreated) population using the three-colour viability staining solution. Similar results (2.8 ± 1.1 apoptotic and 2.3 ± 0.9 necrotic) were found for control cells grown on a glass slide (non-agarose embedded) (images not shown). The comets formed from these cells show only slight DNA migration with an average DNA tail percentage of 9.8 ± 3.9. Also the trypan blue exclusion test shows that the embedded cells are not membrane corrupted and no blebbing is seen (Figure 1, A-5).

Figure 1B shows the effects of the three-colour staining solution on vortexed cells. The cells show a weaker fluorescent blue signal that indicates reduced esterase activity and therefore a decrease in viability (Figure 1, B-1). Both the Annexin-V and E–HD (green and red fluorescence) signals are seen from these cells (Figure 1, B-2 and B-3). This indicates that the cells have corrupted membranes. Under such conditions the PS residues on the internal membranes become accessible to Annexin-V resulting in a green signal and, because the cell is unable to prevent the binding of E–HD to nucleic acid, a red signal is observed, i.e. resulting in a double positive staining indicating necrosis. Control staining with gel embedded cells using trypan blue also indicates corrupted cell membranes. Nearly all cells (>95%) showed corrupted membrane integrity, which was also found in control experiments using non-embedded cells for analysis. Additionally, the trypan blue exclusion test reveals that embedded vortexed cells have a corrupted membrane. Thus, the three-colour viability stain and trypan blue exclusion test show comparable results. The comets developed from these cells did not show any DNA in the ‘head’ but only in the tail region (Figure 1, B-5). The fragmentation was so dispersed that it was not possible to analyse the comet using any conventional imaging systems.

Figure 1C shows the hyperthermically (heat) treated cells. On the left side the agarose embedded cells exhibit a bright green Annexin-V fluorescence indicating apoptosis (Figure 1, C-2). Because of decreased cytosolic esterase activity the blue fluorescence (Figure 1, C-1) was of a weaker intensity compared with the untreated cells (Figure 1A). No red fluorescence was visible which suggests that the cell membranes were intact and that the PS residues were externalized (translocated) and available for binding with Annexin-V (in keeping with the morphological features of cells undergoing apoptosis). Also the trypan blue stained cells embedded in gel show intact cell membranes with no dark cells (Figure 1, C-6). Again, these cells show a homogeneous staining pattern with ~60% of Annexin-V positive cells and ~5% of E–HD positive cells. No difference in the staining pattern was observed between the gel embedded cells and the cells grown on a slide. Morphological changes (e.g. blebbing) were neither visible on heat-treated cells in gel, nor did they show significant membrane rupture (controlled by trypan blue staining). The comets formed from the Annexin-V positive cells and the viable cell show a homogenous pattern of DNA migration (C-5). This could indicate that the detected DNA damage (% Tail DNA) was produced by the heat and not by apoptosis cleavage. The mean percentage DNA in the tail of the comets formed from the Annexin-V negative heat-treated cells was 82 ± 5.9% (mean of medians), whereas the mean percentage DNA in the comets formed from the Annexin-V positive cells after heat treatment was slightly higher (90 ± 5.3). This suggests that the heat treatment alone induces a significant level of DNA migration in the Comet-assay and that this is slightly enhanced by the initial apoptotic chromatin cleavage of DNAses.

Etoposide induced apoptosis

The topoisomerase inhibitor Etoposide was used as an additional (chemical) agent to induce apoptosis. HaCaT cells were stained after embedding in agarose, and alkaline comet assay incorporating the viability staining adaptation was performed. Figure 2 shows HaCaT cells treated with 5 μg/ml Etoposide at different incubation times after exposure. Figure 2 (A-1) shows untreated control cells together with the resulting comets (Figure 2, A-1). These appear viable with very little detectable DNA fragmentation. Three hours after exposure (Figure 2, B-1) the cells show an enhanced green fluorescence (Annexin-V), which indicates PS translocation. Nevertheless, a DNA fragmentation was not detectable (Figure 2, B-2); however, after 8 h (Figure 2, C-1) the cells show compromised viability (no blue staining) but enhanced Annexin-V and E–HD staining indicating late apoptosis with ruptured membranes. These cells also show a high degree DNA fragmentation (Figure 2, C-2).

Enlarged images of typical apo-comet, necro-comets and untreated control comets are shown in Figure 3, D–F. The upper images (Figure 3, D-1, E-1 and F-1) show the merged
result of a three-colour staining, and the lower image show the resulting comets.

Taken together, these results suggest that the staining procedure using calcein-Blue–AM, Annexin-V-Fluos and E–HD can be applied on agarose embedded cells or cells grown on slides. It is also possible to use this method to detect apoptosis and necrosis as well as the DNA fragmentation induced by apoptosis.
DNA damage induction by hyperthermic treatment

Having established that this adaptation to the Comet-assay was effective in identifying DNA damage in cells undergoing apoptosis and necrosis, the method was used to investigate the time course of apoptosis induction and/or normal DNA repair in heat-treated HaCaT cells. Heat treatment was the preferred choice of apoptosis induction for this investigation since HaCaT cells did not respond well to chemical-induced apoptosis. However, this technique was controlled using other cell types and by inducing apoptosis with etoposide and bleomycin (data not shown). Figure 3 illustrates the time taken for HaCaT cells to repair DNA damage or apoptosis induced by heat treatment.

Figure 3 also illustrates the comet types formed by cells undergoing the process of repair or apoptosis over time. In Figure 3A the untreated control cells are shown. No Annexin-V positive cells are visible. The resulting comets show a low degree of DNA fragmentation (mean: 5.9 ± 3.2% DNA in tail). Figure 3B displays cells 1 h after heat treatment. DNA migration was detected using the comet-assay in all cells independent of Annexin-V status. The Annexin-V positive cells did not show a significantly higher level of DNA migration compared with the Annexin-V negative cells, which suggests that the fragmentation detected was caused by the heat treatment and not as a consequence of apoptosis.

Figure 3C shows comets from Annexin-V positive and negative cells 3 h after the Annexin-V status was measured (4 h after the heat treatment). From this sample a mixture of apoptotic and viable cells could be distinguished on the basis of the staining pattern (Annexin-V positive or negative) and by the comets subsequently formed, e.g. the comets formed from the cells in advanced stages of apoptosis were barely visible (ghost comets). The brightness of the two Annexin-V positive cells in this example image has been enhanced to enable clearer visualization of the DNA. Most of the DNA has migrated during electrophoresis leaving barely detectable comets with a high degree of DNA damage (% tail DNA). Such comets cannot be analysed using conventional image analysis software. The first of the Annexin-V positive cells shows total fragmentation (indicated by the white arrow), where in contrast the second comet was only moderately fragmented; this may represent initial chromatin fragmentation. In addition, the Annexin-V negative cells showed a significantly reduced level of % tail DNA 4 h after treatment compared with the comets formed 1 h after treatment. This indicates active repair of the initial DNA damage in a normal (viable) cell. Finally, 6 h after the treatment the Annexin-V negative cells appear to have completed the repair process (Figure 3D). This is in contrast to the Annexin-V positive cells which show complete migration of the DNA (the comet of the Annexin-V positive cell in the upper part of the image is enhanced for clarity and this is indicated by the dotted square).

Taken together, the results of these experiments show that the newly described staining technique can follow the events of apoptosis as well as the fragmentation pattern of comets after apoptotic induction.
Apoptosis, necrosis and clonogenic survival of HaCaT cells after UVA exposure

After the adaptation to the Comet-assay was established the overall time course for apoptosis following UVA in HaCaT cells was investigated. Hence, HaCaT cells grown on a slide and exposed to a single UVA exposure ranging from 0 to 1280 kJ/m² were examined for PS translocation and compromised membrane integrity. These results were used to calculate clonogenic survival.

Apoptosis and necrosis during UVA irradiation

Apoptosis and necrosis were measured in HaCaT cells grown on microscopic slides using Annexin-V/E-HD staining. Figure 4A shows the induction of necrosis (open circles) during UVA exposure using the Annexin-V/E–HD detection method. Cells showing signs of apoptosis (filled circles) were not detectable for up to 40 min UVA exposure or 1280 kJ/m² UVA, respectively. In contrast, the number of necrotic cells increased significantly at fluencies >1000 kJ/m², resulting in the induction of necrosis in >50% of the cells. A sample image of cells stained with Annexin-V/E–HD during UVA exposure is shown in Figure 4D together with unexposed cells (Figure 4C). This result is also reflected in the colony forming plot (Figure 4A, crosses), which shows a decrease in the cell survival rate at doses >1000 kJ/m².

Time course of apoptotic events

From the initial (UVA dose finding) experiments described above a single exposure of 960 kJ/m² of UVA was considered the most appropriate to follow the time course of apoptosis in HaCaT cells, since this dose results in a survival of >80% and an intact membrane in >80% of the cells. Annexin-V and TUNEL-analysis were conducted with cells grown on microscope glass slides. Additionally, DNA fragmentation was measured by the alkaline and neutral Comet-assay at comparable time points (without the three-colour viability staining solution). These measurements were performed for up to 24 h post-irradiation.

Annexin-V. The values of Annexin-V positive cells are plotted in Figure 4B (blue line). A peak of Annexin-V positive cells (24% of the population) was recorded 8 h after exposure. The number of apoptotic cells decreased 12 h after exposure and reached a level of <5% (comparable to the control), as indicated by the Annexin-V staining. The number of necrotic-positive cells was also elevated 8 h after exposure (~7%) (data not shown), although it may be possible that some of these ‘necrotic’ cells were late stage apoptotic cells showing a weak nuclear staining. Typical micrographs for Annexin-V/E–HD stained cells are shown in Figure 4E and F for 5 and 12 h post-exposure.

Neutral and alkaline Comet-assay.

DNA fragmentation induced by either the UVA exposure directly or by the apoptotic nuclease process, on the other hand, was investigated using the alkaline and neutral versions of the Comet-assay. The neutral Comet-assay—a technique that solely detects DSBs—shows a weak peak after UVA exposure direct, which reflects the direct DSBs induced by this radiation (23). A significant increase in DNA fragmentation (% tail DNA) was observed after 12 h with the alkaline Comet-assay (Figure 4B, red line). The initial induction of DSBs was reduced from 31% DNA in tail directly after irradiation to 12% 8 h

Fig. 3. Time course of the induction of apoptosis and the comets formed from cells after heat treatment and Annexin-V staining. The comets formed from Annexin-V positive cells are marked with A+ whereas the comets formed from the Annexin-V negative cells are marked with A− (the images of cells stained with Annexin-V are not shown in this series). (A) Comets formed from untreated control cells (with a negative Annexin-V staining). (B) Comets formed from a group of Annexin-V positive and negative cells one hour after heat treatment. No significant difference in DNA fragmentation patterns was observed. (C) Three hours after heat treatment DNA fragmentation can only be observed in one of the comets formed from the Annexin-V positive cells after image enhancement. The comets formed from the Annexin-V negative cell show a reduction in the amount of migrated DNA. (D) Six hours after the heat treatment the highly fragmented comet tail has migrated away from the head of the comet formed from the Annexin-V positive cell and again requires image enhancement for visualisation. In contrast the comet formed from the Annexin-V negative cell shows very little DNA fragmentation and migration.
post-irradiation. Then a second peak in DNA fragmentation was detected coincidentally with the apoptotic events indicated by the Annexin-V and TUNEL-assays.

The alkaline Comet-assay (black line, Figure 4B) also shows a strong peak directly after irradiation, which corresponds to the detection of DNA damage (direct dsb). This damage is efficiently repaired within the first 5 hours after UVR exposure. A second peak in DNA fragmentation can be found 12 hours post-irradiation, which coincides with apoptosis induction observed with the Annexin-V and TUNEL assays.

**TUNEL-assay.** The TUNEL-assay was performed in order to detect apoptosis after UVA exposure and is dependent on DNA fragmentation caused by DNAses during the apoptosis process. The data for the frequency of TUNEL positive cells are plotted in Figure 4B (green line). For non-irradiated HaCaT cells <1% of TUNEL positive cells were observed. After irradiation this increased to 2.3%. No significant increase in the number of apoptotic cells was recorded for up to 8 hours post-irradiation. An increase in the number of apoptotic cells was observed (19%) 12 hours after exposure but this decreased again after 16 hours to 4.3%. This decrease is probably due to elimination of the apoptotic cells. Typical sample images are shown in Figure 4G and H for control cells and 12 hours post-exposure, respectively.

Although fewer apoptotic cells were observed using the TUNEL-assay compared with Annexin-V staining, TUNEL-positive cells are detected at a later state in the apoptosis process. This late stage occurrence of TUNEL-stained cells
indicates that apoptotic DNA fragmentation, leading to DNA laddering (180–2000 bp), lags behind the Annexin-V translocation and occurs in HaCaT cells ~10–12 h after exposure to a lethal dose of UVA irradiation and cannot therefore interfere with the Comet-assay analysis of cells undergoing DNA repair after treatment.

DNA damage repair kinetics and interference with apoptotic fragmentation

Having established the time course of apoptotic events, the adapted Comet-assay incorporating the three-colour viability staining procedure was used to measure DNA repair following a single UVA exposure. Hence, cells were exposed to UVA, embedded in agarose and then analysed with the viability staining prior to comet lysis and electrophoresis and second analysis. DNA repair was investigated using the ‘normal’ alkaline Comet-assay. Figure 5 shows typical examples of the three-colour staining results and the comets subsequently formed. Control cells show barely detectable DNA fragmentation (Figure 5, 1-2) which becomes more apparent after UVA exposure (Figure 5, 2-2). Also the unexposed (control) cells show a high viability (Figure 5, 1-1), which is indicated in the bright calcein-blue–AM fluorescence (Figure 5, 1-1). In contrast, some of the cells show corrupt membranes directly after exposure, while others are still viable (Figure 5, 2-1). With ongoing repair the DNA fragmentation is reduced (Figure 5, 3-2), again while cells are still viable (Figure 5, 3-1). The cells emit a green Annexin-V signal 8 h post-irradiation indicating the onset of apoptosis (Figure 5, 4-1); in contrast, the corresponding DNA fragmentation has reduced to control levels indicating DNA repair is complete (Figure 5, 4-2). The resulting DNA repair curve is shown in Figure 4B (inset). An exponential fit function indicates a $T_{1/2}$ for the DNA damages of 75 min.

These results show that the DNA repair measurements are not influenced by endogenous DNA fragmentation due to
apoptosis, which is activated in these cells at around 12 h post-exposure (see Figure 4B).

Discussion

The Comet-assay was one of the main techniques used to investigate the time course of apoptosis and necrosis and examine the DNA repair kinetics in HaCaT cells after exposure to UVA radiation. While the Comet-assay in its present form is widely used to investigate DNA damage induced by genotoxins such as UVA radiation, the data produced can be subject to misinterpretation and bias due to comets formed from cells undergoing apoptosis and necrosis. This is because it has not been possible to distinguish comets formed from viable cells from those formed from non-viable (apoptotic or necrotic) cells. The adaptation to the Comet-assay presented here, however, does allow viable, apoptotic and necrotic cells to be identified on the basis of individual cells. This adaptation has been used to study the time path of UVA-induced apoptosis in cultured skin cells in more depth and with more precision than has been reported previously (24–30). The adaptation can also be used to investigate chemical-induced apoptosis, although heat (hyperthermic) treatment was the preferred method of this investigation because it provided a rapid means of inducing apoptosis in spontaneously immortalized cultured human keratinocytes (HaCaT) cells.

From the results it can be concluded that the observed fragmentation pattern for comets formed from apoptotic cells is more dependent on the progress of apoptosis rather than on the apoptosis process itself, i.e. the effect of apoptosis on the Comet-assay measurement of DNA damage depends on the time the cells are assayed after genotoxic exposure and apoptosis induction. Initial chromatin fragmentation during apoptosis can be monitored by the Comet-assay, as described earlier (24), because the technique has a fragment size resolution of ~10–100 kb in the standard alkaline version (31). However, subsequent fragmentation down to the nucleosomal multimers (~200–2000 bp) is outside of the detection limit of standard image analysis, and therefore cells undergoing the final stages of apoptosis with extensive DNA fragmentation produced by DNAses may not provide measurable comets. It should also be noted that this study was unable to produce comets that could (subjectively) be considered ‘hedgehogs’ or ‘clouds’; terms used to describe specific types of comets which have the typical appearance of a small head and large body or comets of low intensity, respectively, and which previously have been associated with cells undergoing apoptosis (32). It is possible, however, that such comet shapes could be associated with cells undergoing late stage apoptosis when DNA fragmentation is at an advanced stage but it is also true that this shape could be mistakenly associated with comets formed from viable cells that have experienced a high degree of DNA fragmentation during the normal repair processes. These results show that without supporting information it is a matter of subjectivity in distinguishing a comet formed from a cell undergoing apoptosis and one formed from a viable cell.

No visual correlation was observed between the fragmentation pattern and the necrotic state of the cell. The appearance of the comet was more influenced by the degree of DNA damage induced by the genotoxic agent than by the occurrence of necrosis. Necrosis is a consequence of compromised cell membrane integrity. This may or may not result in the spillage of intracellular and nuclear components; hence, it is unlikely that comets formed from these cells would provide an identifiable, common DNA fragmentation pattern.

These observations highlight the importance of this combinatorial technique that allows the Comet-assay to be used not only for the quantification of DNA fragmentation but also allows an assessment of the viability status of the cells from which the comets were formed. In respect of this we propose that terms such as ‘apocomet’ and ‘necrocomet’ are more apt than ‘hedgehog’ or ‘cloud’ when describing a comet when it is certain that it has been formed from a cell undergoing apoptosis or necrosis, respectively, as detected by immunostaining and/or cytostaining techniques or morphological analysis.

As mentioned this apo-/necro-Comet-assay technique was used to follow the time course of necrosis and apoptosis following exposure to UVA irradiation in HaCaT cells. The results show that UVA irradiation can induce necrosis and/or apoptosis in cultured human keratinocytes. If high fluencies (>1000 kJ/m²) are used necrosis is detected during irradiation. In contrast to the immediacy of necrosis apoptosis can only be detected ~8 h later. This observation is in keeping with what is known about apoptotic events. For example, the results show that PS translocation, enabling the binding of Annexin-V, was identified as a manifestation of early apoptosis ~8 h post-exposure, during the subsequent 4–6 h DNA fragmentation and membrane blebbing occurred.

Having established that this adaptation to the Comet-assay (apo/necro-Comet-assay) is effective in identifying the DNA fragmentation patterns of comets formed from cells undergoing apoptosis and necrosis, and having also established that the method was effective in helping to determine the DNA fragmentation time course of apoptosis and necrosis following exposure to UVA irradiation, it was also used to address the question of whether DNA fragmentation induced during apoptosis interferes with DNA repair studies and, *inter alia*, other Comet-assay based analysis. The results show that DNA fragmentation from apoptotic cells does not occur until after PS translocation, i.e. more than 8 h after exposure to high (>1000 kJ/km²) UVA doses. Since single strand DNA repair kinetics following exposure to UVA radiation is exponential with a repair half life of 75 min (23), and since it can be seen from the results that cells undergoing repair produce the same comets as that of control cells 8 h post-UVA exposure (suggesting repair has been completed), it can be concluded that comets formed from apoptotic cells do not interfere with the data produced by the DNA repair kinetics experiments reported here. In contrast, the results do show that it is possible for comets formed from cells undergoing necrosis to impact on data analysis, which gives weight to the importance of using the adaptation to the comet assay, i.e. which allows the distinction between non-viable and viable cells to be made before image analysis data processing.

Conclusions

The methods developed for this study provide an effective means of detecting apoptotic and necrotic cells prior to evaluating DNA damage induced by genotoxins, such as UVA radiation using the Comet-assay; a method that until now was sensitive to bias from comets formed from non-viable cells. In principle, this adaptation to the Comet-assay should be applicable to any fluorescent-based staining technique for identifying extracellular and/or intracellular markers. For example, in addition to the application presented here this method has
also been used in conjunction with an antibody-based cell surface dye to discriminate between different cell types in a heterogeneous sample (data not shown), i.e. an extension that can be used to differentiate DNA damage induced in specific cell types that form a heterogeneous population, using whichever version of the Comet-assay protocol is required, i.e. alkaline or neutral conditions.

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