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Cutaneous Biology

Antimicrobial photodynamic therapy: assessment of genotoxic effects on keratinocytes *in vitro*

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Summary

Background Work has shown that cutaneous microbial species associated with skin conditions of microbial aetiology are susceptible to killing by antimicrobial photodynamic therapy (APDT) using visible light and methylene blue.

Objectives To evaluate immediate and delayed genotoxicity of APDT on keratinocytes *in vitro*.

Methods A combination of methylene blue (100 μg mL^{-1}) and visible light (42 mW cm^{-2}), as used in studies of microbe and keratinocyte cytotoxicity, was employed to test a human keratinocyte cell line (H103) for genotoxic damage by comet assay.

Results The comet assay was able to detect genotoxic damage in H_2O_2-treated keratinocytes (positive control). APDT did not cause either immediate or delayed genotoxic damage in keratinocytes following APDT of up to 180 min.

Conclusions APDT sufficient to reduce microbes by seven log cycles showed no detectable genotoxic effects on keratinocytes. APDT applied *in vivo* may represent a useful low-risk alternative to conventional antimicrobial treatment in dermatology.

Key words: antimicrobial photodynamic therapy, comet assay, cutaneous microorganisms, DNA damage, genotoxicity, keratinocytes, methylene blue, safety

Bacterial resistance to antibiotics in humans and animals is causing concern worldwide.[1,2] An alternative therapeutic approach may be to use antimicrobial photodynamic therapy (APDT), which involves killing organisms by light in the presence of a photosensitizer. Excitation of the sensitizer by absorption of light of appropriate wavelength in the presence of oxygen converts the sensitizer to its photoactive triple state, which reacts either with a local substrate (type I reaction) to form cytotoxic radicals, or with molecular oxygen (type II reaction) to produce cytotoxic singlet oxygen ($^1O_2$) and free radicals. The reactive oxygen species generated lead to/induce cell death.[3]

We have shown that APDT sufficient to reduce microbes by seven log cycles has little cytotoxic effect on keratinocytes.[4] This implies that the method may be a useful alternative and/or adjuvant to antibiotics and antiseptics for microbe-associated skin disease. However, prior to testing *in vivo*, it is important to determine possible genotoxic effects of effective APDT on skin cells *in vitro*. To the best of our knowledge, no previous studies have examined the effects of methylene blue (MB) and light on potential DNA damage and repair in keratinocytes.

The present *in vitro* study investigated APDT against keratinocytes using the comet assay to determine genotoxicity. The data may indicate a therapeutic/dosing regimen whereby microbes could be killed effectively without genotoxic damage to keratinocytes.

Materials and methods

Antimicrobial photodynamic therapy

APDT using defined polychromatic visible light (with < 1% spectrum content of ultraviolet or infrared) and

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MB as a photosensitizer, and the methods for keratinocyte culture, have been previously described.[4]

Keratinocyte treatment

Cell suspensions ($\approx 10^6$ cells mL^{-1} final concentration) of the human keratinocyte cell line H103 were prepared in triplicate by adding 2 mL of cells, 1 mL of MB and 17 mL of phosphate-buffered saline to each Petri dish and mixing. Following a 5-min pre-irradiation period with MB, the test was exposed to visible light (42 mW cm^{-2}) and MB for a total of 180 min ($\approx$ 452 J), leading to an approximate 80% kill. The light alone and MB alone controls were exposed to light or MB, respectively, for the same periods of time as the test. Duplicate samples (1 mL) were harvested at times 0, 45, 90 and 180 min of light exposure. One sample was prepared directly for the comet assay to examine immediate effects and another prepared after a 4-h dark incubation period to examine delayed genotoxicity. For the latter, the medium containing MB was removed, cells were harvested by centrifugation (350 *g*, 5 min; Beckman, Luton, U.K.) and resuspended in cell culture medium for 4 h at 37 °C in the dark.

Comet assay

For the preparation of sample slides, 1·2% (w/v) low melting point (LMP) agarose (Sigma-Aldrich, Poole, U.K.) was prepared and dissolved in 10 mL aliquots, and molten agarose pipetted on to frosted microscope slides (1 mL per slide) and left to set at room temperature. Samples of cell suspensions (1 mL) were mixed with an equal amount of molten LMP agarose and pipetted on to the cooled base agarose (one sample per slide).

The slides containing samples were immersed in lysis mix buffer [2·5 mol L^{-1} NaCl, 100 mmol L^{-1} disodium ethylenediamine tetraacetic acid (Na_2EDTA), 10 mmol L^{-1} Tris and 10 mmol L^{-1} NaOH to pH 10, plus 10% dimethylsulphoxide and 1% (v/v) Triton X-100] for 2 h at 4 °C, allowing cell lysis to occur. Slides were left in alkaline buffer (300 mmol L^{-1} NaOH, 1 mmol L^{-1} Na_2EDTA, pH 12·6) for 1 h. Each slide was electrophoresed (Bio-Rad Laboratories Ltd, Hemel Hempstead, U.K.) at 20 V with a current setting of 275 mA for 24 min.

After two 5-min periods of washing with neutralization buffer (400 mmol L^{-1} Tris–HCl, pH 7·4) the slides were dried and stained with ethidium bromide solution (80 μg mL^{-1}) (approximately 0·2 mL per slide). Slides were kept for 1 h in the dark to complete the staining.

The numbers of intact and lysed cells and the extent of the DNA migration from individual lysed cells (comets) were observed through visualization using a confocal microscope (Nikon Europe BV, Badhoevdorp, the Netherlands). The degree of DNA damage from a sample of 200 lysed cells per slide was determined by observation, and categorized using a four-stage comet scoring system,[5] where 0 = no observable migration and 3 = maximum dispersion and migration of the stained DNA. Intact cells that had survived the slide preparation lysis were not included in the comet scoring.

To show that the comet assay was capable of detecting DNA damage, positive controls were included consisting of keratinocytes treated overnight at 4 °C with hydrogen peroxide (600 μmol L^{-1}).

Results

Peroxide-treated keratinocytes (positive control) showed genotoxic damage (Table 1). Most cells (87%) were lysed following slide preparation. Of 200 lysed

Table 1. Comparison of keratinocyte genotoxicity testing (the comet assay) both immediately and following a 4 h recovery period at 37 °C in the dark, using standard light intensity (25 cm light source = 42 mW cm^{-2}) and methylene blue photosensitizer at 100 μg mL^{-1}

	APDT treatment time (min)			
	0	45	90	180
Immediate comet assay				
% cell lysis[a]	90	80	80	90
Grade 0	100	100	100	100
Grade 1	0	0	0	0
Grade 2	0	0	0	0
Grade 3	0	0	0	0
The comet assay after 4 h recovery				
% cell lysis[a]	90	90	90	90
Grade 0	100	100	100	100
Grade 1	0	0	0	0
Grade 2	0	0	0	0
Grade 3	0	0	0	0
Positive control (H_2O_2)[b]				
% cell lysis[a]	87			
Grade 0	0			
Grade 1	0			
Grade 2	5			
Grade 3	95			

APDT, antimicrobial photodynamic therapy. [a]Lysis following slide preparation with lysis buffer. Intact cells (with observationally intact cytoplasmic membrane) were distinct from lysed cells (with a diffuse appearance) and were not comet graded. [b]Overnight incubation with H_2O_2 at 4 °C.

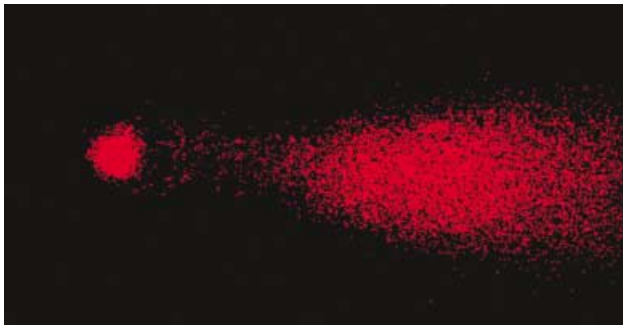

Figure 1. Comet grade 2 (from cells treated with hydrogen peroxide).

Figure 2. Comet grade 0 (from cells treated with antimicrobial photodynamic therapy).

cells, some 95% were comet class 3 and the remainder were typically class 2; no cells scored as class 1 or 0. The typical appearance of a class 2 comet is shown in Figure 1.

Treatment of keratinocytes by APDT followed by the comet assay showed cell lysis at 80–90% (Table 1). The comets were all class 0 for both immediate and 4-h dark-incubated samples and for all treatment times. The typical appearance of class 0 comets is shown in Figure 2.

Discussion

The comet assay used was reported to detect very low levels of DNA damage (both single and double strand) in individual cells.[6] Furthermore, it has been used following a cell recovery period to detect delayed damage (e.g. apoptosis) or repair.[7]

Keratinocytes treated with peroxide showed a high proportion of cells with a typical comet appearance and showed the technique capable of detecting DNA damage. None of the APDT-treated cells showed detectable signs of DNA damage. The cumulative total of scored cells was 600, suggesting that the DNA damage rate was lower than the detection limit of 0·165%. Although DNA damage is the first stage of cancer initiation, the relationship between mutagenicity and DNA strand breakage is not straightforward,[8] and only a small proportion of damaged cells becomes cancerous as most are repaired or killed.[9]

The proposed key toxic species during APDT is singlet oxygen capable of reacting with subcellular targets, including DNA.[3] MB is lipophilic and localizes predominantly in the cytoplasm and membranes.[10,11] As the diffusion distance of singlet oxygen in a cell is $< 0{\cdot}075$ μm,[12] it is unlikely that DNA is the primary target.

The skin contains other cells in addition to keratinocytes and it is possible that these types may be more susceptible to genotoxic damage. APDT using MB caused DNA damage in a human myeloid leukaemic cell line *in vitro*[13] and the genotoxic damage was repaired following dark incubation (4 h at 37 °C).

Those data from microbe[14] and keratinocyte killing[4] with these on genotoxicity suggest that a wide safety margin exists for APDT between bacterial eradication and keratinocyte damage.

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