



Induction of apoptotic cell death by photodynamic therapy in human keratinocytes

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Abstract

The use of photodynamic therapy (PDT) for the treatment of skin and oral cancer has been the subject of several clinical studies but there has been little scientific evaluation of its mechanism of action. Evidence to date suggests that whilst epithelial cell death may be secondary to vascular damage, direct cell killing may occur and may involve an apoptosis-like mechanism. To investigate the mechanism of epithelial cell death following PDT, two cell lines, human epidermal keratinocytes (UP) and oral squamous cell carcinoma-derived cells (H376) were subjected to PDT with aluminium disulphonated phthalocyanine (AlS₂Pc) as the photosensitizer and red laser light at 675 nm. Control groups received red laser light, photosensitizer or neither. The effects of PDT were assessed using an MTS cell-proliferation assay, which showed a significant reduction in viability ($p < 0.01$) for PDT-treated cells compared to controls. For morphological analysis, cells were stained with haematoxylin and eosin and the numbers showing typical apoptotic features counted. The treated cultures showed significantly increased numbers of apoptotic cells. Moreover, the H376 control cultures showed a baseline level of apoptosis of approx. 15%. Apoptosis was confirmed by ultrastructural analysis and by *in situ* end-labelling of DNA fragments. The results show that PDT using AlS₂Pc as a photosensitizer promotes apoptotic cell death in UP and H376 cells *in vitro* and suggest that direct killing of epithelial cells may contribute to tumour necrosis *in vivo*. © 1998 Elsevier Science Ltd. All rights reserved

Key words: Apoptosis, Photodynamic therapy, Epithelial cell lines, Oral squamous cell carcinoma

1. Introduction

Photodynamic therapy is a promising new treatment particularly suited to the management of superficial skin and mucosal cancers. It involves the administration of a photosensitizer followed by illumination of the tumour site with light of an appropriate wavelength. On exposure, the sensitizer becomes activated, leading to cell damage by a process thought to involve the singlet oxygen species (Moan and Berg, 1991). The

most widely used photosensitizing agents have been porphyrin derivatives (Pass, 1993), but these are not ideal as, after sensitization, they persist in normal skin leading to prolonged photosensitivity. Newer agents that are not retained for as long are becoming available. 5-Aminolaevulinic acid, a naturally occurring porphyrin precursor, has been used to stimulate endogenous production of protoporphyrin IX (Kennedy *et al.*, 1990), and phthalocyanines (Barr *et al.*, 1987, 1990; Tralau *et al.*, 1987) have been used chelated with metals such as aluminium or zinc to enhance their cytotoxicity (Rosenthal, 1991). In the mouth, *in vivo* studies using Photofrin 2 to treat early squamous-cell carcinomas have shown a massive inflammatory response, with superficial necrosis of both the tumour and adjacent normal tissues with loss of endothelial cells and damage to the small blood vessels and micro-

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Abbreviations: AlS₂Pc, aluminium disulphonated phthalocyanine, ELISA, enzyme-linked immunosorbent assay, HPV, human papillomavirus, KGM, keratinocyte growth medium, PBS, phosphate-buffered saline.

vasculature (Grant *et al.*, 1992). In an experimental model of gastric mucosa, the photodynamic effect following photosensitization with 5-aminolaevulinic acid was restricted to the mucosa rather than the underlying layers (Loh *et al.*, 1992). Use of AlS_2Pc led to full-thickness mucosal damage in experimental gastric (Loh *et al.*, 1992), colonic (Barr *et al.*, 1987, 1990) and bladder (Pope and Bown, 1991) carcinoma, although the underlying muscle appeared relatively insensitive to photodynamic therapy. However, because of the degree of inflammation, the extent to which tumour ablation was achieved by direct damage to the malignant cells or indirectly, as a consequence of damage to the microvasculature, could not be determined.

One approach to the resolution of this question has been to study the effect of photodynamic therapy on epithelial cell lines *in vitro*. Epidermal keratinocyte cultures sensitized with AlS_2Pc showed a marked reduction in vitality in response to photodynamic therapy (Soukos *et al.*, 1994). Apoptosis, in contrast to necrosis, is an active, calcium-dependent process. Chinese hamster ovary cells loaded with chloraluminium phthalocyanine were, on exposure to light, killed in a dose- and calcium-dependent manner (Ben-Hur *et al.*, 1991). In a range of human- and rodent-derived cell lines, cell death following photodynamic therapy may be due to apoptosis, whilst in others the decrease in the number of viable cells involves a non-apoptotic, necrosis-like mechanism (Oleinick *et al.*, 1993). When Photofrin was used as the photosensitizer, human prostate carcinoma and rat mammary carcinoma cells exposed to photodynamic therapy exhibited cell death with features of apoptosis. In contrast, a human lung carcinoma cell line was killed by photodynamic therapy, but without any apoptotic features (He *et al.*, 1994). Thus, some, but not all cell lines undergo cell death by apoptosis in response to photodynamic therapy.

In order to investigate the mechanism of photodynamic therapy on oral carcinoma-derived cells *in vitro*, its effect after sensitization with AlS_2Pc was studied in H376, an oral carcinoma-derived cell line and compared with UP, an immortalized line derived from keratinocytes for which more baseline data were available (Soukos *et al.*, 1994).

2. Materials and methods

2.1. Tissue culture

H376, a cell line derived from an oral squamous-cell carcinoma (Prime *et al.*, 1990) and UP, a transformed epidermal keratinocyte-derived line (Pei *et al.*, 1991), were recovered from frozen stock and cultured to confluence in a standard KGM as described by Soukos *et al.* (1994).

When confluent, cells were released with 0.25% trypsin and 0.05 M EDTA and counted in a haemocytometer. All experiments were repeated at least twice.

2.2. Photosensitization of cells

Cells were seeded into 96-well tissue-culture plates at a density of 3×10^3 cells/well (0.32 cm^2) in $300 \mu\text{l}$ of KGM. Cells were allowed to attach for 24 hr before being washed twice with PBS, then photosensitized by the addition of serum-free medium without additives to which $25 \mu\text{g/ml}$ AlS_2Pc had been added. Control wells contained medium without AlS_2Pc . Plates were incubated at 37°C in 5% CO_2 in the dark for 24 hr, the wells were then washed twice with PBS and the medium replaced with $300 \mu\text{l}$ of colourless PBS. Wells containing cells treated with AlS_2Pc and control wells were given 1 J/cm^2 laser light from a diode laser via a 5-mm liquid light guide, output 10 mW/cm^2 , at a wavelength of 675 nm. Other wells, with and without cells treated with AlS_2Pc , were not irradiated and were used as additional controls. The PBS was then replaced with $300 \mu\text{l}$ of fresh KGM and the preparation returned to the incubator for a further 24 hr.

Cell viability was assessed with a commercially available kit using a colorimetric method based on the cellular conversion of a tetrazolium salt into a formazan in the presence of phenazine methosulphate (MTS assay; Promega). Absorbance, measured with an ELISA plate reader at 490 nm, is directly proportional to the number of living cells in the culture.

2.3. Light microscopy

Cells were seeded into 8-well 'Nunc' glass tissue-culture slides (Gibco/BRL) at 10^4 or 5×10^4 cells/well (0.8 cm^2). Cells were left to attach for 24 hr in $400 \mu\text{l}$ of KGM before being washed twice with PBS and photosensitized as described for the assay of cell viability. Following photosensitization, the slides were returned to a 5% CO_2 , 37°C incubator for a further 24 hr and then fixed in 4% freshly prepared, ice-cold paraformaldehyde and air-dried. Slides were then stained with haematoxylin and eosin before being examined by light microscopy. Control cultures that were photosensitized but not treated with laser light, treated with laser light but not photosensitized with AlS_2Pc , or neither photosensitized nor treated with AlS_2Pc , were also prepared.

Cells were classified as apoptotic if they showed four features of apoptosis readily observed under the light microscope: a reduction in cell volume, an eosinophilic cytoplasm, dense basophilic or fragmented nuclei and an increased nuclear:cytoplasmic ratio. The total number of cells and the number of apoptotic cells per high-

power field were counted. Six high-power fields, selected in a semirandom fashion, were counted. The first field was selected randomly and subsequent adjacent fields were chosen by moving clockwise round the well. Each experiment was carried out on at least six wells.

2.4. Electron microscopy

Cells were released with 0.25% trypsin/0.05 M EDTA, pelleted and resuspended in PBS. An equal volume of 2% 'EM Grade' glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.4, was added, and the cells were allowed to fix for 30 min at 4°C. The cells were then pelleted again, the glutaraldehyde fixative removed, followed by postfixation in 1% osmium tetroxide at 4°C for 2 hr. The pellet was then dehydrated through a series of graded alcohols, infiltrated with propylene oxide and Araldite, and embedded in Araldite. Sections were cut at 100 nm, post-stained with uranyl acetate and Reynold's lead citrate, and examined in a JEOL 100/CX electron microscope.

2.5. In situ visualization of DNA fragmentation

DNA fragmentation was visualized using a modification of the TUNEL technique (Gavrieli *et al.*, 1992). Fluorescein-labelled nucleotides were incorporated on to free 3'-OH ends of DNA associated with strand breaks using terminal deoxynucleotidyl transferase in a commercially available kit (Boehringer Mannheim). Incorporated fluorescein was detected by fluorescence microscopy. Cells exhibiting apoptotic features were readily recognizable by intense, granular nuclear staining and were photographed with a Zeiss immunofluorescence microscope.

2.6. Statistical analysis

Unpaired *t*-tests were used to compare absorbance values for photosensitized samples with controls.

3. Results

The number of viable cells in cultures photosensitized with AlS_2Pc then irradiated with laser light was significantly reduced compared to control groups for both the UP ($p < 0.05$) and H376 ($p < 0.05$) cell lines (Fig. 1).

Morphological analysis on haematoxylin and eosin-stained sections showed dramatic changes in treated cultures of both H376 and UP cells photosensitized with AlS_2Pc then treated with laser light. The control UP cultures were characterized by a subconfluent monolayer of spindle or plump cuboidal cells with

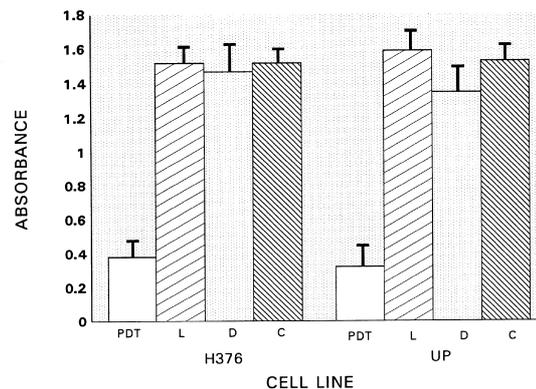


Fig. 1. Histogram comparing the number of viable cells in experimental and control cultures assessed using the MTS assay of cell vitality. Results are expressed as the mean (\pm SD) of absorbance for 12 microwells. The results for two experiments have been pooled. PDT, cells cultured in AlS_2Pc before exposure to laser light; L, cultures exposed to laser light but not photosensitizer; D, cultures that received photosensitizer but not laser light; C, cultures receiving neither laser light nor photosensitizer.

relatively pale-staining nuclei and prominent nucleoli (Fig. 2A). Apoptotic cells were rarely seen. Control H376 cultures were much more heterogeneous with respect to nuclear and cellular morphology (Fig. 2C). Irregular mitotic figures and apoptotic bodies were occasionally seen. In the treated cultures there was loss of adherence and apoptotic cells predominated (Fig. 2B, D). Table 1 gives the ratio of the number of apoptotic cells to the total number of cells counted for UP and H376 cells. Whilst apoptotic cells were rarely encountered in control UP cultures, a definite basal level of apoptosis of about 15% was noted in the H376 controls. In sensitized UP cells, photodynamic therapy caused an unequivocal and overwhelming increase in the number of apoptotic bodies. Although less dramatic, a similar significant ($p < 0.01$) increase in sensitized H376 cells was also seen.

To provide further evidence that photosensitized cells subjected to photodynamic therapy were exhibiting features of programmed cell death, *in situ* end-labelling was carried out on treated and control cultures. A marked increase in fluorescence was observed in treated cultures compared to controls (Fig. 3). This had an intranuclear distribution with a granular or focal pattern. Cells showing this intranuclear pattern were occasionally seen in control H376 cultures but not in UP. These qualitative observations are summarized in Table 2.

Ultrastructural examination of treated cells (Fig. 4) showed many cells with sparse cytoplasm and a marked alteration in nuclear:cytoplasmic ratio. The cytoplasmic membrane and organelles were present

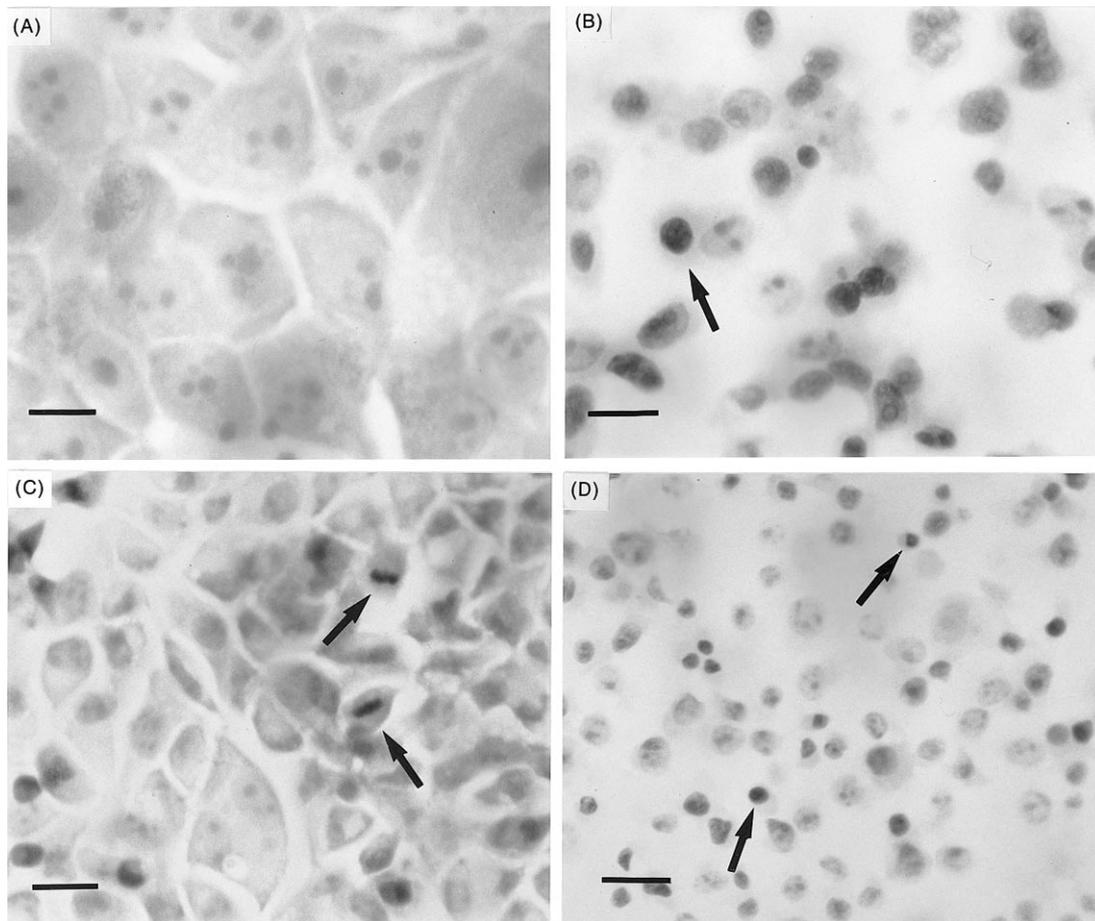


Fig. 2. H & E-stained photomicrographs comparing control cultures of UP cells (A) with experimental UP cells cultured in ALS₂Pc and exposed to laser light (B). Bar 70 μ m. Control H376 cultures (C) are compared with experimental H376 cultures treated with ALS₂Pc and exposed to laser light (D). Bar 250 μ m. In the control cultures (A and C) note the size of the nuclei and the prominent nucleoli. Examples of mitotic figures are arrowed. In the experimental cultures (B and D), examples of apoptotic cells showing shrinkage, a small dense basophilic nucleus and a reduced nuclear:cytoplasmic ratio are arrowed.

Table 1

Proportion of apoptotic cells (number of apoptotic cells/total number of cells) per high-power field

	H376	UP
PDT	0.27 (0.12)	0.62 (0.22)
L	0.17 (0.06)	O
D	0.09 (0.06)	O
C	0.13 (0.05)	O

Figures are a mean (SD) of 12 high-power fields; area of high-power field 16 mm².

PDT, cells cultured in ALS₂Pc and exposed to laser light; L, cultures exposed to laser light but not photosensitizer; D, cultures that received photosensitizer but not laser light; C, cultures receiving neither laser light nor photosensitizer.

and intact, and surface blebbing was occasionally visible. Intranuclear chromatin condensation was widespread with a variable distribution. In some nuclei, clumps of chromatin were visible throughout, whilst in others they were more prominent adjacent to the nuclear membrane. These features were much less prominent in control cultures.

4. Discussion

This study demonstrates that photodynamic therapy following photosensitization using ALS₂Pc is able directly to kill H376 oral squamous carcinoma cells *in vitro*, and confirms a previous study demonstrating

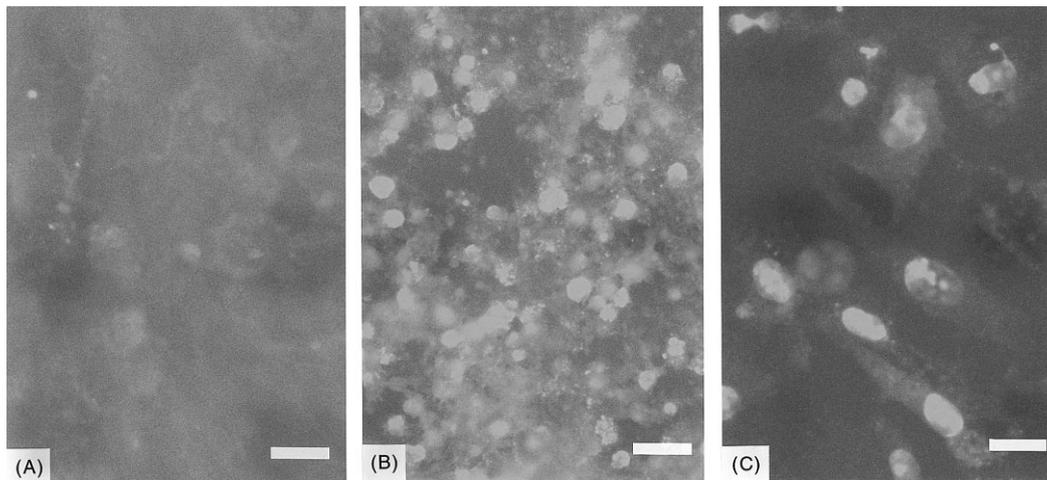


Fig. 3. Fluorescent photomicrographs of H376 cells following *in situ* 'end labelling' of DNA strand breaks in control cultures (A) and cultures treated with AIS₂Pc and exposed to laser light (B and C). Bar 100 μ m (A and B) and 50 μ m (C).

that this photosensitizer is effective in killing UP epidermal keratinocytes (Soukos *et al.*, 1994).

In addition, we demonstrate that for both cell lines, cell death occurs by an apoptotic mechanism. Although several characteristic features of programmed cell death are well documented, a single feature on its own may not be indicative of the state, and recognition of apoptosis depends on identification of several features in an appropriate context. Possibly the principal indicator of apoptosis is the light-microscopic appearance and five easily recognizable histological features have been defined (Wyllie and Duvall, 1992). These include loss of specialized surface structures such as microvilli and contact regions so that the cell ceases to be adherent to its neighbours, a reduction of cell volume with an increase in the nuclear:cytoplasmic ratio, nuclear shrinkage with chromatin condensation giving the nucleus a pyknotic appearance, and cell fragmentation giving rise to apoptotic bodies that may be phagocytosed by a neighbour. At an ultrastructural level, integrity of the cell membrane is maintained, the changes in nuclear:cytoplasmic ratio are readily

demonstrable, and there are characteristic patterns of intranuclear chromatin condensation. A much greater proportion of cells in the treated groups sensitized with AIS₂Pc and exposed to laser light showed these features than in controls. Thus we have demonstrated that photodynamic therapy using AIS₂Pc induces cell death in UP and H376 cells by apoptosis. An additional feature of apoptosis is fragmentation of DNA into small nucleotide chains about 180 bp in length (Wyllie, 1980; Duke *et al.*, 1983; Arends *et al.*, 1990). These fragments can be 'end-labelled' *in situ* and visualized with a fluorescent or chromatographic dye (Gavrieli *et al.*, 1992; Ansari *et al.*, 1993), and this technique is particularly suited to the recognition of DNA strand breaks in situations where only a small number of cells are available. Using this 'end-labelling' technique we found extensive evidence of intranuclear labelling in the treated groups. Furthermore, this labelling had a granular appearance suggesting co-distribution with the chromatin condensation. This provides further evidence that cell death observed in the treated cells was apoptotic in nature.

There has always been some controversy about the mechanism of photodynamic therapy *in vivo* as to whether cell death occurs by direct action on the epithelial component or indirectly, following endothelial damage and thrombosis of the small blood vessels (Pass, 1993). We demonstrate that malignant oral epithelial cells respond primarily by an apoptotic mechanism following photosensitization with AIS₂Pc *in vitro*. The extent to which this observation can be applied *in vivo* remains largely uninvestigated. In one study of normal colon (Barr *et al.*, 1987), a scattering of cells with pyknotic nuclei as observed following AIS₂Pc photodynamic therapy. Although details of the cell types involved were not given, the presence of pyknotic

Table 2

A semiquantitative assessment of DNA fragmentation using *in situ* 'end labelling'

	H376	UP
PDT	***	***
L	*	—
D	*	—
C	*	—

* < 5 fluorescent cells per high-power field.

** 5–10 fluorescent cells per high-power field.

*** > 10 fluorescent cells per high-power field.

Area of high-power field 0.16 cm².

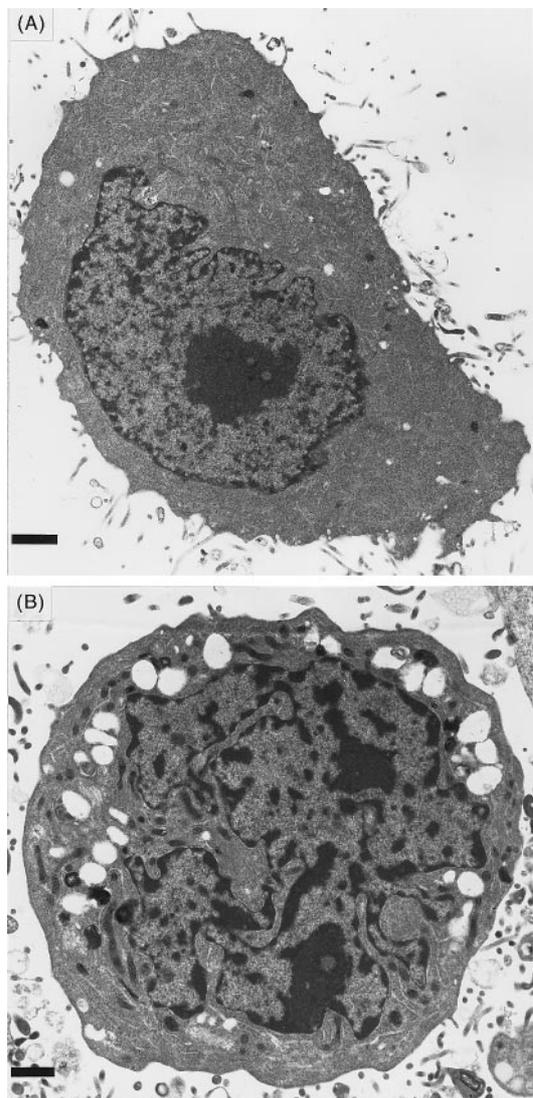


Fig. 4. Representative transmission electron micrograph of a control H376 cell (A) compared to an experimental H376 cell cultured in AIS₂Pc and exposed to laser light (B). The latter shows features of apoptosis: an intact cytoplasmic membrane, a shrunken condensed nucleus, nuclear chromatin condensations, and an increased nuclear:cytoplasmic ratio. Bar 1.1 μ m (A) and 1.9 μ m (B).

nuclei suggests that an apoptotic mechanism may have been involved.

In addition to the marked differences between the treated and control groups, some more general differences were observed between the cell lines. Some baseline apoptotic activity was noted in the H376 but not the UP control groups. The cause of this is unknown. However, it has been proposed that a common mechanism involving expression of *c-myc* may drive both mitosis and apoptosis (Evan *et al.*, 1994). Many

tumours, including oral squamous-cell carcinomas, over express *c-myc* (Scully, 1993), so it is possible that in H376, an oral squamous cell carcinoma-derived line, disordered regulation of the cell cycle is associated with disturbance in the regulation of apoptosis.

The H376 and UP cell lines differed widely in their response to photodynamic therapy. Although this could be due to factors such as the uptake of photosensitizer, it might alternatively reflect a protective effect against DNA damage. Mammary epithelial cells immortalized with HPV express wild-type p53 and appear to be more sensitive to apoptotic stimuli than carcinoma cells expressing mutant p53 (Xu *et al.* 1995). The UP line is an HPV16-transformed line (Pei *et al.*, 1991) and the H376 line is carcinoma-derived, so it is possible that a similar phenomenon is being observed here. The possibility of using photodynamic therapy selectively to stimulate apoptosis in malignant oral epithelium remains. However, before this goal is achieved, further *in vitro* studies are required to understand the mechanisms of cytotoxicity in photodynamic therapy and to help determine the optimal conditions for inducing apoptosis *in vivo*.

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