

Photobiology

Histological changes and involvement of apoptosis after photodynamic therapy for actinic keratoses

H. NAKASEKO, M. KOBAYASHI, Y. AKITA, Y. TAMADA AND Y. MATSUMOTO

Department of Dermatology, Aichi Medical University, Nagakute, Aichi 480–1195, Japan

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Summary

Background Photodynamic therapy (PDT), which employs a combination of a tumour-localizing photosensitizer and visible light, has been used to treat superficial malignancies in the epidermis.

Objectives To examine histological changes and the role of apoptosis in lesions of actinic keratosis (AK) after PDT using 5-aminolaevulinic acid (ALA) and excimer dye laser.

Methods After topical ALA-PDT, biopsy specimens were collected from 18 skin lesions in 15 patients with AK. Paraffin-embedded sections of the skin specimens were stained with haematoxylin and eosin. The detection of apoptosis was performed using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labelling (TUNEL) method, antiactivated caspase-3 antibody and anti-Fas antibody.

Results One hour after PDT, cells with eosinophilic cytoplasm and markedly stained nuclei were found, and vacuolation of some tumour cells was noted in the lower layer of the epidermis. An infiltrate of lymphocytes and neutrophils was observed in the upper layer of the dermis. One day after PDT, all layers of the epidermis exhibited slightly degenerative necrosis, with shadow cell formation and chromatin condensation around the nuclear membrane in the lower layer of the epidermis. Necrosis in all layers of the epidermis and lymphocyte infiltration in the dermis were found 3 days after PDT. Tumour cells had disappeared and regenerative thickening of the epidermis was observed 7 days after PDT. TUNEL staining revealed apoptosis-positive cells in the epidermis in 8 of 11 specimens obtained 1 day after PDT. Activated caspase-3 expression was noted in the lower layer of the epidermis in four of these eight TUNEL-positive specimens.

Conclusions Results suggested that apoptosis is involved in tumour cell death after PDT in patients with AK, and that it occurs within 1 day after PDT.

Key words: 5-aminolaevulinic acid, actinic keratoses, apoptosis, caspase-3, photodynamic therapy, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labelling method

Photodynamic therapy (PDT) for malignancies is a widely used modality based on photochemical sensitization induced by a combination of a tumour-localizing photosensitizer and visible light.^{1–4} In the field of dermatology, PDT consisting of light irradiation at a specific wavelength after topical application of 5-aminolaevulinic acid (ALA), a porphyrin precursor, has frequently been used in the treatment of actinic

keratosis (AK), basal cell carcinoma and Bowen's disease.^{3–5} Although the action mechanism of PDT remains unclear, several studies using cultured cells and experimental animals have suggested the involvement of apoptosis in tumour cell death after PDT. However, few clinical studies have been performed.

In this study, we performed PDT in patients with AKs, and examined serial histopathological changes. To investigate the involvement of apoptosis in tumour cell death after PDT, the terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate

Correspondence: Hirono Nakaseko.
E-mail: i09810@gk.amu.aichi-med-u.ac.jp

(dUTP)-biotin nick end labelling (TUNEL) method was performed. Furthermore, we investigated the expression of caspase-3, a lower caspase that acts in the process of apoptosis, and the involvement of Fas, which is a signal receptor for apoptosis.

Materials and methods

Patients and lesions

Specimens were collected from 18 skin lesions (16 on the face, one on the chest and one in the postauricular area) in 15 patients (4 men and 11 women; age range 58–94 years, mean 86) who were pathologically diagnosed as having AKs and were treated with PDT at the out patient clinic of the Department of Dermatology, Aichi Medical University Hospital, between April and December 2000. Skin biopsy specimens were obtained 1, 3 and 7 days after PDT from 11 lesions in eight patients, 3 h after PDT from four lesions in four patients and 1 h after PDT from three lesions in three patients.

Informed consent was obtained from all patients about the objectives, technical details and side-effects of PDT.

Photodynamic therapy

The skin surface was treated with a 20% oil-in-water emulsion (Japanese Pharmacopoeia, Merck Hoei Ltd, Osaka, Japan) of ALA (Sigma, St Louis, MO, U.S.A.), occluded with food-grade cling film and covered with aluminium foil for light protection. About 5–6 h after application of ALA, light irradiation of 100 J cm^{-2} at 100 mW cm^{-2} was given to the lesion using an excimer dye laser (PDT EDL-1, Hamamatsu Photonics K.K., Hamamatsu, Japan) emitting 630-nm laser light.

Pathological examination after haematoxylin and eosin staining

Paraffin-embedded sections of the skin specimens obtained after PDT were stained with haematoxylin and eosin and observed under a light microscope.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labelling method

Skin specimens were stained using the TUNEL technique, as previously described.⁶ After deparaffinization and hydration, tissue sections were treated with

$20 \mu\text{g mL}^{-1}$ of proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature for 15 min. After inactivation of endogenous peroxidase with 2% H_2O_2 , tissue sections were washed with distilled water and placed in TdT buffer solution (30 mmol L^{-1} Trizma base, pH 7.2, 140 mmol L^{-1} sodium cacodylate, 1 mmol L^{-1} cobalt chloride). Sections were then reacted with a mixture of TdT (Takara Shuzo Co Ltd, Otsu, Japan) and biotinylated dUTP (Boehringer Mannheim) under humid conditions at 37°C for 60 min.

The reaction was terminated by placing sections in TB buffer solution (300 mmol L^{-1} sodium chloride, 30 mmol L^{-1} sodium citrate) at room temperature for 15 min. Tissue sections were washed with distilled water, exposed to a solution of 2% bovine serum albumin in water at room temperature for 10 min, washed with distilled water again and placed in phosphate-buffered saline (PBS) for 5 min. Tissue sections were then reacted with ExtrAvidin (peroxidase conjugate) (Sigma) under humid conditions at 37°C for 30 min and stained with diaminobenzidine (DAB) accompanied by nuclear staining with methyl green.

Immunohistochemical examination

Paraffin-embedded specimens were serially cut at $5 \mu\text{m}$, and were stained immunohistochemically to detect the distribution of activated caspase-3 and Fas. Immunohistochemical staining was performed using the avidin–biotin–peroxidase method. Sections were deparaffinized in xylene, and hydrated through a graded series of ethanol. In the case of anti-Fas antibody, sections were placed in citrate buffer and heated at 500°W five times each for 10 min in a microwave oven. Sections were incubated in 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase activity. Sections were then washed in PBS and incubated in 10% normal goat serum for 20 min to reduce nonspecific binding. For evaluation of immunoreactivity, sections were reacted with antibodies against activated caspase-3 (1 : 250 dilution; Promega, Madison, WI, U.S.A.) and Fas (1 : 80 dilution; Novocastra Laboratories, Inc., U.S.A.). Sections were incubated for 60 min at room temperature with the primary antibodies, and the second antibody, biotinylated goat antirabbit IgG, was applied for 10 min at room temperature. After washing three times in PBS, sections were treated with avidin–biotin–peroxidase complex reagent. Finally, the reaction products were visualized with DAB and H_2O_2 and lightly

counterstained with haematoxylin. Negative controls were performed by replacing the primary antibodies with PBS.

Results

Clinical findings

Each AK improved with one treatment of PDT, and the skin lesions almost disappeared over several months (Fig. 1a,b).

Histopathological findings

One hour after PDT, cells with eosinophilic cytoplasm and markedly stained nuclei were observed in the basal layer of the epidermis, and vacuolation of some tumour cells was noted in the lower layer of the epidermis. A scanty infiltrate of lymphocytes and neutrophils was observed in the upper layer of the dermis (Fig. 2a,b).

Three hours after PDT, the number of cells with eosinophilic cytoplasm and markedly stained nuclei increased and vacuolation of tumour cells became more evident. There was a marked neutrophilic infiltrate in the upper layer of the dermis and lower layer of the epidermis (Fig. 2c).

One day after PDT, all layers of the epidermis exhibited necrosis. Shadow cell formation and chromatin condensation around the periphery of nuclei in the tumour area were noted. A marked infiltrate of neutrophils and some eosinophils was observed in the epidermis and upper layer of the dermis (Fig. 2d).

Three days after PDT, necrosis was observed in all layers of the epidermis. A large number of shadow cells was also noted in the epidermis. Lymphocytes and some neutrophils were observed in the upper layer of the dermis (Fig. 2e).

Seven days after PDT, tumour cells in the epidermis disappeared and regenerative thickening of the epidermis was seen. A lymphocytic infiltrate was noted in the upper layer of the dermis (Fig. 2f).



Figure 1. Actinic keratosis on the right cheek. Clinical appearance (a) before and (b) 2 months after photodynamic therapy.

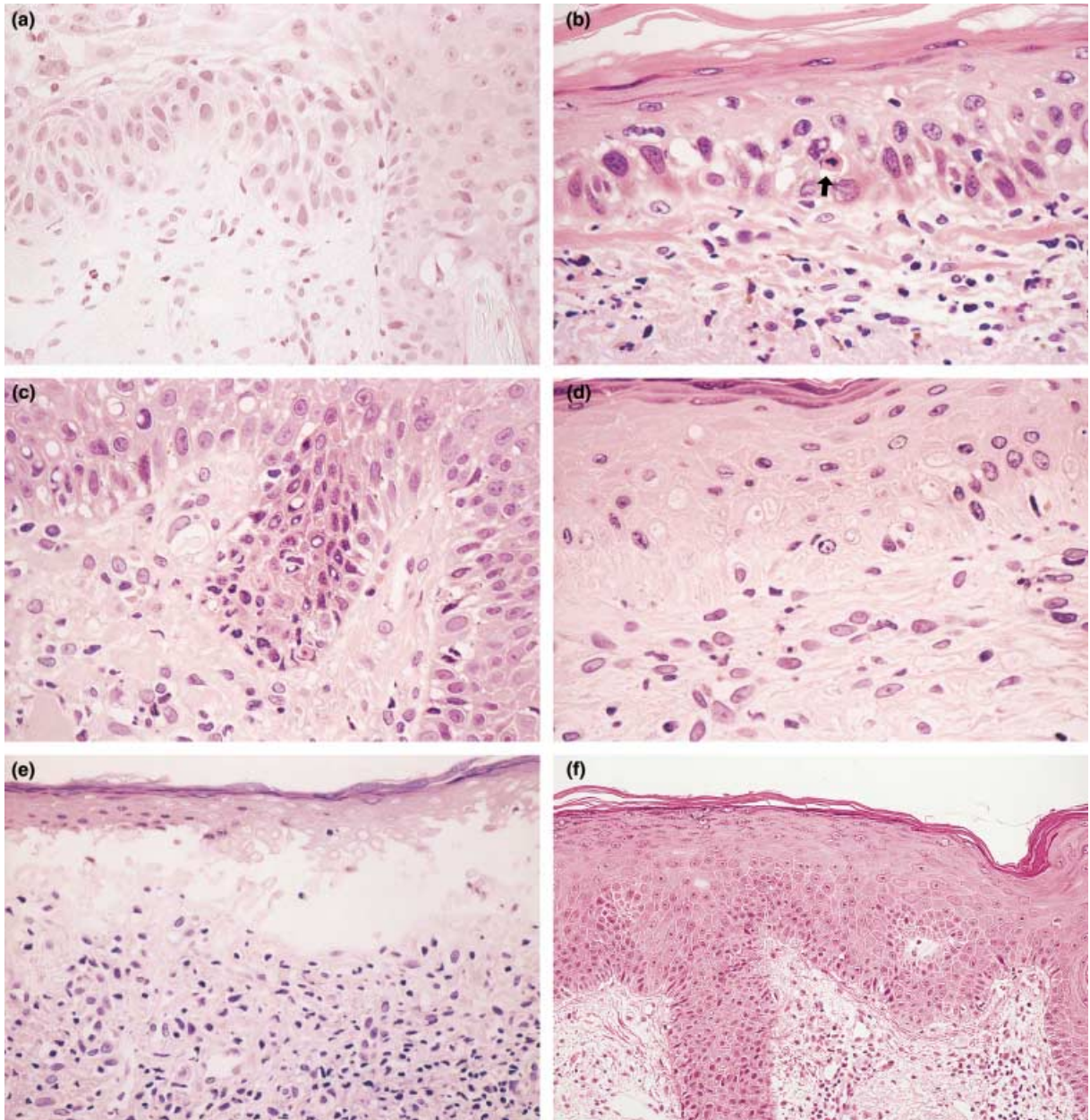


Figure 2. Histopathological findings. (a) Atypical cells are seen in the lower layer of the epidermis before photodynamic therapy (PDT). (b) One hour after PDT, cells with eosinophilic cytoplasm and markedly stained nuclei, assumed to be sunburn cells (arrow), were observed and vacuolation of some tumour cells was noted in the lower layer of the epidermis. (c) Three hours after PDT, the number of cells with eosinophilic cytoplasm and markedly stained nuclei increased and vacuolation of tumour cells became more evident. (d) One day after PDT, all layers of the epidermis exhibited necrosis. Shadow cell formation and chromatin condensation around the periphery of the nuclei were noted in the tumour area. (e) Three days after PDT, necrosis was observed in all layers of the epidermis. A large number of shadow cells was also noted in the epidermis. (f) Seven days after PDT, tumour cells in the epidermis had disappeared and regenerative thickening of the epidermis was seen (haematoxylin and eosin; original magnification: a, c, $\times 132$; b, d, $\times 160$; e, f, $\times 80$).

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labelling

One day after PDT, positive staining was observed corresponding to atypical cells in 8 of 11 specimens

(Fig. 3a). Three days after PDT, positive cells were found in 3 of 11 specimens (Fig. 3b). Positive cells were not detected in any specimens obtained 1 h, 3 h or 7 days after PDT.

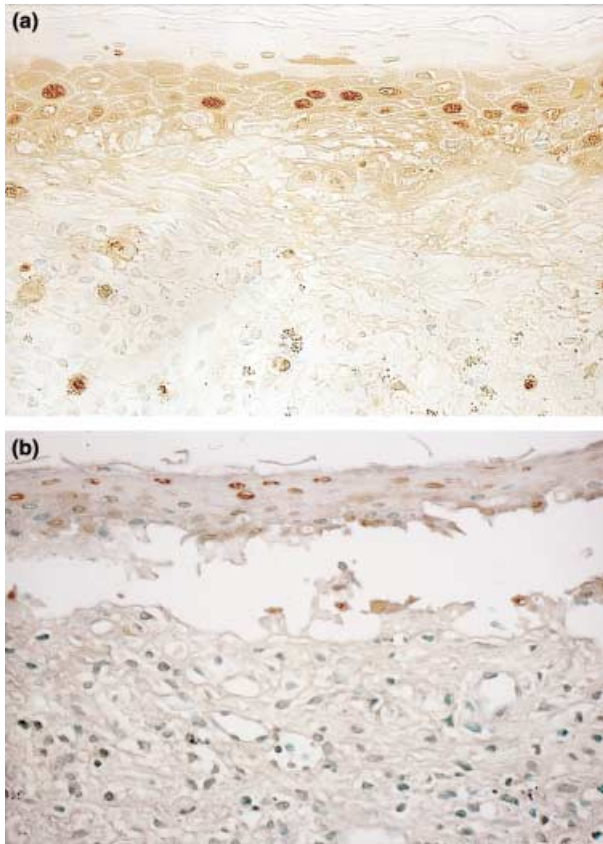


Figure 3. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labelling. (a) One day after photodynamic therapy (PDT), positive staining was observed, corresponding to atypical cells in the epidermis. (b) Three days after PDT, positive cells were found in the epidermis (original magnification: a, b, $\times 132$).

Immunohistochemical findings

Immunohistochemical examination with antiactivated caspase-3 antibody was performed in 18 sections taken 1 h, 3 h and 1 day after PDT. No positive results were obtained in specimens obtained 1 h and 3 h after PDT. A positive result was noted, corresponding to tumour cells in the lower layer of the epidermis, in four of eight specimens that showed positive staining with the TUNEL method, obtained 1 day after PDT (Fig. 4).

Immunohistochemical examination with anti-Fas antibody was performed in specimens obtained 1 h, 3 h and 1 day after PDT. No positive results were obtained.

Discussion

The principle of PDT is as follows. When exposed to visible light, the photosensitizer localized in tumour

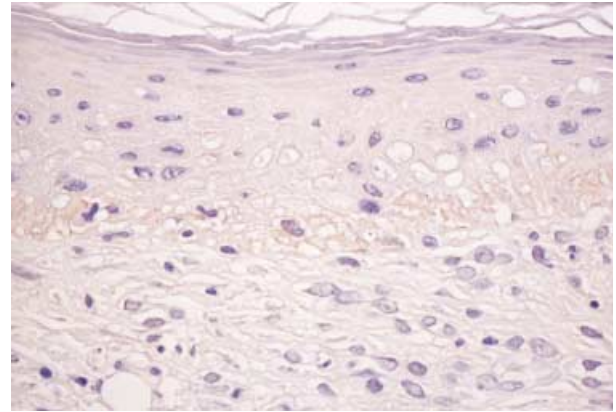


Figure 4. Immunohistochemical examination with antiactivated caspase-3 antibody. One day after photodynamic therapy, positive staining corresponded to tumour cells in the lower layer of the epidermis (original magnification $\times 132$).

tissue accepts light energy and is transformed into an excited state (singlet state). Energy conversion while returning to the basal state from the excited state causes damage to intracellular respiration, resulting in degeneration and necrosis of tumour cells.^{1,2} Some researchers consider that cellular necrosis is caused by disturbed blood flow due to damaged vascular endothelial cells or thrombus formation,^{1,7} although the mechanism of PDT-mediated tumour cell death remains unclear.

Using a mouse lymphoma cell line, Agarwal *et al.*⁸ showed that apoptosis was induced between 30 min and 2 h after PDT. Matsumoto *et al.*⁹ reported the induction of apoptosis 2 h after PDT in cultured human squamous cell carcinoma cells and Tajiri *et al.*¹⁰ noted PDT-induced apoptosis in cultured pancreatic carcinoma cells. Early induction of apoptosis is considered to be characteristic for PDT.

In the present study, cells with eosinophilic cytoplasm and markedly stained nuclei, assumed to be sunburn cells, were histologically detected 1 h and 3 h after PDT. Necrosis in all layers of the epidermis, shadow cell formation and chromatin condensation around the periphery of the nuclei in the tumour area were noted 1 day after PDT. An electron microscopic study by Agarwal *et al.*⁸ and a phase-contrast microscopic study by Matsumoto *et al.*⁹ showed that chromatin condensation around the nuclear membrane was a characteristic finding of apoptosis induced by PDT. Our observations of nuclear morphology 1 day after PDT, which are consistent with their results, suggest the induction of apoptosis.

Positive staining with the TUNEL method was observed in 8 of 11 specimens obtained 1 day after PDT and 3 of 11 specimens obtained 3 days after PDT. The TUNEL method enables the differentiation of apoptosis from necrosis because DNA fragments produced by apoptosis are exclusively labelled with the TUNEL method.^{11,12} Therefore, apoptosis appears to be involved in tumour cell death in the epidermis after PDT.

Caspase-3, a cysteine proteinase, plays an essential role in the induction of apoptosis. Among caspases, caspase-3 has been most widely investigated. Human apoptotic cells are specifically stained by antiactivated caspase-3 antibody, while nonapoptotic cells are not. In the present study, tumour cells in the lower layer of the epidermis showed positive staining for the antibody 1 day after PDT.

We found vacuolated changes in the lower layer of the epidermis, with nuclear atypia. However, degeneration was not detected in the superficial area of the epidermis without nuclear atypia, or at nonlesional sites. Furthermore, in normal skin, which was biopsied as a control, there were no reactions on TUNEL or caspase-3 analysis. Accordingly, there is no evidence that the biopsy influenced the results of the study. The above results suggest that apoptosis is involved in tumour cell death after topical ALA-PDT within 1 day after PDT in patients with AKs.

Ahmad *et al.*¹³ suggested that apoptosis after PDT was induced via the signal transduction pathway of Fas. Using immunoblot analysis, they showed a significant time-dependent increase in the protein expression of Fas at 5, 15, 30 and 60 min after PDT, followed by a decrease at later time-points (2 and 3 h after PDT). In the present study, positive staining for anti-Fas antibody was not noted in specimens obtained 1 h, 3 h and 1 day after PDT. The participation of the Fas pathway in the induction of apoptosis remains unknown.

Godar¹⁴ recently reported that singlet oxygen directly damaged the mitochondrial membrane in the early process of apoptosis after PDT and ultraviolet A radiation. In addition, using a Western blotting method, Granville *et al.*¹⁵ showed that after PDT an increase in mitochondrial cytochrome c and apoptosis-inducing factor levels were detected in the cytosol

immediately, and that their levels increased steadily up to 2 h. Further studies are required to clarify whether or not the signal transduction pathway of apoptosis is located in mitochondria after PDT.

Acknowledgments

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