Pathologic Changes After Photodynamic Therapy for Basal Cell Carcinoma and Bowen Disease

A Histologic and Immunohistochemical Investigation

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Objective: To investigate the in vivo reactions and the mechanisms of cell death after photodynamic therapy (PDT) for cutaneous carcinomas. Photodynamic therapy is a new treatment modality for nonmelanoma skin cancers. Its effects on target tissue have been well investigated in vitro, where apoptosis appears to be the main effector mechanism, but its effects remain undefined in vivo.

Design: Skin biopsy specimens were obtained sequentially after PDT for basal cell carcinoma and in situ squamous cell carcinoma (Bowen disease). Evidence from routine histologic evaluation was compared with a panel of apoptosis-related (TUNEL, caspase-3, and Bcl-2) and inflammatory (CD4, CD8, CD20, CD68, and CD56) markers. We used electron microscopy to evaluate cell damage at the ultrastructural level.

Main Outcome Measures: Evidence of the mechanisms of tumor cell damage after PDT, detection of histologic and/or immunohistochemical signs of apoptosis, and time course of the tumor destruction and inflammatory reaction.

Results: Early epidermal damage and an acute dermal inflammatory response were detected 15 minutes after PDT. In basal cell carcinoma, nodule damage progressed from scant apoptotic cells seen at the dermal-epithelial junction to massive destruction seen after 1 and 2 days. The periphery of the basaloid nodules consistently showed earlier and predominant damage, as demonstrated by the perfect coincidence of histologic and immunohistochemical evidence with apoptotic markers (TUNEL and caspase-3 staining). Fibrosis and lentigo-like changes were seen in late biopsy specimens.

Conclusions: This study defines the time course and characteristics of the skin tumor response to PDT. Taken together, our observations suggest that direct damage to cancer cells is the main effector mechanism leading to PDT response. The involvement of apoptosis is demonstrated by the simultaneous appearance of histologic, immunohistochemical, and ultrastructural markers that occur in the early phases of the cutaneous reaction to PDT. These observations could help to develop future refinements of the PDT technique.

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Photodynamic therapy (PDT) has been increasingly used in the treatment of nonmelanoma skin cancers. Topical application of the porphyrin precursor 5-aminolevulinic acid or its lipophilic ester methylaminolevulinate, followed by light irradiation at appropriate wavelengths, has proved to be a simple, selective, and effective therapy for cutaneous superficial premalignant and malignant conditions such as actinic keratosis, Bowen disease (BD), and basal cell carcinoma (BCC). Photodynamic therapy is based on the principle of energy transfer from light to a photosensitizer to tissue: 5-aminolevulinic acid is preferentially taken up and converted into protoporphyrin IX (PpIX) by neoplastic cells, and successive activation of PpIX by light results in selective damage of tumor tissue, with sparing of healthy tissue. The intracellular pathways leading to neoplastic cell damage have been intensely investigated; after uptake of exogenous 5-aminolevulinic acid, PpIX accumulates in mitochondria, and highly reactive free radicals and oxygen species originate as excited PpIX returns to the ground state and interacts with molecular oxygen. Several in vitro models in normal and neoplastic cell lines have also addressed the final mechanism leading to tumor destruction, and substantial evi-
dence supports the activation of programmed cell death (apoptosis) as a dominant, albeit not exclusive, damage pathway.

On the contrary, in vivo investigations so far have focused mainly on the clinical efficacy of PDT, with only a few reports addressing the pathologic response of cutaneous neoplasms to PDT. Understanding the actual in vivo mechanisms leading to tumor destruction is crucial for improving PDT strategies and clinical responses. To evaluate the in vivo reactions and the mechanisms of cell death in the cutaneous responses to PDT, we investigated the pathologic changes in BCC and BD in serial biopsy specimens obtained after PDT with methylaminolevulinate. Routine histologic findings were compared with immunohistochemical findings using apoptotic (TUNEL [terminal deoxynucleotidyl transferase–mediated biotin–deoxyuridine triphosphate nick end labeling] and activated caspase-3) and antiapoptotic (Bcl-2) markers. We used electron microscopy to evaluate the early tissue changes at the cellular level. Moreover, the inflammatory response was analyzed by means of histochemistry and immunohistochemistry.

**METHODS**

**PATIENTS AND LESIONS**

The study design was reviewed and approved by our ethics committee. Fifty-one skin biopsy specimens were obtained from the lesional skin of 28 patients (18 men and 10 women; age range, 53-95 years). Three patients had multiple lesions; in 23, a single lesion larger than 1 cm underwent biopsy twice. Lesions consisted of 31 BCC (7 nodular and 24 superficial; pigmented and morphic BCC were excluded) and 4 BD. Informed consent was obtained from each patient before biopsy. The diagnosis was histologically confirmed before treatment. Basal cell carcinoma lesions were located on the head and neck region in 6 patients and on the trunk in 23. The BD lesions were all located on the face. Clinical outcome after treatment was confirmed by histologic findings (in 17 patients) or by prolonged clinical follow-up, revealing the persistence of nodular BCC in 3 patients and BD in 2 patients.

**METHYLMALOEVULINATE TREATMENT AND PDT**

After the skin lesions were photographically documented and cleansed with sterile saline solution, hyperkeratotic areas were removed with curetage. No debulking of the lesions was performed. A 1-mm-thick layer of methylaminolevulinate cream (16 mg/mL; Metvix; Galderma, Milano, Italy) was then applied to the lesions, 3 to 5 mm beyond the visible lesional borders, and covered with an occlusive dressing. After 3 hours, the dressings were removed, and the lesions were cleansed with isotonic sodium chloride solution and irradiated with an incoherent narrowband light-emitting diode lamp (Aktivite CL128; Photocure ASA, Oslo, Norway) at a wavelength of 630 nm (total light dose, 37 J/cm²; irradiation field, 180 × 80 mm; irradiance, 50 mW/cm² at 50 mm from the lesion). As negative control specimens, 2 BCC lesions from patients scheduled for excisional surgery were occluded for 3 hours with methylaminolevulinate cream without irradiation and 2 other BCC lesions were irradiated without previous cream application. The control specimens were then excised and processed for routine histologic evaluation.

**BIOPSY SPECIMENS**

Four-millimeter punch biopsy specimens were obtained under local anesthesia from lesional skin before PDT (n=16) and at the following indicated times: for BCC, 15 minutes, 1 and 2 hours, 1 and 2 days (n=2 each), and 1 (n=4), 2 (n=2), 4 (n=7), and 8 (n=7) weeks after PDT; for BD, 1 (n=2), 2 (n=1), and 7 (n=2) days after PDT. The routine treatment in our institutions consists of 2 PDT sessions, 1 week apart. The early biopsy specimens were obtained after the first PDT session. The 1-week biopsy specimens were obtained immediately before the second PDT session. Samples were fixed in formalin; a few tissue samples were frozen to compare the immunohistochemical staining results in frozen and fixed tissue. Routine histologic analysis was performed on paraffin-embedded sections stained with hematoxylin–eosin; toluidine blue O was used for the staining of mast cells in a few sections. TUNEL and activated caspase-3 staining was applied to untreated BCC specimens and to tissue samples obtained 1 to 2 hours and 1 to 2 days after PDT. We used immunohistochemical studies to analyze the inflammatory infiltrate at 1 hour in representative samples, whereas electron microscopy was performed on 2 BCC biopsy specimens that were obtained before and 1 day after PDT.

**TUNEL STAINING**

Paraffin-embedded specimens were cut at 5 µm. Staining was performed using an in situ cell death detection kit (Roche Diagnostics GmbH, Basel, Switzerland) according to the manufacturer’s instructions. Briefly, after deparaffinization and rehydration, tissue sections were treated with 20-µg/mL proteinase K at room temperature for 30 minutes. Tissue sections were then washed with phosphate-buffered saline solution and incubated with fluorescein-labeled nucleotides and terminal deoxynucleotidyl transferase at 37°C for 60 minutes in the dark, after which they were washed and incubated with converter alkaline phosphatase (antifluorescein antibody conjugated with the reporter enzyme alkaline phosphatase) for 30 minutes at room temperature. The reaction was developed with Fast Red tablets in naphthol phosphate substrate (Laboratory Vision Corporation, Fremont, California).

**IMMUNOHISTOCHEMICAL ANALYSIS**

Immunohistochemical analysis was performed on deparaffinized and rehydrated 4-µm sections. A panel of primary antibodies was applied; these antibodies are listed in the eTable (available at http://www.archdermatol.com). Briefly, sections were incubated for 5 minutes with a blocking solution (Ultra V Block; Laboratory Vision Corporation) to stop the endogenous peroxidase or alkaline phosphatase activity. For activated caspase-3, a staining protocol was performed using a second-generation polymer detection system and a chromogen kit (UltraVision LP Detection System AP Polymer and Fast Red, respectively; Laboratory Vision Corporation) according to the manufacturer's instruction. The signal was amplified with ready-to-use antibody (UltraVision Primary Antibody Enhancer and UltraVision AP Polymer; Laboratory Vision Corporation) for 20 and 30 minutes, respectively, at room temperature. Visualization was obtained with chromogen tablets (Fast Red) in naphthol phosphate substrate for 10 to 20 minutes. For all other antibodies, we used a streptavidin-biotin complex method with an automated immunostainer (Benchmark; Ventana, Tucson, Arizona). The reaction products were visualized with diaminobenzidine and hydrogen peroxide and lightly counterstained with hematoxylin of Carazzi. Negative controls were
obtained by replacing the primary antibody with phosphate-buffered saline solution.

**ELECTRON MICROSCOPY**

Biopsy specimens were fixed in 2.5% glutaraldehyde in Tyrode buffer for 24 hours, postfixed in 1% osmium tetroxide at room temperature for 3 hours, then dehydrated through a series of graded alcohols, infiltrated with propylene oxide and resin (Durcupan; Fluka, Buchs, Switzerland), and embedded in the resin. Semithin sections were cut at 0.5 µm. Final sections were diamond cut at 50 nm, poststained with 7% uranyl acetate in methanol and 1% Reynold lead citrate, and examined in a JEOL 2011 electron microscope (JEOL, Peabody, Massachusetts) at 200 keV.

**RESULTS**

**HISTOLOGIC FINDINGS**

Epidermal and dermal changes after PDT were similar in BCC and BD and are therefore described together (Figures 1, 2, and 3). As early as 15 minutes after PDT, a diffuse epidermal damage (ie, edema, homogenization, and keratinocyte vacuolization) was apparent in BCC and BD tissue. The upper and middle dermis showed intense acute inflammation with edema, vasodilation, red blood cell extravasation, and mixed diffuse perivascular and interstitial inflammatory infiltrate consisting of polymorphonuclear cells (neutrophils and eosinophils) and mononuclear cells (lymphocytes, monocytes, and plasma cells). The BCC nodules were mostly unaffected (Figure 1A). These features correlated well with the clinical response, which showed an intense inflammatory reaction during and immediately after PDT, with a progressive increase in inflammation in the first few hours.

At 1 hour, the signs of acute inflammation were more pronounced. The epidermis showed striking full-thickness damage (similar to that after a burn or ischemic injury) with homogenization, loss of cell borders, the appearance of ghost cells with pyknotic nuclei, perinuclear vacuolization, and shrunken cytoplasm, and marked basal vacuolar degeneration (Figure 1B). In BCC, the peritumoral spaces were enlarged, separating the infiltrating nodules from an edematous dermis. Individual cells in the tumor nodules, preferentially located

![Figure 1. Sequential histologic alterations after photodynamic therapy (PDT) for basal cell carcinoma (BCC). A, Fifteen minutes after PDT, epidermal damage and dermal inflammatory reaction are prominent (original magnification ×100). B, One hour after PDT (original magnification ×200). C, A BCC nodule 1 hour after PDT (original magnification ×200). Massive chromatin condensation is seen in the peripheral cells (arrows). D, Two hours after PDT, apoptotic cells are increased in BCC nodules (original magnification ×200). E, One day after PDT, epidermal shedding (arrows) is seen. Asterisks indicate BCC nodules (original magnification ×200). F, After 1 day, a few cells of BCC nodules appear still undamaged (asterisk) (original magnification ×200). G, Two days after PDT, a small superficial BCC nodule is seen, with necrotic peripheral palisading (arrows) and normal-appearing inner cells (asterisk) (original magnification ×100). H, Eight weeks after PDT, epidermal lentigolike changes and dermal fibrosis are evident (original magnification ×100).](https://archderm.jamanetwork.com/)
at the borders, showed morphological alterations consistent with apoptosis (ie, chromatin condensation, pyknotic nuclei, eosinophilic cytoplasm, and an increased nuclear to cytoplasmic ratio) (Figure 1C).

At 2 hours, along with acute inflammation, an initial fibroblast response could be appreciated in the dermis. In BCC specimens, the number of apoptotic neoplastic cells appeared markedly increased, mainly at the periphery of the BCC islands and as individual cells at the center (Figure 1D).

In both BCC and BD specimens, the epidermis was completely necrotic after 1 day (Figure 2A), and an initial epidermal shedding, with basal cleavage, could be detected in several areas, leaving 1 to 2 layers of regenerating basal epidermis (Figure 1E). In BCC, most of the neoplastic cells in the tumor nodules appeared to be damaged (ie, showing pyknotic nuclei and eosinophilic homogeneous cytoplasm), but some apparently undamaged foci were present, preferentially at the center of the tumor islands (Figure 1F). The inflammatory dermal response began to fade slightly, with a parallel increase of the fibroblasts. At this time, the treated lesions clinically showed acute inflammation with peeling or complete loss of the epidermis and exudation.

At 2 days, the full-thickness necrotic epidermis persisted (Figure 2C) or showed extensive shedding and regrowth of the reepithelium (Figure 2D). The epidermis was not completely necrotic at this time (Figure 2E). Remnants of the necrotic epidermis are seen in parts E and F (asterisks). Arrows in part F indicate the dermoepidermal junction (original magnification ×100).

**Figure 2.** Histologic findings (A, C, and E) and results of TUNEL (terminal deoxynucleotidyl transferase-mediated biotin–deoxyuridine triphosphate nick-end labeling) staining (B, D, and F) of Bowen disease specimens after photodynamic therapy (PDT). A and B, One day after PDT; C and D, 2 days after PDT; and E and F, 7 days after PDT. Remnants of the necrotic epidermis are seen in parts E and F (asterisks). Arrows in part F indicate the dermoepidermal junction (original magnification ×100).
generation (Figure 1G). Dermal healing was prominent, with fibroblastic response and verticalization of superficial vessels. The BCC nodules exhibited marked damage, but some areas of apparently undamaged cells were present at the core of the nodules, well separated from the necrotic, still visible peripheral palisading. The persistence of foci of undamaged neoplastic cells was rather independent of the depth of the tumor as it was also present in tumor nests at the papillary dermis level (Figure 1G).

At 7 days, a well-formed, regenerated epidermis was completed in both BCC and BD specimens (Figure 2E). The dermis showed fibrocytic increase, new collagen formation, and persistence of mononuclear inflammatory infiltrate. In BCC, necrotic remnants could be seen, divided by clefts from the surrounding dermis. Occasionally, undamaged remnants of BCC were found in the superficial and deep dermis, surrounded by an intense mononuclear infiltrate. A band of unaffected dermis separated the deep tumor islands from the surface. Clinically, the treated lesions showed an almost complete epithelial regeneration, with scant residual erosions and crusting.

After 2, 4, and 8 weeks, the histologic pictures were similar. In the patients with tumor disappearance, the dermis showed fibrosis, and the epidermis was completely reconstituted with flattening of the rete ridges induced by the fibrosis or with lentigolike features such as hyperplasia, basal layer pigmentation, and melanophages in the papillary dermis (Figure 1H). Clinically, a fibrotic area surrounded by peripheral hyperpigmentation was noted. When present, BCC persistence showed no peculiar histologic characteristics, as was the case in 3 of 17 patients with later biopsy specimens (Figure 3A).

No signs of mast cell increase could be detected in toluidine blue O–stained samples obtained 1 hour and 1 day after PDT (data not shown). Histologic findings from negative controls showed no alteration.

**TUNEL STAINING**

In general, the TUNEL reaction paralleled the histologic signs of apoptosis. In untreated tumors, TUNEL staining was nearly absent in the epidermis and present in sporadic cells in BCC nodules (Figure 4A). One hour after PDT, diffuse, weak staining at all epidermal levels was apparent, whereas, in the dermal tumor nodules, individual cells that were mainly located at the periphery began to show clear TUNEL staining (Figure 4C). The
TUNEL-positive neoplastic cells in BCC specimens greatly increased 2 hours after PDT (Figure 4E). One and 2 days after PDT, a diffuse staining consistent with extensive cell damage was present in the epidermis of the BCC and BD lesions (Figure 2B and D) and in wide sheets of BCC cells at the peripheral regions of tumor islands, whereas the centers of the nodules often remained unstained (Figure 4G). Scant or no TUNEL staining was present in the BCC nodules that persisted after PDT (Figure 3B).

**IMMUNOHISTOCHEMICAL ANALYSIS**

**Activated Caspase-3**

Caspases are a family of cytosolic proteases in which sequential activation constitutes the basic enzymatic mechanism of apoptosis. In particular, caspase-3 is called the executioner caspase because it is activated during the final apoptotic pathway. Sporadic caspase-3–positive cells were detected in untreated BCC nodules, whereas none were observed in untreated epidermis (Figure 4B). Caspase-3 staining increased 1 hour after PDT in the epidermis as well as in the BCC nodules, both in frozen and in formalin-fixed sections. Single positively stained cells in a peripheral (basal) location were again apparent, with only a few positive cells in the center of epithelial islands (Figure 4D). The number of positive cells progressively increased 2 and 24 hours after PDT (Figure 4F and H), whereas it appeared reduced after 48 hours, matching the appearance of the untreated tumors.

**Bcl-2 Staining**

Staining with Bcl-2 gave no consistent results in BCC, before or after PDT. In some samples tumor islands were unstained, whereas in others as many as 50% of the cells were positive for Bcl-2 staining, irrespective of the time after PDT. No Bcl-2–positive cells were seen in the epidermis of BD specimens after 1, 2, and 7 days (data not shown).

**ELECTRON MICROSCOPY**

Samples obtained 1 day after PDT showed marked ultrastructural alterations in the epithelial cells from the epidermis and the BCC nodules. These alterations included a wide range of degenerative changes, from nuclear and cytoplasmic features indicative of apoptosis (eg, chromatin margination along an intact nuclear membrane, chromatin condensation, swollen mitochondria with fragmentation of the mitochondrial crests, an increased number of cytoplasmic vacuoles, and conservation of the membranes) to advanced damage (eg, isolated, destroyed cell remnants) (Figure 5).

**INFLAMMATORY INFILTRATE**

The immunohistochemical analysis of the early inflammatory infiltrate showed a prevalence of CD4-positive cells, whereas CD8 and CD20 lymphocytes were less represented, and CD68 stained isolated cells. No CD56-positive cells were detected (Figure 6). Significant results are summarized in the Table.

**COMMENT**

Photodynamic therapy is a recent, promising treatment option for superficial nonmelanoma skin cancers, and its effectiveness has been considered comparable to that of traditional therapies in several studies. Even if most PDT failures seem related to tumor depth, a number of BD and thin BCC lesions escape PDT treatment for unknown reasons. Knowledge of the mechanisms of PDT action on skin tumors could help us to better focus PDT treatment on those lesions that have the best chances to respond. It is believed that tumor ablation by PDT may result from direct, vascular, and/or immunologic mechanisms. The contribution of each of them can be studied only in vivo. In an effort to highlight the actual pathways of PDT action in vivo, we investigated the pathologic alterations in BCC and BD after PDT using sequential histologic analysis. Moreover, the apoptotic markers caspase-3 and TUNEL, as well as electron microscopy, were used in parallel with histologic evaluation, because definite identification of apoptosis relies on the recognition of different hallmarks. Our results help to define the time course and characteristics of the cutaneous response to PDT. Pathologic changes parallel the clinical evidence after PDT: skin inflammation with erosions and crusts, followed by healing with fibrosis and, sometimes, hyperpigmentation. Photodynamic therapy induces early, intense damage to the epithelial cells, strongly suggesting a direct cytotoxic effect as the main mechanism of tumor cell death. In fact, the histologic signs of cell injury can be detected as early as 15 minutes after PDT, increasing progressively for the first 2 days. Photodynamic damage ultimately leads to a complete renewal of the epidermis and to the destruction of the tumor nodules. The dermal microvasculature showed vasodilation, but no evidence of a vascular mechanism...
(ie, histologic signs of true vasculitis and/or thrombosis), implied in the tumor destruction could be found. This finding is consistent with the topical delivery of the lipophilic photosensitizer, which targets the intracellular compartments (mitochondria) and bypasses the cutaneous vascular system. Indeed, hydrophobic photosensitizers such as PpIX are supposed to destroy tumor cells, primarily through direct action.14

The inflammatory response was immediate and intense, but it appeared histologically and immunohistochemically unspecific, with an initial recruitment of granulocytes, T and B lymphocytes, plasma cells, and monocyte macrophages. No clear evidence of early tumor aggression was found. These observations suggest that the early inflammatory infiltrate plays a subsidiary role in immediate tumor destruction. Nevertheless, inflammation may trigger a specific antitumor immune response that could be relevant to long-term tumor control15,16; this possibility cannot be ruled out in our experimental setting.

We obtained definite evidence of activation of the apoptotic mechanism in tumor cells, with a striking consistency between our histologic and immunohistochemical observations. Single apoptotic cells, identified by histologic studies and TUNEL staining, could be observed in the BCC nodules as early as 1 hour after irradiation, with a progressive increase of the number of apoptotic cells in the first 48 h. No signs of ongoing apoptotic reaction are seen 7 d after PDT.

In BCC, apoptosis takes place primarily and predominantly at the periphery of the dermal nodules, often leaving islands of apparent tumor resistance in the center.

Table. Main Results of the Study

1. Full-thickness epidermal damage and a massive dermal inflammatory infiltrate are detected as early as 15 min after PDT irradiation in BCC and BD.
2. The damaged epidermis is shed in the days after PDT, whereas progressive epidermal regeneration takes place. A well-formed, multilayered epidermis is present 7 d after PDT.
3. The inflammatory infiltrate gradually fades in the dermis, switching from mixed/acute to chronic/mononuclear and surrounding the tumor remnants.
4. Dermal healing involves a fibroblastic response with final fibrosis and/or lentigolike changes.
5. In the BCC nodules, histologic, immunohistochemical, and electron microscopic evidence of apoptosis is detected as early as 1 hour after irradiation, with a progressive increase of the number of apoptotic cells in the first 48 h. No signs of ongoing apoptotic reaction are seen 7 d after PDT.
6. In BCC, apoptosis takes place primarily and predominantly at the periphery of the dermal nodules, often leaving islands of apparent tumor resistance in the center.
7. No altered histologic or immunohistochemical features were detected in cases of tumor persistence 2, 4, or 8 wk after PDT.

Abbreviations: BCC, basal cell carcinoma; BD, Bowen disease; PDT, photodynamic therapy.
mor cells could be observed after 1 week. Early activation of the apoptosis-inducing enzyme caspase-3 and the ultrastructural observations correlate well with these findings. These results differ in part from the observations of Tosca et al., who found signs of epidermal necrosis progressing from the first to the fourth day after PDT and evidence of necrosis in BCC islands at the fourth day.

Nakaseko et al. found histologic evidence of epidermal damage (cells with eosinophilic cytoplasm and hyperchromatic nuclei and cell vacuolization) at 1 and 3 hours after δ-aminolevulinic acid and PDT for actinic keratoses. In addition, they found full-thickness epidermal necrosis 1 day after therapy. These authors did not observe TUNEL or activated caspase-3 staining until 1 day after PDT; the samples obtained 1 and 3 hours after PDT yielded negative results. Different protocols in both PDT and immunohistochemical techniques can explain these discrepancies. In fact, the onset and progression of tumor damage by PDT depends on a variety of different technical factors that are related to the types of photosensitizer and irradiation. In the previous studies, different concentrations of δ-aminolevulinic acid were irradiated with broadband lamps at lower fluences than those used in our investigation. This difference could be responsible for the delayed onset of tumor damage, compared with our experimental model. Moreover, it is conceivable that the parallel investigation in our study, which used histologic and immunohistochemical analyses in the same samples, enabled us to detect even the slightest signs of epithelial cell damage that might otherwise have been missed. Indeed, the perfectly matched time course and the colocalization of histologic and immunohistochemical hallmarks make the activation of apoptosis immediately after PDT unquestionable. Furthermore, these results are consistent with evidence obtained in experimental tumors in vivo, showing an early, progressive increase in apoptotic cell numbers after PDT. 5

The contributions of apoptosis vs necrosis as death-effector mechanisms in PDT are still debated. In in vitro investigations, prevalence of one of these mechanisms is largely dependent on the experimental setting. In general, the subcellular localization of the photosensitizer strongly influences the cell response after PDT, and photosensitizers localized in the mitochondria, such as PpIX, are potent inducers of an apoptotic response. 17 Differentiation between cell death mechanisms is much easier in vitro, at defined photosensitizer concentrations and light fluences, than in vivo. Indeed, in the latter case, the situation is much more complex because of progressive photobleaching of the sensitizer, ongoing inflammatory responses, and suboptimal irradiation in deep tumor regions; thus, tissue destruction is likely caused by a combination of apoptosis and necrosis. 18 Our histologic observations cannot exclude that a necrotic mechanism is involved in the epidermal damage because of the immediate and massive degeneration at the epidermal tissue level that occurs after PDT. In this case, the TUNEL staining of the entire epidermis may reflect keratinocyte death with heavy DNA degeneration. Indeed, TUNEL staining, which is generally considered a reliable apoptotic marker, may yield positive results in necrotic cells. 19

On the contrary, apoptosis appears to be the main effector mechanism in the BCC nodules because it takes place progressively at the single cell level and because no signs of generalized early destruction are apparent. This hypothesis is consistent with previous investigations showing that the type of cell death switches from apoptosis to necrosis with the increased intensity of the insult. 20 In our case, the injury would be maximal at the epidermis and progressively lower in the dermis.

A consistent observation was that apoptosis begins and predominates at the periphery of the BCC nodules, whereas the innermost tumor cells sometimes appear to be unaffected by PDT, as was seen in our histologic and immunohistochemical studies. The depth of the tumor nodules did not seem to correlate with the degree of damage because some of the inner cells of the subepidermal BCC nodules also appeared unchanged. This finding suggests that the photosensitizer penetration from the dermal-epithelial junction into the tumor nodules is crucial for reaching an effective damage threshold. A number of previous studies 4,7 used fluorescence microscopy to evaluate the distribution of PpIX in BCC, but the results appear rather inconsistent, ranging from homogeneous fluorescence of superficial and nodular BCC treated with δ-aminolevulinic acid 21 to full- or partial-thickness fluorescence in the superficial part of nodular BCC. 22,23 One study reported the most intense PpIX fluorescence after δ-aminolevulinic acid pretreatment in the tumor regions adjacent to the dermis, 24 which correlates well with our observations. Nevertheless, similar studies with methylaminolevulinate also showed a selective and homogeneous distribution of the photosensitizer in thick BCC. 25 Another possible explanation is that the peripheral cells exert a protective action toward the center of the tumor nodules, for example, by reducing the availability of tissue oxygen and, therefore, the oxidative stress. Nevertheless, our experimental model does not allow confirmation of any relationship between the size of the tumor nodules and the PDT response, and further investigations would be required to address this issue.

The Bcl-2 family of proteins is a potent regulator of the mitochondrial (intrinsic) pathway of apoptosis and comprises both proapoptotic and antiapoptotic molecules. The antiapoptotic Bcl-2 protein is a potentially interesting molecule to investigate for its protective role against PDT injury and its variations after therapy that may influence cell death or rescue. Overexpression of Bcl-2 induces resistance against PDT-induced apoptosis, 26 whereas transfection with an antisense Bcl-2 sequence increases cell line sensitivity to PDT. 27,28 Moreover, it has been reported that Bcl-2 is specifically photodamaged by PDT 29 and that Bcl-2 expression decreases in BCC during other apoptosis-activating treatment, such as imiquimod therapy. 30 We were not able to find any clear-cut variation in Bcl-2 expression in BCC before and after PDT. A variable immunohistochemical staining for Bcl-2 was evident at various times after PDT in BCC nodules; a PDT-induced Bcl-2 destruction was not apparent in our model. Specific studies comparing a higher number of pretreatment with posttreatment samples are necessary to evaluate a possible in vivo variation, taking into ac-
count the variability in Bcl-2 expression in untreated BCC (reported at 67%-100%\(^{19}\)).

In conclusion, our results contribute to the definition of the pathologic events after PDT for skin cancers, help to highlight the mechanisms leading to tumor destruction, and provide evidence of a key role for apoptosis. These results should be considered for future technical refinements to improve the effectiveness of PDT and to state its position clearly among the presently available therapies.

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Author Contributions: Dr Fantini had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Fantini and Pincelli. Acquisition of data: Fantini, Greco, Surrenti, Peris, and Vaschieri. Analysis and interpretation of data: Fantini, Cesinaro, Marconi, and Giannetti. Drafting of the manuscript: Fantini, Vaschieri, and Marconi. Critical revision of the manuscript for important intellectual content: Greco, Cesinaro, Surrenti, Peris, Giannetti, and Pincelli. Obtained funding: Pincelli. Administrative, technical, and material support: Greco, Surrenti, and Vaschieri. Study supervision: Pincelli.

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Additional Information: The eTable is available at http://www.archdermatol.com.

REFERENCES


### eTable. Panel of Antibodies Used in the Study

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