Vitamin D3 Enhances the Apoptotic Response of Epithelial Tumors to Aminolevulinate-Based Photodynamic Therapy

Sanjay Anand1,2, Clara Wilson2, Tayyaba Hasan3, and Edward V. Maytin1,2,3

Abstract

Photodynamic therapy, mediated by exogenously administered aminolevulinic acid (ALA-PDT), followed by exposure to a laser or broadband light source, is a promising modality for treatment of many types of cancers; however, it remains inadequate to treat large, deep, solid tumors. In this article, we report that calcitriol, the active form of vitamin D3, can be administered before ALA as a nontoxic preconditioning regimen to markedly increase the efficacy of ALA-PDT. Using mouse models of squamous cell skin cancer for preclinical proof of concept, we showed that calcitriol, delivered topically or intraperitoneally, increased tumoral accumulation of the PDT-activated ALA product protoporphyrin-IX (PpIX) up to 10-fold, mainly by altering expression of the porphyrin-synthesis enzymes coproporphyrinogen oxidase (increased) and ferrochelatase (decreased). Calcitriol-pretreated tumors underwent enhanced apoptotic cell death after ALA-based PDT. Mechanistic studies have documented activation of the extrinsic apoptotic pathway, with specific cleavage of caspase-8 and increased production of TNF-α in tumors preconditioned by calcitriol treatment before receiving ALA-PDT. Very low doses of calcitriol (0.1–1 μg/kg body weight) were sufficient to elicit tumor-selective enhancement to ALA-PDT efficacy, rendering toxicity concerns negligible. Our findings define a simple, nontoxic, and highly effective preconditioning regimen to enhance the response of epithelial tumors to ALA-PDT, possibly broadening its clinical applications by selectively enhancing accumulation of photosensitizer PpIX together with TNF-α in tumors. Cancer Res; 71(18): 6040–50. ©2011 AACR.

Introduction

Photodynamic therapy (PDT) is a cancer treatment that combines a photosensitizer, light, and oxygen to achieve selective cell killing (1, 2). PDT offers the advantage of dual targeting, because both the photosensitizer and the visible light can be effectively localized to the tumor (1). Excellent selectivity can be achieved with 5-aminolevulinic acid (5-ALA; ALA), a small-molecule precursor for the synthesis of endogenous porphyrins, most notably protoporphyrin IX (PpIX) within cells (3). Because PpIX, not ALA, is the actual photosensitizer and because pathways that regulate ALA uptake and conversion into PpIX are generally more active in tumor cells than in normal cells, tumor-selective PpIX accumulation to very high levels can be obtained (4). PDT, mediated by exogenously administered aminolevulinic acid (ALA-PDT), has proven clinically useful for wide-field treatment of carcinoma in situ of the skin (4–7) and cervix (8). ALA-PDT is also useful for image-guided surgical resection of urinary tract dysplasia (9) and high-grade gliomas in the brain (10, 11). However, when employed as a monotherapy, ALA-PDT remains unsatisfactory for treatment of large and deep tumors, with incomplete response rates and recurrence rates that are often less than ideal (reviewed in ref. 4).

To improve the efficacy of ALA-PDT, our goal has been to develop new combination regimens based on the observation that certain pharmacologic agents, which we call "differentiating agents," can enhance PpIX accumulation in a variety of epithelial cancer cells (12–14). For example, we recently reported that methotrexate, administered systemically as a 3-day preconditioning regimen prior to ALA-PDT, significantly enhances the accumulation of PpIX within squamous tumor cell lines both in vitro and in vivo (14, 15). Remarkably, methotrexate exerts these effects at subnanomolar concentrations (i.e., 100- to 1,000-fold lower than those typically used in the clinic; refs. 14, 15), clearly too low to be tumoricidal yet sufficient to trigger differentiation in choriocarcinoma cells (16, 17), normal keratinocytes (18), and A431 carcinoma cells (15). At these same low levels, methotrexate was shown to enhance PpIX accumulation through a mechanism involving increased expression of coproporphyrinogen oxidase (CPO), one of the porphyrin-synthetic enzymes (15).

In the current article, another class of molecule, vitamin D, is shown to selectively enhance PDT by preferentially increasing...
PpIX accumulation in preneoplastic and neoplastic cells in vivo, while causing very little damage to normal tissue. Vitamin D belongs to a family of steroid hormones that regulate bone and mineral metabolism, calcium homeostasis, and cellular differentiation (reviewed in refs. 19–21). Although pathways for synthesis and processing of the various isofoms of vitamin D are rather complex, it is clear that the major isofom that interacts with nuclear receptors and mediates hormonal effects at the end organs is the trihydroxylated molecule 1α,25-dihydroxy-vitamin D3 (or calcitriol; ref. 20). The first indication that vitamin D might be useful for enhancing PDT was our finding that calcitriol and synthetic vitamin D analogues can stimulate increases in PpIX levels in LNCaP prostate cancer cells (13). In other cells, however, this phenomenon did not appear entirely consistent. For example, when vitamin D was added to cultures of skin keratinocytes, no PpIX-elevating effect was observed in monolayer cell culture (22). However, when the same cells were exposed to calcitriol in a 3-dimensional (3D; organotypic) culture system, they responded by accumulating significantly higher levels of PpIX (22). The remarkable potency of calcitriol (in the picomolar range) for stimulating PpIX accumulation in 3D cultures, coupled with indications that such effects require a native tissue environment, encouraged us to examine vitamin D preconditioning in vivo. In this article, we describe an investigation of the ability of vitamin D to affect PpIX accumulation in fully formed epithelial tumors in vivo. Two different tumor models were used to represent premalignant dysplasia and malignant carcinoma. We confirmed that vitamin D confers a tumor-specific increase in photosensitizer (protoporphyrin) levels, and showed the underlying mechanism, namely, a change in the expression of 2 rate-limiting porphyrin-synthetic enzymes. Furthermore, we showed that vitamin D preconditioning selectively stimulates increased tumor cell killing after ALA-PDT, relative to unconditioned controls. Finally, we explored the mechanism of increased cell death and showed that vitamin D encourages activation of the extrinsic apoptotic pathway after ALA-PDT, accompanied by massive increases in TNF-α production.

Materials and Methods

Reagents

Vitamin D ointments Dovonex (calcipotriene, 0.005%) and Vectical (calcitriol; 3 μg/g) were obtained from Warner Chilcott Inc., and Galderma Laboratories, respectively. Calcitriol (1α,25-dihydroxy-vitamin D3) was obtained from Sigma-Aldrich.

Generation of tumor models in vivo

Superficial tumors in SKH-1 mice were generated by topical application of 7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) as described previously (15). To generate deep s.c. tumors, immunocompromised nude mice (obtained from Charles River Laboratories) were injected with 2 × 106 A431 cells in the flanks, as described previously (15). The A431 human squamous cell carcinoma cell line was purchased from the American Type Culture Collection where the line was characterized by morphologic appearance during growth and recovery, cytochrome C oxidase I PCR for species specificity, and human origin by short tandem repeat (STR) profiling.

Preconditioning regimen using vitamin D

Superficial tumors were preconditioned with topical Vectical, Dovonex, or petrolatum (control) once daily for 3 days followed by topical application of ALA (Levulan Kerastick; DUSA Pharmaceuticals) for 4 hours on the fourth day. Deep tumors were preconditioned via systemic vitamin D in PBS (1 μg/kg) or PBS alone, delivered daily for 3 days by intraperitoneal (i.p.) route. ALA was administered as described previously (15).

Analysis of PpIX fluorescence and histologic markers in tumors

For analysis of PpIX levels, tumors were harvested, embedded for frozen sectioning, and analyzed on a Leica confocal microscope as previously described (15). Standard hematoxylin and eosin (H&E) staining was done on formalin-fixed/paraffin-embedded tissues, and immunohistochemical staining was carried out as previously described (23). Sources and dilutions of antibodies were as follows: active caspase-3 (BioVision; dilution 1:50), ferrochelatase (FC; 1:100), E-Cad (Santa Cruz Biotechnology; 1:100), Ki-67 (NeoMarkers; 1:250), GAPDH (Santa Cruz Biotechnology; 1:100), TNF-α (BioXcell; 1:100), CY3, or fluorescein isothiocyanate (FITC)-conjugated Donkey anti-Rabbit (Jackson ImmunoResearch; 1:1,500). To assess cell proliferation in vivo, 5-ethyl-2′-deoxyuridine (EdU) was injected (100 μg/mouse, i.p.) 1 hour before the animals were sacrificed. Formalin-fixed tissues were sectioned and incubated with the Click-IT reaction cocktail according to the manufacturer’s instructions (Invitrogen). To estimate cell death, the terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay was conducted on paraffin sections according to the manufacturer’s (Roche Applied Science) directions.

Light exposures for PDT in vivo

Tumors were irradiated at a fluence of 100 J/cm² using a 633-nm noncoherent light source (LumaCare Products). The source was calibrated using a FieldMate laser power meter (Coherent).

Western blot analyses

Frozen tumors were powdered in a tissue pulverizer (Fisher Scientific), dissolved in urea lysis buffer, and analyzed by the Western blot technique, as described previously (15). Primary antibodies were directed against ALA dehydratase (ALAD Abnova; 1:1,000); Active/Cleaved caspase-8 (Novus Biologicals; 1:5,000); Cleaved caspase-9 (Cell Signaling Technology; 1:1,000); caspase-12 (Santa Cruz Biotechnology; 1:1,000); FC (a gift of Dr. Harry Dailey; 1:5,000); glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology; 1:5,000); porphobilinogen deaminase (Abnova; 1:1,000); TNF-α (BioXcell; 1:1,000); and α-tubulin (Sigma-Aldrich; 1:10,000). Secondary
Results

To test the hypothesis that vitamin D, delivered as a preconditioning regimen, may enhance the production of intracellular protoporphyrin and subsequent responses to ALA-PDT, 2 in vivo epithelial tumor models were employed: (i) superficial, low-grade squamous cell carcinomas (papillomas) generated by chemical carcinogenesis in hairless mice (Fig. 1A), and (ii) a malignant carcinoma model in which human squamous carcinoma cells (A431) were implanted s.c. into immunocompromised nude mice (Fig. 1B).

Topical preconditioning of superficial tumors with vitamin D analogues enhances the accumulation of PpIX photosensitizer

Superficial skin tumors were pretreated topically with inert vehicle (petrolatum), or with an ointment containing 1 of 2 different active vitamin D analogues (calcitriol or calcipotriene), daily for 3 days. The mice were then treated with topical ALA for 4 hours, and tumors were harvested 4 days after the initiation of vitamin D treatment. Frozen tumor sections were examined by confocal microscopy under conditions that selectively excite the PpIX molecule and allow detection of PpIX-specific fluorescence (Fig. 1C). This technique was shown previously to correlate closely with biochemical measurements of PpIX in cells and tissues (15). In vitamin D–preconditioned tumors, PpIX was more abundant (as documented by fluorescent intensity measurements averaged over the entire tumor; Fig. 1D) and was also qualitatively present at greater depths within the tumor, as compared with nonconditioned (control) tumors. This was true whether calcitriol (Fig. 1C) or calcipotriene (Fig. 1D) was used. With either of the vitamin D analogues, the ~3-fold increase in PpIX observed within tumors was statistically significant (Fig. 1D), whereas no differential effect upon PpIX was observed in normal skin (data not shown).

Systemically delivered vitamin D enhances PpIX accumulation in deep squamous cell (A431) tumors

To examine effects of vitamin D in an invasive tumor model, squamous carcinoma cells (A431) were implanted in nude mice. Animals with visible tumors received systemic preconditioning with calcitriol or vehicle alone (saline) over a 3-day period. On the fourth day, ALA was given intramuscularly, and 24 hours later, tumors were harvested and analyzed for PpIX levels (Fig. 1E). Calcitriol pretreatment caused a marked increase in PpIX accumulation throughout the tumor (Fig. 1E; compare top vs. bottom panels). A dose–response study (Fig. 1F) revealed a significant increase in PpIX (4-fold) at a dose of 0.1 μg/kg calcitriol, and PpIX levels reached maximal levels (10-fold) at 0.5 μg/kg calcitriol and more (Fig. 1F). Local (perilesional) injection of calcitriol into the skin was also effective for delivery (Fig. 1F, bar 7). Interestingly, intratumor injection of calcitriol at a site distant from the tumor also exerted a PpIX-elevating effect, although less pronounced (Fig. 1F, bar 8). Measurements of the serum calcitriol concentration at 1 hour after injection or topical application revealed calcitriol levels between 6 and 9 ng/mL; residual levels at day 4 (after 3 preconditioning doses) were indistinguishable from normal physiologic levels of calcitriol (Supplementary Table S1).

Changes in porphyrin-synthetic enzyme expression contribute to the increase in PpIX accumulation in vitamin D–pretreated tumors

To explore the mechanism of increased PpIX accumulation after calcitriol pretreatment, A431 tumor lysates from mice preconditioned with calcitriol (1 μg/kg) or saline alone were examined for changes in levels of the 4 porphyrin-synthetic enzymes [ALAD, porphobilinogen deaminase (PBGD), coproporphyrinogen oxidase (CPO), and FC] that have been described as potentially rate-limiting under various circumstances in different biologic systems (24–29). Two enzymes were substantially altered after vitamin D pretreatment (Fig. 2A). CPO was increased ~5-fold, and FC was decreased ~4-fold (Fig. 2B). Each of these changes (higher CPO levels and lower FC levels) favors an increase in accumulation of PpIX because the enzymes lie immediately upstream and downstream of PpIX, respectively.

Vitamin D pretreatment elicits preferential changes in cellular differentiation and proliferation within tumors in vivo

In our previous studies in various epithelial cancer cells in vitro (12–14) and in vivo (15), we had shown that differentiation-promoting agents such as methotrexate cause an increase in PpIX accumulation that is tightly correlated with the enhancement of terminal differentiation. Methotrexate always caused growth arrest, but in the case of vitamin D, the effects upon growth were less clear. For example, in an organotypic keratinocyte model, calcitriol caused enhanced differentiation while having no apparent effect upon proliferation (22).

To examine the effects of vitamin D upon epithelial tumors in vivo, mice bearing superficial papillomas or deep A431 tumors were preconditioned with vitamin D by topical or systemic delivery, respectively, and markers of cellular differentiation (E-cadherin) and proliferation (Ki-67) were examined histologically (Fig. 3). After vitamin D pretreatment, E-cadherin expression was increased 4- to 6-fold in both types of tumors, as compared with an insignificant increase (<1.5-fold) in normal skin (Fig. 3A). Vitamin D pretreatment also increased expression of the proliferative marker (Ki-67) in both
Figure 1. Tumor models used in this study. A, superficial carcinogen-induced papillomas. B, s.c. A431 human squamous cell carcinoma 10 days after subdermal injection of 2 million A431 cells. C, PpIX levels in superficial tumors, after 3 days of daily pretreatment with topical calcitriol (3 μg/g) or inert vehicle (petrolatum), followed by administration of 5-ALA on day 4 to stimulate PpIX synthesis. Confocal micrographs of frozen tumor sections are shown. D, digital quantification of PpIX-specific fluorescence in papillomas pretreated with either topical calcipotriene (0.005%) or calcitriol. Mean ± SEM from 3 to 6 tumors per condition; fold increases relative to inert vehicle. E, PpIX in deep A431 tumors after 3 days of pretreatment with i.p. calcitriol (1 μg/kg) or with saline, followed by 5-ALA on day 4. F, digital quantification of PpIX-specific fluorescence in A431 tumors subjected to increasing doses of calcitriol (μg/kg body weight), delivered either by an i.p. route or by injection into the skin at the site of the lesion. In the latter case, another tumor located ~4 cm away from the first was analyzed to examine the systemic effect of intracutaneous injection (distal). Mean ± SEM from 6 tumors per condition. The P values from unpaired 2-sided t-tests are indicated above the brackets. Scale bar, 50 μm.
tumor types, as compared with no significant increase in normal skin (Fig. 3B). To confirm that vitamin D stimulates tumor cell proliferation in vivo, a second assay was conducted in which EdU was injected into mice to measure incorporation into newly synthesized DNA. The latter experiments confirmed that DNA synthesis is stimulated in tumors by pretreatment with vitamin D (Fig. 3C).

To determine which tumor cell populations express the highest levels of PpIX following vitamin D pretreatment, colocalization experiments were carried out wherein adjacent histologic sections were examined for PpIX (Supplementary Fig. S1A), E-cadherin (Supplementary Fig. S1B), and Ki-67 (Supplementary Fig. S1C). The data show that the highest levels of PpIX in the A431 tumors correspond to differentiating areas, and these are almost completely distinct from regions that express the proliferation marker, Ki-67 (Supplementary Fig. S1A–C). The differentiating tumor areas are also the sites of TNF-α production following light exposure (Supplementary Fig. S1D), which is described in more detail later in this article.

**PDT-mediated cell death is enhanced in vitamin D–preconditioned tumors**

Tumors were preconditioned with calcitriol for 3 days, treated with ALA and exposed to light, and then harvested at various time points for analysis of cell death (Fig. 4). With the use of the TUNEL assay on A431 tumors, a large increase in cell killing was observed in vitamin D–pretreated tumors (relative to vehicle alone) at 1, 6, and 24 hours, representing a ~6-fold difference between the 2 conditions at all time points (Fig. 4A and B). In the squamous cell papilloma model, a similar preferential enhancement of cell death in vitamin D–preconditioned tumors was observed (Fig. 4C). To better examine the microanatomic extent of destruction, tumors were also examined using H&E staining and scored by morphologic criteria, primarily the complete loss of cells (Fig. 4D; Supplementary Fig. S2A and B). Although evidence of cell death was observable in control tumors by 6 hours of ALA-PDT, significantly more areas of cell death were seen by 1 hour in vitamin D–preconditioned tumors, rising to a significantly larger fraction of the total tumor area (~250% larger) at 24 hours (Fig. 4E). Enhancement of cell killing occurred preferentially in vitamin D–preconditioned tumor tissue as compared with adjacent normal skin, indicating tumor selectivity of the vitamin D effect (Supplementary Fig. S2C).

**Enhancement of PDT-mediated cell death by vitamin D involves cleavage of caspase-3 and caspase-8**

Because positive TUNEL labeling indicates (but does not prove) that an apoptotic pathway is activated after ALA-PDT, histologic sections from papillomas (Fig. 5A) or A431 tumors (Fig. 5B) were probed with an antibody specific for the activated (cleaved) form of caspase-3. The results show that caspase-3 is activated by ALA-PDT by 6 hours in both systems, and vitamin D appears to cause a preferential increase in caspase-3 activation. To learn which upstream proteolytic pathway is responsible for caspase-3 activation (30), tumor lysates were evaluated by Western blot analysis; no activation of caspase-9 (Supplementary Fig. S3A; associated with the mitochondrial pathway) (30), nor of caspase-12 (Supplementary Fig. S3B; associated with the endoplasmic reticulum pathway; ref. 31) could be shown. In contrast, a plasma membrane-associated caspase (caspase-8) was significantly affected by vitamin D pretreatment (Fig. 5C). Expression of the precursor form (Fig. 5D) and levels of the cleaved forms, p41 and p43 (Fig. 5E), were all increased significantly. Together, these results indicate that caspase-8–mediated apoptosis plays a major role in vitamin D enhancement of tumor cell death after ALA-PDT.

**The combination of vitamin D preconditioning and ALA-PDT significantly increases production of TNF-α within tumors in vivo**

The fact that caspase-8 is preferentially cleaved in vitamin D–preconditioned tumors after ALA-PDT (Fig. 5) suggests that a membrane-associated death receptor, such as TNF receptor (TNF-R), might be activated in this process. To test this possibility, levels of TNF-α in calcitriol-preconditioned
tumors were compared with TNF-α levels in nonconditioned tumors; a small, less than 2-fold increase in the baseline level was observed in the vitamin D subset (Fig. 6A). Following ALA-PDT alone (in the absence of vitamin D), a robust increase in TNF-α levels of ~10- to 15-fold was seen at 1 hour and remained nearly constant up to 24 hours (Fig. 6B and C, left). In contrast, ALA-PDT delivered after calcitriol preconditioning led to a dramatic and significant increase in TNF-α levels, reaching a ~70-fold elevation by 24 hours (Fig. 6B and C, right). Immunostaining of PDT-treated tumor tissue revealed that the highest TNF-α expression was found in linear, isolated regions (pockets) near the central portions
of the tumors, which appear to represent vascular structures (Fig. 6D; Supplementary Fig. S1D). However, the majority of TNF-α staining co-localized within tumor cells that also expressed E-cadherin, that is, in the differentiated regions of vitamin D–preconditioned tumors (Supplementary Fig. S1D).

Discussion

Our data show that epithelial skin tumors in vivo, whether premalignant or malignant, accumulate higher intracellular levels of PpIX when preconditioned with vitamin D (calcitriol). These PpIX increases occur preferentially in neoplastic versus normal tissues. The underlying biochemical basis for these observations is an altered expression of 2 enzymes in the porphyrin synthesis pathway after vitamin D preconditioning. CPO is increased whereas FC is decreased; the net effect is a greater accumulation of PpIX. As a consequence, vitamin D–preconditioned tumors show an enhanced cytotoxic response to ALA-PDT.

Our data suggest that, in addition to a selective increase in PpIX, other mechanisms contribute to vitamin D–mediated enhancement of tumor killing. In the vitamin D–pretreated tumors prior to ALA-PDT, a small (~2-fold) increase in TNF-α can be detected. However, following exposure to ALA-PDT, TNF-α levels become preferentially elevated in the vitamin D–preconditioned tumors (~70-fold above baseline), mostly within the differentiated tumor cells but also within vascular structures, compared with unconditioned tumors (~10-fold above baseline). Notably, expression of TNF-α within blood vessels and endothelial cells in inflamed tissues has been reported previously (32, 33). High TNF-α levels are often
associated with activation of the extrinsic apoptotic pathway, in which pro-caspase-8 cleavage occurs after ligation of death receptors such as TNF-R and TNF-related apoptosis-inducing ligand (TRAIL-R1) on the plasma membrane of cells (34, 35). The extrinsic pathway is involved after PDT, on the basis that caspase-8 and caspase-3 are preferentially activated, whereas caspase-9 and caspase-12 are not. In the few studies in the literature that examined mechanisms of cell death following aminolevulinate-based PDT, caspase activation in leukemia and lymphoma cells was either undetectable (36) or involved caspase-9 (37). However, human oral (epithelial) cancer cells showed activation of both caspase-8 and caspase-9 after PDT using 5-ALA (38). The latter report is consistent with our observation of ALA-PDT–mediated activation of caspase-8 in vitamin D–pretreated A431 epithelial tumors in vivo.

Although the ability of vitamin D to elevate PpIX levels in cultured cells was described previously (13, 22, 39), our new data are significant because they establish a tumor-selective PpIX-inducing effect of vitamin D in vivo. The magnitude of PpIX enhancement achievable in tumors in vivo equals or exceeds the effects obtainable in vitro—an observation that may indicate a role for paracrine signaling between epithelial and stromal cells. The 3- to 10-fold rise in PpIX accumulation in benign papillomas and A431 s.c. tumors compares very favorably to calcitriol-induced elevations in PpIX reported previously in cultured cell lines. For example, PpIX was elevated 1.5-fold in cultured A431 cell monolayers (39), 2.3-fold in organotypic rat epidermal keratinocytes (REK; ref. 22), and 10-fold in LNCaP prostate carcinoma cells (13), when those cultures were preincubated with calcitriol (pmol/L to nmol/L concentration range, 24- to 96-hour incubation) followed by ALA or hexyl-ALA.

The finding that vitamin D leads to activation of the extrinsic pathway and increased TNF-α production is particularly compelling because the literature suggests a beneficial link between high TNF-α levels and increased PDT efficacy. Bellnier showed that, for s.c. implanted adenocarcinomas, injection of a single dose of recombinant TNF-α 3 hours before PDT (using Photofrin, from Axcan Pharma, as the photosensitizer) led to an improved tumor response, with no deleterious effects upon normal skin photosensitization (40). The same group showed that preadministration of 5,6-dimethylxanthenone-4 acetic acid (DMXAA), a drug that elevates TNF-α levels, causes significant enhancement in tumor responsiveness to Photofrin-mediated PDT in mice with s.c. RIF-1 fibrosarcomas (41). Furthermore, DMXAA

Figure 5. Changes in expression of caspase-3 and caspase-8, in vitamin D-pretreated (+Vit D) versus nonpretreated (−Vit D) tumors, as a function of time after ALA-PDT. Immunodetection of activated caspase-3 (insets) and quantitative counts of activated caspase-3 (graphs; mean ± SD of 2 tumors, 3 sections/tumor) in histologic specimens from, A, squamous papillomas and, B, A431 tumors. White bars, no preconditioning; Gray bars, vitamin D–preconditioned. C, Western blot analysis of pro-caspase-8 expression (top-half; 6 μg protein/lane), and cleaved forms of caspase-8 (bottom-half; 25 μg protein/lane) from A431 tumors. D, densitometric scans of the pro-caspase blot in C, mean ± range. E, densitometric analysis of 2 Western blotting experiments that analyzed cleaved caspase-8; mean ± SEM, n = 4 tumors per point. Asterisks, significantly higher than the corresponding time point in nonpretreated tumors; *, P < 0.05; **, P < 0.005.
displayed a similar PDT-enhancing effect using a different tumor model (colon carcinoma cells) and photosensitizer (Photochlor; HPPH; ref. 42). Although the photosensitizer used in those studies was not 5-ALA, the results suggest that any agent capable of increasing intratumoral TNF-α levels at the time PDT is administered might help to increase overall cell killing. Preconditioning with vitamin D represents a novel way to achieve this end.

Vitamin D now joins methotrexate in a growing arsenal of agents that effectively promote PpIX accumulation. However, methotrexate stimulates tumor cell differentiation and inhibits proliferation (15), whereas vitamin D appears to stimulate both differentiation and proliferation (Fig. 3; Supplementary Fig. S1). This brings up 2 important questions: (i) what is the cellular basis for vitamin D–stimulated proliferation in these areas, and (ii) what are the possible consequences for the therapeutic response to PDT? The fact that vitamin D causes increased proliferation in the normal epidermis of mice (Fig. 3C) is actually a well-reported phenomenon (43, 44) for which the molecular basis remains controversial. A common misconception is that vitamin D always causes epithelial growth arrest, but Gniadecki and colleagues showed that, in human keratinocytes, exposure to calcitriol (at pmol/L or nmol/L concentrations) can cause either growth arrest or hyperproliferation, based on whether cells are undifferentiated or committed to terminal differentiation (45). In breast cancer cell lines (46), calcitriol stimulated cell growth at low concentrations (0.1 nmol/L). In that situation, the balance between proliferation and growth arrest appeared to depend upon levels of a pro-proliferative molecule (a degradation product of calcitriol) that accumulates through the action of an oxidative enzyme, CYP24 (46). Taking everything discussed above into account, the observation that some regions of epithelial tumors in vivo exhibit a pro-proliferative response to calcitriol whereas other regions exhibit a differentiation response appears to make sense.

The more pressing question, of course, is how the differentiated versus proliferative regions of vitamin D–pretreated tumors respond to ALA-PDT, and whether the regions with low PpIX could be a problem if surviving cells were to continue to proliferate after treatment. Our data showed a robust cell kill in the central zone of nodular tumors (Supplementary Fig. S2) that was consistent with a combined effect of ALA-PDT and hypoxia in these regions. However, the surviving rim of cells observed at the tumor periphery is a reminder that this report is only an initial description of vitamin D and ALA-PDT; no judgment about preclinical efficacy can be made until detailed animal studies are conducted using higher fluences of light, multiple PDT treatments, and longer-term analyses of tumor regression and regrowth. The literature, however, offers indications that hyperproliferation in response to vitamin D could represent an advantage rather than a disadvantage. Tumor cells may become more susceptible to PDT when forced to actively proliferate; this was shown in studies (with either hematoporphyrin or PpIX) examining relationships between cell-cycle stage and PDT-responsiveness, which

Figure 6. TNF-α expression is increased in PDT-damaged A431 tumors as a result of vitamin D preconditioning (+ Vit D). A, in the absence of PDT, TNF-α levels in tumors are increased ~2-fold as a result of preconditioning with vitamin D; Western blot of individual tumor lysates. B, TNF-α levels are elevated at various times after ALA-PDT, and even more when PDT is combined with vitamin D preconditioning. C, quantitation of the relative changes in TNF-α as determined by densitometry of 2 pooled experiments (n = 4 tumors per point), mean ± SEM. **, significantly higher than corresponding time point in nonpreconditioned tumors, P < 0.0005. D, immunochemical demonstration of elevated TNF-α in vitamin D–preconditioned tumors, versus nonconditioned tumors, at 6 hours post-PDT. Inset: Control, no primary antibody. Scale bar, 50 µm.
showed that cells are usually most sensitive in S-phase, that is, while undergoing active DNA synthesis (47–49). Furthermore, as suggested by Gniadecki and colleagues (45), epithelial cells with a hyperproliferative response to vitamin D may actually represent a population already committed to terminal differentiation, thereby having a relatively low growth potential. A final point is that TNF-α released within vitamin D–preconditioned tumors may produce a bystander effect, promoting apoptosis in cells that manage to escape direct photocytotoxicity.

Thinking ahead to the clinic, it will be important to address the advantages and disadvantages of vitamin D preconditioning compared with preconditioning with methotrexate, another clinically used drug that has shown efficacy in increasing levels of ALA-induced PpIX (15). The potential for undesirable side effects, such as liver toxicity (with methotrexate) and hypercalcemia (with vitamin D), may be relatively small due to the short duration and low doses used to achieve transient PpIX-enhancement before PDT. However, only clinical trials can establish this for sure. In a pilot study to address the question of hypercalcemia in mice, peak and trough serum levels of calcitriol were measured (Supplementary Table S1) and compared with values from the literature (50). Calcitriol serum levels were measured 1 hour after the following conditions: (i) injection of a 1-μg/kg dose; (ii) injection of an 8-μg/kg dose, replicating the study of Muindi and colleagues (50); and (iii) a single application of calcitriol ointment. We found that peak levels were similar for all conditions (6–9 ng/mL), within the range reported by Muindi and colleagues (50). No evidence for a residual calcitriol elevation was found, that is, the serum trough levels after 3 days of preconditioning were indistinguishable from physiologic background levels in mice (50). This suggests a reasonable safety margin, given that Muindi and colleagues (50) employed calcitriol doses at least 8 times higher than those used in our study, and the doses used were sufficient to exert antitumor effects without any detectable side effects in mice (50). In humans, whether or not calcitriol can offer a safe and effective adjuvant approach for PDT remains an open question. Alternatively, could one use another form of vitamin D, such as 25-OH vitamin D3 (calcidiol, the major isof orm of vitamin D found in health food supplements) that requires subsequent conversion to calcitriol? These will be interesting questions for future studies.

Disclosure of Potential Conflicts of Interest

No conflicts of interest were disclosed.

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