Research Article

1α,25(OH)2-Vitamin D and a Nongenomic Vitamin D Analogue Inhibit Ultraviolet Radiation–Induced Skin Carcinogenesis

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Abstract

Exposure to ultraviolet radiation (UVR) can lead to a range of deleterious responses in the skin. An important form of damage is the DNA photolesion cyclobutane pyrimidine dimer (CPD). CPDs can be highly mutagenic if not repaired prior to cell division and can lead to UV-induced immunosuppression, making them potentially carcinogenic. UVR exposure also produces vitamin D, a prehormone. Different shapes of the steroid hormone 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3] can produce biological responses through binding either to its cognate nuclear receptor (VDR) to regulate gene transcription or to the VDR associated with plasma membrane caveolae to produce, via signal transduction, nongenomic physiologic responses. Here, we show that both 1,25(OH)2D3 and 1α,25(OH)2-lumisterol (JN), a conformationally restricted analogue that can generate only nongenomic responses, are effective inhibitors of UV damage in an immunocompetent mouse (Skh:hr1) model susceptible to UV-induced tumors. Both 1,25(OH)2D3 and JN significantly reduced UVR-induced CPD, apoptotic sunburn cells, and immunosuppression. Furthermore, these compounds inhibited skin tumor development, both papillomas and squamous cell carcinomas, in these mice. The observed reduction of these UV-induced effects by 1,25(OH)2D3 and JN suggests a role for these compounds in prevention against skin carcinogenesis. To the best of our knowledge, this is the first comprehensive report of an in vivo long-term biological response generated by chronic dosing with a nongenomic-selective vitamin D steroid. Cancer Prev Res; 4(9); 1485–94. ©2011 AACR.

Introduction

Ultraviolet radiation (UVR) causes many biological responses in the skin, including sunburn inflammation (erythema), apoptosis, photo immunosuppression, and mutagenic DNA damage. One of the most common DNA photolesions is the cyclobutane pyrimidine dimer (CPD), formed generally when 2 adjacent pyrimidines undergo an opening of the 5-6 double bond and form a stable ring structure following absorption of photons from UVR wavelengths in the UVB range, though other mechanisms may also contribute to CPD formation (1, 2). Other DNA photolesions can be formed, including the 6-4 photoproduct (6-4PP), but these lesions are repaired more efficiently and are less prevalent than CPDs (3). CPDs are highly mutagenic, and if they are not repaired before cell replication they may ultimately lead to skin carcinogenesis (4). Repair of CPDs also prevents immunosuppression, as CPD formation is a direct molecular trigger of immunosuppression (5). Despite these detrimental effects of UVR, it is required to produce vitamin D3 in skin cells. Vitamin D3 is produced in the skin following exposure of its precursor, 7-dehydrocholesterol, to UVR (6). Following 2 successive hydroxylation reactions, the biologically active steroid hormone 1α,25-dihydroxyvitamin D3 [1,25(OH)2D3] is formed.

Steroid hormones can serve as chemical messengers in a wide number of species and target tissues to transmit signals that result in both genomic and nongenomic responses (7). Although it is clear that steroid hormone regulation of gene transcription is mediated by formation of a ligand–receptor complex with a cognate receptor of the superfamily of steroid hormone nuclear receptors (8), substantial evidence indicates that the nongenomic responses are mediated by a variety of receptor types located near or associated with the plasma membrane (7). Thus, the same 1,25(OH)2D3 nuclear receptor (VDR) has been found in the plasma...
membrane caveolae of target cells where it is engaged in mediating nongenomic responses (9, 10). The VDR ligand 1,25(OH)₂D₃ is highly conformationally flexible, and it has been shown that one shape of 1,25(OH)₂D₃ is preferred for genomic responses whereas another shape is preferred by the VDR for nongenomic responses (11–13). The 6-s-cis conformationally restricted analogue 1α,25(OH)₂-lumisterol (JN) shows little ability to increase transcription of the osteocalcin gene in bone cells compared with 1,25(OH)₂D₃ (14) but generates nongenomic actions in pancreatic β cells and endothelial cells (15, 16).

Our previous studies, and those of others, provide compelling evidence for novel photoprotective effects of vitamin D compounds in human skin cells and in hairless mice (17–24). These studies were limited to investigation of the effects of an application of these compounds in acute UVR-induced skin damage, including cell loss and formation of DNA lesions in the form of CPDs and preliminary studies on photoimmunosuppression. We previously proposed that 2 mechanisms might contribute to protection from UVR-induced DNA damage: a further enhancement by 1,25(OH)₂D₃ of nuclear p53 expression after UVR exposure and a decrease in nitric oxide (NO) products (17). The increase in p53 could facilitate DNA repair (25). Because increased NO inhibits DNA repair (26, 27), the 1,25(OH)₂D₃-induced decrease in NO products may also contribute to better DNA repair. Photocarcinogenesis, however, is the result of chronic UVR exposure. Consequently, although the data provide evidence for photoprotection by vitamin D compounds in terms of acute damage, they do not necessarily imply that the compounds inhibit skin carcinogenesis.

The photocarcinogenesis protocol in albinos hairless mice delivers an established carcinogenic dose resulting in initiation of benign mouse skin lesions and subsequently progression to squamous cell carcinomas (SCC; refs. 28–31). Mouse skin in the initiation stage shows persistent general hyperplasia and the formation of multiple discrete benign papillomas (32). The onset of such benign papillomas in mice is analogous to the appearance of actinic keratosis (AK) in human skin. AKs are thought to be the key event in the evolution from photo-aged skin to SCC (33). Similar to skin papilloma formation in mice, the causative factor of AKs is believed to be chronic/long-term UVR exposure. Previous studies have supported a protective role for 1,25(OH)₂D₃ and some related analogues in response to chemical-induced carcinogenesis, also initiated through papilloma formation (34, 35).

Although nongenomic responses to 1,25(OH)₂D₃ and other steroid hormones in various cell types have been described by many groups, their overall significance in physiologic responses is unclear and to date no long-term nongenomic response in vivo has been definitively linked to 1,25(OH)₂D₃. Our preliminary studies in human skin cells in culture were consistent with the proposal that the photoprotective effects of 1,25(OH)₂D₃ occur via a nongenomic signal transduction-mediated pathway. This report describes the use of the conformationally flexible steroid hormone form of vitamin D₃, 1,25(OH)₂D₃, and a conformationally restricted structural analogue JN to inhibit UVR-induced skin carcinogenesis.

Materials and Methods

Cell culture and treatments

Studies in human skin cells were approved by the University of Sydney Human Ethics Committee (reference 1866). Keratinocytes were cultured from neonatal foreskins as previously described (36), and cells of passages 2 to 5 were seeded onto poly-L-lysine–coated glass coverslips in 96-well plates for p53 studies or directly into 96-well plates for nitrotyrosine studies. The active metabolite 1,25(OH)₂D₃ was purchased from Sigma-Aldrich, whereas both JN and 1β,25-dihydroxyvitamin D₃ (HL) were synthesized in the laboratory of Prof. A.W. Norman (University of California, Riverside, CA). 25-Dehydro-1α-hydroxyvitamin D₃-26,23S-lactone (TEL-9647) was a gift from Dr. Seiichi Ishizuka (Teijin Institute for Biomedical Research, Tokyo, Japan). Cells were treated with vehicle, 1,25(OH)₂D₃, or JN immediately after UVR exposure. For the study with HL and TEL-9647, these compounds were added for 24 hours prior to irradiation alone or in combination with 1,25(OH)₂D₃ and again immediately after UVR exposure. The UVR source for in vitro studies consisted of a UVA and a UVB fluorescent tube (Phillips), providing irradiance of 1,168 mJ/cm² UVA and 203 mJ/cm² UVB, filtered through 0.5-mm cellulose triacetate (Eastman Chemical Products) to remove wavelengths below 290 nm.

Detection of p53, CPD, and 3-nitrotyrosine

For detection of p53 and CPD, cells were fixed with methanol (100%) at 4°C for 10 minutes at 3 and 6 hours post–UVR exposure. Immunocytochemistry, using the D07 mouse monoclonal IgG2B anti-p53 antibody (Dako-cytomation) or the mouse monoclonal IgG1 λ anti-thymine dimer antibody (Affitech; ref. 37), with image analysis was carried out as previously described (17). For whole-cell ELISA detection of 3-nitrotyrosine, peroxynitrite (420 μmol/L) was added to positive control wells 1 hour before fixation with formaldehyde [3.7% (v/v) in PBS] at room temperature for 10 minutes. Briefly, cells were washed 3× with PBS containing 0.1% Triton X-100, followed by blocking with H₂O₂ (1% in PBS) and then PBS (5% in PBS). 3-Nitrotyrosine was detected by incubation with the mouse monoclonal IgG2b primary antibody (Abcam) overnight at 4°C at a concentration of 2 μg/mL, followed by goat anti-mouse IgG-HRP (Cell Signaling Technology) for 1 hour at room temperature. Color development was initiated with a citrate phosphate buffer containing tetramethylbenzidine (0.1 mg/mL) and H₂O₂. Following development (30–40 minutes), the reaction was stopped by the addition of H₂SO₄ (2 mol/L). Absorbance was read at 450 nm on a Polarstar Galaxy microplate reader (BMG Labtech).
Murine studies

Female Skh:hr1 hairless mice were maintained in wire-topped plastic boxes at 23°C to 25°C on compressed paper bedding from Fibrecycle Pty. Ltd. The project was approved by the Animal Ethics Committee of the University of Sydney (K22/3-2005/4/4089). Mice were fed Gordon rat and mouse pellets and tap water ad libitum. The solar-simulated UVR source was 1 fluorescent UVB tube (Philips TL40W 12R/S) flanked by 6 UVA tubes (Hitachi 40W F40T 10/BL) and was filtered through 0.125-mm cellulose acetate sheeting (Grafix Plastics) as described in our previous studies (23). Animals were divided into treatment groups of 5 for immunosuppression studies and groups of 3 for studies involving histologic analyses. UV-irradiated mice were subjected to a single exposure equal to 3 times the minimal erythemal dose (MED) of UVR (3.98 kJ/m² UVB and 63.8 kJ/m² UVA) for histologic and immunosuppression studies.

Immediately after irradiation, mice were treated topically on the dorsal surface with vehicle, 1,25(OH)₂D₃ or JN. Stock solutions of test compounds were dissolved in ethanol and diluted in a base lotion containing ethanol, propylene glycol, and water to a final solvent ratio of 2:1:1, respectively. Treatments were applied topically on the highest part of the back as 100 μL of each solution (base lotion) only, or vehicle containing 1,25(OH)₂D₃ (22.8 r mol/cm²), or JN (22.8 r mol/cm²), as indicated in results.

Histology

Biopsies were taken from UV-irradiated dorsal skin 3 or 24 hours post–UVR exposure and fixed in Histochoice fixative (Amresco) for 6 hours. Skin samples were paraffin-embedded and 5-μm sections were cut for all analyses. Sections (24 hours after UVR exposure) were subjected to routine hematoxylin and eosin (H&E) staining for visualization of sunburn cells (SBC). The stained sections were examined under a Zeiss-Axioplan light microscope at 40× magnification, and the number of sunburn cells per linear millimeter of skin section was recorded. Immunohistochemistry and image analysis were carried out for CPD detection (3 hours after UVR exposure) as described previously (17). The antibody was specific for thymine dimers, the major subset of CPDs (38). Analysis of p53 expression in human keratinocytes was carried out as previously described (17).

Contact hypersensitivity

The contact hypersensitivity response was tested to investigate the effects of 1,25(OH)₂D₃ and JN on UVR-induced systemic immunosuppression. Mice were sensitized approximately 1 week after irradiation with 100 μL of 2% oxazolone [Sigma; (w/v)] in absolute alcohol, applied to the nonirradiated abdominal skin. Sensitization was repeated on the subsequent day. The sensitized mice were challenged 2 weeks after irradiation by application of 5 μL 2% oxazolone to each surface of both pinnae so that each mouse received 20 μL in total. Ear thickness measurements were recorded before challenge and at 16 hours after challenge.

Photocarcinogenesis model

For photocarcinogenesis studies, groups of 20 mice were exposed to a suberythemal dose of UVR on 5 d/wk for 10 weeks. The solar-simulated UVR source was identical to that used in our previous studies (23), as described earlier for acute exposures, but the daily dose was lowered to mimic chronic human sunlight exposure without burning. The daily dose provided 0.658 kJ/m² UVB and 20.30 kJ/m² UVA, which is approximately one MED. Erythema in the hairless mouse is difficult to observe, and the term MED strictly refers to minimal edematous dose because it is the edema by which the inflammatory reaction can be quantified. A fan, situated equidistant from each box of mice during irradiation, was used to control temperature.

Immediately after irradiation, mice were treated topically over 7 cm² on the irradiated dorsal skin with 100 μL of either base lotion, 1,25(OH)₂D₃ (22.8 or 4.6 pmol/cm²), or JN (22.8 pmol/cm²). Mice receiving 1,25(OH)₂D₃ (22.8 pmol/cm²) showed slight but nonstatistically significant weight reductions compared with mice in other treatment groups at 2 weeks. As this was likely to be due to dehydration caused by hypercalcemia/hypercalcuria, the dose was lowered to 11.4 pmol/cm² at the commencement of week 3 for the remainder of the irradiation/treatment period. Hypercalcemia results in increased urinary calcium, which then causes a diuresis and subsequent weight loss. Calcium levels were not directly measured because of the practical difficulty of obtaining blood without morbidity or mortality, but mice were monitored for weight loss, as it is a sensitive sign of hypercalcuria. Weight loss was not observed in mice treated with JN. After the dose of 1,25(OH)₂D₃ was lowered, no further weight loss occurred—average mouse weight did not differ significantly from that of other groups. The highest dose of 1,25(OH)₂D₃ is given as 11.4 pmol/cm² in the text, figures, and legends, as this dose was used for most of the study.

The time of appearance and the location of tumors were mapped for each mouse over the first 10 weeks of UVR/treatment and the following 30 weeks of tumor monitoring. Skin tumor formation, as evidenced by an outgrowth 1-mm or greater in diameter, was expressed as both the average tumor incidence (% of mice affected) and the average tumor multiplicity (average number of tumors per tumor-bearing mouse) for each treatment group. All tumors were biopsied and examined histologically to confirm the diagnosis. The minimum criterion for diagnosis of a SCC was local invasion into the dermis.

Statistical analyses

The Mantel–Haenszel log-rank test (39) was used to analyze incidence data—as the progressive probability of acquiring a tumor, comparing all treatments with control over the entire time course of the study.
In CPD, SBC, and immunosuppression studies, results are based on 3 to 5 mice per group. Experiments were carried out at least twice with similar results. Results are expressed as mean ± 1 SD or ± 1 SEM from multiple experiments as indicated. Comparisons between treatment groups were made by ANOVA, followed by Dunnett’s or Tukey–Kramer post-test, using the GraphPad Instat statistical program (GraphPad Software Inc.).

Results

UVR-induced CPDs in mouse skin measured 3 hours after UVR exposure (Fig. 1A and B) were reduced to a similar extent as a result of topical treatment with either 1,25(OH)2D3 or JN applied immediately after UVR exposure. Sunburn cells (apoptotic keratinocytes) assessed at 24 hours were also reduced to a similar extent in mice by both 1,25(OH)2D3 and JN (Fig. 1C). Along with this, skin edema after UV, measured after 48 hours, was also reduced by 1,25(OH)2D3 and both doses of JN (Fig. 1D). We have previously shown in preliminary studies that UVR-induced immunosuppression is reduced by 1,25(OH)2D3 and a high dose of JN.

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Mice began to develop papillomas toward the end of the 10-week period of UVR and treatment. Figure 3A shows typical histology of the tumors recorded during the study, and Figure 3B shows photographs of some of the skin tumors formed. The photographs in Figure 3B (i) and (ii) show papillomas and Figure 3B (iii) and (iv) show both SCCs and papillomas.

The period to onset of tumor (papilloma) formation in mice varied between treatment groups. The average latency in the vehicle-treated group was 22.5 ± 2.9 weeks from the first UVR exposure and topical treatment. This increased significantly compared with vehicle in 1,25(OH)2D3-treated mice (11.4 pmol/cm2) to 28.3 ± 8.3 weeks (P < 0.05), but it was not different than that for vehicle with any other treatment (data not shown).

Tumor incidence was calculated as the percentage of mice in each group bearing 1 or more tumors, recorded on a weekly basis throughout the 40-week study. All treatments were compared with vehicle control over the entire time course of the study, using the Mantel–Haenszel log-rank test. Treatment with 1,25(OH)2D3 (11.4 pmol/cm2) resulted in highly significantly lower tumor incidence (P = 5.7 × 10⁻¹¹) and significantly lower tumor incidence with 1,25(OH)2D3 4.6 pmol/cm2 (P < 0.01) than with vehicle treatment (Fig. 4A). There was also a significantly lower risk of developing a tumor in mice treated with the low-calcemic nongenomic agonist JN (22.8 pmol/cm2; P < 0.01; Fig. 4A). It can be seen in this figure that tumor incidence in all groups increased over time. Decreases in tumor incidence at some time points occurred because of tumor regressions.

In the vehicle group, there were a total of 22 tumor regressions; 23% of tumors completely regressed. The percentage of tumor regressions was not significantly higher in the 1,25(OH)2D3 11.4 pmol/cm² group (26%, a total of 8 regressions) or any of the other treatment groups.

Tumor multiplicity was calculated as the average number of tumors per tumor-bearing mouse. Figure 4B shows the average tumor multiplicity throughout the 40-week course of study. Significant reductions were observed with 1,25(OH)2D3 (11.4 and 4.6 pmol/cm²) and JN (22.8 pmol/cm²). Average tumor multiplicity was significantly reduced from 1.3 ± 0.3 in vehicle-treated mice to 0.3 ± 0.2 (P < 0.05) in mice treated with 1,25(OH)2D3 (11.4 pmol/cm²) at 23 weeks following the first UVR exposure and remained significantly reduced for the entire study thereafter. At week 27, a further reduction in multiplicity was noted in the 1,25(OH)2D3-treated group, from 2.5 ± 0.4 in the vehicle group to 0.8 ± 0.2 (P < 0.01). JN was also effective in reducing tumor multiplicity from 26 weeks onward. Average tumor multiplicity was reduced from 2.0 ± 0.4 in vehicle-treated mice to 0.9 ± 0.2 (P < 0.05) in JN-treated mice.

Some of the papillomas progressed to SCCs throughout the course of the study. Figure 4C shows incidence of SCC over the period of study. When analyzed by the Mantel–Haenszel log-rank test, which tests the progressive probability of acquiring a tumor over the entire time course of the study (based on weekly data), the progressive risk of developing a tumor over the entire time course was reduced by 1,25(OH)2D3 and JN. Treatment with both doses of 1,25(OH)2D3 (11.4 and 4.6 pmol/cm²) resulted in significantly lower incidence of SCC over the period of the study, compared with vehicle, as did treatment with JN (22.8 pmol/cm²; Fig. 4C). Figure 4D shows incidence of SCC at 40 weeks. In the
vehicle-treated group 42% of mice developed SCCs at this time point, compared with only 17% of mice treated with 11.4 pmol/cm² 1,25(OH)₂D₃, 26% with 4.6 pmol/cm² 1,25(OH)₂D₃, and 33% of mice with SCCs in the group treated with JN (Fig. 4D). When the risk of developing an SCC was analyzed over the whole time course, this risk was significantly reduced by 1,25(OH)₂D₃ and JN (Fig. 4D).

Figure 1. 1,25(OH)₂D₃ and JN reduce UVR-induced CPD, SBCs, and immunosuppression in Skh:hr1 mouse skin. Mice were exposed to 1 × 3 MEDs of solar-simulated UVR (3.98 kJ/m² UVB and 63.8 kJ/m² UVA) and were treated topically on the UV-irradiated dorsal surface immediately after UVR exposure with 100 μL of vehicle only, 1,25(OH)₂D₃, or JN. A, histology: skin biopsies were collected and fixed 3 hours after UVR exposure for immunohistochemical and image analysis to quantify positively stained nuclei (arrows) as a proportion of total nuclei. Darkly stained nuclei (arrows) indicate the presence of CPD (thymine dimers). i, negative control; ii, vehicle; iii, vehicle + UVR; iv, 1,25(OH)₂D₃ + UVR; and v, JN + UVR. B, image analysis results of 3 hours post-UVR skin biopsies presented as the mean of 3 skin sections ± 1 SD. Area of positively stained nuclei expressed as the percentage of area of total nuclei. Significantly different from vehicle-treated UV-irradiated mice. P < 0.01, n = 3. C, skin biopsies were taken 24 hours after UVR exposure for routine H&E staining. Sunburn cells were identified under a light microscope as cells with pyknotic nuclei surrounded by an eosinophilic cytoplasm and were counted along the length of each skin section. Results presented as the mean number of sunburn cells per 1,000 linear micrometer of skin section; n = 3 (mice per group). Significantly different from vehicle: **, P < 0.01; n = 3. D, edema was measured as dorsal skin-fold thickness in the mice 48 hours after UVR exposure. Significantly different from vehicle-treated UV-irradiated mice. **, P < 0.01; n = 5. E, mice were sensitized on nonirradiated abdominal skin 7 and 8 days after UVR exposure with 2% oxazolone and then challenged on their ears 7 days after sensitization and ear swelling recorded 16 hours later. Results were calculated as the difference between pre- and postchallenge ear thickness measurements of nonirradiated mice as a proportion of the difference between pre- and postchallenge ear thickness measurements of irradiated mice for each treatment. Immunosuppression expressed as 100% minus this value ± SEM. ***, P < 0.001 significantly different from vehicle; ###, P < 0.001 significantly different from 1,25(OH)₂D₃ 11.4 pmol/cm² and JN 22.8 pmol/cm²; n = 5.

Vitamin D Compounds Inhibit Photocarcinogenesis

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The data presented in this study support our previous findings on protection by 1,25(OH)₂D₃ against acute UVR-induced events associated with initiation of carcinogenesis, such as DNA damage, and extend these findings to show protection against chronic UVR-induced skin carcinogenesis. We have previously proposed that photoprotection by 1,25(OH)₂D₃ is likely to be mediated through the non-genomic pathway (20, 22, 23), in part because of the acute effects of JN and because the photoprotective actions of 1,25(OH)₂D₃ in fibroblasts were completely reversed by a nongenomic antagonist but not by an antagonist of the genomic pathway (23). In the current study, we show the reversal of the protective effect of 1,25(OH)₂D₃ in normal skin keratinocytes by the nongenomic antagonist HL but not by the genomic pathway antagonist 25-dehydro-1α,26,23S-lactone (TEI-9647; P = non-significant, comparison of 1,25(OH)₂D₃ vs. 1,25(OH)₂D₃ + TEI-9647 by 1-way ANOVA, followed by the Tukey–Kramer test). However, a small effect of the genomic pathway in this assay cannot be excluded. Furthermore, we show that JN, a 6-s-cis-locked analogue that is only capable of acting via the nongenomic pathway (14–16), mimicked at least to some extent the photoprotective effects of 1,25(OH)₂D₃ in a murine in vivo model. Two doses of 1,25(OH)₂D₃ were tested in the photocarcinogenesis model, based on its effects in earlier acute studies. The original concentration for the higher dose of 1,25(OH)₂D₃ was 22.8 pmol/cm². However, mice receiving this dose of 1,25(OH)₂D₃ daily, 5 d/wk, showed significant weight reductions compared with mice in other...
treatment groups. As this was likely to be due to dehydration caused by hypercalcemia/hypercalcuria, the dose was lowered to 11.4 pmol/cm² at the commencement of week 3 for the remainder of the irradiation/treatment period. No further weight loss occurred, and the new dose of 11.4 pmol/cm² was still effective in reducing skin carcinogenesis. In our previous studies in which mice were treated with a dose of 22.8 pmol/cm², the treatment was administered only once immediately after UVR exposure and did not cause any adverse effects (23).

We previously showed JN to have a similar potency to that of 1,25(OH)₂D₃ in reducing UVR-induced CPD and cell loss, 24 hours after irradiation, in cultured human skin cells (20, 23). As shown in the current study, treatment with this analogue also reduced UVR-induced CPD 3 hours post-UVR exposure and sunburn cells, skin edema, and immunosuppression in mice with a potency similar to that of 1,25(OH)₂D₃. Topical application of JN also resulted in significant protection from photocarcinogenesis, though not to the same level as that conferred by even half the dose of 1,25(OH)₂D₃. The finding that 1,25(OH)₂D₃, even when used at lower doses, is more effective than JN may well be due to its ability to act via 2 pathways, including genomic responses, whereas JN can activate only the non-genomic pathway. For example, induction of the oxygen radical scavenger metallothionein was observed in the skin of UVB-irradiated mice that had been treated topically with 1,25(OH)₂D₃ (18). Moreover, 1,25(OH)₂D₃ has been shown to regulate metallothionein gene expression (40) and this may contribute to photoprotection. Comprehensive studies on the effects of JN on other early molecular markers of skin carcinogenesis may prove useful in determining why this compound was less effective than 1,25(OH)₂D₃ in reducing skin carcinogenesis. In this study, the low-calcemic compound JN did not cause hypercalcemia. Future studies in which higher doses of JN are tested may perhaps show a stronger protective role for this compound.

The results of this study showed that topical application of 1,25(OH)₂D₃ or JN immediately after daily UVR significantly reduced tumorigenesis. The ability of 1,25(OH)₂D₃ and JN to inhibit tumor formation in mice may be attributed, in part, to their ability to reduce CPD (17, 20, 22, 23). Although thymine (T=T) dimers are not reportedly mutagenic (though this has been disputed), T>T dimers are proportional to other CPD (2). Supporting this view are separate studies by our group showing fewer CPD post-UVR exposure with 1,25(OH)₂D₃, using a method in which DNA was extracted from cultured skin cells and digested with T4N5 endonuclease followed by alkaline gel electrophoresis (24), as described by Ananthaswamy and Fisher (41). It has been proposed that CPD formation is responsible for induction of the major mutational hotspots in UVR-induced mouse skin tumors (42).

The mechanism for reduced CPD with vitamin D compounds is not clear but may involve enhanced DNA repair. In an earlier publication, we showed further increases in p53 expression with 1,25(OH)₂D₃ treatment as seen after
UVR alone. In the current study, JN also enhanced p53 nuclear expression to a similar extent. p53 has been implicated in the facilitation of DNA repair (25). We also showed that 1,25(OH)_{2}D_{3} reduced NO products measured by the insensitive Griess assay. Here, using a more sensitive measure of nitrative damage, 3-NT (43), we show a clear reduction in 3-NT with both 1,25(OH)_{2}D_{3} and similar concentrations of JN. These results complement those of Ellison and colleagues, who showed that VDR-knockout mice are much more susceptible to photocarcinogenesis (44). The VDR has been shown to be essential for non-genomic effects of vitamin D compounds (9, 10, 13, 44). In VDR-knockout mice, thymine dimer repair was significantly reduced (44). In those studies, there was no increase in chemical-induced carcinogenesis in 1α-hydroxylase-knockdown mice, which are unable to produce 1,25(OH)_{2}D_{3}. These authors concluded that the unliganded VDR, rather than vitamin D metabolites, is important for protection from chemical-induced skin carcinogenesis, but UVR-induced carcinogenesis in 1α-hydroxylase-knockdown mice was not examined. Our results and those of Wood and colleagues (34) and Kensing and colleagues (35) provide direct evidence that 1,25(OH)_{2}D_{3} itself and related compounds protect against skin carcinogenesis. An alternative explanation for the results of the study conducted by Ellison and colleagues (44) is that photoprotection in mice seems to be mediated through a nongenomic pathway, which is still likely to involve the VDR but which may be activated by compounds other than the classical hormone (10).

Although we added vitamin D compounds topically after UV and showed photoprotection, it is likely that vitamin D and metabolites produced physiologically in skin after UVR exposure contribute to photoprotection also. Production of vitamin D_{3} in skin following UVR exposure takes several hours and subsequent conversion to 1,25(OH)_{2}D_{3} requires several more hours (45). This time course makes it more likely that vitamin D compounds produced locally in skin, some of which may stay in skin (45), can provide protection against subsequent exposures to UVR rather than the UVR exposure that resulted in their production. This is analogous to increased pigmentation and cornification that occur as a result of UVR damage and contribute to photoprotection (46). It is known that continued UVR results in the degradation of vitamin D_{3}, leading to the production of overirradiated products (47, 48). Because the
structures of some of these overirradiation products are conformationally restricted non secos-teroids, it is possible to speculate that they too might act via nongenomic mechanisms to provide endogenous photoprotection, such as analogue JN, as described here. One of these compounds is, in fact, lumisterol-D$_3$, and pathways for the metabolism of lumisterol to JN have been reported [49, 50].

The data presented in this study raise the possibility that vitamin D analogues, including nongenomic analogues, may be useful in the prevention of UVR-induced skin carcinogenesis, though dose-ranging studies would be necessary. In particular, because vitamin D compounds are effective immediately after UV exposure, they represent the possibility of a second chance at UV protection.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Cancer Prevention Research

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