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1,25-Dihydroxyvitamin D and three low-calcemic analogs decrease UV-induced DNA damage via the rapid response pathway☆

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Abstract

1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] is anti-apoptotic in human keratinocytes, melanocytes and fibroblasts after ultraviolet (UV)-exposure. To date, there is no published data on the effects of 1,25$(OH)_2D_3$ or its analogs on DNA damage in irradiated skin cells. In these skin cells, 24h pre-treatment with 1,25$(OH)_2D_3$ dose-dependently (10^{-12} to 10^{-8} M) decreased CPD damage by up to 60%. This photoprotective effect was also seen if the 1,25$(OH)_2D_3$ was added immediately after irradiation and was mimicked by QW-1624F2-2 (QW), a low-calcemic 1β-hydroxymethyl-3-epi-16-ene-24,24-difluoro-26,27-bis homo hybrid analog. The well-studied low calcemic, rapid acting agonist analogs 1α,25$(OH)_2$lumisterol$_3$ (JN) and 1α,25$(OH)_2$-7-dehydrocholesterol (JM) also protected skin cells from UV-induced cell loss and CPD damage to an extent comparable with that of 1,25$(OH)_2D_3$. In contrast, the rapid response antagonist analog 1β,25$(OH)_2D_3$ (HL) completely abolished the photoprotective effects (reduced cell loss and reduced CPD damage) produced by treatment with 1,25$(OH)_2D_3$, JN, JM and QW. Evidence for involvement of the nitric oxide pathway in the protection from CPD damage by 1,25$(OH)_2D_3$ was obtained. These data provide further evidence for a role of the vitamin D pathway in the intrinsic skin defenses against UV damage. The data also support the hypothesis that the photoprotective effects of 1,25$(OH)_2D_3$ are mediated via the rapid response pathway(s).

Keywords: Photoprotection; Pyrimidine dimers; 1,25-Dihydroxyvitamin D; Vitamin D analogs; Skin cells; Nitric oxide

1. Introduction

Vitamin D_3 is formed in skin by the action of ultraviolet irradiation (UVR) on 7-dehydrocholesterol. It is likely that further metabolites, 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), are also produced locally in skin cells [1,2]. These vitamin D compounds are involved in regulating the normal differentiation of keratinocytes [1], hair follicle development [3] and in some circumstances, pigmentation [4]. Vitamin D compounds may also have a role in protection of skin cells from UVR. The active vitamin D hormone, 1,25$(OH)_2D_3$ has been reported to reduce UV-induced apoptosis in keratinocytes [5,6] and melanocytes [7]. If 1,25$(OH)_2D_3$ reduces skin cell death after exposure to UVR, the question then arises whether these actions allow more cells with severe DNA damage to survive. That would clearly be unhelpful in a teleological sense. Cyclobutane pyrimidine dimers (CPD) are signature UVR-induced forms of DNA damage [8] which can be detected in skin and skin cells by a sensitive, specific and well characterized antibody [9].

The mechanism of the anti-apoptotic effect of 1,25$(OH)_2D_3$ is not clear. As is the case for other steroid hormones, 1,25$(OH)_2D_3$ appears to exert the full spectrum of functional effects through two main pathways. One is the classical pathway, in which 1,25$(OH)_2D_3$ binds to its receptor, dimerizes with the retinoid X receptor and binds to the vitamin D response elements on target genes, resulting in an increase or decrease in transcription of those target genes [10]. The second major pathway is known as the rapid or non-classical pathway and involves the hormone binding

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to an ill-defined receptor and stimulating intracellular signalling pathways such as MAP kinase, intracellular calcium, cAMP or protein kinase C, some or all of which may be implicated in the particular response generated [10]. Vitamin D analogs with different conformations have been used to dissect these pathways [10]. In particular, the *cis*-locked, low calcemic analogs 1α,25(OH)$_2$lumisterol$_3$ (JN) and 1α,25(OH)$_2$-7-dehydrocholesterol (JM) appear to activate the rapid, non-genomic pathway [10], while the analog 1β,25(OH)$_2$D$_3$ (HL) is an antagonist of this. In contrast, the low calcemic QW-1624F2-2 (QW) compound, 1β-hydroxymethyl-3-epi-16-ene-24,24-difluoro-26,27-bis homo hybrid analog has some transactivating capacity [11]. In this study, we show that vitamin D compounds, including three low calcemic analogs are also anti-apoptotic and reduce CPD in irradiated skin cells.

2. Materials and methods

Melanocytes and keratinocytes were cultured from human neonatal foreskins as previously described with some modifications [12]. Fibroblasts were cultured in DMEM with 5% (v/v) FBS. Experiments were carried out at approximately 60–80% confluence and culture media changed to DMEM without EGF and cholera toxin for keratinocytes and to Eagles MEM without PMA and cholera toxin for melanocytes for at least 2 days before experiments to allow cell signal pathways to normalize [13]. Cells were treated with 1,25(OH)$_2$D$_3$ or other agents 24 h prior to UVR, unless otherwise stated, and media containing treatments were added immediately after irradiation. Media were changed to a Martinez buffer solution for irradiation. Viable cell counts were performed 24 h after UVR. The UV source was a FS20T12 UVB lamp and a FL20SBL UVA lamp (Phillips, Amsterdam, Holland) filtered through cellulose tri-acetate (Eastman Chemical Products, Kingsport, TN) to remove wavelengths below 290 nm as previously described [12]. Irradiance was 200 mJ/cm^2 UVB and 1170 mJ/cm^2 UVA. Sham-irradiated cells were subjected to similar procedures but were shielded during the irradiation.

Cells were fixed with 100% methanol 3 h after UV irradiation unless otherwise indicated. Antigen retrieval involved nuclear DNA denaturation with 70 mmol/l NaOH diluted in 100% ethanol, followed by proteolytic digestion with proteinase K 1 μg/ml. Non-specific antibody blocking was achieved using 50% normal human serum diluted in PBS. Immunohistochemistry was performed using the DAKO LSAB Plus kit (DAKO Corp., Tokyo, Japan) with mouse monoclonal IgG$_1$ lambda anti-thymine dimer antibody [9] (Affitech, Oslo, Norway) for CPD detection or an isotype control. Diaminobenzidine (DAB) was the chromogen used for visualisation of keratinocytes and fibroblasts and 3-amino ethyl carbimazole (AEC) for melanocytes. Stained coverslips were analyzed in a Zeiss Axioplan light microscope at 10 times magnification for positive nuclei as a proportion of total nuclei using image analysis after capture of random fields using a Sony Progressive colour CCD camera (Sony, Tokyo, Japan) into a Zeiss KS400 (Carl Zeiss Vision, Munich, Germany) image analysis system.

The Griess assay quantifies nitrite (a stable metabolite of NO) in solution and was performed as described [14]. All experiments shown were repeated 2–6 times with similar results. Results are expressed as mean ± 1 standard deviation (1 S.D.) or ± standard error of mean (1 S.E.M.). Significant differences between means were determined by Student's unpaired *t*-test or one-way analysis of variance (ANOVA) using GraphPad Instat program (GraphPad Software Inc., San Diego, CA) where appropriate.

3. Results

As shown in Fig. 1, the dose-response for reduction in UVR-induced fibroblast cell death after treatment with the rapid response analog JN was almost identical to that seen with 1,25(OH)$_2$D$_3$. JN at a dose of 10^{-9} M, like 1,25(OH)$_2$D$_3$, also reduced UVR-induced cell death in melanocytes from 32 ± 8% with vehicle to 3.5 ± 1% ($P <$ 0.01) and in keratinocytes from 28 ± 4% with vehicle to 4 ± 1% ($P < 0.01$). QW at 10^{-9} M also reduced fibroblast loss from 20 ± 4% with vehicle to 2 ± 3% ($P < 0.05$). The anti-apoptotic dose response curve for QW in fibroblasts was also very similar to that of 1,25(OH)$_2$D$_3$ (data not shown). The analog JB (1α25-dihydroxytachysterol$_3$), which is only a weak agonist of both the genomic and non-genomic pathways [10] had no significant anti-apoptotic effect. The reduction in cell death after UVR by 10^{-9} M 1,25(OH)$_2$D$_3$ added immediately after UVR was similar to that achieved

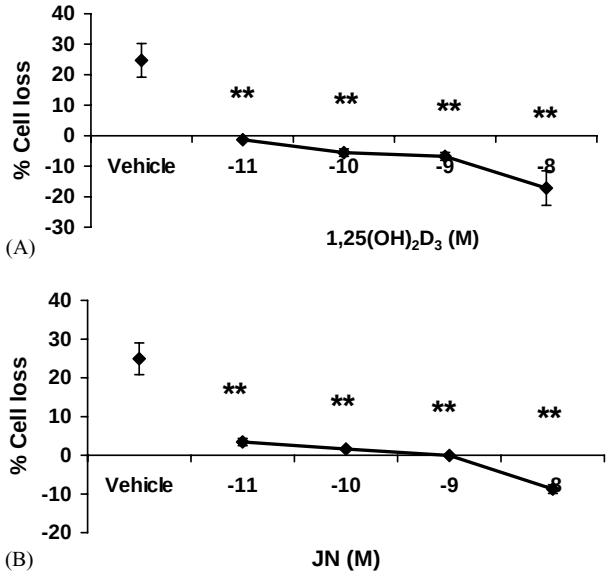

Fig. 1. Dose-dependent inhibition of UVR-induced fibroblast death by 1,25(OH)$_2$D$_3$ (A) or JN (B). Significantly different from vehicle-treated controls: $^{**}P < 0.001$.

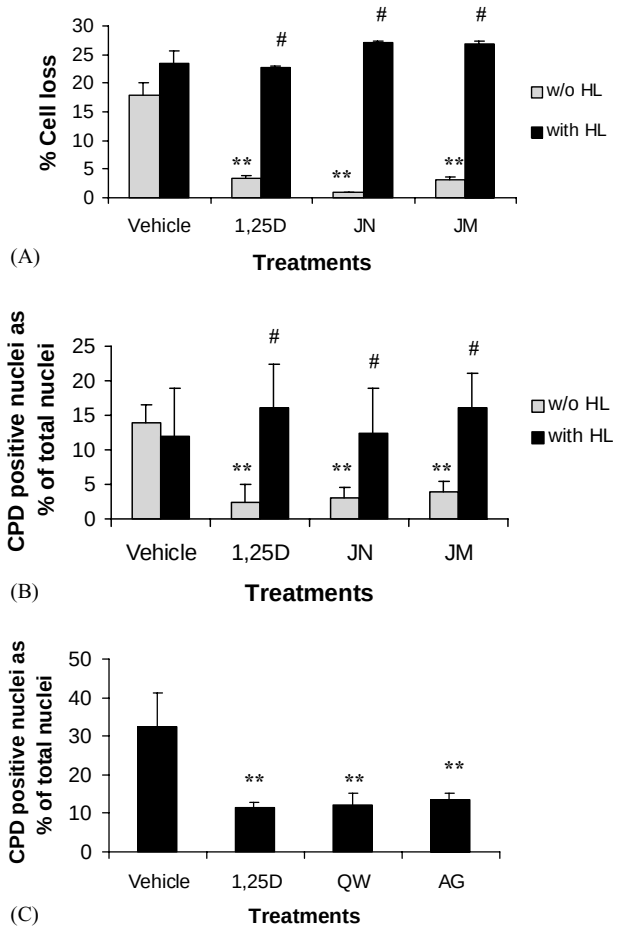

Fig. 2. (A) Reduction in UVR-induced fibroblast death by 10^{-9} M $1,25(OH)_2D_3$, JN or JM and reversal by 10^{-7} M HL. Significantly different from vehicle-treated controls: $^{**}P < 0.01$; significantly different from cultures without HL $\#P < 0.01$. (B) Reduction in UVR-induced fibroblast CPD by 10^{-9} M $1,25(OH)_2D_3$, JN or JM and reversal by 10^{-7} M HL. Significantly different from vehicle-treated controls: $^{**}P < 0.01$; significantly different from cultures without HL $\#P < 0.01$. (C) Reduction in keratinocyte CPD by 10^{-9} M $1,25(OH)_2D_3$, 10^{-9} M QW or 2 mM aminoguanidine (AG). All treatments added immediately after UVR. Significantly different from vehicle-treated controls: $^{**}P < 0.01$.

with the same dose of the compound added 24 h before UVR. Cell loss was $40 \pm 7\%$ with vehicle, $15 \pm 3.5\%$ with pre-treatment and $19 \pm 1\%$ post-treatment (treatment versus vehicle, $P < 0.05$, pre-treatment versus post-treatment, $P =$ ns).

The analog HL, a specific antagonist of the rapid response pathway, at a dose of 10^{-7} M, had little anti-apoptotic effect on its own, but completely reversed that of the low calcemic, rapid response analogs JN and JM at concentrations of 10^{-9} M in fibroblasts (Fig. 2A) and melanocytes (data not shown). HL also reversed the anti-apoptotic effect of QW, from cell loss of $2 \pm 3\%$ back to $33 \pm 4\%$ ($P < 0.01$).

The reduction in CPD after UVR in the presence of 10^{-9} M $1,25(OH)_2D_3$, JN and JM is shown in Fig. 2B. These compounds all had a similar effect on reduction of CPD. HL at 10^{-7} M had no significant effect on its own, but reversed the CPD reduction seen with $1,25(OH)_2D_3$, JN and JM. The results shown in Fig. 2C show a significant reduction in CPD in keratinocytes treated immediately after irradiation with 10^{-9} M $1,25(OH)_2D_3$ or QW. A similar reduction was seen in the presence of the nitric oxide synthase inhibitor aminoguanidine (AG) at a concentration of 2 mM (Fig. 2C). Both aminoguanidine and 10^{-9} M $1,25(OH)_2D_3$, reduced the concentration of the stable nitric oxide metabolite, nitrite, to a similar extent. Nitrite concentrations were 180 ± 40 mg/l in UVR-treated wells, compared with 70 ± 20 mg/l in sham-irradiated wells and this was decreased to 166 ± 4 mg/l ($P < 0.05$ versus vehicle) with 10^{-9} M $1,25(OH)_2D_3$ and to 158 ± 4 mg/l ($P < 0.01$ versus vehicle) with 2 mM.

4. Discussion

It is clear from these data that the anti-apoptotic effect of $1,25(OH)_2D_3$ after UVR, previously reported in keratinocytes and melanocytes, also extends to dermal fibroblasts in culture. The studies also show that this effect occurs with rather lower concentrations of $1,25(OH)_2D_3$ than reported in some other studies [5]. Based on the production rates demonstrated in human skin equivalents and in human skin using microdialysis [2,15], it can be calculated that local concentrations of $1,25(OH)_2D_3$ could rise to the low nanomolar range. The photoprotective effects of $1,25(OH)_2D_3$ were present with doses of this order or less.

The anti-apoptotic effect previously described, on its own, could allow a large proportion of skin cells with severe DNA damage to survive UVR, which might increase the risk of subsequent neoplasia. Crucial to the case that vitamin D compounds are photoprotective, is the demonstration that at least one form of DNA damage, CPD, is not increased in the surviving cells, but is significantly reduced, as demonstrated in this study. In related work, we have demonstrated dose-dependent reduction in CPD in keratinocytes, melanocytes and fibroblasts at various times after UVR from 0.5 to 48 h [16].

The evidence from the work so far, which showed that the rapid response agonists JN and JM have a similar photoprotective effect to that of $1,25(OH)_2D_3$, and the complete reversal of that effect by the rapid response antagonist HL are entirely consistent with a photoprotective effect mediated through the rapid response pathway. Further studies to examine whether the genomic pathway antagonist TEI-9647 [17] has any effect on the $1,25(OH)_2D_3$ response would help to test this hypothesis. Studies in fibroblasts from vitamin D receptor knockout mice and their normal littermates would also help to clarify the cellular pathway involved. At least two possible mechanisms for the anti-apoptotic effect of $1,25(OH)_2D_3$ have been proposed. Lee and Youn [6] demonstrated an increase in metallothionein in the skin of $1,25(OH)_2D_3$-treated mice exposed to UVR, which showed reduced sunburn cell formation. Manggau

et al. [5] proposed an increase in sphingosine-1-phosphate, but did not report a significant anti-apoptotic effect below 100 nM $1,25(OH)_2D_3$. Neither metallothionein nor sphingosine-1-phosphate have been linked to CPD. The demonstration that $1,25(OH)_2D_3$ reduced the stable nitric oxide product, nitrite, in these cultures to the same extent as a known nitric oxide synthase inhibitor, aminoguanidine was unexpected. Nevertheless, nitric oxide derivatives have been reported to inhibit CPD repair [18] and aminoguanidine reduced CPD in the skin cells to a similar extent to $1,25(OH)_2D_3$.

The observations reported here that $1,25(OH)_2D_3$ reduces skin cell apoptosis after UVR and also reduces a major form of DNA damage, CPD formation, even when added after UVR, together with other data indicating local skin synthesis of $1,25(OH)_2D_3$ as a result of UVR exposure [2], provides compelling evidence for the contribution of the vitamin D system to intrinsic skin photoprotection. The demonstration that vitamin D analogs which have little ability to raise serum calcium concentrations, nevertheless have similar photoprotective effects to the parent compound, raises the possibility of pharmacological enhancement of this protective pathway.

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