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TOPICAL REVIEW

Vitamin D and systemic cancer: is this relevant to malignant melanoma?

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Summary 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] is a well-known potent regulator of cell growth and differentiation and there is recent evidence of an effect on cell death, tumour invasion and angiogenesis, which makes it a candidate agent for cancer regulation. The classical synthetic pathway of $1,25(OH)_2D_3$ involves 25- and 1α-hydroxylation of vitamin D_3, in the liver and kidney, respectively, of absorbed or skin-synthesized vitamin D_3. There is recent focus on the importance in growth control of local metabolism of $1,25(OH)_2D_3$, which is a function of local tissue synthetic hydroxylases and particularly the principal catabolizing enzyme, 24-hydroxylase. The classical signalling pathway of $1,25(OH)_2D_3$ employs the vitamin D nuclear receptor (VDR), which is a transcription factor for $1,25(OH)_2D_3$ target genes. Effects of this pathway include inhibition of cellular growth and invasion. Cytoplasmic signalling pathways are increasingly being recognized, which similarly may regulate growth and differentiation but also apoptosis.

$1,25(OH)_2D_3$ has a major inhibitory effect on the G_1/S checkpoint of the cell cycle by upregulating the cyclin dependent kinase inhibitors p27 and p21, and by inhibiting cyclin D1. Indirect mechanisms include upregulation of transforming growth factor-β and downregulation of the epidermal growth factor receptor. $1,25(OH)_2D_3$ may induce apoptosis either indirectly through effects on the insulin-like growth receptor and tumour necrosis factor-α or more directly via the Bcl-2 family system, the ceramide pathway, the death receptors (e.g. Fas) and the stress-activated protein kinase pathways (Jun N terminal kinase and p38). Inhibition of tumour invasion and metastasis potential has been demonstrated and mechanisms include inhibition of serine proteinases, metalloproteinases and angiogenesis.

The lines of evidence for an effect of vitamin D_3 in systemic cancer are the laboratory demonstration of relevant effects on cellular growth, differentiation, apoptosis, malignant cell invasion and metastasis; epidemiological findings of an association of the occurrence and outcome of cancers with derangements of vitamin $D_3/1,25(OH)_2D_3$ and the association of functional polymorphisms of the *VDR* with the occurrence of certain cancers. In addition, vitamin D_3 analogues are being developed as cancer chemotherapy agents.

There is accumulating evidence that the vitamin $D_3/1,25(OH)_2D_3$/VDR axis is similarly important in malignant melanoma (MM). MM cells express the VDR, and the antiproliferative and prodifferentiation effects of $1,25(OH)_2D_3$ have been shown in cultured melanocytes, MM cells and MM xenografts. Recently, an inhibitory effect on the spread of MM cells has been demonstrated, low serum levels of $1,25(OH)_2D_3$ have been reported in MM patients and the VDR polymorphisms have been shown to be associated with both the occurrence and outcome of MM.

The relationship between solar irradiation and MM is more complex than for the systemic cancers. As in other cancers, there is evidence of a protective effect of vitamin D_3 in MM, but ultraviolet radiation, which is a principal source of vitamin D_3, is mutagenic. Further work is

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necessary on the influence of serum vitamin D_3 levels on the occurrence and prognosis of MM, the effects of sun protection measures on serum vitamin D_3 levels in temperate climates and epidemiological studies on geographical factors and skin type on the prognosis of MM. Meanwhile, it would seem mandatory to ensure an adequate vitamin D_3 status if sun exposure were seriously curtailed, certainly in relation to carcinoma of breast, prostate and colon and probably also MM.

Introduction

1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] or calcitriol, the hormonal derivative of vitamin D_3, has been established since the 1980s as an antiproliferative and prodifferentiation agent, and more recently as a proapoptotic agent and an inhibitor of cell migration, which may imply an inhibitory effect in cancer. More direct evidence of such a protective influence has emerged in a number of systemic cancers, particularly colon, prostate and breast. This is based on epidemiological studies related to diet, skin type and geographical factors, serum $1,25(OH)_2D_3$ levels and, recently, functionally significant polymorphisms of the vitamin D nuclear receptor (VDR) gene. There is now accumulating evidence for a similar role of $1,25(OH)_2D_3$ in malignant melanoma (MM).

The aims of this review are to summarize the relevant metabolism of vitamin D_3 and $1,25(OH)_2D_3$; to describe the effect of $1,25(OH)_2D_3$ on pertinent cellular molecular mechanisms; to document the evidence for an association of $1,25(OH)_2D_3$ with systemic cancer; to present the case for a similar relationship between $1,25(OH)_2D_3$ and MM; and to discuss the implications of this.

Synthesis of vitamin D_3 and 1,25-dihydroxyvitamin D_3

Vitamin D_3 is derived from a cholesterol-like precursor, 7-dehydrocholesterol, found in the skin. The direct action of sunlight on this precursor produces previtamin D_3 by photolysis. This is rapidly transformed by a rearrangement of double bonds to form vitamin D_3. The steroid hormone $1,25(OH)_2D_3$ is produced by 25-hydroxylation of vitamin D_3 in the liver followed by 1α-hydroxylation in the kidney. Subsequently, $1,25(OH)_2D_3$ is catabolized by hydrolysis, the important first step being 24-hydroxylation. Blood levels of $1,25(OH)_2D_3$ are influenced by the availability of vitamin D_3, a major determinant of which is skin exposure to ultraviolet (UV) B radiation, the integrity and activity of the 1α- and 25-hydroxylases, and 24-hydroxylase. Homeostatic mechanisms include parathyroid activity, serum calcium and serum $1,25(OH)_2D_3$ itself.

Tissue levels of $1,25(OH)_2D_3$ are a new focus of interest, particularly in relation to local cellular growth regulation. Tissue concentration of $1,25(OH)_2D_3$ is a function of activity of the local hydroxylases and availability of substrate. The presence of 1α-hydroxylase has been reported recently in a wide range of extrarenal tissues, including colon, pancreas, adrenal medulla, brain, placenta and lymph nodes,[1] and both 1α- and 25-hydroxylase have been reported in keratinocytes.[2] Thus $1,25(OH)_2D_3$ may be synthesized in various peripheral tissues, using either 25-hydroxyvitamin D_3 [$25(OH)D_3$] or even vitamin D_3 as substrate.[1–3] It has recently been suggested that higher circulating levels of $25(OH)D_3$ are necessary to satisfy local cell growth requirements than for bone and calcium homeostasis.[4] 24-Hydroxylase activity is high in the skin, resulting in low basal $1,25(OH)_2D_3$ levels.[5] Increased activity of 24-hydroxylase has recently been recognized as a cause of lack of growth regulation by $1,25(OH)_2D_3$ in a $1,25(OH)_2D_3$-resistant malignant prostatic cell line.[6]

Intracellular signalling pathways of vitamin D_3

The 'classical' signalling pathway, as for other steroid molecules, is via a nuclear receptor, VDR, which is a transcription factor. However, there is increasing evidence for the involvement of cytoplasmic pathways. These may be invoked by a proposed cytoplasmic membrane receptor, and hence be independent of the nuclear VDR, or occasionally by upregulation of transcription of the gene of a constituent protein in the pathway, via the VDR. Stimulation of these pathways classically results in post-translational effects, which occur rapidly within minutes, but may also lead to genomic effects, which occur after hours to days.

The vitamin D nuclear receptor

The VDR, a member of the steroid nuclear receptor superfamily, was first discovered in chicken intestine, and subsequently in a wide variety of cells and tissues.

It has also been demonstrated in a broad range of tumours and malignant cell types. A full coding sequence for the human *VDR* was determined in 1988.[7] There are highly conserved nuclear and ligand-binding domains (Fig. 1). The VDR gene maps to chromosome 12q14[8] and contains eight exons that are invariably translated (exons 2–9) and six that are alternatively spliced (1a–1f). There are two potential translation start codons situated in exon 2, which result in a protein of either 424 or 427 amino acids. Expression of the gene is modulated by a wide range of stimuli including oestrogens, cytokines, growth factors and peptide hormones; and mRNA levels are also influenced by the cell cycle and the differentiation state of the cell.[9]

The VDR is believed to be located in the nucleus prior to activation by $1,25(OH)_2D_3$, which dissociates from the serum vitamin D-binding protein, enters the cell by diffusion and binds with the VDR. Ligand binding produces conformational changes in the VDR, exposing surfaces for coactivating factor binding and dimerization. The dimerization partner is a retinoid receptor, usually retinoid X receptor, which is a subordinate but obligate partner for full transactivation of the VDR. The effect of dimerization is to enable high-affinity interaction with the target gene promoter at the vitamin D response element (VDRE). Coactivating proteins, carried on the VDR, then initiate transcription. As yet, only a few genes exhibiting functional VDREs are recognized, and include bone-related genes (*osteocalcin*, *osteopontin*, *bone sialoprotein*, *calbindin-D28K*, *calbindin-D9K*, *fructose 1,6-bisphosphate*, *PTH*, *PTHrP*) and, of more relevance here, *24-hydroxylase*, *protein lipase* (*PL*) *Cγ*, the cell cycle regulating protein *p21*, *transforming growth factor* (*TGF*)-β*2*, *fibronectin*, *urokinase plasminogen activator* and β*3 integrin*.

Polymorphism of the vitamin D nuclear receptor

Interest in the *VDR* polymorphisms was first generated by a reported association with bone mineral density.[10,11] Recently, there has been reported a polymorphism of *VDR* in exon 2 (the *Fok*I restriction site) and a cluster of polymorphisms towards the 3′ end of the gene. These are situated in the last intron (*Bsm*I, *Apa*I) and an adjacent site in exon 9 (*Taq*I) and in the 3′ untranslated region, a length polymorphism of a polyadenyl (polyA) microsatellite, classified into *long* (*L*) and *short* (*S*) variants. The 3′ end polymorphisms are in mutual tight linkage disequilibrium (*L* linked with *b*, *a*, *T*). Both 3′ and 5′ polymorphisms have functional significance. The polymorphism at the *Fok*I restriction site (C → T transition) alters an ACG codon 10 base pairs upstream from the translation start codon, resulting in a further start codon. Translation initiation from this alternative site (ATG restriction site present, *f* and variant allele) results in a longer protein of 427 amino acids,[12] which is less transcriptionally active.[13] However, others have failed to detect an effect on function, but it was admitted that their methods could be insensitive.[14] The 3′ end polymorphisms are thought to be associated with decreased transcription of the VDR. In transfected fibroblast lines, the *L* (wild-type) alleles were associated with greater endogenous VDR activity than the *S* alleles.[13] The net effect of polymorphisms at these sites is a potential decrease in the intracellular activity of $1,25(OH_2)D_3$.

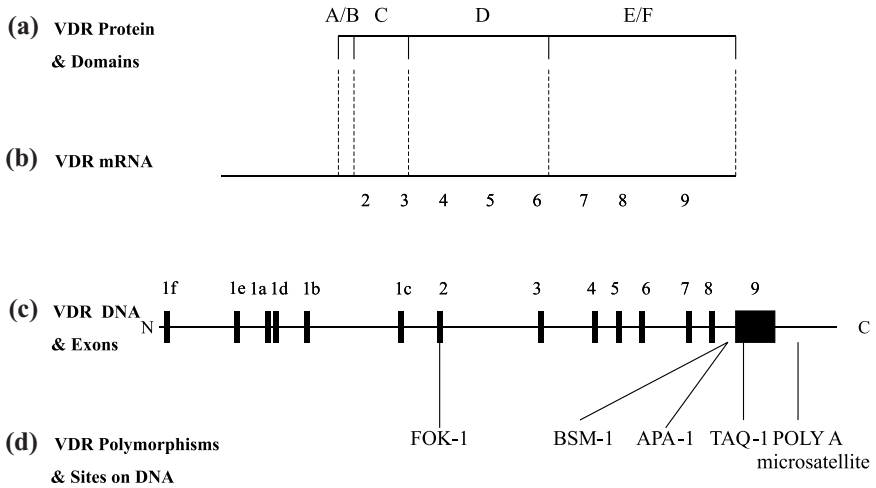

Figure 1. The domains of the vitamin D nuclear receptor (VDR) protein (a), their mRNA (b) and exonal derivations (c) and polymorphic sites on the *VDR* gene (d). A/B domain is encoded by exon 2 and is abbreviated compared with other nuclear proteins. C is the DNA-binding domain and is also involved in dimerization. It is the most conserved domain between nuclear receptors and contains two zinc co-ordination sites and two peptide loops (zinc fingers) essential for binding. D is the 'flexible hinge', which allows marked bending of the protein. E/F is the ligand-binding domain and is also involved in dimerization and transactivation.

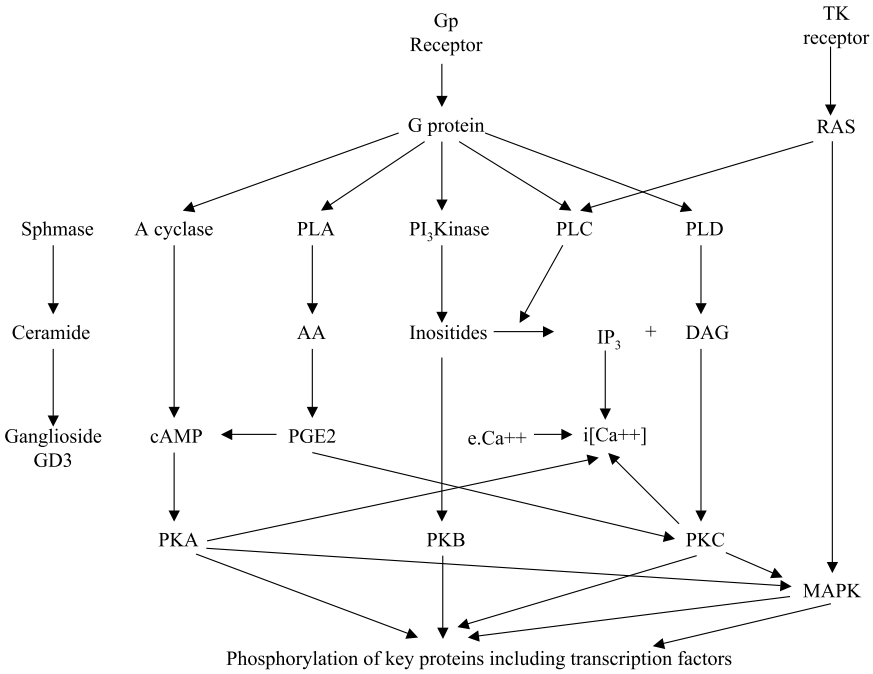

Figure 2. Cytoplasmic signalling pathways relevant to the actions of 1,25-dihydroxyvitamin D_3. Gp, G protein; TK, tyrosine kinase; Sphmase, neutral sphingomyelinase; A, adenylyl; PL, protein lipase; PI, phosphatidyl inositol; AA, arachidonic acid; IP_3, inositol triphosphate; DAG, diacylglycerol; PG, prostaglandin; e.Ca^{++}, extracellular calcium; i[Ca^{++}], intracellular free calcium; PK, protein kinase; MAPK, mitogen-activated PK.

Non-genomic signalling pathways of 1,25-dihydroxyvitamin D_3

Since 1975, a series of observations has suggested that some of the biological responses generated by $1,25(OH)_2D_3$ occur too rapidly to be compatible with a genomic mechanism, e.g. $1,25(OH)_2D_3$ can stimulate the transport of Ca^{2+} across the intestine within 1–2 min[15] (now known as transcaltachia). In 1981, it was first suggested that there was a membrane receptor for $1,25(OH)_2D_3$, which mediated these responses,[16] but this still awaits characterization. Subsequently, $1,25(OH)_2D_3$ has been shown to activate a number of cytoplasmic signalling pathways (Fig. 2), some of which affect cellular growth, differentiation or apoptosis and are therefore of relevance here. Also of importance is that these pathways may co-operate with the classical genomic pathway, through transactivation, by phosphorylation of the nuclear VDR.[17] Activation of pathways is usually direct and rapid, e.g. the activation of G proteins and the direct activation of protein kinase (PK) C by $1,25(OH)_2D_3$ itself,[18] but on occasion the transcription of a pathway protein is upregulated via the VDR, e.g. *PLC*γ has the VDRE. The end result of activation of the signalling pathways is often rapid, e.g. changes in cytosolic calcium concentration and calcium fluxes in various tissues[19] and phosphorylations of key proteins (which may result in their activation or deactivation) such as Bad, Bcl-2 and the transcription factor c-Jun. However, it may result in an effect on gene transcription[20] and this genomic effect is thus independent of direct VDR interaction with the target gene.

There have been several studies on the involvement of $1,25(OH)_2D_3$ in the signalling of G protein → inositol triphosphate (IP_3) and → PKC[21–23] (Fig. 2). There is good evidence for the involvement of G protein in the $1,25(OH)_2D_3$-induced pathway.[24,25] $1,25(OH)_2D_3$-induced phosphatidylinoside hydrolysis results in IP_3, which in turn causes redistribution of intracellular Ca^{2+} from endoplasmic reticulum stores followed by opening of calcium release-activated Ca^{2+} channels, and diacylglycerol (DAG). There is also evidence for involvement of all or part of the G protein → PLA pathway,[26–28] playing an important part in Ca^{2+} flux[26,28] and mitogenic signalling,[27] and G protein → prostaglandin E_2,[29–31] affecting differentiation (e.g. of monocyte lines[32]). $1,25(OH)_2D_3$ may also have a negative effect on the pathway, inhibiting tumour necrosis factor (TNF)-α-induced apoptosis.[33] PKC is a key regulatory enzyme in the mechanism of action of $1,25(OH)_2D_3$.[27,34] $1,25(OH)_2D_3$ may upregulate PKC via several routes, by direct binding to phorbol ester binding sites on PKC[18] or via DAG, either through PLC, as described above, or PLD acting on phosphatidylcholine,[35] or via the prostaglandin pathway.[36] PKC is an important mediator of $1,25(OH)_2D_3$ action on cell proliferation and differentiation, via a direct action on

DNA synthesis and mitogen-activated PKs (MAPKs),[37] and also affects calcium fluxes.[19,38] Intracellular free Ca^{2+} concentration (i[Ca^{2+}]) is also recognized as a $1,25(OH)_2D_3$ signal transduction messenger.[39] $1,25(OH)_2D_3$ may increase i[Ca^{2+}] by causing ingress of extracellular Ca^{2+}, through voltage-sensitive channels (VSCC), e.g. in enterocytes,[39] or voltage-insensitive channels (VICC), e.g. breast cancer cells,[40] or by mobilization of intracellular endoplasmic reticulum stores through IP_3 as described above. A co-ordinate action of the PKC and PKA systems is also involved in $1,25(OH)_2D_3$-regulated calcium channel Ca^{2+} influx,[19] and PKA is involved in membrane protein phosphorylation, which is concurrent with Ca^{2+} influx in myocytes.[41] i[Ca^{2+}], modified via VSCC, is thought to mediate transcaltachia[39] and, via VICC, $1,25(OH)_2D_3$-induced apoptosis in breast cancer cells.[40] The effect of $1,25(OH)_2D_3$ on keratinocyte differentiation has been partially ascribed to an increase in i[Ca^{2+}], although the intermediary mechanisms increasing i[Ca^{2+}] are not clear.[42] The MAPKs integrate multiple intracellular signals and consist of two main cascades, the extracellular signal-regulated kinases (ERKs) and the stress-activated PKs (SAPKs), which include the Jun N terminal kinase (JNK) and p38 pathways. The activity of the ERKs is stimulated by growth factors, e.g. epidermal growth factor (EGF), and cytokines, through tyrosine kinase receptors, and they convey proliferation and differentiation signals via phosphorylations of key proteins, including transcription factors such as c-Myc. There is evidence that another phosphorylation target is the VDR, thus impacting transactivation, which is then an example of co-operative signalling between the cytoplasmic and classical nuclear pathways, referred to above.[17] The SAPKs are activated by cellular stresses such as UV and osmotic change and proinflammatory cytokines, e.g. TNF-α and interleukin-1, and these are also regulators of growth but also apoptosis. $1,25(OH)_2D_3$ may engage the ERK pathway at different points, e.g. at the level of the EGF receptor, which it may downregulate,[43] at the level of Ras activation, via rapid phosphorylation of an adaptor protein,[44] or, via PKC, downstream of Ras and Raf.[37] $1,25(OH)_2D_3$ (or its analogues) may downregulate ERK, resulting in decreased proliferation,[43,45] but paradoxically it may also be mitogenic via this pathway.[44] There are reports of upregulation of SAPK by $1,25(OH)_2D_3$ or an analogue,[45,46] resulting in apoptosis. It is long established that $1,25(OH)_2D_3$ may downregulate c-myc expression,[47,48] and this is one mechanism whereby $1,25(OH)_2D_3$ may affect the G_1/S cell cycle checkpoint

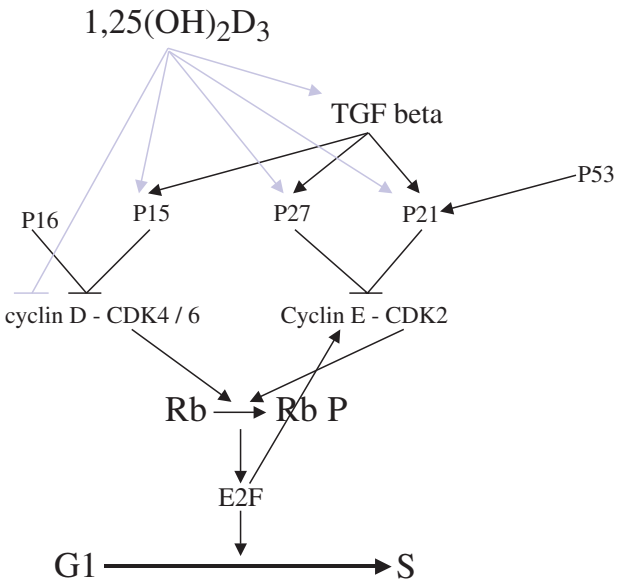

Figure 3. The G_1–S checkpoint of the cell cycle and the effect of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. Rb, retinoblastoma protein; RbP, phosphorylated retinoblastoma protein; CDK, cyclin dependent kinase; TGF, transforming growth factor.

(see below), but the mechanism is not fully elucidated. Another cytoplasmic signalling pathway activated by $1,25(OH)_2D_3$, via sphingomyelin hydrolysis, is the ceramide pathway,[49] which is relevant to apoptosis and differentiation, as discussed later.

Molecular mechanisms by which vitamin D affects cellular 'growth' and malignant cell invasion

Since the 1980s, $1,25(OH)_2D_3$ has been recognized as a potent cellular antiproliferative and prodifferentiation agent. More recently, there has also been intense interest in effects of $1,25(OH)_2D_3$ on apoptosis, malignant cell invasion and metastasis.

Cellular proliferation

$1,25(OH)_2D_3$ has antiproliferative and prodifferentiation effects on a number of cell types that express the VDR. Central to the effect of $1,25(OH)_2D_3$ on proliferation is the G_1/S checkpoint of the cell cycle. It is generally accepted that in most, if not all, cancers there are aberrations in the G_1/S checkpoint. Proliferating cells progress through the cell cycle (Fig. 3), which comprises the G_0/G_1 phase (most differentiated, nondividing cells are in the G_1 phase), the S phase in which new DNA is synthesized, and the G_2 phase, which is

followed by mitosis (M phase) whereupon the cells re-enter the G_0/G_1 phase. The cell cycle in mammalian cells is punctuated with inherent blocks that are overcome by the transient formation of cyclin–cyclin-dependent kinase (CDK) complexes. When a cell is in the G_1 phase, the G_1/S checkpoint is blocked by the non-phosphorylated form of the retinoblastoma (Rb) family of proteins (Fig. 3), which bind and inactivate transcription factors, e.g. E2F, which are essential for DNA synthesis in the S phase and are necessary for the upregulation of further proteins required for G_1–S progression. Phosphorylation of Rb by CDK4, 6 and 2 removes the inhibitory effect and liberates the transcription factors. The CDKs are activated sequentially by combination with cyclins, cyclins D and E for CDK4/6 and CDK2, respectively. The CDKs are inhibited by two classes of CDK inhibitors, derivatives of the INK4a locus (e.g. p16 and p15), which displace cyclin from the cyclin–CDK complexes, and the Cip/Kip proteins (e.g. p27 and p21), which form inhibitory ternary complexes with the cyclin–CDKs. The cyclins are activated transcriptionally by growth factors, e.g. EGF.

Mutations of *cyclin D1*, *p16* and *CDK4* are frequent in systemic cancer. This is also relevant to MM, where germline or MM cell line mutation or loss of function of *p16*,[50–53] and rarely *p15*[54,55] and the *CDK4* gene,[51,56] have been reported.

There is now abundant evidence that $1,25(OH)_2D_3$ at pharmacological levels has an inhibitory effect on the G_1/S checkpoint and may produce complete arrest of the cell cycle at that point. There is a decrease in the proportion of cells in the S phase when cancer cells of various types are exposed to $1,25(OH)_2D_3$. The main effect of $1,25(OH)_2D_3$ is to upregulate inhibitors of CDKs,[57] and there is some evidence of downregulation of activators.[58] *p27* and *p21* are upregulated at the transcriptional level, a VDRE is located in the promotor of p21,[58,59] and/or at the post-translation level.[60] There is also evidence that the effect of $1,25(OH)_2D_3$ on p21 may be secondary to an effect on TGF-β1.[58] An intermediary role of TGF-β2 (via increased secretion and synthesis) in the $1,25(OH)_2D_3$ control of human bone and keratinocyte growth has been described.[61] *p15* may also be upregulated.[62] The effects of $1,25(OH)_2D_3$ on the cell cycle have been reported in malignant cells in culture[57–60] and *in vivo* in a murine squamous cell carcinoma (SCC) model.[63] We have shown that calcipotriol, a $1,25(OH)_2D_3$ analogue, increased the expression of *p21* in normal human skin.[64] A further effect of $1,25(OH)_2D_3$ is to inhibit *cyclin D1* at the transcriptional level.[58] There is also evidence, in normal keratinocytes and colonic tumour cells, that $1,25(OH)_2D_3$ downregulates the EGF receptor and hence may specifically counteract EGF-stimulated tumour cell growth.[43,48]

Differentiation

In normal cells, $1,25(OH)_2D_3$ promotes the differentiation of keratinocytes from epidermal precursors,[65] monocytes–macrophages from myelopoietic progenitors/stem cells[66] and osteoclasts from mononuclear precursors.[67] Differentiation is obviously linked to the cell cycle and is associated with slowing of the cell cycle, e.g. upregulation of p21 has an inhibitory effect on cell proliferation but may promote differentiation.[62] However, some maintain that the antiproliferative and prodifferentiation effects of $1,25(OH)_2D_3$ are not intimately linked, as the smaller concentrations of $1,25(OH)_2D_3$ sufficient to induce keratinocyte differentiation may induce, rather than inhibit, proliferation.[42] There is little known of specific differentiation pathways in most tissues. Of interest to dermatologists are mechanisms involving keratinocytes. It is known that, *in vitro,* cultured keratinocytes proliferate readily in low Ca^{2+} media but differentiate in higher Ca^{2+} media[68] and *in vivo*, a calcium gradient exists in the epidermis,[69] which might provide the driving force for differentiation in intact dermis. i[Ca^{2+}] has been proposed as the intermediary of the effect of extracellular Ca^{2+} on differentiation.[70] Furthermore, Ca^{2+}-responsive regulatory elements in genes involved in keratinocyte differentiation, such as *involucrin*[71] and

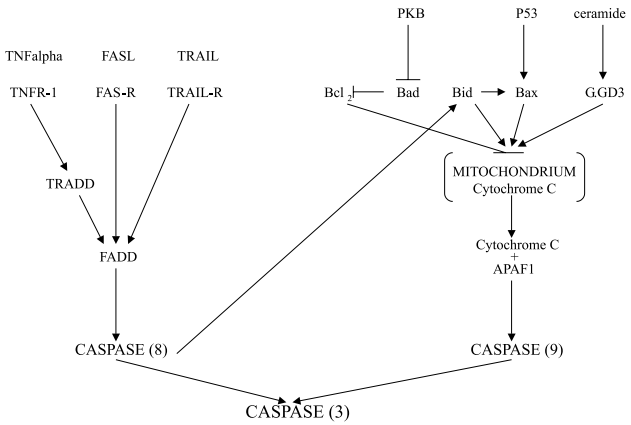

Figure 4. Apoptotic pathways. PK, protein kinase; TNF, tumour necrosis factor; TNFR-1, TNF receptor 1; FASL, Fas ligand; FAS-R, Fas receptor; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; G.GD3, galactoside GD3; TRADD, TNFR-1-associated death domain protein; FADD, FAS-associated death domain protein.

transglutaminase type 1,[72] have been identified. Similarly, $1,25(OH)_2D_3$ may increase i[Ca^{2+}], and it has been concluded that this is a partial explanation for $1,25(OH)_2D_3$-induced keratinocyte differentiation.[42] Another downstream target of PLC, PKC, has also been implicated.[73] Of particular interest is that *PLC*γ contains a VDRE.[42] It has also been suggested that keratinocyte differentiation is at least partially regulated by ceramide,[74] which is better known for its apoptotic action (see below). A proposed pathway is ceramide upregulation of apoptosis signal-regulating kinase 1, which is a component of the SAPK cascades (JNK and p38), and induction of the differentiation markers transglutaminase-1, loricrin and involucrin.[75]

Apoptosis

The underlying processes by which $1,25(OH)_2D_3$ induces apoptosis are poorly characterized, but multiple mechanisms have been demonstrated. These can be classified as indirect, via an effect on a growth factor or cytokine or their receptors, or direct, where there is an effect on a molecule that is part of the signalling pathway, resulting in apoptosis. Insulin-like growth factor (IGF) is a powerful constitutive antiapoptotic agent, and upregulation is an important factor in certain cancers, e.g. breast.[76] $1,25(OH)_2D_3$ antagonizes the antiapoptotic effect of IGF, mechanisms including downregulation of the IGF receptor,[77] increase in the IGF-binding protein[78,79] and a proposed downregulation of the IP_3 kinase $\rightarrow$ PKB pathway (Fig. 2) (which downregulates the proapoptotic factor Bad by phosphorylation).[76] TNF-α is a well-known proapoptotic agent and $1,25(OH)_2D_3$ has been shown to potentiate its killing power.[80]

A final common pathway in apoptotic signalling is the caspase cascade (Fig. 4). The caspases are a family of evolutionary conserved cysteine proteinases cleaving vital cytoskeletal and nuclear proteins, e.g. poly ADP-ribose polymerase, which results in cell disassembly. The pathways activating caspases may be divided into two main groups, those involving death receptors and those involving the mitochondrium. A well-documented mitochondrial system involves the Bcl-2 family, in which there are both antiapoptotic (e.g. Bcl-2 and Bcl-x) and proapoptotic (e.g. Bax, Bid and Bad) members. The effects of Bax are the opening of the mitochondrial permeability transition (PT) pore, reduction of mitochondrial transmembrane potential, loss of calcium retention and generation of reactive oxygen species. Consequently there is release of cytochrome c into the cytosol, which, in association with APAF1, results in activation of the effector caspases. Bcl-2 encodes an inhibitor of the PT pore, and there are several reports of increase in the Bax/Bcl-2 ratio by $1,25(OH)_2D_3$ or its analogues, which may be via downregulation of Bcl-2[45,81] or via translocation of Bax to the mitochondrium.[82,83] The sphingomyelin–ceramide–ganglioside GD3 signalling pathway is also known to induce apoptosis, ganglioside GD3 affecting the PT pore complex. $1,25(OH)_2D_3$ is reported to be an agonist of this pathway.[49,84]

The apoptotic signal of the familiar death receptors Fas and TNF-α, which may more directly activate caspases, can be inhibited by IEX-1 protein, and the transcription of the *IEX-1* gene may be downregulated by $1,25(OH)_2D_3$.[85] The killing pathways of TNF-α also include PLA2, and it has been suggested that $1,25(OH)_2D_3$ may modulate TNF-α apoptosis via an effect on this phopholipase.[76] The impact of SAPK and the other MAPK pathways on apoptosis is poorly understood, but upregulation of SAPKs is reported to promote apoptosis.[86,87] Mechanisms include phosphorylation (and inactivation) of Bcl-2 and Bcl-x[88] and inhibition by c-Jun of the association of p53 with the p21 promoter[89] (the effect of upregulated p53 is to inhibit cell cycle progression through p21 or to stimulate apoptosis, which is favoured if interaction with p21 is inhibited). It has been suggested that $1,25(OH)_2D_3$ (or an analogue) may produce apoptosis via upregulation of p38[45] and a component of the JNK cascade, MEKK-1.[46] Just as cytosolic calcium concentration is important in differentiation, raised i[Ca^{2+}] is implicated in apoptosis,[40] although the downstream effector system is unknown. $1,25(OH)_2D_3$ has been shown to trigger apoptosis by causing mobilization of intracellular Ca^{2+} stores and increase in Ca^{2+} entry through VICC.[90]

In addition to the undoubted proapoptotic effect, there are circumstances when $1,25(OH)_2D_3$ is antiapoptotic such as the effect of increased cell survival following UV trauma,[91,92] and protection from the apoptotic effects of cytotoxics in cancer cell cultures.[93] The mechanisms for this are not fully elucidated, but upregulation of metallothionein[91] or downregulation of JNK and partial G_1/S arrest in the cell cycle are possible explanations. Various physiological and environmental physical cellular stresses upregulate JNK, and this is inhibited by $1,25(OH)_2D_3$,[94] which is a proposed explanation of the protective effect of $1,25(OH)_2D_3$ on cell survival following stress. An inhibitory effect on TNF-α-induced apoptosis via inhi-

Laboratory	Effect on normal/malignant cell proliferation, apoptosis, malignant cell invasion and angiogenesis of $1,25(OH)_2D_3$ (*in vitro* and *in vivo*)
	Effect on experimental carcinogenesis of $1,25(OH)_2D_3$/vitamin D_3
Epidemiology/clinical	Effect on tumour occurrence/outcome of:
	low dietary vitamin D_3
	decreased vitamin D_3 synthesis from lack of sun exposure
	indirect estimates from studies on latitude and dark skin
	direct estimates of sun exposure
	low serum $1,25(OH)_2D_3$
	lack of cellular effect of $1,25(OH)_2D_3$
	functional polymorphisms of the VDR

Table 1. Lines of evidence for an effect on cancer of vitamin D_3

$1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; VDR, vitamin D nuclear receptor.

bition of TNF-α-induced transcription and translocation of PLA2 by $1,25(OH)_2D_3$ has also been described.[33] It has been demonstrated that when there is a G_1–S block in the cell cycle, cells are relatively immune to apoptotic signals,[93] and G_1–S block is an important effect of $1,25(OH)_2D_3$, as described above. Inhibition of the ceramide pathway has also been demonstrated, where $1,25(OH)_2D_3$ upregulates sphingosine kinase and the resulting sphingosine-1-phosphate antagonizes the apoptotic effect of ceramide.[95]

Malignant cell invasion

In an *in vitro* bioassay of cell invasion (such as Boyden chamber and Amgel), treatment with $1,25(OH)_2D_3$ at physiological concentrations has been shown to inhibit a number of cultured malignant cell types, such as breast,[96] prostate[97] and lung.[98] *In vivo*, intravesical instillation of $1,25(OH)_2D_3$ inhibited invasion in experimentally induced bladder cancer in rats.[99]

In relation to mechanisms, $1,25(OH)_2D_3$ has been shown to inhibit certain serine proteinases, e.g. components of the plasminogen activator (PA) system, and certain matrix metalloproteinases (MMPs) (and their inhibitors), which are important determinants of invasion. Decreased expression of urokinase PA and tissue-type PA and increased expression of PA inhibitor in response to $1,25(OH)_2D_3$ have been described in breast cancer cells,[100] and decreased expression of MMP-2 in prostate cancer cells[97] and MMP-9 in breast and prostate.[97,100] In addition, a VDRE was demonstrated in the *urokinase PA* promotor.[100] Tenascin-C is an extracellular matrix protein with growth-, invasion- and angiogenesis-promoting activities, which is upregulated in many cell types during tumorigenesis. Transcription of *tenascin-C* has been shown to be inhibited by $1,25(OH)_2D_3$ in a variety of mouse and human normal and malignant epithelial cell lines.[101]

An essential component of tumour growth is angiogenesis and recently it been suggested that this is inhibited by $1,25(OH)_2D_3$. *In vitro*, $1,25(OH)_2D_3$ has been shown to inhibit the proliferation of at least some types of tumour-derived endothelial cells,[102] to inhibit vascular endothelial growth factor-induced sprouting and elongation of endothelial cells[103] and to cause regression of sprouting elongated cells (via apoptosis).[103] Similarly, *in vivo*, in immunosuppressed mice, angiogenesis induced by intradermal injection of human tumour cell lines of different origins was inhibited by $1,25(OH)_2D_3$, administered both systemically to the animals and *in vitro* to the tumour cells.[104]

Vitamin D_3 and systemic cancer

The first observation relating vitamin D_3 to cancer was the demonstration that cultured human breast cancer cells express the VDR.[105] Subsequently, the first demonstration that $1,25(OH)_2D_3$ is capable of inhibiting the growth of human cancer cells was made with MM cells.[106] There is now good evidence that $1,25(OH)_2D_3$ has an anticancer effect in several systemic cancers; the lines of evidence are summarized in Table 1. *In vitro* evidence includes inhibition of growth by $1,25(OH)_2D_3$ of cultured malignant cells of prostate,[107–109] breast,[110–112] colon,[113–115] bladder,[99] leukaemic[116] and murine SCC cells,[63] and promotion of differentiation.[117–120] Furthermore, the level of differentiation in colon cancer has been reported to be correlated with the level of VDR expression.[114] In addition to malignant cell culture, $1,25(OH)_2D_3$ has been shown to inhibit tumour growth in xenografts.[121,122] It is notable that in certain breast carcinoma lines the $1,25(OH)_2D_3$ analogue EB 1089 is equipotent to tamoxifen as a growth inhibitor.[83] $1,25(OH)_2D_3$ induces apoptosis of various cancer cells, including breast,[40,123] prostate[124] and colon.[120] This

has been demonstrated in the main in culture but also *in vivo* in xenografts with a vitamin D_3 analogue.[125–127] Inhibition of experimental carcinogenesis by dietary vitamin D supplementation and $1,25(OH)_2D_3$ administration has also been demonstrated *in vivo* in animals.[99,128–131] The inhibitory effect of $1,25(OH)_2D_3$ on tumour invasion has been described above and inhibition of metastasis has been demonstrated in xenografts.[98,122] The force of these arguments, particularly *in vitro*, has to be tempered by the fact that the concentrations of $1,25(OH)_2D_3$ were usually in the range 1–5 nmol L^{-1}, whereas the physiological concentration is approximately 0.05 nmol L^{-1}, excepting some *in vitro* studies on proliferation,[112] differentiation[47,65,70,118] and tumour invasion.[96,97]

Epidemiologically, derangement of the vitamin $D_3/1,25(OH)_2D_3$ status has been reported to be associated with carcinoma of the breast, prostate and colon. Furst *et al.*[132] reported decreased aneuploidy associated with high dietary intake of vitamin D. Several studies have suggested an inverse relationship between dietary vitamin D intake and colorectal cancer,[133,134] although others have not confirmed this finding.[135] Recently, the carcinoma of the breast NHANES 1 epidemiological study suggested that optimal vitamin D_3 nutrition affords protection against breast cancer.

Increasing latitude from the equator and increased racial skin pigmentation have been shown to correlate with increased incidence and/or increased aggressiveness in a number of studies, and it has been assumed that the mechanism is decreased exposure to effective solar irradiation and subsequent decreased cutaneous synthesis of vitamin D.[136–141] The incidence of cancer of the breast is higher in countries located at high latitudes,[136,140] and there is an increased mortality rate associated with acid haze air pollution.[140] There are several reports of geographical differences, which correlate with availability of sunlight, in the incidence of invasive prostate cancer,[138,139] but this is not the case for *in situ* tumours. When compared with black men in Nigeria, black men in America have a sixfold increased risk of developing clinically detectable prostate cancer.[142] In Asians, the risk of prostate cancer is increased in migrants, and it has been suggested that this is due to the diet being less rich in fish oils. Both the risk of colon cancer and the mortality increase with increasing latitude and decreasing sunlight intensity,[141] and in urban areas associated with acid haze pollution.[140] A notable exception is Japan, which has relatively little colon cancer, but is located at relatively high latitude. A potential explanation suggested by some investigators is that the Japanese diet is very rich in vitamin D.

Black American women have a poorer prognosis than white American women for carcinoma of the breast, and Hispanic women have less aggressive disease than black women, but more aggressive than white women.[143] In this study the larger tumour size and increased frequency of nodal involvement were associated with increased skin pigmentation. Black American men also have a higher incidence of prostate cancer than white American men,[144] while the latent forms of prostate cancer are equally common in black and in white men.[145]

The evidence cited above is indirect in that the effects of change in latitude from the equator and racial skin pigmentation are assumed to influence effective sun exposure, which in turn affects vitamin D synthesis. However, recently the effects of more direct measures of sun exposure were assessed in one racial group at one location in England.[146] Regular foreign holidays, sunbathing score, and higher exposure to UV radiation were protective against the development of prostate cancer. Paradoxically, in relation to MM, the greatest protection was from regular sunburn in childhood (odds ratio 0.18, 95% confidence interval 0.08–0.38, $P = 0.0001$)! Furthermore, cumulative sun exposure and outdoor occupation have been found to be associated with reduced risk of advanced stage prostatic tumours but not with metastasis *per se*.[147]

Low mean serum levels of $1,25(OH)_2D_3$, or its precursors, have been reported in postmenopausal women with early breast cancer[148] and, furthermore, decreasing serum $1,25(OH)_2D_3$ levels have been shown to correlate with disease progression and metastasis.[149] Lower serum $1,25(OH)_2D_3$ has also been reported as a risk factor for prostate cancer[150] and cancer of the colon.[151,152]

Polymorphisms of the VDR have been reported to be associated with cancer of the prostate and breast. The *Fok*I restriction fragment length polymorphism has been reported to be associated with breast cancer,[153] where the *FF* genotype was associated with a decreased risk of breast cancer of $\sim$ 50% in African-Americans. The polyA polymorphism has been associated with altered risk of breast[153] and prostate cancer.[154,155] In breast cancer, the presence of *L* alleles was also associated with a $\sim$ 50% reduction in risk.[153] However, in prostate cancer, the presence of *L* or *T* was associated with a three- to fivefold increased risk of prostate cancer.[154,155] However, the *bL* haplotype was

found to be protective against advanced cancer of the prostate in African-Americans,[156] and the *ff* haplotype predisposed to metastasis in a British population.[147] The findings in cancer of the breast are exactly as would be anticipated, given that *F* and *L* alleles are associated with a more effective VDR. The findings in occurrence of cancer of the prostate are difficult to interpret but the associations with advanced disease are as would be expected.

On the basis of this evidence, vitamin D_3 is apparently an important endogenous cancer protective agent and vitamin D_3 analogues are promising anticancer therapeutic agents. Hypercalcaemia and hypercalciuria limit their clinical use but various strategies are being devised to obviate this, such as less calcaemic synthetic analogues and natural prohormones, e.g. $25(OH)D_3$, from which target tissues, such as prostate,[157] synthesize active analogues locally. $1,25(OH)_2D_3$ has also been demonstrated to enhance significantly the antitumour efficacy of other anticancer drugs in *in vitro* and *in vivo* model systems. Dexamethasone potentiates the antitumour effect of $1,25(OH)_2D_3$ and decreases $1,25(OH)_2D_3$-induced hypercalcaemia.[81] Clinical trials have been initiated involving $1,25(OH)_2D_3$ or its analogues alone, in combination with steroids and with either carboplatin or paclitaxel.[158] Trials of systemic $1,25(OH)_2D_3$ and vitamin D_3 analogues are also now under way in patients with advanced pancreatic, hepatocellular, colorectal and breast cancer and in acute myeloid leukaemia/myelodysplastic syndromes.[159]

A comparison of the roles of vitamin D_3 in systemic cancer and malignant melanoma

There is now accumulating evidence that the vitamin $D_3/1,25(OH)_2D_3$/VDR axis is a determinant of MM, as it is of other systemic cancers cited above. MM cells express the VDR and the antiproliferative and prodifferentiation effects of $1,25(OH)_2D_3$ have been shown in human MM cells,[106,110,160] the first malignancy in which this effect was demonstrated. In addition, in both melanocytes and MM cell lines, stimulation of tyrosinase activity, which is a specific prodifferentiation stimulus for melanocytes, has been reported for $1,25(OH)_2D_3$,[161,162] although this was not the case in some reports.[163] As is the case of oestrogen receptors in human breast cancer lines, not all melanoma cell lines express the VDR, or it may be expressed at very low levels, and in such cases there has been failure to demonstrate cell growth inhibition or upregulation of melanogenesis.[160,164] *In vivo*, $1,25(OH)_2D_3$ has been shown to suppress growth in human MM (expressing the VDR) derived xenografts in immune-suppressed mice,[121] but not in a VDR-negative MM cell line. $1,25(OH)_2D_3$ has been shown to induce apoptosis in a human MM cell line *in vitro*, although one cell line, despite expressing the VDR, was unresponsive.[165] Recently, an inhibitory effect on the spread of MM cells has been demonstrated *in vitro*.[166] Pretreatment of mouse melanoma cells with $1,25(OH)_2D_3$ resulted in inhibition of migration through extracellular matrix, the adherence of the cells to reconstituted basement membrane (Matrigel), type IV collagenolysis and the formation of lung metastases in animals inoculated with the vitamin D_3-treated melanoma cells. In a human MM cell line, $1,25(OH)_2D_3$ has been shown to downregulate, at the transcriptional level, the $\alpha 6$ subunit of the integrin laminin receptor, which resulted in reduced ability of the cells to adhere to an artificial basement membrane, in a human MM cell line.[167]

Low serum levels of $1,25(OH)_2D_3$ have been reported in MM patients, but this did not reach statistical significance.[168] We have shown that polymorphism at the *Fok*I restriction site of the VDR is associated with increased susceptibility to MM but a stronger association was found with MM outcome, as predicted by Breslow thickness.[169] The risk reduction for MM attributable to the *FF* genotype was estimated at 33.6%, and the combined *ttff* genotype, and to a lesser degree heterozygote genotypes, were associated with thicker tumours, particularly those > 3.5 mm thick. The effect on tumour thickness could be the result of an effect on cell proliferation or alternatively on tumour cell invasion. These findings for MM are closely similar to those reported for the occurrence of carcinoma of the breast,[153] and outcome, but not occurrence, of cancer of the prostate,[147] cited above. There are thus close parallels in the evidence of the involvement of vitamin D_3 in MM and certain systemic cancers, in terms of VDR expression, growth and death responses of malignant cells in culture, malignant cell migration and metastasis, association with low circulating levels of vitamin D_3 and the effect of *VDR* polymorphisms.

However, in MM there are no studies showing an effect of dietary vitamin D_3 and there is an apparent opposite effect of solar radiation in terms of geographical factors and skin colour on its occurrence. An obvious difference between skin and internal organs is that skin is exposed to UV, which is both potentially indirectly protective (via vitamin D_3) and directly damaging (via mutagenesis). The final effect may be a

balance of the two. Many studies have shown that the incidence of MM increases with decreasing latitude towards the equator[170] and therefore there is a clear difference from the internal cancers discussed above. This suggests that the mutagenic effect of solar radiation outweighs any protective effect of increased cutaneous synthesis of vitamin D_3. However, in higher less extreme latitudes, with individuals having limited sun exposure, it is possible that the protective/harmful balance of solar radiation could change and in Europe, for example, a reverse relationship with latitude has been found.[171] An alternative explanation for this is a range of skin colour from dark in the South to light in the North. There are very few data on the effect of latitude on MM outcome and it is notable that a VDR polymorphism study in MM[169] suggested that vitamin D_3 is more influential on outcome than occurrence. An Australian study showed that, although people living closer to the equator had an increased incidence of MM, they more often had *in situ* and thin invasive lesions than thicker lesions.[172] There is also no doubt that the incidence of MM is less in dark-skinned races, again representing a difference from the aforementioned internal malignancies. Also, within white races, fairer skinned individuals have an increased chance of developing MM.[173–175] However, in relation to outcome it is of interest that the rare MM in blacks is extremely aggressive. There is no evidence on outcome in less markedly dark skin and studies on the impact of skin type on prognosis would be of interest.

Assessment of sun exposure parameters has consistently shown an association with short-term intense exposure,[175–178] particularly burning in childhood,[177,178] and the development of MM. However, more chronic less intense exposure has not been found to be a risk factor and in fact has been found to be protective by some.[175–177] An alteration in the protective effect of vitamin D_3 because of change in cutaneous synthesis might at least partly explain such protection. An argument against this is that very short and limited cutaneous solar exposure is necessary to achieve 'adequate' vitamin D_3 synthesis at latitudes such as Boston, U.S.A. However, Boston is on the same latitude as northern Spain, and is therefore sunnier than northern Europe. Furthermore, it has been suggested that the physiological levels of vitamin D_3 metabolites required for cancer protection are greater than for its mineral effects.[4] Altered vitamin D_3 status could also be a factor in the debate on the association of MM with sunscreen use.[179,180] However, in Australia no association was found between vitamin D_3 levels and sunscreen use[181] but similar studies have not been undertaken in more temperate climates where ambient UV radiation is reduced.

Therefore the relationship between solar irradiation and MM is more complex than for the systemic cancers previously described. On the one hand there is evidence of an association between the incidence of MM and sun exposure, and on the other there is the potentially deleterious effect of lack of vitamin D_3 due to limitation of sun exposure. That UV is mutagenic is not an argument against a protective role of vitamin D_3. Further work is necessary on the influence of serum vitamin D_3 levels on the occurrence and prognosis of MM, the effects of sun protection measures on serum vitamin D_3 levels in temperate climates and epidemiological studies on geographical factors and skin type on the prognosis of MM. It would seem mandatory to ensure an adequate vitamin D_3 status if sun exposure is seriously curtailed, certainly in relation to carcinoma of breast, prostate and colon and probably also MM.

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