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Vitamin D Control of Hematopoietic Cell Differentiation and Leukemia

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ABSTRACT

It is now well known that in the mammalian body vitamin D is converted by successive hydroxylations to 1,25-dihydroxyvitamin D (1,25D), a steroid-like hormone with pleiotropic properties. These include important contributions to the control of cell proliferation, survival and differentiation, as well as the regulation of immune responses in disease. Here, we present recent advances in current understanding of the role of 1,25D in myelopoiesis and lymphopoiesis, and the potential of 1,25D and analogs (vitamin D derivatives; VDDs) for the control of hematopoietic malignancies. The reasons for the unimpressive results of most clinical studies of the therapeutic effects of VDDs in leukemia and related diseases may include the lack of a precise rationale for the conduct of these studies. Further, clinical trials to date have generally used extremely heterogeneous patient populations and, in many cases, small numbers of patients, generally without controls. Although low calcemic VDDs have been used and combined with agents that can increase the leukemia cell killing or differentiation effects in acute leukemias, the sequencing of agents used for combination therapy should to be more clearly delineated. Most importantly, it is recommended that in future clinical trials the rationale for the basis of the enhancing action of drug combinations should be clearly articulated and the effects on anticancer immunity should also be evaluated. J. Cell. Biochem. 116: 1500–1512, 2015.

KEY WORDS: VITAMIN D; CALCITRIOL; HEMATOPOIESIS; LEUKEMIA

Pluripotent hematopoietic stem cells in the bone marrow give rise to two major classes of leukocytes—myeloid and lymphoid cells. Orchestrated by tightly regulated context-dependent developmental cues, hematopoietic stem cells differentiate into monocytes/macrophages, eosinophils, basophils, neutrophils, myeloid dendritic cells, red blood cells and platelets (myeloid origin), as well as B cells, T cells, plasmacytoid dendritic cells and NK cells (lymphoid origin) to constitute a fully functional repertoire of diverse cell types with specialized functions.

In recent years vitamin D has emerged as a pivotal regulator of multiple cell events including cell proliferation, survival, differentiation, immune activities and regulation of the cytokine milieu. These investigations were largely spurred by the discovery that vitamin D receptor (VDR) is expressed in a variety of mammalian cells including the immune cells, as are the enzymes that catalyzes the conversion of vitamin D from its inactive form to its biologically active form (1,25-dihydroxyvitamin D_3—both vitamin D_3 or plant derived vitamin D_2 are abbreviated here as 1,25D), also known as calcitriol.

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Thus, not only are mammalian cells capable of sensing systemic 1,25D levels, but can also modulate the localized concentrations of 1,25D [Chun et al., 2014].

Here we discuss our current understanding of vitamin D regulation of myelopoiesis and lymphopoiesis, as discovered using both in vitro and in vivo models of vitamin D deficiency or supplementation during normal hematopoiesis and hematopoietic malignancies. While supporting epidemiological studies were also important in documenting the health relevance of vitamin D [Giovannucci et al., 2006; Hart et al., 2011; Grant, 2014; Shui and Giovannucci, 2014], relatively little information is available on the role of vitamin D in hematopoiesis from these studies.

VITAMIN D AND NORMAL HEMATOPOIESIS

MYELOPOIESIS

The bioactive forms of vitamin D predominantly exert their biological effects through the VDR, as determined in model systems of genetically modified mice and cultured leukemia cells. Mice with genetic deletion (KO) of VDR serve as a robust model of human vitamin D deficiency, which replicate clinical manifestations of hypocalcemia, hyperparathyroidism, hypophosphatemia, rickets and osteomalacia. Interestingly, VDR KO mice have normal hematopoiesis, with normal relative numbers of red and white blood cells [O'Kelly et al., 2002]. However, vitamin D derivatives (VDDs), including 1,25D, are known to influence later stages of hematopoiesis by cellular signaling cascades. Since neoplasia can be perceived as a disease of communication not only between but also within cells, it is of fundamental importance to tease out the networks of these signaling pathways.

Thus, studies using human myeloid leukemia cells have established the beneficial effects of 1,25D in promoting monocytic differentiation and modulating immune functions, and are inspiring further detailed mechanistic studies to identify specific molecular targets for driving terminal differentiation and/or apoptosis of leukemic cells.

Vitamin D-regulated MAPK signaling pathways in hematopoietic cells. The mitogen-activated protein kinase (MAPK) pathways are key components of the signal transduction cascades which link diverse extracellular stimuli to proliferation, differentiation, and survival of different cell types, including hematopoietic cells [Davis, 1993; Lewis et al., 1998; McCubrey et al., 2007; Geest and Coffer, 2009]. Numerous studies have shown that dysregulated activation of MAPKs plays an important role in the transformation of myeloid cells to leukemic blasts, and MAPK pathways also play an important role in the regulation by 1,25D of cell proliferation, differentiation and apoptosis in AML [Studzinski et al., 2006; Gocek and Studzinski, 2009]. The members of the MAPKs family are serine/threonine kinases which participate in four major signaling pathways: the extracellular signal-regulated protein kinase (ERK), the p38 kinase, the c-Jun N-terminal kinase (JNK), and the ERK5 cascades (Fig. 1) [Dhillon et al., 2007]. Most MAPKs exist as different isoforms, such as ERK1/2; p38α, p38β, p38γ, and p38δ [Krishna and Narang, 2008]; and JNK1, JNK2 and JNK3 [Karin and Gallagher, 2005; Krishna and Narang, 2008]. There are at least three main tiers of each MAPK signaling cascade, a MAPK kinase kinase (MAP3K), a MAPK kinase (MAP2K), and a MAPK. Upstream MAPK kinases phosphorylate and activate downstream MAPKs, which in turn phosphorylate various downstream proteins, such as transcription factors (e.g., c-Jun, Elk-1, C/EBPs, and MEF2C) [Yordy and Muise-Helmericks, 2000; Marcinkowska et al., 2006; Kasza, 2013; Wang et al., 2014] and apoptosis-related proteins (e.g., the pro-apoptotic protein BIM and the anti-apoptotic protein MCL-1) [Weston et al., 2003; Ewings et al., 2007; McCubrey et al., 2007; Nishioka et al., 2010], thereby promoting the survival of cancer cells. These MAPK pathways can interact and cooperate with each other to transmit signals to the downstream regulators and determine cell fate [Krishna and Narang, 2008].

The ERK1/2 pathway is the best studied and is involved in the regulation of the proliferation, survival and differentiation of hematopoietic cells [Davis, 1993; Geest and Coffer, 2009]. The evidence for a role of this MAPK pathway in AML includes constitutive activation of ERK in a subset of primary AML cell samples, and the activation of MEK which contributes to constitutive ERK activation [Towatari et al., 1997; Milella et al., 2001; Ricciardi et al., 2005; Prijic et al., 2014]. The MEK1/ERK1/2 pathway can be activated through a wide range of extracellular signals such as cytokine and growth factor stimuli, which sequentially activate Raf1, MEK1/2, and then ERK1/2. Of clinical interest, activation of the RAF/MEK/ERK pathway conveys a poor prognosis, and constitutes a promising target for therapeutic intervention [Ricciardi et al., 2012].

Activated ERK translocates into the nucleus and activates a number of nuclear transcription factors that are important for myeloid differentiation, such as Elk-1, MEF2C and C/EBPs [Marcinkowska et al., 2006; Kasza, 2013; Wang et al., 2014]. ERK1/2 can also be activated through the formation of a signaling complex with multiple kinases with the help of a scaffold protein, the Kinase Suppressor of Ras-1 (KSR-1) [Wang and Studzinski, 2004]. The MEK1/2-ERK1/2 cascade is an important regulator pathway of monocytic differentiation in AML cells, and can act as a biomarker of early monocytic differentiation [Wang and Studzinski, 2001].

The p38 and JNK MAPKs pathways are activated by pro-inflammatory cytokines or cellular stresses, and JNK also by VDDs [Studzinski et al., 2005]. The p38 MAPK family consists of four isoforms: p38α, β, γ, and δ which display tissue-specific patterns of expression [Hale et al., 1999]. p38α and β are widely expressed among all tissues, whereas the expression of p38γ and δ appears to be more specific to certain tissues, for example, p38γ in skeletal muscle, and p38δ in endocrine glands [Uddin et al., 2004]. The p38 MAPKs are activated by the phosphorylation by upstream kinases MKK3 and MKK6, while activated p38 MAPKs phosphorylate and activate downstream targets, such as MAPKAP kinase 2 [Rane et al., 2001]. Based on sequence homology and substrate specificities, the p38MAPK family can be divided into two subsets with p38α and p38β in one set and p38γ and p38δ in the other set. Importantly for vitamin D research, the inhibition of p38α and p38β actually potentiates the differentiating action of 1,25D, and is associated with a compensatory upregulation of p38γ and p38δ in AML cells. This effect is increased by the presence of carnosic acid (CA), a plant-derived polyphenol with antioxidant properties [Studzinski et al., 2005; Wang et al., 2005a], and suggests that one or more downstream targets of p38 α and β exert negative feedback on the activity of an upstream regulator of several branches of MAPK pathways (JNK,

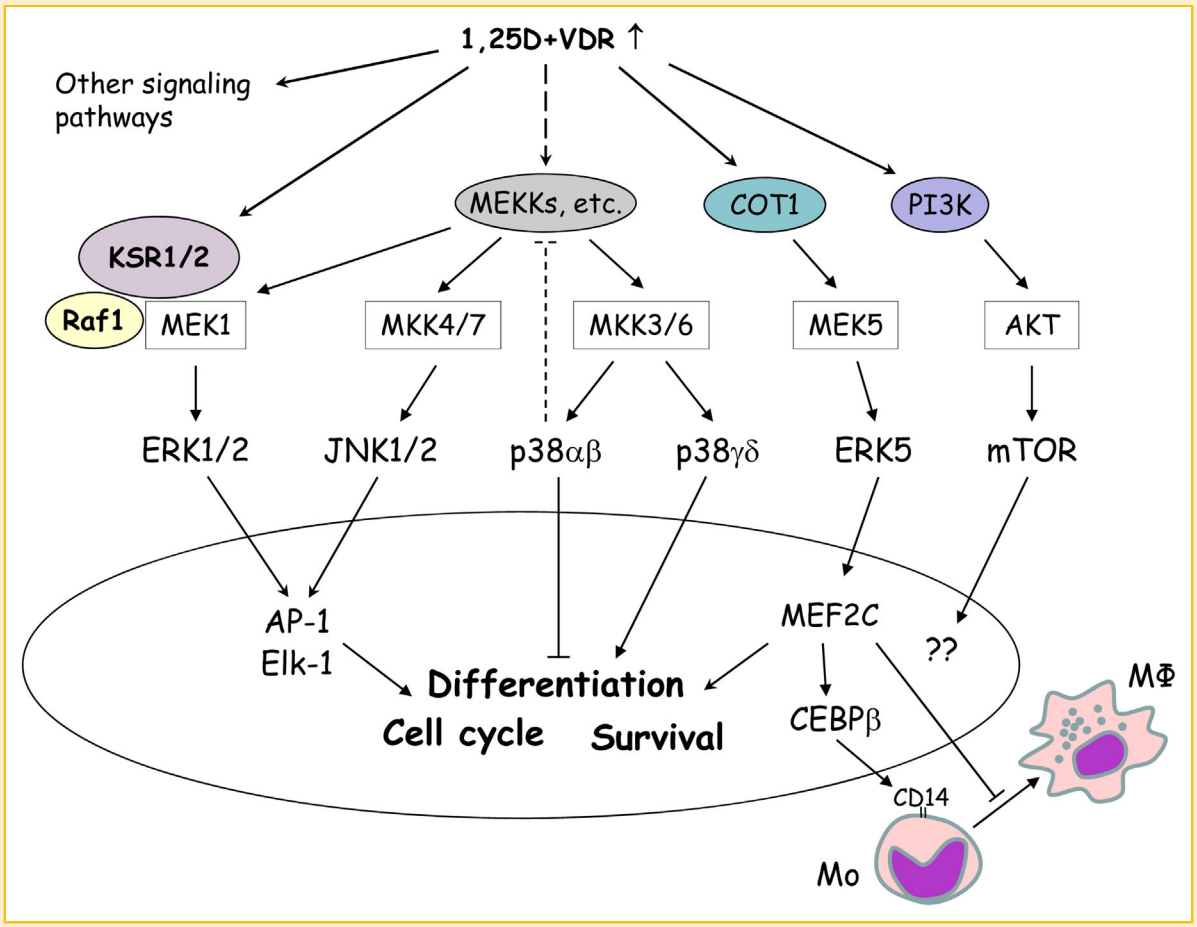

Fig. 1. Intracellular signaling pathways induced by 1,25D in AML cells. The MAPK and other signaling pathways discussed in the text are outlined here. In these cells the effects of 1,25D are initially mediated by an upregulation of the levels of vitamin D receptor (VDR) and activation of the upper echelons of kinase cascades, resulting in cell differentiation, arrest of the cell cycle, and changes in cell survival control, including apoptosis. Dotted lines depict suggested interactions. The regular arrows show activation, blunt ended arrows inhibition. The latter includes the inhibition by ERK5 of the progression of the monocyte (Mo) differentiated by 1,25D to the macrophage (MΦ).

ERK1/2, p38), while a reduction of ROS levels by CA [Danilenko et al., 2003] appears to favor this effect.

The JNK kinases are important members of MAPK superfamily, often referred to as stress-activated protein kinases (SAPK). This family includes three members: JNK1, JNK2 and JNK3 [Fleming et al., 2000; Karin and Gallagher, 2005]. JNKs are activated by upstream kinases, such as MKK4 and MKK7, and regulate target gene expression through a variety of transcription factors, such as c-Jun, ATF-2, and Elk-1, to mediate cell proliferation, differentiation or survival [Kyriakis et al., 1994; Lee et al., 2003]. c-Jun is essential for monocytic differentiation of human AML cells, as a part of the AP-1 transcription factor [Wang and Studzinski, 2001, 2006]. Studies with AML model systems have indicated a regulatory function of JNK activity in 1,25D-induced differentiation and the consequent phosphorylation of the c-Jun protein [Wang et al., 2003]. Another study concluded that JNK2 is a negative regulator of monocytic differentiation, by JNK2 antagonizing the signaling of differentiation by JNK1 in human AML cells resistant to VDDs [Chen-Deutsch et al., 2009].

While the three main MAPK pathways discussed above have been extensively studied in relation to AML, the role of the "Big MAPK" ERK5 (also known as BMK1) has only rarely been explored in relation to hematopoiesis or AML therapy. Like the other MAPKs this pathway has been implicated in cell proliferation, differentiation and survival, but ERK5 signaling has some distinct effects from the other MAPK pathways [Wang et al., 2015]. The upstream kinases MEKK2 and MEKK3 activate MEK5, which then phosphorylates and activates ERK5 on the N-terminal TEY sequence. The activated ERK5 subsequently undergoes autophosphorylation on the C-terminal transcriptional activation domain [Mody et al., 2003; Morimoto et al., 2007]. The sequential phosphorylation allows the activated ERK5 to translocate into the nucleus and to activate transcription factors, such as the myocyte enhancer factor 2C (MEF2C) [Kato et al., 1997; Wang et al., 2014; Zheng et al., 2014]. It has been shown that reduction in ERK5 MAPK activity by inhibition of Cot1 kinase, which can also activate ERK5 [Chiariello et al., 2000], enhances the expression of 1,25D-induced differentiation-promoting factors and cell cycle regulators such as p27Kip1, leading to cell cycle arrest [Wang

et al., 2010]. ERK5 can also regulate C/EBPβ expression via MEF2C [Zheng et al., 2014], which controls the expression of monocytic differentiation marker CD14, and thus promotes monocytic differentiation. On the other hand, a recent study has demonstrated that while playing a positive role in monocytic differentiation, ERK5, but not ERK1/2, inhibits the progression of monocytic phenotype to the terminal differentiation into functioning macrophages by negatively regulating the expression of macrophage colony stimulating factor receptor (M-CSFR) in AML cells (Fig. 1) [Wang et al., 2015]. Since tumor-associated macrophages (TAMs) are considered to have generally a protumoral role by promoting key processes in tumor progression [De Palma and Lewis, 2013; Noy and Pollard, 2014], the 1,25D-augmented ERK5 function [Wang et al., 2014] illustrated in Figure 1, may explain the beneficial effects of 1,25D on some solid tumors [Kaler et al., 2009; Kaler et al., 2010; Zhang et al., 2014].

Vitamin D has also been reported to affect both the enhancement of and the resistance to the differentiating action of VDDs by modulating the activity of MAPK pathways. For instance, CA combined with SB202190, a p38α/β MAPK inhibitor, increased the potency of 1,25D in HL60 cells [Wang et al., 2005a]. In these experiments, determination of the activity of MAPK pathways showed that increased differentiation was associated with enhanced activity of JNK pathway in all responding cell AML subtypes [Wang et al., 2005a,b]. Other examples of 1,25D combinations with other compounds that have a synergistic effect with 1,25D, which involves at least in part the activation of MAPK cascades, are provided by studies in a number of different laboratories. For example, it is reported that non-specific COX inhibitors acetyl salicylic acid or indomethacin signal by Raf1 but not ERK1/2 pathway [Jamshidi et al., 2008], ceramide derivatives signal via PI3-K/PKC/JNK/ERK pathway [Kim et al., 2007], nargenicin via PKCβ1/MAPK pathway [Kim et al., 2009], and all show synergy with VDDs by these similar but not identical MAPK signaling pathways. Also, iron chelating agents which induce iron deprivation can potentiate the VDR pathways and magnify the activation of JNK induced by VDDs [Callens et al., 2010]. More recently, epidermal growth factor receptor (EGFR) inhibitors erlotinib and gefitinib were reported to be potent enhancers of differentiation of AML cells, presumably by the effects of MAPKs downstream of EGFR [Lainey et al., 2013].

Conversely, MAPKs can also be involved in resistance to 1,25D. An example is provided by HL60-40AF cells, a subclone of HL60 cells resistant to 1,25D [Studzinski et al., 1996], in which protein levels of Hematopoietic Progenitor Kinase 1 (HPK1), an upstream MAP4K serine/threonine kinase, are dramatically increased. The HPK1 protein is further increased when the 1,25D resistance of 40AF cells is partially reversed by the addition of carnosic acid and p38MAPK inhibitor SB202190. Knockdown of HPK1 reduces 1,25D/VDD-induced differentiation of both 1,25D-sensitive HL60 and U937 cells and 1,25D-resistant 40AF cells. Molecular basis for this was shown to be that the full-length HPK1 protein is a positive regulator of VDD-induced differentiation in AML cells, but the cleaved HPK1 fragment inhibits differentiation. Thus, high HPK1 cleavage activity contributes to 1,25D/VDD resistance [Chen-Deutsch and Studzinski, 2012].

Other vitamin D-regulated signaling pathways in hematopoietic cells. Although the regulation of vitamin D-induced differentiation and anti-proliferative effects in hematopoietic cells is especially well documented for the MAPK pathways, VDDs can also stimulate, and occasionally inhibit, activation of several other signal transduction circuits, including the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR), nuclear factor kappa B (NF-κB) and Janus kinase and signal transducer and activator of transcription (JAK-STAT) pathways [Muthian et al., 2006; Stoffels et al., 2006; Janjetovic et al., 2010; Lisse and Hewison, 2011; Datta-Mitra et al., 2013].

It is now well established that the PI3K/AKT/mTOR signaling axis plays a central role in cell proliferation and survival under physiological conditions, and aberrant PI3K/AKT/mTOR signaling has been implicated in many human cancers, including AML. An early observation in the field was that the AKT pathway is activated by 1,25D and participates in its anti-apoptotic effect and cell cycle control in differentiating HL60 cells [Zhang et al., 2006]. Wang and others [Wang et al., 2009] suggested that AKT regulates 1,25D-induced leukemia cell differentiation via the RAF/MEK/ERK signaling, linking the AKT and the MAPK pathways. It was also reported that inhibition of mTOR by the rapamycin analog everolimus potentates the effects of 1,25D on U937 AML cells [Yang et al., 2010], and that an analog of 1,25D ("BE") induces immunosuppression through the PI3K/AKT/mTOR cascade [Datta-Mitra et al., 2013]. The 1,25D-stimulated increase in steroid sulphatase activity in AML cell lines is also achieved via the PI3Kα pathway [Hughes et al., 2008]. In addition, the AKT/mTOR pathway has been reported to transduce signals provided by VDDs to diverse cell types, including skeletal muscle [Buitrago et al., 2012; Salles et al., 2013], primary human keratinocytes [De Haes et al., 2004], squamous cell carcinoma cells [Ma et al., 2006], and breast, prostate and leukemia cell lines [O'Kelly et al., 2006]. Collectively, these reports are in keeping with the suggestion that mTOR may be a general integrator of both immunomodulatory and antiproliferative activities of 1,25D [Lisse and Hewison, 2011].

A link between PI3K/AKT and NF-κB activation was reported in HL60 cells exposed to 1,25D by showing PI3K/AKT-dependent degradation of IκB, and was suggested to be contributory to vitamin D-mediated immune regulation [Tse et al., 2007]. Also, inhibition of NF-κB activity by antisense oligonucleotides to its various subunits [Sokoloski et al., 1998] or by phytochemicals, such as curcumin, β-carotene and parthenolide [Kang et al., 2002; Sokoloski et al., 1997a,b] enhance 1,25D-induced differentiation of HL60 cells into monocytes. Perhaps related, is the finding that combined activation of the JAK-STAT, p38 MAPK, and NF-κB pathways is necessary to regulate the expression of 1α-hydroxylase in monocytes in a synergistic way, with C/EBPβ being a likely downstream target [Stoffels et al., 2006]. It is also known that 1,25D modulates the JAK-STAT pathway in IL-12/IFN gamma axis leading to Th1 response in experimental allergic encephalomyelitis [Muthian et al., 2006], and that 1,25D blocks TNF-induced monocytic tissue factor expression by the inhibition of activation pathways for transcription factors AP-1 and NF-κB [Chung et al., 2007]. It is therefore clear that there is considerable cross talk between pathways that propagate signals provided to hematopoietic cells by vitamin D and its metabolites.

Thus, studies using human myeloid leukemia cells have established the beneficial effects of 1,25D in promoting monocytic differentiation and modulating immune functions, and are inspiring further detailed mechanistic studies to identify specific molecular targets for driving terminal differentiation and/or apoptosis of AML and other leukemic cells.

LYMPHOPOIESIS

Studies using VDR KO mice lacking vitamin D signaling have also identified a critical role of vitamin D in the thymic development of functional invariant Natural Killer T (iNKT) cells bearing the Vα14-Jα18 rearranged T cell receptor (TCR) [Yu and Cantorna, 2008]. Vitamin D deficiency resulted in the absence of the expression of T-bet transcription factor in developing iNKT cells, and was associated with compromised expression of the surface glycoprotein CD1d in the thymus of VDR KO mice, suggesting ineffective antigen presentation and selection of iNKT cells in the thymus. Notably, the numbers of TCRαβ CD8αα intraepithelial lymphocytes (IELs) were also compromised in the VDR KO mice [Yu et al., 2008]. In vitro studies of hematopoiesis have identified an inhibitory effect of vitamin D on NK cell development, while promoting myeloid differentiation [Weeres et al., 2014]. However, the differentiation of mature conventional TCRαβ CD8αα T cells remains largely unaltered in the absence of 1,25D signals. Analysis of CD4 and CD8 single- and double-positive and double-negative T cells in the thymus identified no differences between WT and VDR KO mice. Likewise, natural regulatory T cell numbers are also largely unaltered in the absence of vitamin D signaling under homeostatic conditions [Yu and Cantorna, 2008, 2011].

The iNKT cells are known for their ability to rapidly produce inflammatory and immune stimulatory cytokines such as IFN-γ, TNF-α and IL-2, and their deficiency is associated with development of autoimmune disorders such as diabetes, inflammatory bowel disease (IBD) and atherosclerosis [Wobke et al., 2014; Yin and Agrawal, 2014]. Likewise, CD8αα cells are also implicated in suppressing gastrointestinal inflammation [Cheroutre and Lambolez, 2008]. Thus, compromised development of iNKT and CD8αα T cells in the absence of vitamin D signaling may be responsible in part for the increased susceptibility to IBD in human populations with low vitamin D status. Indeed, IBD patients are associated with low vitamin D status [Cantorna and Mahon, 2004], and polymorphisms in the *vdr* gene are associated with increased susceptibility for Crohn's disease and ulcerative colitis [Simmons et al., 2000; Dresner-Pollak et al., 2004].

Collectively, these findings, and other ongoing studies, can provide a new understanding of dysregulated differentiation in human hematopoietic leukemias.

VITAMIN D AND MALIGNANCIES

Accumulating evidence indicates that inadequate vitamin D levels are associated with increased risk of several types of cancer [Shui and Giovannucci, 2014]. With regard to hematopoietic malignancies, an inverse correlation has been observed between plasma concentrations of 25-hydroxyvitamin D_3 (25D) and a risk and disease progression of CLL [Shanafelt et al., 2011; Molica et al., 2012; Luczynska et al., 2013]. Also, low 25D levels were found to be associated with adverse outcomes in patients with AML [Lee et al., 2014]. The totality of existing in vitro, in vivo, epidemiological, and clinical studies demonstrate anti-proliferative and differentiation-inducing effects of VDDs on many cancer cell types, and strongly indicate a role for vitamin D in the reduction of total human mortality [Autier and Gandini, 2007].

ANTI-LEUKEMIC EFFECTS OF 1,25D AND OTHER VITAMIN D DERIVATIVES IN MYELOID MALIGNANCIES

The potential therapeutic and chemopreventive significance in myeloid leukemias of VDDs acting alone. The demonstration of a marked antiproliferative and differentiation-inducing activity of 1,25D and its synthetic low-calcemic analogs in various cancer cell lines including AML, patient-derived primary cell cultures and animal models has suggested a potential therapeutic and chemopreventive significance of VDDs [Abe et al., 1981; Koeffler et al., 1984; Studzinski et al., 1985]. To date, a number of clinical trials of VDDs have been conducted in various types of solid cancers. However, apart from some encouraging results, these compounds have not yet shown consistent clinical responses, and hypercalcemia still remains the major limiting factor.

Currently, analogs of the plant-derived vitamin D_2 are among the best candidates for an eventual use. Doxercalciferol (1α-hydroxyvitamin D_2, trade name Hectorol) and paricalcitol (19-nor-1,25-$(OH)_2$-vitamin D_2, trade name Zemplar) are approved in the USA for human use to suppress parathyroid hormone synthesis in dialysis patients. However, the concentrations used for this purpose are unlikely to have an effect on AML blasts, as shown by the negligible therapeutic effect of the administration of doxercalciferol to patients with MDS, a preleukemic condition [Petrich et al., 2008]. Recent laboratory studies also show that modification of the vitamin D analog structure including the removal of the 20-methyl group from the side chain of the analog selectively eliminates bone calcium mobilization activity [Barycki et al., 2009]. This was followed by other studies including the synthesis of 20-hydroxyvitamin D_2, a novel analog of vitamin D_2, also showing a modification at C20, and the demonstration that this analog had reduced calcemic activity [Slominski et al., 2011]. While 20-hydroxy vitamin D_2 was shown to have more potent anti-proliferative and pro-differentiation effects on epidermal cells, its activity on myeloid leukemia cells tested (K562-CML and HL60-AML) was markedly lower. Thus, while the calcemic activity of this analog was shown to be reduced in short term (7 days) experiments in rats [Slominski et al., 2011], its prospects for clinical application to human leukemia are at best uncertain at this time. An important concern regarding treatment of neoplastic diseases with VDDs alone is the possibility that prolonged treatment with 1,25D can result in the development of a resistant and more aggressive disease associated with increased distant organ metastasis as recently shown in a mouse model of prostate cancer [Ajibade et al., 2014].

Thus, as summarized in recent reviews [Harrison and Bershadskiy, 2012; Kim et al., 2012; Marchwicka et al., 2014] despite several examples of minor clinical efficacy, barriers remain to the successful application of VDDs in the treatment of MDS and AML. These barriers include: (i) The lack of definition of a sensitive target sub-population of AML patients because clinical trials conducted so far have

generally used extremely heterogeneous patient populations and, in many cases, small numbers of patients, usually without controls. (ii) The still unknown optimal choice of a vitamin D analog and the dosing schedule. (iii) Most importantly, contrary to some optimistic expectations [Barycki et al., 2009], there is still no solid evidence that calcium mobilizing properties of the thousands of vitamin D analogs synthesized and tested to date can be dissociated from their differentiation-inducing actions to the extent that they can be useful in the clinic.

Anti-myeloid leukemia effects of 1,25D/VDDs combinations with other non-cytotoxic agents. Since the discovery of their anticancer activity in experimental models, the idea of administering 1,25D and its analogs either as adjuvants or as integral part of conventional chemotherapy have attracted much interest [Kasukabe et al., 1987; Kumagai et al., 2005; Studzinski et al., 1986]. Evidence accumulated over more than 30 years provides the basis for a combination strategy for vitamin D-based cancer therapy which may prove more effective compared with VDDs administered as single agents [Ma et al., 2010]. Initial observations made in 1983 by Japanese and Swedish research groups demonstrated a marked synergistic induction of differentiation in AML cell lines by near physiological concentrations of 1,25D combined with the synthetic glucocorticoid dexamethasone [Miyaura et al., 1983] or the vitamin A derivative all-trans retinoic acid (ATRA) [Olsson et al., 1983]. Since that time, numerous studies have confirmed the ability of 1,25D and its analogs to cooperate in the anticancer effects with various agents, in different experimental models of neoplasia (reviewed in [Danilenko and Studzinski, 2004; Luong and Koeffler, 2005; Ma et al., 2010]) and in some clinical studies described below and elsewhere. These include differentiation inducers and enhancers, most prominently ATRA [Bastie et al., 2004, 2005; Danilenko and Studzinski, 2004; Takahashi et al., 2014], anti-inflammatory agents [Sokoloski and Sartorelli, 1998; Krishnan et al., 2007; Jamshidi et al., 2008; Laverny et al., 2009] and, importantly, phytochemicals [Sokoloski et al., 1997a; Danilenko et al., 2001; Kang et al., 2001; Wang et al., 2005b; Shabtay et al., 2008; Bobilev et al., 2011].

On the encouraging side, several recent clinical studies in which VDDs were combined with other drugs showed promising outcomes. For instance, in a retrospective case–control study the therapeutic effects of a combination treatment with 25D and the iron-chelating agent deferasirox (DFX) were demonstrated in elderly patients with AML who failed to respond to demethylating agents [Paubelle et al., 2013]. Median survival of patients treated with this combination was significantly increased in comparison with matched patients receiving best supportive care alone. Interestingly, the only factor associated with an increased overall survival was the level of serum 25D. The prognostic role of 25D was also demonstrated in another study where its serum levels were evaluated in newly diagnosed patients with AML who were intensively treated with conventional chemotherapy [Lee et al., 2014]. This study demonstrated that insufficient/deficient 25D levels were associated with worse relapse-free survival compared with normal 25D levels. Another study evaluated a 4-year maintenance therapy with low-dose chemotherapy combined with 1,25D and 13-*cis* retinoic acid in poor-prognosis elderly patients with AML and MDS who were ineligible for allografts [Ferrero et al., 2014]. This treatment resulted in a lower relapse incidence and a longer disease-free survival compared to the control patients who did not receive maintenance treatment. A 5-year overall survival of the treated patients was also prolonged in the control group. The results suggest that this strategy of combination maintenance therapy may improve the outcome of poor-risk AML and MDS patients.

Anti-myeloid leukemia effects of 1,25D/VDDs in combination with cytotoxic and cytostatic drugs. A number of preclinical studies have shown that VDDs can potentiate cytotoxicity of chemotherapeutic agents, such as 1-β-D-arabinofuranosyl cytosine (Ara-C) in AML cells [Studzinski et al., 1991]. These VDDs cooperated with several cytotoxic agents, such as doxorubicin, cisplatin, imatinib and docetaxel in growth arrest of different cancer cell types [Pelczynska et al., 2006; Wietrzyk et al., 2007; Switalska et al., 2012]. Both 1,25D [Rogers et al., 2014] and paricalcitol [Kumagai et al., 2005] potentiated the cytotoxic effect of arsenic trioxide on AML cells. Inhibition of mTORC1 by the rapamycin analog everolimus potentiated the growth-inhibitory and differentiation-inducing effects of 1,25D in AML cells both in vitro and in vivo [Yang et al., 2010]. In another study, 1,25D enhanced the apoptotic activity of MDM2 antagonist nutlin-3a in AML cells expressing wild-type p53 [Thompson et al., 2010]. Several studies have indicated the ability of 1,25D to cooperate in the induction of differentiation and growth arrest with epigenetically active drugs, such as the demethylating agents 5-aza-2′-deoxycytidine [Niitsu et al., 2001] and decitabine [Koschmieder et al., 2007] and the histone deacetylase (HDAC) inhibitor sodium butyrate [Hoessly et al., 1989]. These data suggest that the transcriptional activity of VDR may be epigenetically suppressed in leukemic cells, thus providing the mechanistic basis for potential epigenetic leukemia therapy involving VDDs.

VITAMIN D AND ALL

Acute lymphoblastic leukemias (ALLs) are the most common form of pediatric malignancy with bimodal peak incidence between 2–5 years and after 50 years. B cell ALLs (B-ALL) involve arrested development at the very early common lymphoid progenitor stage, before commitment to the B cell lineage. Thus, no B cell progenitors or B cell specific gene expression is observed. T cell ALLs (T-ALL) involves arrested differentiation of thymocytes. While a large body of work supports beneficial effects of VDD-dependent differentiation in treatment of AMLs, very little is known about the role of 1,25D in regulating lymphopoiesis and ALLs. This may be ascribed to a study involving in vitro bone marrow cultures under conditions of myelopoiesis or B lymphopoiesis, which identified specific role of 1,25D in regulating myelopoiesis, but not B lymphopoiesis [Dorshkind et al., 1989]. In addition, Antony et al. [2012] showed that 1,25D had no effect on lymphoblastic leukemia cell proliferation, and actually had a modest effect of impairing dexamethasone cytotoxicity and induction of apoptosis. On the contrary, Consolini et al. [2001] reported significant inhibition of the growth of normal and malignant lymphoid progenitors by 1,25D, despite lack of detectable VDR expression on leukemic blast cells, suggesting a leukemia cell-extrinsic function of 1,25D in regulating leukemogenesis. Considering the tightly controlled yet multifactorial variability in these in vitro experiments, further detailed investigations into leukemia cell-

intrinsic and -extrinsic effects of VDDs in the physiologically relevant in vivo setting of human malignancies and animal models are strongly warranted.

Even in the case of differentiation therapy, there are potentially promising newer avenues of research that will benefit from systematic studies in the future. Studies by Chen et al. [2007] demonstrate an inhibitory effect of 1,25D on antibody production, plasma cell differentiation and memory B cell development, suggesting that it is possible to manipulate the B cell differentiation state through 1,25D signal control. Differentiation of mature peripheral B cells into plasma cells or memory cells is tightly coordinated by a set of well-defined transcription factors, such as Blimp-1, XBP-1, IRF-4, which drive plasma cell differentiation, and Pax5 and MITF, which define B cell lineage specific gene expression and are also associated with memory B cell state [Kalia et al., 2006]. Pax5, the B cell lineage transcription factor is commonly mutated in B-ALL [Mullighan et al., 2007], and its ectopic expression in mature peripheral B cells leads to de-differentiation into uncommitted progenitors and the rescue of T cell development in the thymus [Cobaleda et al., 2007]. There is increasing evidence demonstrating 1,25D signaling cross-talk with a variety of transcription factors, such as Bcl-6 [Nurminen et al., 2014], HIF-1α [Nolan et al., 2015], C/EBPα [Dhawan et al., 2014], STATs [Lange et al., 2014], and RUNX2 [Stephens and Morrison, 2014]. Therefore, we expect that the growing systems biology approach towards delineating 1,25D-dependent gene regulatory networks [Carlberg, 2014] will identify novel molecular targets for manipulating lymphoid differentiation during leukemia which will propel the development of a full immune repertoire in 1,25D-based differentiation therapy.

VITAMIN D ROLE IN IMMUNE MODULATION

In addition to its effects on hematopoiesis, vitamin D is being increasingly recognized for its post-developmental immune-modulatory effects on functional properties of mature immune cells. While development of mature T cells is largely unaffected in VDR KO mice, in wild type mice VDR expression is increased in activated T cells [Bhalla et al., 1983; Provvedini et al., 1983]. Also, 1,25D inhibits lymphocyte proliferation [Lemire et al., 1984, 1985; Vanham et al., 1989; Chen et al., 2014] and suppresses T helper cell differentiation into inflammatory subsets (such as TH1 and TH17) [Lemire et al., 1995; Joshi et al., 2011; Palmer et al., 2011]. Consistent with this, 1,25D has been shown to reduce the production of IFN-γ, TNF-α, IL-2 and IL-17 by CD4 and CD8 T cells in vitro, and enhance the secretion of immunosuppressive cytokines such as IL-10 and TGF-β [Willheim et al., 1999; Thien et al., 2005; Prabhu Anand et al., 2009]. 1,25D also regulates migration of T cells to specialized niches such as the GI tract [Yu et al., 2008] and the skin by altering the expression of skin-homing receptors [Sigmundsdottir et al., 2007], thereby modulating the tissue microenvironment.

In the periphery, 1,25D also promotes development of CTLA-4- and FOXP3-expressing regulatory T cells [Jeffery et al., 2009] through direct effects on T cells as well as indirect effects on tolerogenic dendritic cells [Griffin et al., 2001]. Collectively, these anti-inflammatory and immunosuppressive activities of vitamin D imply a beneficial role for 1,25D supplementation during allogeneic bone marrow stem cell transplantation, the only, albeit hazardous, curative therapy for AML [Zuckerman and Rowe, 2014]. It is intriguing to speculate that 1,25D supplementation during bone marrow transplantation may induce the development of tolerogenic DCs and immunosuppressive T regulatory cells (Tregs). These may act synergistically with the anti-inflammatory and anti-proliferative effects of 1,25D on effector T cells to curb graft-versus-host disease (GVHD), a common complication of allogeneic stem cell transplantation. While somewhat simplistic associations between low vitamin D body status and GVHD have been made [Benrashid et al., 2012; van der Meij et al., 2013] more direct, systematic investigations in murine and monkey models are warranted to clarify potential clinical benefits.

Several in vitro and clinical studies implicate a critical function of 1,25D in reducing inflammation [Zanetti et al., 2014]. Since inflammation impacts all aspects of tumor biology, including initiation, growth, angiogenesis, and metastasis [Baumgarten and Frasor, 2012], beneficial effects of 1,25D supplementation in cancer control may be mediated through reduction of inflammation. Thus, detailed delineation of inflammatory targets of 1,25D signaling that intersect with leukemia growth and immune control represent an exciting area of future investigation. Additionally, adoptive T cell therapeutic approach holds much promise in the treatment of ALLs [Hochberg et al., 2014]. Indeed, success with ALL regression following CD19 targeted chimeric antigen receptor (CAR) T cell therapy has reinvigorated efforts at molecularly defining the mechanisms by which effector and memory CD8 T cell responses can be precision regulated [Jensen and Riddell, 2014]. With recent studies identifying critical requirement of 1,25D signals in promoting the development and survival of potent antigen-specific effector and memory CD8 T cells [Yuzefpolskiy et al., 2014], it is enticing to predict that vitamin D supplementation may provide dual benefits of mediating direct anti-proliferative effects on cancer cells, while also of enhancing anti-cancer immunity at the same time. Closer evaluations of 1,25D manipulation in controlling the functional properties of anti-cancer CD8 T cells in the future are supported by the reported increase in intratumoral activated CD8 T cells following 1,25D supplementation in a mouse model of Lewis lung carcinoma [Young et al., 1993] and patients with head and neck squamous cell carcinoma [Walsh et al., 2010; Starska et al., 2011a,b].

OUTLOOK

Our knowledge of the health aspects of vitamin D actions has been growing exponentially in the past 30 years or so. Having been regarded as an important guardian of calcium homeostasis and bone health, it was gradually realized that the actions of vitamin D in the mammalian body also include the control of important components of cell proliferation and differentiation, inflammation and immunity. Clearly, an understanding of these controls is likely to have important consequences for cancer prevention, and potentially for treatment of cancer and immune-based diseases. Most recently, the effects of vitamin D on metabolism and cardiovascular system are coming under scientific scrutiny. Thus, in a true sense, the physiological form

of vitamin D can be regarded as a molecule with holistic properties. Unfortunately, according to many in the field, the medical establishment has been slow to accept this, and for years recommendations for adequate daily requirements for vitamin D have been principally based on its bone health value. This "Prospect" is focused on hematopoietic neoplasms, and it is argued that the future in differentiation-based therapy of leukemia is most likely to lie in the use of rational combinations with compounds that enhance its actions. This will depend on the still developing basic knowledge underlying the clinical manifestations of vitamin D insufficiency, and the recently emerging successes in clinical trials may encourage society to increase the resources for research in this promising field of translational studies.

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