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Vitamin D and differentiation in cancer

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Abstract

This paper reviews the current understanding of the vitamin D-induced differentiation of neoplastic cells, which results in the generation of cells that acquire near-normal, mature phenotype. Examples of the criteria by which differentiation is recognized in each cell type are provided, and only those effects of 1α,25-dihydroxyvitamin D_3 (1,25D) on cell proliferation and survival that are associated with the differentiation process are emphasized. The existing knowledge, often fragmentary, of the signaling pathways that lead to vitamin D-induced differentiation of colon, breast, prostate, squamous cell carcinoma, osteosarcoma, and myeloid leukemia cancer cells is outlined. The important distinctions between the different mechanisms of 1,25D-induced differentiation that are cell-type and cell-context specific are pointed out where known. There is a considerable body of evidence that the principal human cancer cells can be suitable candidates for chemoprevention or differentiation therapy with vitamin D. However, further studies are needed to fully understand the underlying mechanisms in order to improve the therapeutic approaches.

Keywords

Differentiation; myeloid leukemia; signaling pathways; solid tumors; vitamin D

Introduction

In general, differentiation is a term that signifies the structural and functional changes that lead to maturation of cells during development of various lineages. Cancer cells are unable, to varying degrees, to achieve such maturation, and thus malignant neoplastic cells show a lack of, or only partial evidence of, differentiation, known as anaplasia. Since the basic underlying cause for the failure to differentiate can be attributed to structural changes in the cell's DNA, *i.e.* mutations, which are essentially irreversible, it is remarkable that some compounds can induce several types of malignant cells to undergo differentiation toward the more mature phenotypes. The physiological form of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25D), is one

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such compound, and the importance of this finding is that it offers the potential to be an alternative to, or to provide an adjunctive intervention to, the therapy, as well as to act in the prevention of neoplastic diseases.

The feasibility of differentiation therapy of cancer is supported by the early observations that some cases of neuroblastoma, a childhood malignancy, can spontaneously differentiate into tumors that are composed of normal-appearing neuronal cells, and the child's life is spared (1,2). The reasons for this conversion have not been elucidated, but it seems reasonable to assume that, as the child matures, the endocrine and immune systems become more efficient, and one or more of such factors are able to induce differentiation of neural precursor cells to the more mature, non-invasive forms.

An example of an already successful interventional approach to differentiation therapy of a neoplastic disease is the use of all-trans retinoic acid (ATRA) for the treatment of acute promyelocytic leukemia (APL) and perhaps other leukemias (3–5). Additionally, a synthetic analog of ATRA, Fenretinide, can potentially serve as an agent that can prevent breast cancer in women(6), illustrating the fact that a demonstration of a clear clinical therapeutic effect of a differentiation agent opens up the possibility that it may also serve as a cancer chemopreventive compound.

While the role of 1,25D in cancer chemotherapy and cancer chemoprevention is only beginning to be established, there are several reasons to believe that its promise will be fulfilled. These reasons include the fact that 1,25D is a naturally occurring physiological substance and thus unlikely to cause the adverse reactions that occur when xenobiotics are administered to patients, unless it is given in very high concentrations. Second, the issue of hypercalcemia, which occurs when the concentrations of 1,25D greatly exceed the physiological range and has previously limited its clinical applications (7,8), can be addressed by the dual strategy of developing analogs of 1,25D with reduced calcium-mobilizing properties (9–12),and combining these with other compounds that enhance the differentiation-inducing actions of 1,25D or its analogs (13–15). Also, progress is being made in understanding the mechanisms responsible for 1,25D-induced differentiation, summarized later in this review, and although this understanding is by no means complete, it is likely that insights will be obtained that can be translated into clinical applications.

Differentiation of neoplastic cells induced by 1,25D and other agents rarely, if ever, results in the generation of completely normal, functioning cells. Indeed, the appearance of cells resulting from induced progenitors has been aptly described as resembling "caricatures" rather than normal cells. Such cells may exhibit, and are recognized by, some features of the normal, mature cells of the particular developmental lineage but seldom function like the mature normal cells. However, this is not the major objective of differentiation therapy of neoplastic diseases; the real benefits are due to the cessation of the proliferation of these cells, which is a consequence of cell cycle arrest associated with differentiation(16–19) and in some cases to the reduced survival of the differentiated cells. For instance, 1,25D-induced monocytic differentiation of myeloid leukemia cells can result in the G1 phase cell cycle block, resulting in cessation of cell proliferation(19), while 1,25D treatment of breast or prostate cancer cells can induce cell death by apoptosis as well as by differentiation(20–22).

An important consideration in the area of 1,25D-induced differentiation is cell type and cell context specificity. For instance, in contrast to breast and prostate cancer cells, which are induced to undergo apoptosis, in myeloid leukemia cells, 1,25D-induced differentiation is accompanied by increased cell survival(23,24). The pathways that are known to signal 1,25D-induced differentiation and the associated cell cycle and survival effects also differ, though they may overlap, in different cell types. This may be complicated further by the type of

mutations that are responsible for the block of differentiation and the resulting uncontrolled proliferation of the neoplastic cells. We therefore discuss separately the principal cancer cell types known to be candidates for differentiation therapy or chemoprevention by 1,25D.

Solid tumors

Colon cancer

It is well established that colon cancer cells in culture can undergo differentiation to a more mature phenotype, and the inducing agents include the short-chain fatty acid butyrate and 1,25D. The evidence for differentiation has traditionally been the expression of the hydrolytic enzyme alkaline phosphatase (Alk Pase), which can be demonstrated on the microvilli and tubulovacuolar system of the surface "principal cells" of the colon mucosa(25,26) but is poorly expressed in proliferating colon cancer cells(27). More recently, other markers of colonic epithelial cell differentiation have been identified, and these include changes in "transepithelial electrical resistance" and ubiquitin, as based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS). The latter procedure generates specific mass spectral fingerprints characteristic of cell differentiation, and it was suggested that ubiquitin can be a marker of differentiation of the T84 human colon carcinoma cell line(28). In another colon cancer cell line, SW80, 1,25D was shown to induce easily recognizable morphological changes indicative of differentiated epithelial-like phenotype(29). These morphological changes include consequences of the adherence to the culture substratum, which make the cells look flat and polygonal, and it was demonstrated that these cells have reduced tumorigenicity when implanted into athymic mice. Thus, the epidemiological data that indicate that 1,25D has a negative effect on the incidence of human colorectal cancer(30,31) are well supported by the *in vitro* studies of 1,25D-induced differentiation of colon carcinoma cell lines.

How 1,25D signals differentiation of colon cancer cells is not entirely clear, but several groups of key molecules have been identified that appear to govern this process, and an outline of their postulated interactions is integrated in Figure 1. One mechanism that can explain the reduced cell proliferation that accompanies differentiation is the marked inhibitory effect of 1,25D on the expression of epidermal growth factor receptor (EGFR), apparent at both mRNA and protein levels in CaCo-2 cells(32). The accumulated data also suggest that the central role in 1,25D-induced differentiation is played by the vitamin D receptor (VDR). An early study demonstrated that 1,25D has a protective effect on chemically induced rat colon carcinogenesis (33), and others showed that VDR can be a marker for colon cancer cell differentiation(34, 35). This was followed up by Cross and colleagues in a series of experiments that showed that VDR levels increased in early stages of carcinogenesis, or in human colonic mucosa during early tumor development, but that VDR levels were low in poorly differentiated late-stage carcinomas(36,37). This suggested that VDR levels have a restraining effect on the growth of colon cells. A mechanism that can explain the increased levels of VDR in differentiated colon cells was provided by the indication that, in CaCo-2 cells, 1,25D causes an increased activity of the AP-1 transcription factor(27), which is downstream from the mitogen-activated protein kinases (MAPK) pathways and can transactivate VDR gene expression(38). The consequent up-regulation of VDR may further be increased in the presence of 1,25D by stabilization of the VDR protein(39), but the nature of the initial activation of MAPK pathways in colon cancer cells is not entirely clear. The suggested calcium-induced activation of protein kinase C alpha (PKC α) as an upstream event in MAPK activation (27,40) appears to be feasible, as an influx of calcium into the cells is known to occur after 1,25D exposure of many types of cells including colon cancer(41), but this pathway remains to be further investigated. Nonetheless, the importance of VDR in colon cancer cell differentiation is further underscored by the suggestion that butyrate-induced differentiation of CaCo-2 cells is mediated by VDR(42) and by the recent report that decreased recruitment of VDR to the vitamin D response elements (VDRE)

contributes to the reduced transcriptional responsiveness of proliferating CaCo-2 cells to 1,25D (43).

An emerging role for VDR, other than its function as a transcription factor that binds to VDRE in the promoter regions of 1,25D-responsive genes, is exemplified by the finding that VDR can interact with β-catenin and thereby repress its oncogenic gene-regulatory activity in colon cells(29). The transrepression of β-catenin signaling is not limited to an interaction with VDR, as such interactions can take place with other nuclear receptors, such as the retinoic acid receptor (RAR) and the androgen receptor(29,44). This interaction has been shown to involve also the co-activator p300, a histone acetyl transferase(45). The recently reported repression of the VDR gene by the transcription factor SNAIL(46) and the repression by 1,25D of the Wingless-related MMTV integration site (Wnt) antagonist DICKOPF-4(47) may also be important for the inhibition of Wnt/ β-catenin signaling by 1,25D and for its induction of differentiation in colon cancer cells.

Signaling by β-catenin can also be repressed by the 1,25D-induced up-regulation of the expression of E-cadherin(29), a transmembrane protein that plays a major role in the maintenance of the adhesive and polarized phenotype of epithelial cells(48). The presence of E-cadherin can promote nuclear export of β-catenin, and this may be augmented by direct VDR/β-catenin interaction(48). Since β-catenin/T-cell transcription factor 4 (TCF-4) complex is the nuclear effector of the Wnt growth-signaling pathway, responsible for the expression of c-myc and other growth promoting genes(49), the repressive effects of 1,25D on the growth of colon cancer cells may be explained by the ability of 1,25D to regulate the expression of VDR, E-cadherin and the activity of the β-catenin/TCF pathway, as illustrated in Figure 1.

In addition to protein-protein complex formation with β-catenin, VDR has also been reported to interact with the transcription factor-specificity protein 1 (Sp1) in SW 620 human cancer cells and thus to induce the expression of p27/Kip1 inhibitor of the cell cycle(50). However, it is not clear precisely how this is achieved given the ubiquitous nature of Sp1 binding sites in gene promoters. Nonetheless, the direct binding of VDR to other proteins, which may be ligand independent, is an area that deserves further study and has been reported to occur in cells types other than colon carcinoma, such as osteoblastic cells and myeloid leukemia, as discussed later.

Breast cancer

The induction of differentiation of breast cancer cell lines by 1,25D and the role of 1,25D in normal development of rodent mammary tissue are well established. For instance, studies of VDR knock-out mice have shown that 1,25D participates in the growth inhibition of the normal mammary gland(51). Further, the disruption of 1,25D/VDR signaling leads to distorted morphology of murine mammary gland with duct abnormalities and increased numbers of preneoplastic lesions, suggesting that 1,25D-liganded VDR serves to maintain differentiation of normal mammary epithelium(52).

Induction of differentiation of breast cancer cells by 1,25D can be demonstrated by β-casein production(53) or by a change in overall cell size and shape, associated with changed cytoarchitecture of actin filaments and microtubules in MDA-MB-453 cells (54). Treatment of these cells with 1,25D resulted in accumulation of integrins, paxillin, and focal adhesion kinase as well as their phosphorylation. In contrast, the mesenchymal marker N-cadherin and the myoepithelial marker P-cadherin were down-regulated, suggesting that 1,25D reverses the myoepithelial features associated with the aggressive forms of human breast cancer. However, it is to be noted that not all breast cancer cell lines respond to 1,25D. In many cases this can be attributed to the lack of or low VDR expression or function(55,56), but it may also be due

to alterations in 1,25D-metabolizing enzymes, which can reduce the levels of 1,25D below its effective concentration(57).

Among the breast cancer cell lines that do respond to 1,25D, a range of phenotype alterations has been reported(58), emphasizing that the mechanistic basis for the differentiating effects of 1,25D in the breast cancer cell system will be very complex. Together with the uncertainty over whether induced differentiation of breast cancer cells, *per se*, has potential clinical significance, mechanistic studies in this system have been largely directed to the antiproliferative effects of 1,25D on breast cancer cells. These studies revealed that induction of apoptosis and G1 cell cycle arrest result in inhibition of tumor cell growth in several types of breast cancer cells(20,57,59), but the relationship of these biological effects to differentiation is not obvious. Nonetheless, some hints did result from those studies, as detailed below.

An interesting set of candidate 1,25D-target proteins was identified by proteomic screening of a breast cancer cell line sensitive to 1,25D (MCF-7) and from a subclone of these cells derived by resistance to 1,25D (MCF-7/DRES)(60); and some of these proteins can be related to differentiation and associated phenotypic cellular changes. Examples are Rho-GDI and Rock-DI, known to participate in the formation of focal adhesions and stress fibers, which contribute to the adhesive epithelial phenotype and changes in cell shape(60). Proteins previously linked to pathways involved in 1,25D-induced differentiation, such as phospho-p38, MEK2, and RAS-GAP, were also noted in this screen(52). In a tissue culture study, the JNK pathway, also known to contribute to 1,25D-induced differentiation of colon and myeloid cells(61), was shown to cooperate with the p38 pathway to transactivate VDR in breast cancer cells, but it was proposed that this contributes to the anti-proliferative rather than the differentiation-inducing effects of 1,25D in these cells(38). The antiproliferative effects of 1,25D can also be explained by the reduction in EGFR mRNA and protein, but this is seen in only some breast cancer cell lines(62,63).

Another suggested link to differentiation in 1,25D-treated breast cancer cells is that VDR and estrogen receptor (ER) pathways converge to regulate BRCA-1, thus controlling the balance between signaling of differentiation and proliferation(64). Since ER is important for mammary gland differentiation, studies that pursue this concept would be very valuable, and it already appears that the over-expression of ER and VDR is not sufficient to make ER-negative breast cancer cells responsive to 1α,hydroxy-vitamin D_5, a vitamin D analog known to mediate differentiation in a manner similar to that of 1,25D(65,66).

Prostate cancer

Similar to breast cancer, prostate cancer originates in hormone-dependent epithelial cells, and, as in breast cancer cell lines, 1,25D has anti-proliferative effects in some, but not all, established prostate cancer cell lines. The anti-proliferative action of 1,25D is, to a variable degree, due to the induction of cell death by apoptosis(67) and to cell cycle arrest(68), but to what extent these are associated with differentiation is uncertain.

The evidence of prostate cancer cell differentiation includes the release of prostate specific antigen (PSA) from cells treated with a differentiating agent, such as 1,25D(69–71). This can be useful in cultured cells, but, in patients, the increasing PSA levels suggest progressive disease, making it difficult to acquire data on the role of differentiation in clinical trials(72). A study of the role of 1,25D in the differentiation of the normal rat prostate gland was based on morphological characteristics, which included an increased abundance of cytoplasmic secretory vesicles(73). This characteristic has been used as a differentiation marker, along with the expression of keratins 8, 17, and 18, in human prostate cancer PC-3 cells(74). In other studies(75,76), the increased expression of E-cadherin was used as a marker of differentiation. However, although many reports on the effects of 1,25D on prostate cancer cells include the

word “differentiation,” the documentation most often focuses on the anti-proliferative effects of 1,25D exposure, which may or may not be associated with phenotypic differentiation.

In a recent microarray analysis of 1,25D regulation of gene expression in LNCaP cells, Krishman *et al.*(77) reported several findings that appear relevant to 1,25D-induced differentiation. In addition to the major up-regulation of the expression of the insulin-like growth factor binding protein-3 (IGFBP-3), which functions to inhibit cell proliferation by up-regulating p21/Cip1(78), it was noted that among about a dozen genes up-regulated by 1,25D was the “prostate differentiation factor,” a member of the bone morphogenetic protein (BMP) family, which is generally involved in growth and differentiation of both embryonic and adult tissues(79). Also interesting was the finding that, in these cells, 1,25D regulates those genes that are androgen-responsive as well as the genes that encode the enzymes involved in androgen catabolism.

Furthermore, it has been shown that 1,25D up-regulates the expression and activity of the androgen receptor (AR)(80,81), raising the possibility that the differentiation effects of 1,25D on prostate cells are not direct but due to modifications in the level or activity of AR. Interestingly, it has also been suggested that androgens up-regulate the expression of VDR (82); thus, a positive feedback loop that includes 1,25D activation of VDR could be a factor in inducing differentiation of cancer cells derived from the hormonally regulated tissues (Figure 2), while, in normal cells, the sex hormone (androgen or estrogen) is sufficient to promote differentiation. Since 1,25D has an established anti-cancer activity in prostate cells, it can be assumed that, in this scenario, VDR selectively enhances the AR-mediated androgenic pro-differentiation but not the proliferation-enhancing activity (Figure 2). In addition, it is likely that nuclear receptors for retinoids, glucocorticoids, and PPAR affect the signaling pathways, directly or indirectly. Whether the demonstrated 1,25D-induced decrease in the expression of COX-2 and increase in 15-PGDH in prostate cancer cells(77,83) has any relationship to cell differentiation remains to be established.

Prostate cancer cells are also known to undergo “trans-differentiation” toaneuroendocrinephenotype, and when this phenotype is found in human tumors, it may indicate an aggressive form of the disease(84). Although, currently, 1,25D has no known role in this form of differentiation, this may be a promising area of future research, since recent studies point to a key role for NFκB, as well as IL-6, in this process(85,86). This suggestion is based on the finding that, in some cells, 1,25D up-regulates the expression of C/EBP β(87), which cooperates with NFκB in regulation of the secretion of the cytokine IL-6 in neuroendocrine human prostate cancer cells(85).

Keratinocytes and Squamous cell carcinoma cells

While there is extensive evidence of 1,25D-induced differentiation in normal keratinocytes, the studies of the induction of differentiation in squamous cell carcinomas (SCC), composed essentially of neoplastic keratinocytes, are less conclusive. Differentiation can be detected by the presence of various components of the keratinizing cells, such as cytokeratins K1 and K10, cornifin-beta, involucrin, and transglutaminase, considered to be a late marker of squamous cell differentiation to normal epidermal keratinocytes(88). The expression of target genes of 1,25D and analogs can also be taken as evidence that SCC cell lines can be driven to differentiation by these compounds(89). Such genes include N-cadherin, which, when over-expressed, restores the epithelial phenotype also in prostate cancer cells(90), cystatin M, protease M, type XIII collagen, and desmoglein 3(89). Bikle and colleagues have presented persuasive models for induction of keratinocyte differentiation by increased calcium levels and by calcium-1,25D interactions(91,92). The key features of calcium-induced human keratinocyte differentiation appear to include the recruitment of phosphatidylinositol 3-kinase (PI3K) to a complex at the cell plasma membrane consisting of E-cadherin, β-catenin, and

p120-catenin. This complex is postulated to activate PI3K, leading to the accumulation of phosphatidylinositol 3,4,5-triphosphate (PIP_3), which binds to and activates phospholipase C gamma-1 (PLC-γ1)(93,94). The activated phospholipase generates inositol triphosphate (IP_3), which stimulates the release of calcium from the intracellular stores in the endoplasmic reticulum, and diacylglycerol, which, together with increased intracellular calcium, activates PKC. PKC, and perhaps calcium activation of other enzymes, then initiate signaling cascades that impinge on nuclear transcription factors such as AP-1, which lead to differentiation (95)

How much of this description applies to the 1,25D-induced differentiation is less clear, but Bikle *et al.*(91) presented a plausible model in which 1,25D interacts with calcium to induce keratinocyte differentiation. This model also includes a G-protein-coupled calcium-sensing surface receptor (CaR), which, when activated by 1,25D leads to the activation of PKC, with consequences described above. The associated influx of calcium, which occurs in human keratinocytes after exposure to 1,25D, has been recently shown to be mediated, at least in part, by the calcium-selective channel TRPV6 up-regulated at the mRNA and protein levels by 1,25D(96). A cohesive picture of 1,25D-induced keratinocyte differentiation is quite well, but perhaps not completely, developed. For instance, regulation of AP-1 activity in cultured human keratinocytes by 1,25D was reported to be independent of PKC(97), in contrast to the model presented by Bikle *et al.*(91). Takahashi *et al.*(98) reported that treatment of normal human keratinocytes with 1,25D increases the expression of cystatin A, a cysteine protease inhibitor, that is a component of the cornified envelope, and that it is the suppression of the Raf-1/MEK-1/ERK signaling pathway that is responsible for this effect. However, cystatin A expression is stimulated by the Ras/MEKK-1MKK7/JNK pathway(99), consistent with the schematic model of Bikle *et al.*(91), explaining why PKC activation may not be essential for AP-1 activation in this cell system.

An enigmatic role of caspase-14 in keratinocyte differentiation induced by 1,25D has been reported(100), and it was suggested that the absence of caspase-14 contributes to the psoriatic phenotype. Since caspase-14 is a nonapoptotic protein, it is unclear if this is related to the report that 1,25D protects keratinocytes from apoptosis(101). On the other hand, the identification of Kruppel-like factor 4 (KLF-4) and c-fos as 1,25D-responsive genes in gene expression profiling of 1,25D-treated keratinocytes(102) fits in well with the existing knowledge of differentiation signaling, as c-fos is a component of the AP-1 transcription factor, and KLF-4 is a transcription factor with a major role in cell fate decisions(103–105). Recently, it was reported that yet another transcription factor, PPAR-gamma, also has a major role in 1,25D-induced differentiation of keratinocytes(106). In these studies, dominant negative (dn) PPAR-gamma inhibited the expression of involucrin (a differentiation marker), suppressed AP-1 binding to DNA, and prevented the 1,25D-induced phosphorylation of p38. Thus, the keratinocyte system provided a wealth of interesting information on 1,25D as a differentiation-promoting and survival-regulating agent.

Transformed keratinocytes, which give rise to SCC, tend to be resistant to the differentiation-inducing action of 1,25D(107,108), even though apoptosis and cell cycle arrest induced by 1,25D have been demonstrated in models of SCC(109,110). While VDR expression is required for 1,25D-induced differentiation, the resistance of SCCs to 1,25D is not due to the lack of functional VDR(111). The possible explanations for the 1,25D resistance include the finding that the VDRE in the human PLCγ-1 gene is not functional(111). Another explanation for this resistance is that increased serine phosphorylation of retinoid X receptor alpha (RXRα) by the Ras/MAPK pathway leads to its degradation, and thus VDR loses its heterodimeric partner for gene transactivation(112). Yet another possibility is that VDR co-activators in SCCs are not appropriate for transactivation of differentiation-inducing genes(95). Specifically, it was suggested that the expression of differentiation markers required a complex of VDR with the Src family of co-activators(113), but in SCC the DRIP co-activator complex is over-expressed,

and there is a failure of SCCs to switch from DRIP to Src, resulting in inability to express genes required for differentiation. It would be interesting to learn if this model has a wider applicability.

Osteosarcoma and osteoblasts

Differentiation as well as growth inhibition have been documented in 1,25D-treated human and rat osteosarcoma cells(114,115). The differentiation was recognized by a morphological change to the chondrocyte phenotype and by increased Alk Pase staining. The presence of Alk Pase or osteocalcin could also be detected at the mRNA level(115). In a human fetal osteoblastic cell line responsive to 1,25D, mineralized nodules were detected(116), demonstrating that an advanced degree of differentiation can be achieved in this cell system. Interestingly, 1,25D-induced differentiation in osteoblasts and osteocytes is accompanied by an increase in the potential for cell survival through enhanced anti-apoptotic signaling(117). It is possible that this is mediated by EGFR-relayed signals, as in contrast to other cell types (32,62,118), 1,25D-treated osteoblastic cells show increased levels of EGFR mRNA(119).

Recent studies suggest that the anti-apoptotic effects of 1,25D on osteoblasts and osteocytes are mediated by Src, PI3K, and JNK kinases(117). The suggested mechanisms include an association of Src with VDR, though transcriptional mechanisms were required, as shown by an inhibition of the biological effect by exposure to actinomycin D or cycloheximide. The association of VDR with other proteins may be particularly important in osteoblasts induced to differentiate by 1,25D, as another group reported that IGF-binding protein-5 (IBP-5) interacts with VDR and blocks the RXR/VDR heterodimerization in the nuclei of MG-63 and U2-OS cells, thus attenuating the expression of bone differentiation markers(120). Also, in ROS 17/28 cells the NFκB p65 subunit integrates into the VDR transcription complex and disrupts VDR binding to its co-activator Src-1(121). Although protein-protein binding between VDR and p65 subunit has not been demonstrated, this remains a possibility, further highlighting the importance of this mode of control of VDR activity.

Leukemias

Hematological malignancies are a diverse group of diseases but can be divided into two major groups, lymphocytic and myeloid leukemias. Although normal activated B and T lymphocytes express VDR, and 1,25D has antiproliferative effects on these cell types(122,123), this does not appear to alter their differentiation state, and lymphocytic leukemia cells do not respond to 1,25D. In contrast, 1,25D has been known since 1981 to induce maturation of mouse myeloid leukemia cells(124), and this can also take place in a wide variety of human myeloid leukemia cell lines, with the exception of the lines derived from the most immature acute myeloid leukemia (AML) blast cells (125–127).

Differentiation induced by 1,25D usually results in a monocyte-like phenotype, but prolonged exposure to 1,25D confers cell surface changes that result in adherence to the substratum, making the differentiated cells macrophage-like(124,128). The monocyte characteristics are recognized by changes related to phagocytosis, such as the ability to break down esters, assayed by the “non-specific esterase” (NSE) cytochemical reaction, also known as “monocytespecific esterase” (MSE) since, in hematopoietic cells, this esterase is specific for monocytes and macrophages(129). Also related to phagocytosis is the ability to generate reactive oxygen species (ROS), including superoxide, usually recognized by nitro bluetetrazolium (NBT) orcytochromereduction(130,131). The availability of flow cytometry (FC) for the recognition of surface proteins has made the study of the differentiating effects of 1,25D on myeloid leukemia cells quite simple, using CD14, a receptor for complexes of lipopolysaccarides (LPS) and LPS-binding protein(132), a near-definitive marker of the monocytic phenotype. This is

usually supplemented by the FC determination of CD11b or another subunit of the human neutrophil surface protein that mediates cellular adherence(133).

In contrast to myeloid cells induced to differentiate by the phorbol ester TPA, in 1,25D-treated cells, the ability to adhere develops more slowly than the ability to phagocytose. Consequently, 1,25D treatment results in an earlier appearance of the CD14 antigen, usually accompanied in parallel by MSE positivity, than the appearance of CD11b and NBT positivity(134,135). Generally, at least two of the above parameters are measured to demonstrate monocytic differentiation, and FC methods require the use of paired isotypic IgG controls for each test sample to avoid obtaining false-positive data. Exposure of AML cells to 1,25D also results in G1 phase cell cycle arrest, which follows, rather than precedes, the phenotypic differentiation (134) and is often taken as the confirmatory evidence that differentiation has taken place. However, in contrast to cells from most solid tumors, monocytic differentiation of AML cells is accompanied by increased expression of anti-apoptotic proteins, and, consequently, 1,25D-treated myeloid cells have an increased cell survival potential(136–140).

The topic of 1,25D-induced leukemia cell differentiation has been extensively studied in many laboratories. These include several groups in Japan(141–145) and a group in Birmingham, England(146,147), who made many valuable contributions to the field. Notably, combined basic and clinical studies of 1,25D-induced leukemia cell differentiation were very comprehensively developed by Koeffler and his various collaborators(148–151). What follows in the remainder of this section is an outline of the signaling mechanisms of AML cells that have occupied the attention of the corresponding author's laboratory.

In these studies, we have focused on HL60 cells, a widely available cell line derived from a patient with promyeloblastic leukemia, with the objective of achieving with the currently available tools as clear a picture as possible of the signaling of monocytic differentiation. In this model, outlined in Figure 3(A and B), a plausible sequence of events is presented, but it is likely that other pathways are also operative but remain to be convincingly demonstrated. The details of the scheme are described below.

Signaling of monocytic differentiation by MAPK and parallel pathways

Early in our investigations, we recognized that 1,25D-induced monocytic differentiation is not a single continuous process but a series of events that can be divided into at least two overlapping phases. In the first phase, which lasts 24–48 h, the cells continue in the normal cell cycle while expressing markers of monocytic phenotype, such as CD14 and NSE. In the next phase, the G1 to S phase cell cycle block becomes apparent, and the expression of CD11b is also prominent, indicating a beginning of the transition to the macrophage phenotype. The first phase is characterized by high levels of ERKs activated by phosphorylation, and these levels decrease as the cells enter the second phase, while the levels of the cell cycle inhibitor p27KIp1 increase at that time. Serum-starved HL60 cells or cells treated with the MAPK inhibitor PD 98059 have a reduced growth rate and a slower rate of differentiation, but the G1 block under these conditions also coincides with decreased levels of activated ERK1/2(152). Our data suggested that the MEK/ERK pathway maintains cell proliferation during the early stages of differentiation and that the consequent G1 block leads to "terminal" differentiation. Using a different experimental design, similar results were obtained by Marcinkowska (153).

We also demonstrated that the JNK pathway, as shown by the increased phosphorylation of c-jun, plays a role in the induction of differentiation of HL60 cells by 1,25D. The data showed that 1,25D-induced differentiation of a stable clone of U937 cells transfected with a dominant negative construct of JNK-1 was reduced, as compared to cells transfected with a control construct(154), and potentiation of 1,25D-induced differentiation by the plant anti-oxidants curcumin and silibinin increased the phosphorylation of c-jun(155). This suggested that the

JNK-jun pathway is involved in 1,25D-induced differentiation, which was further established in experiments that showed that the AP-1 transcription factor complex is required for this process since c-jun, together with ATF-2, is the principal component of this complex (140). This appears to be of wider significance, as c-jun expression was also reported to enhance macrophage differentiation of U937 cells(156).

However, it seems clear that the ERK and JNK MAPK pathways are not the only ones involved in signaling of 1,25D-induced differentiation. For instance, compounds SB203580 and SB2902190, reported to be specific inhibitors of the alpha and beta isoforms of signaling protein p38 MAP kinase(157), were found to markedly accelerate monocytic differentiation of HL60 cells induced by low concentrations of 1,25D(158). Paradoxically, these compounds also induced a sustained enhancement of p38 phosphorylation and of its activity in cell extracts in the absence of added inhibitor, which raised the possibility of a lack of specificity of SB compounds in this cell system or of an up-regulation of the upstream components of the p38 pathway. In addition, SB 203580 or SB 202190 treatment of HL60 cells resulted in prolonged activation of the JNK and ERK MAPK pathways(158). SB203580 treatment of HL60, HT93, and ML-1 human myeloid leukemia cell lines also increased cellular ERK activity(159). These data are consistent with the hypothesis that in HL60 cells an interruption of a negative feedback loop from a p38 target activates a common regulator of multiple MAPK pathways, but it is also possible that SB203580 has an additional unknown action.

Another signaling cascade known to be activated by 1,25D in human AML cells is the PI3K-AKT pathway, which is often envisaged to signal from the cell membrane to the intracellular regulators in parallel with the MAPK pathways (160). Further, monocytic leukemia cells THP-1 exposed to 1,25D in serum-free medium show a rapid and transient increase in PI3K activity, which was attributed to the formation of a VDR-PI3K protein complex (161). However, it is not clear if the lack of growth factors normally provided by the serum contributes to the observed effects. The role of the PI3K pathway in 1,25D induced differentiation was also studied by Marcinkowska and colleagues(162–164), who showed that the activation of PI3K by 1,25D can also be demonstrated in HL60 cells and that the signal is transmitted to AKT. This function of AKT may contribute to the differentiation-related increase in 1,25D-induced cell survival(139). An additional role of the PI3K, as well as of the Ras/Raf/ERK, pathway in human leukemia cells is the stimulation of steroid sulfatase, an enzyme that converts inactive estrogen and androgen precursors to the active sex hormones(147). If this is also operative in breast and/or prostate tissues, it could offer an explanation for the mutual activation of VDR and the estrogen and androgen nuclear receptors, as shown in Figure 2.

The mechanisms of the up-regulation of MAPK pathways in the initial phase of 1,25D action on leukemia cells are still unclear. The very rapid effects of 1,25D on the MAPK pathway in intestinal cells that result in rapid calcium transport (“transcaltachia”) have been attributed to a cell membrane receptor (“mVDR”)(165–167), but whether direct, non-genomic action of such mVDR can initiate or enhance the activity of MAPK pathways in leukemia cells has not been well documented. In non-starved leukemia cells, 1,25D elicits less rapid (hours rather than minutes) activation of the MAPKs. One possibility is that this is achieved by the transcriptional up-regulation of Kinase Suppressor of Ras-1 (KSR-1), a membrane-associated kinase/molecular scaffold also known as ceramide-activated protein kinase (168,169). Although a kinase activity associated with KSR-1 has been reported(170–172), the best established function of KSR-1 is to provide a platform for Raf-1 kinase to phosphorylate and thus activate its downstream targets in the MAPK pathways(173,174). Thus, since KSR-1 has been shown to have a functional DNA element regulated by VDR (VDRE)(175), the activation of the MAPKs may be a direct “genomic” action of 1,25D, as depicted in Figure 3A, rather than signaling initiation at the membrane and “non-genomic.”

Our studies(169,176), combined with those of Marcinkowska and colleagues(164,177), suggest that leukemia cell differentiation is initiated when 1,25D promotes nuclear translocation of liganded VDR, which dimerizes with RXR and transactivates several VDRE-regulated genes, including KSR-1 and KSR-2. The latter appears to play a role in increasing the survival potential of differentiating monocytic cells(24), while KSR-1 acts as a scaffold that, by simultaneously binding to Ras and Raf-1 (and perhaps to other proteins) facilitates or redirects the signaling cascade, at least initially, to MEK/ERK and thus amplifies the signal that initiates monocytic differentiation (Figure 3A).

Raf-1 participation has been shown to be required for the later stages of differentiation, when an impairment in cell cycle progression becomes apparent, and at this more advanced point in the differentiation process, MEK/ERK signaling does not appear to be involved(178,179). While this requires further study, the current model, also supported by observations in other differentiation signaling systems(180–182), suggests that Raf-1 can signal p90RSK activation independently of MEK and ERK, as outlined in Figure 3B.

A rather speculative mechanism describing how MEK/ERK signaling is diminished in the later stages of differentiation, when cell proliferation becomes arrested, is presented below.

p35/Cdk5, a protein kinase system that may interface differentiation processes with cell cycle arrest

After 24–48 h of exposure of myeloid leukemia cells to moderate concentrations of 1,25D (1–10 nM), cell cycle progression becomes progressively arrested, principally due to a G1 to S phase block, although a G2 phase block can also be observed(183). Several mechanisms could explain these cell cycle effects, including activation of cyclin-dependent kinase 5 (Cdk5).

Cdk5 is a proline-directed serine-threonine kinase with sequence homology to the cyclin-activated kinases that regulate cell cycle progression, but its best-known function is participation in differentiation of neuronal cells(184). When combined with a “cyclin-like” neuronal Cdk5 activator (Nck5a) 35 kDa protein (p35/Nck5a, or p35), the p35/Cdk5 complex functions in monocytic cells and plays an important role in normal, and possibly abnormal, development of this hematopoietic lineage. Our initial observations were that, in HL60 cells treated with 1,25D, the monocytic phenotype and expression of Cdk5 appear in parallel. Both active and inactive Cdk5 were associated with cyclin D1 protein, and the inhibition of Cdk5 expression by an antisense oligonucleotide construct reduced the intensity of 1,25D -induced expression of the monocytic marker CD14(185). This finding demonstrated a novel (other than neuronal) cellular type for Cdk5 activity and a concomitant enhancement of monocytic differentiation.

The above study showed that protein levels and kinase activity of Cdk5 increase in HL60 cells induced to monocytic differentiation by 1,25D, but did not establish the specificity of the association of Cdk5 with the monocytic phenotype. Therefore, we showed in a subsequent study that the up-regulation of Cdk5 does not occur in granulocytic differentiation, whereas an inhibition of Cdk5 activity by the pharmacological inhibitor olomoucine, or of its expression by a plasmid construct expressing antisense Cdk5, switches the 1,25D–induced monocytic phenotype (a combination of the positive NSE reaction, the expression of the CD14 marker, and morphology) to a general myeloid phenotype (a positive NBT reaction, the CD11b marker, and morphology)(186). These findings showed that, in human myeloid cells, the up-regulation of Cdk5 is specifically associated with the monocytic phenotype.

The Nck5a 35 kDa protein has hitherto been considered to be exclusively expressed in neuronal cells, as its name implies(187). However, since we had clear evidence that Cdk5 is an active kinase in human leukemia cells HL60 and U937 induced to differentiate with 1,25D, and since

the "classical" cyclins (*e.g.* cyclin D1, cyclin E) are not known to activate Cdk5, we investigated whether p35 can be detected in cells with active Cdk5. Indeed, we demonstrated that p35 is expressed in normal human monocytes and in leukemic cells induced to differentiate toward the monocytic lineage but not in lymphocytes, or cells induced to granulocytic differentiation by retinoic acid. The activator p35 is present in a complex with Cdk5 that has protein kinase activity, and when ectopically expressed together with Cdk5 in undifferentiated HL60 cells it induces the expression of CD14 and NSE markers of the monocytic phenotype(188). These observations not only indicate a functional relationship between Cdk5 and p35 but also support a role for this complex in monocytic differentiation.

A likely link to the diminution of ERK MAPK pathway activity at the onset of phase 2 of 1,25D-induced differentiation is provided by the EGR-1→ p35/Cdk5 ---| MEK 1/2 pathway, which was elucidated in leukemia cells by this laboratory (189). The schematic representation is shown in Figure 3B, and the supporting data can be summarized as follows.

Control of p35 expression by the EGR-1 transcription factor

The evidence in support of a role for EGR-1 in regulating the expression of p35 includes the co-ordinate expression of EGR-1 along with Cdk5, and the co-inhibition of the 1,25D –induced up-regulation of these proteins by PD 98059, an inhibitor of the MEK/ERK1/2 pathway (171,190). Further, the promoter region of human p35 has an EGR-1 binding site that overlaps with an Sp1 site, and a gel shift assay showed that a double-stranded oligonucleotide that contained this sequence bound proteins in nuclear extracts from 1,25D-treated HL60 cells. The EGR-1-site binding proteins were competed with most efficiently by an anti-EGR-1 antibody, though some competition was also observed with an anti-Sp1 antibody, but no competition was observed with an irrelevant antibody, *e.g.* anti-VDR. The data suggested that EGR-1, and perhaps Sp1 proteins, regulate the expression of p35 and contribute to induction of the monocytic phenotype. A "decoy" EGR-1 response element oligonucleotide inhibited both 1,25D-induced p35 expression and monocytic differentiation (189).

The Cdk5/p35 complex phosphorylates MEK

We also found that the Cdk5/p35 can phosphorylate MEK in cell extracts (189). If this can be demonstrated to occur in leukemia cells, it will provide a potential mechanism for the inhibition of the MAPK/ERK pathway seen in the later stages of differentiation (48 h after the addition of 1,25D to the cultures) since phosphorylation of MEK by p35/Cdk5 inhibits its kinase activity. Intriguingly, up-regulation of p35 (which activates Cdk5) is observed *pari passu* as ERK 1/2 phosphorylation is waning, consistent with a cause-effect relationship. We have thus proposed a mechanism that can shut down cell proliferation, possibly by allowing p27Kip1 to accumulate in the cell nucleus due to a decline in ERK 1/2 activity, since it has been reported that the ERK pathway can increase nuclear export of p27Kip1(191).

C/EBP β transcription factor as an effector of monocytic differentiation

One of the downstream targets of the MAPK-RSK pathway is a nuclear transcription factor, the CAAT and Enhancer Binding Protein β (C/EBP β). This transcription factor has been reported to be activated by phosphorylation both by ERK(192) and by RSK(193) and can interact directly with the promoter of CD14, one of the principal markers of monocytic differentiation(194), as illustrated in Figure 3B. We showed that the expression of C/EBP β is increased by 1,25D in parallel with markers of differentiation; conversely, the knockdown of its expression by antisense oligonucleotides, or of its transcriptional activity by "decoy" promoter competition, inhibited 1,25D-induced differentiation(195). In an additional study, the data suggested that 1,25D induced phosphorylation of C/EBP β isoforms on Thr235, and that the C/EBP β-2 isoform is one of the principal differentiation-related transcription factors in this system(87).

These findings suggest that 1,25D can induce leukemic progenitor cells, which have the potential to differentiate into several hematopoietic lineages, to become non-proliferating monocyte-like cells by changing the ratio of nuclear transcription factors in a manner that permits this form of differentiation(196). In this scenario, the event that initiates leukemic transformation, such as a mutation, alters the proper balance of transcription factor activity necessary for normal granulocytic cell differentiation. However, 1,25D-induced expression of C/EBP β then allows the cells to bypass this block to granulocytic differentiation by becoming monocyte-like cells instead (Figure 4).

Interestingly, 1,25D has also been reported to have a negative effect on differentiation, as it inhibits IL-4/GM-CSF-induced differentiation of human monocytes into dendritic cells, and this contributes to 1,25D immunosuppressive activity(197,198). The data also suggested that 1,25D specifically down-regulates the expression of CSF-1 and promotes spontaneous apoptosis of mature dendritic cells, further demonstrating the pleiotropic effects of 1,25D and the cell-type specificity of the outcomes.

Conclusions

The signaling pathways presented here are shown to control the activity of several transcription factors, such as the ubiquitous AP-1 complex, the nuclear receptor VDR, and the lineage-determining C/EBP family of transcription factors. While these clearly play a role in 1,25D-induced differentiation of HL60 cells, there may be redundancy of important cellular regulators, and other pathways and transcription factors are likely to be involved. The initial steps that activate the differentiation-inducing actions of 1,25D are not entirely clear, and while cell membrane-associated events have a role, these events are not necessarily rapid, but they are sustained. It is likely that micro-RNAs will be found to further control or modulate 1,25D signaling, as retinoic acid-induced differentiation of NB4 AML cells has been shown to be associated with the up-regulation of a number of micro-RNAs, and the down-regulation of micro-RNA 181b(199). Thus, extensive additional investigations are warranted to provide a basis for the design of improved therapies of leukemia and solid tumors.

Abbreviations and Glossary

1,25D	1a,25-dihydroxyvitamin D_3
24OHase	24-hydroxylase
A	androgen
AKT	serine/threonine-specific, protein kinase B
Alk-Pase	alkaline phosphatase
AML	acute myeloid leukemia
AP-1	activating protein-1
APC	adenomatous polyposis coli
APL	acute promyelocytic leukemia
AR	androgen receptor
ATRA	all-trans retinoic acid
BMP	bone morphogenetic protein
BRCA-1	(Breast Cancer-1)-breast cancer tumor suppressor gene
CaCo-2	human epithelial colorectal adenocarcinoma cell line

CaR	calcium-sensing surface receptor
Cdk5	cyclin-dependent kinase 5
C/EBP	CCAAT/enhancer binding protein
CoA	co-activator
COX	Cyclooxygenase
DRIP	Vitamin D Receptor-Interacting Protein
E_2	estrogen
EGFR	epidermal growth factor receptor
EGR-1	early growth response protein-1
EP	early progenitor
ER	estrogen receptor
ERK	extracellular-signal regulated kinase
FC	flow cytometry
GF	growth factor
GFR	growth factor receptor
GM-CSF	granulocyte macrophage-colony stimulating factor
hOC	human osteocalcin
hOP	human osteopontin
IBP-5	IGF binding protein-5
IGF	Insulin-like Growth Factor
IGFBP-3	insulin-like growth factor binding protein-3
IL-4	Interleukin-4
IP3	inositol triphosphate
JNK	Jun N-terminal kinase
KLF-4	Kruppel-like factor 4
KSR-1	kinase suppressor of Ras-1
LPS	lipopolysaccharides
MALDI-TOFMS	matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MAPK	mitogen-activated protein kinase
MG-63	Human osteosarcoma cell line
MSE	monocyte-specific esterase (“non-specific” esterase)
NBT	nitroblue tetrazolium
Nck5a	“cyclin-like” neuronal Cdk5 activator
NFkappaB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NR	nuclear receptor
NSE	non-specific esterase

P	progenitor
PGDH	Prostaglandin Dehydrogenase
p90RSK	ribosomal S6 kinase (MAPK-activated protein kinase-1)
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol 3, 4, 5-triphosphate
PKC	protein kinase C
PLC-g1	phospholipase C gamma-1
PPAR	Peroxisome Proliferator-Activated Receptors
Rb	retinoblastoma protein
PSA	prostate-specific antigen
RAR	retinoic acid receptor
ROS	reactive oxygen species
RXRa	retinoid X receptor alpha
SCC	squamous cell carcinoma
Sp-1	specificity protein-1
Src	Non-Receptor Protein Tyrosine Kinase
TCF4	T-cell transcription factor-4
Wnt	Wingless-related MMTV integration site
TPA	12-O-tetradecanoylphorbol-13-acetate
U2-OS	Human osteosarcoma cell line expressing wild type p53 and Rb, but lacking p16
VDR	vitamin D receptor
VDRE	vitamin D_3 response element

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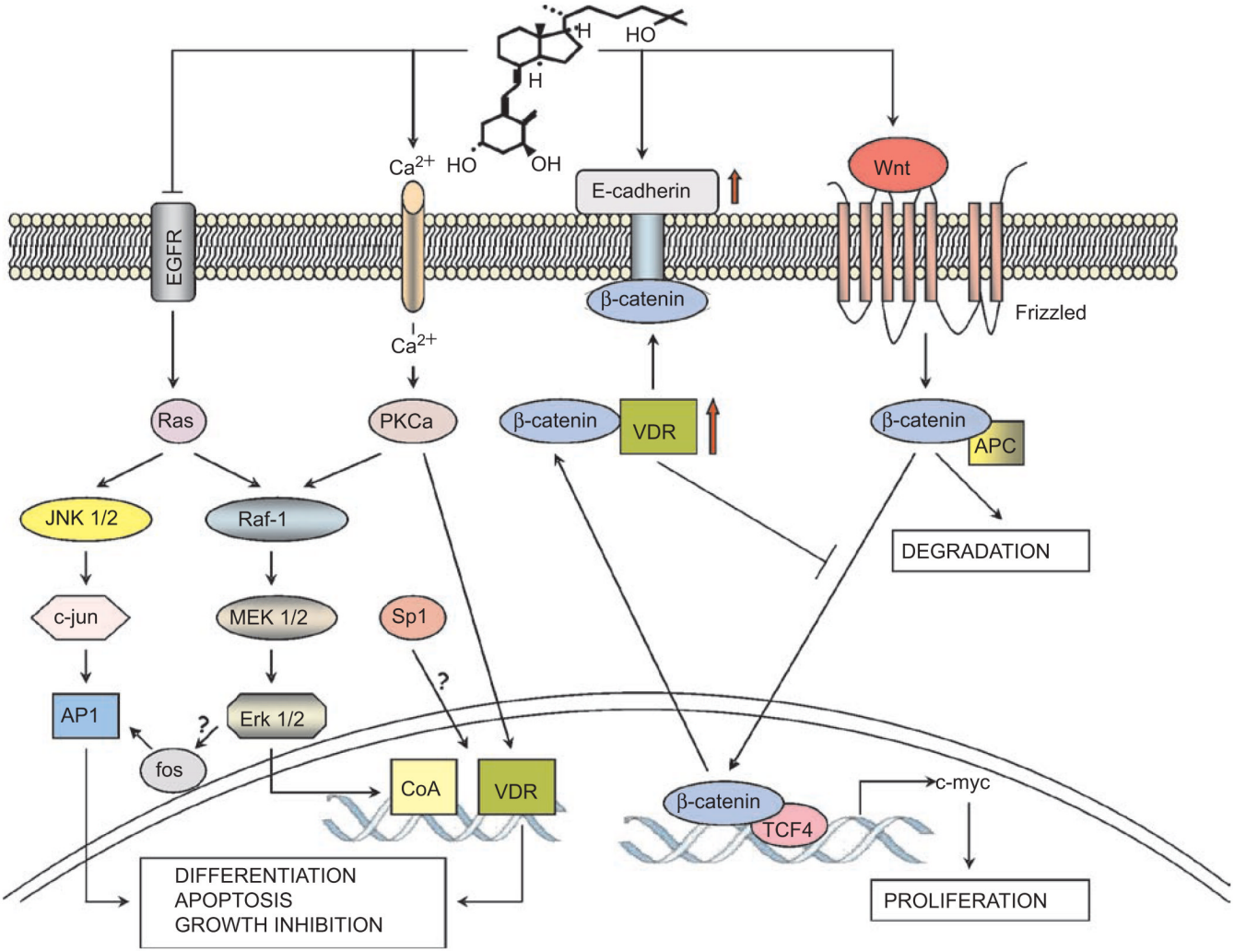

Figure 1.

The suggested pathways of 1,25D-induced differentiation in colon cancer. In proliferating colon epithelial cells the β-catenin complexed with TCF-4 drives the expression of growth promoting genes such as c-myc. This is under the control of Wnt and its surface receptor Frizzled, which inactivate GSK-3β (not shown) and allow the accumulation of β-catenin and thus growth promotion. Binding of β-catenin by VDR, or by other proteins, including E-cadherin, the expression of which is induced by 1,25D (formula shown) leads to the loss of β-catenin from the transcriptional complex in the nucleus, and, as a consequence, to decreased cell proliferation. Also shown is the activation of PKCα by 1,25D-induced influx of calcium (Ca^{2+}), which can activate by phosphorylation the transcriptional activity of VDR and repression of EGFR by 1,25D in colon-derived cells.

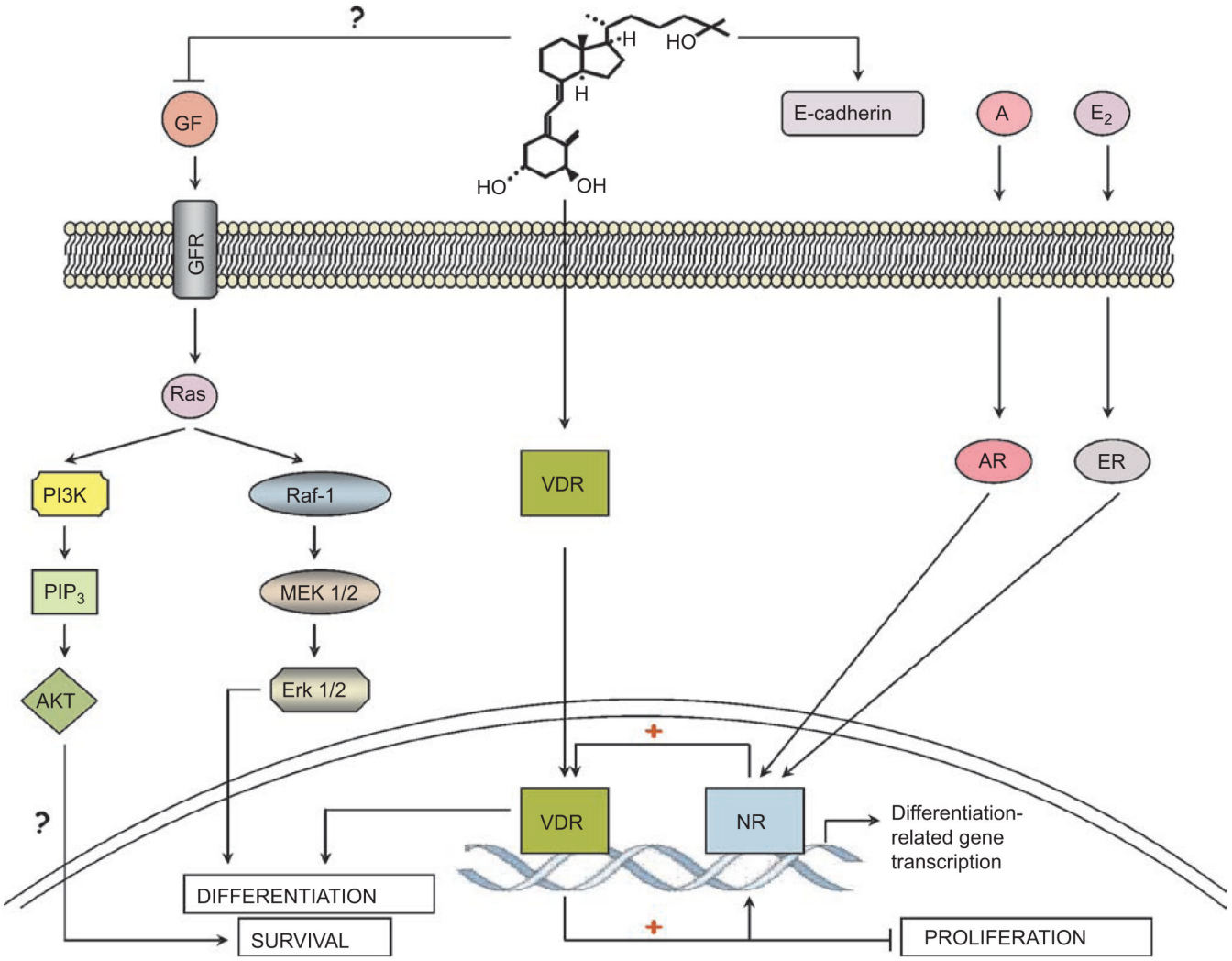

Figure 2.

Signaling of differentiation by 1,25D in hormone-dependent cancer cells. This schematic illustrates the hypothesis that in normal breast or prostate cells, estrogen (E_2) or androgen (A) is sufficient to induce differentiation, respectively. In cancer cells the differentiation signal provided by the hormone-liganded nuclear receptor (NR) may need to be amplified by the cooperation with 1,25D-activated VDR to induce differentiation. Since cells also receive signals from growth factors (GF), several of which activate Ras, the presence of a Ras-activated signaling pathways is exemplified by the AKT and ERK cascades, though the role of these pathways in the differentiation of hormone-dependent cells is uncertain.

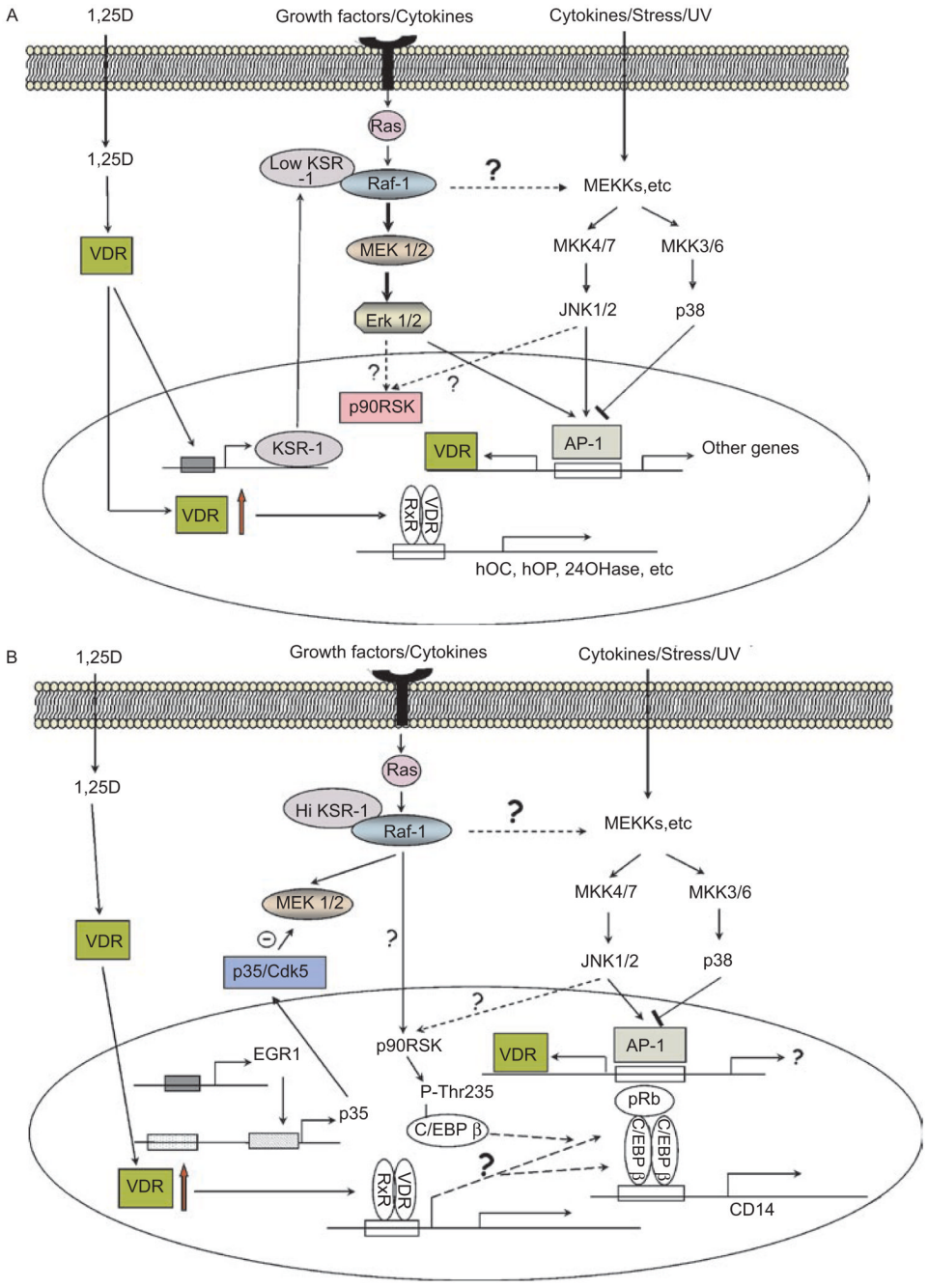

Figure 3.

(A) Suggested signaling of the early stages of 1,25D-induced monocytic differentiation. Binding of 1,25D to VDR stimulates its translocation to the cell nucleus where it heterodimerizes with RXR, and in myeloid precursor cells, it transactivates genes containing VDREs in their promoter regions. These include genes that encode proteins involved in calcium homeostasis and bone integrity, such as osteocalcin (hOC), osteopontin (hOP), and the 1,25D-catabolic enzyme 24-hydroxylase (24OHase). It is postulated that the regulators of signaling pathways, *e.g.* KSR-1, are also up-regulated in myeloid cells and alter Ras signaling from the cell membrane so that signaling by MAPKs (MEKs, ERKs, and JNKs) increases the AP-1 activity. This can have a positive feedback effect on differentiation by increasing VDR

abundance. It is also suggested that a potential negative feedback mechanism is provided by p38 MAPK, as inhibition of its signaling by SB203580 enhances 1,25D-induced monocytic differentiation. (B) Later stages of 1,25D-induced differentiation. This figure illustrates that the transcription factor EGR-1, known to be up-regulated by 1,25D (189), can increase the expression of p35/Nck5a (p35) activator of Cdk5. Cdk5 activated by p35 then can phosphorylate MEK on Thr286, a site that inactivates it (200), as shown by the Θ symbol. This diminishes ERK1/2 activity downstream from MEK (not shown here), but Raf-1 can activate p90RSK directly, which, in turn, activates the transcription factor C/EBP β, perhaps bound to pRb, and increases the expression of CD14, as part of monocytic differentiation. The activation of p90RSK may also be increased by the JNK pathway, which also activates AP-1, and may lead to VDR expression. The interplay between the signaling by 1,25D, growth factor, and stress add to the overall complexity of the induction of the monocytic phenotype.

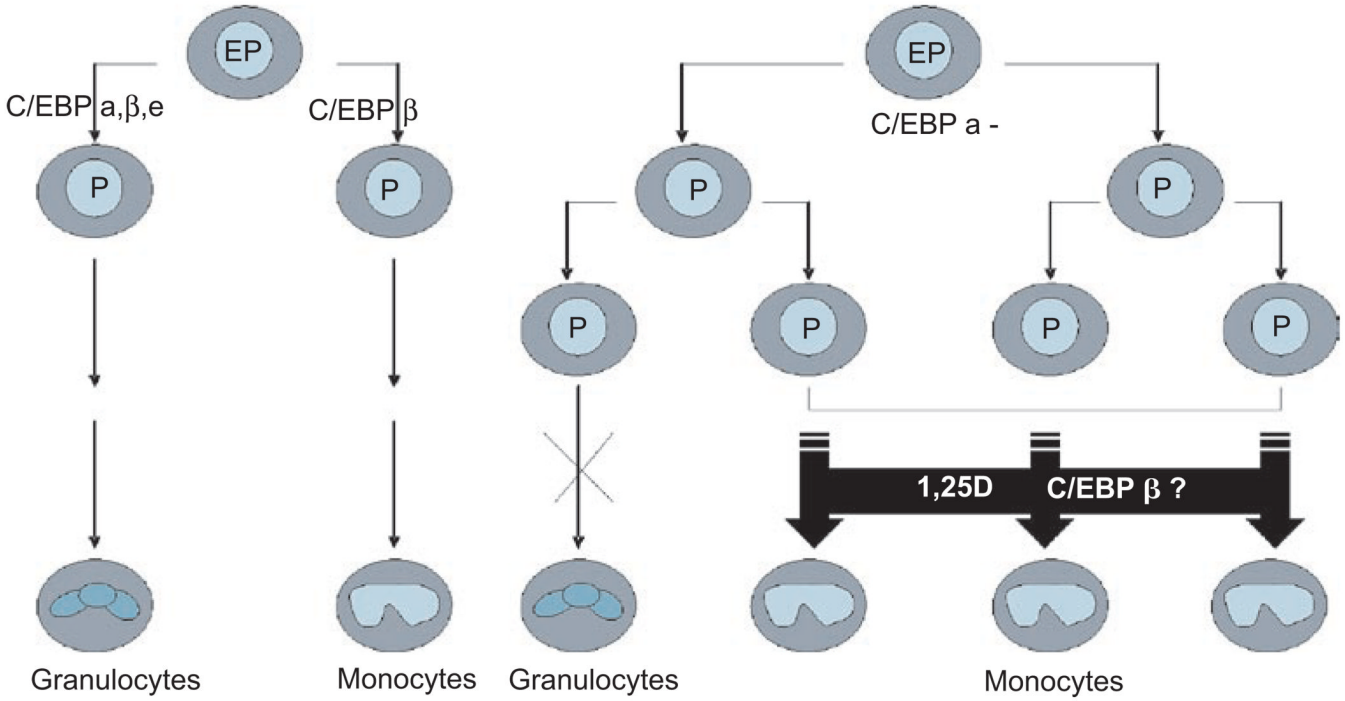

Figure 4.

The suggested role of CAAT/Enhancer Binding Protein β in 1,25D-induced bypass of the differentiation block in leukemia cells. In this scenario, C/EBP α is indispensable for normal granulopoiesis, while C/EBP β regulates monocytic differentiation. When C/EBP α is mutated or inactivated and granulopoiesis is blocked, immature myeloid cells accumulate in the bone marrow and appear in the peripheral blood, resulting in AML. 1,25D-induced expression of C/EBP β may allow the cells to bypass this block to granulocytic differentiation by switching the lineage of cell differentiation from granulocytes to monocytes.