Role of cell cycle regulatory molecules in retinoic acid- and vitamin D3-induced differentiation of acute myeloid leukaemia cells

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Abstract

The important role of cell cycle regulatory molecules in all trans-retinoic acid (ATRA)- and vitamin D3-induced growth inhibition and differentiation induction has been intensively studied in both acute myeloid leukaemia primary cells and a variety of leukaemia cell lines. Cyclin-dependent kinases (CDK)-activating kinase has been demonstrated to interact with retinoic acid receptor (RAR) α in acute promyelocytic leukaemia cells, and inhibition of CDK-activating kinase by ATRA causes hypophosphorylation of PML-RAR α , leading to myeloid differentiation. In many cases, downregulation of CDK activity by ATRA and vitamin D3 is a result of elevated p21- and p27-bound CDKs. Activation of p21 is regulated at the transcriptional level, whereas elevated p27 results from both (indirectly) transcriptional activation and post-translational modifications. CDK inhibitors (CKIs) of the INK family, such as p15, p16 and p18, are mainly involved in inhibition of cell proliferation, whereas CIP/KIP members, such as p21, regulate both growth arrest and induction of differentiation. ATRA and vitamin D3 can also downregulate expression of G1 CDKs, especially CDK2 and CDK6. Inhibition of cyclin E expression has only been observed in ATRA- but not in vitamin D3-treated leukaemic cells. *In vitro*, not only dephosphorylation of pRb but also elevation of total pRb is required for ATRA and vitamin D3 to suppress growth and trigger their differentiation. Finally,

sharp reduction in c-Myc has been observed in several leukaemia cell lines treated with ATRA, which may regulate expression of CDKs and CKIs.

Introduction

Acute myeloid leukaemia (AML) is a malignancy of the myeloid line of blood cells. A characteristic abnormality of AML cells is that they become blocked at an early stage of their development and fail to differentiate into functional mature cells. Acute promyelocytic leukaemia (APL) is a subtype of AML characterized by t(15;17) chromosomal translocation and expression of abnormal PML-RAR α fusion protein. Abnormal PML-RAR α complex blocks leukaemia cell differentiation and causes accumulation of immature cells. Thus, induction of cell differentiation is a major strategy for anti-AML therapy.

Since the 1980s, all trans-retinoic acid (ATRA), a metabolite of vitamin A, and 1,25(OH) $_2$ D $_3$, a derivative of vitamin D3, have been used clinically as an anti-leukaemia therapy. The mechanisms responsible for ATRA- and 1,25(OH) $_2$ D $_3$ -induced differentiations have been studied intensively in a variety of AML cell lines. The first evidence of ATRA-induced differentiation of leukaemia HL-60 cells was reported in 1980 (1). There, ATRA, at physiological concentration, induced terminal differentiation to granulocytes in 90% of the primary leukaemia cells in culture. Subsequent studies demonstrated that ATRA was specifically effective in APL cells (2). Thus, to enhance efficiency of ATRA in treatment of non-APL leukaemia, a combination of ATRA with one or more other molecule(s) is often used. Although the primary role of vitamin D3 has long been believed to maintain calcium and phosphate homeostasis in humans and other vertebrate organisms, cumulative studies *in vitro* suggest that it also has multiple types of anti-cancer activity. In 1981, it was found that mouse M1 myeloid cells could be induced to become

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macrophages by $1,25(\text{OH})_2\text{D}_3$ (3). Two years later, its role in induction of differentiation was observed in mouse leukaemia cells (4). Subsequently, vitamin D₃-induced differentiation has been observed in various types of human AML cells, including HL-60 (5–7), U937 (8), NB4 (9), THP-1 (10) and KG-1 cells (11).

Biological effects of ATRA and $1,25(\text{OH})_2\text{D}_3$ are mainly mediated by retinoic acid receptor (RAR) and vitamin D receptor (VDR) respectively. In humans, there are three types of RAR and RXR: α , β and γ and ATRA-induced granulocytic differentiation of HL-60 cells is mediated primarily through RAR α (12,13). In APL patients, presence of an abnormal PML-RAR α fusion protein is directly linked to the disease (14,15). The PML-RAR α /RXR complex inhibits gene transcription and blocks differentiation of leukaemia cells at the promyelocyte stage, leading to accumulation of their (16–18). As a further member of the same nuclear receptor family, the VDR also needs to bind to RXR to form a heterodimer. This is followed by conformational changes that allow the heterodimer to bind to VDR elements (VDREs) in the promoter region of target genes. The heterodimer then recruits several coactivators. As a result of interactions of these molecules, DNA becomes accessible to transcription factors and RNA polymerase for activation. In the absence of $1,25(\text{OH})_2\text{D}_3$, the VDR-RXR heterodimer binds to co-repressors, recruiting histone deacetylases (HDACs), and resulting in transcriptional repression.

All trans-retinoic acid- and $1,25(\text{OH})_2\text{D}_3$ -induced cell differentiation is usually accompanied by cell cycle arrest. All growing cells undergo cell cycle changes. In mammals, the cell cycle is controlled by a group of proteins termed cyclin-dependent kinases (CDKs) (19). Mammalian cells have as many as nine CDKS, among which four of them (CDK1, CDK2, CDK4 and CDK6) have been identified to regulate cell cycle progression. CDKs only become active when they bind to a regulatory subunit called cyclin. Cell cycle progression from G2 into M phase is driven by CDK1 (cdc2) complexed to cyclin B (also termed 'G2 checkpoint kinase'). G1 cyclin-CDK complexes (also termed 'G1 checkpoint kinases') regulate progression of the cell cycle through G1 to DNA replication (S phase). There are at least five major G1 cyclins, termed cyclins D1, D2, D3, A and E. Each of these can associate with one or more of the G1 CDK family (CDK2, CDK4 and CDK6). All cyclin-CDK complexes are generally considered to act on DNA replication machinery in the nucleus. Full activation of all CDKs in eukaryotic cells requires phosphorylation at a conserved threonine (or serine) residue within their activation segment (T-loop), as well as cyclin binding. CDK-activating kinase (CAK) is an enzyme com-

plex that is capable of phosphorylating CDKs at the T-loop and is essential for G1 and G2 CDK activities (20–23). The CAK is composed of CDK7, cyclin H and Mat 1 (24). Cyclin H is a regulatory subunit of CDK7 and Mat 1 is an assembly factor for the complex (25–27) and regulates CAK substrate specificity (28,29). In contrast, activity of CDKs is counterbalanced by CDK inhibitors (CKIs). All CKIs directly bind to the cyclin-CDK complex and repress its activity, leading to arrest of cell cycle. Thus, CKIs play an important role in negative control of cell cycle progression, which prevents overgrowth of cells and so tumour formation. It is not surprising that genes encoding these CKIs are often found mutated in some human cancers. This review summarizes our current understanding of the role of cell cycle regulatory molecules in ATRA- and $1,25(\text{OH})_2\text{D}_3$ -mediated growth inhibition and differentiation of human myeloid leukaemia cells with a focus on AML and APL leukaemic cells.

Cell cycle regulatory molecules and ATRA

CDK-activating kinase

There has been a variety of proposed mechanisms by which ATRA exerts its anti-cancer functions. Among these, importance of CAK in ATRA-induced cell differentiation has been demonstrated in a number of studies in a variety of cell types. First, CDK7 is able to interact with and phosphorylate RAR α *in vitro* and *in vivo* (30,31). Second, it has been observed that mouse cells lacking Mat1 lose the ability to enter S phase and exhibit defects in phosphorylation of RNA polymerase II (32). Reduced CAK phosphorylation of RAR is accompanied by the differentiation process that occurs in leukaemic HL-60 and NB-4 cells treated with ATRA (33,34). In APL cells, formation of PML-RAR α blocks myeloid differentiation and suppresses apoptosis (35,36), whereas ATRA disrupts the PML-RAR α -RXR complex and restores RAR α -RXR signalling (36,37). The role of CAK in APL cells in response to ATRA has been described in detail in two further reports in which APL cells treated with ATRA exhibited dissociation of PML/RAR α from CAK. As a result of dissociation, MAT1 is degraded and PML/RAR α is converted from hyperphosphorylation to hypophosphorylation, which leads to G1 arrest and cell differentiation (33,38). As CAK regulates CDK activity, it was expected that degradation of MAT1 would cause G1 arrest. However, the question is how this could induce transcription activation and cell differentiation. Further studies exposed the link between phosphorylation status of RAR and co-activators/co-repressors. Hypophosphorylated RAR α

reduces binding to retinoic acid responsive elements (RARE) and enhances transcriptional activity of RA target genes, which correlate with RAR α dissociation from co-repressors, and association with coactivators (39). These data suggest that (i) dissociation of RAR α from RARE facilitates its dissociation from co-repressors, to its interaction with co-activators (NcoA-3); (ii) recruitment of NcoA-3 by hypophosphorylated RAR α may promote localization of transcription factor II H (TFIIH) to the promoter of RA target genes to activate transcription (39). Briefly, CAK coordinates gene expression induced by ATRA, resulting in commitment to differentiation.

Previous studies have shown that RAR/RXR heterodimers can operate as either transcriptional repressors or activators (40) and several models have been presented to explain the mechanisms responsible for ATRA-induced differentiation. From a classical point of view, without a ligand, DNA-bound RAR α /RXR inhibits transcription by recruiting co-repressors NCOR1, SMRT (NCOR2), which in turn, recruits HDAC. In the presence of ATRA, binding of RAR ligand induces a conformational change, which reduces association of co-repressors and allows recruitment of coactivators, histone acetyltransferases, and activates transcription (41). Several other studies suggest that transcriptional activation by ATRA is also involved in degradation of the hetero-receptors (either RAR α or PML-RAR α) through the ubiquitin-proteasome system, that is, heterodimerization of RAR α /RXR or PML-RAR α /RXR triggers degradation of the receptors and promotes transcription (37,42–44). Identification of the role of CAK in ATRA-induced differentiation of leukaemia cells, in several laboratories, as described above, is a supplement to these existing modes. However, the link between MAT1 degradation-RAR α hypophosphorylation and differentiation-dependent gene transcription triggered by binding of TFIIH is still unclear. As CAK is also a kinase subunit of TFIIH, most likely, TFIIH would lose its biological activity, causing inhibition of gene transcription if MAT1 is degraded by ATRA. Future studies will address this question.

The role of CAK is not limited to leukaemia cells, and a similar effect of MAT1 on cell population growth and differentiation has also been observed in neuroblastoma (NB) CHP126 cells. It was found that 9sRA-induced transition of proliferation/differentiation in CHP126 cells correlates with reduced CAK activity. Interestingly, in these NB cells, CAK interacts with Rb protein (pRB) and RXR α . As a result of 9sRA treatment, CAK hyperphosphorylation of pRb and RXR α switched to hypophosphorylation of pRb and RXR α , resulting in differentiation of the CHP126 cells (45).

CDK inhibitors

As described in the Introduction, CDKs promote cell cycle progression, and inhibition of CDK activity is usually observed when a CKI binds to CDK during cell growth arrest in response to an inhibitory signal. In general, CKIs can be divided into two families: INK and KIP/CIP. The INK family includes p16, p15, p18 and p19 and the KIP/CIP family is composed of three members: p21, p27 and p57. Members of the INK family are able to associate with and inhibit cyclin D-CDK4/CDK6 kinase activity, whereas the KIP/CIP family members bind to and inhibit cyclin D-CDK4/6 as well as cyclin E/A-CDK2 activities. Involvement of CKIs in ATRA-induced G1 arrest and cell differentiation has been demonstrated in a variety of cell lines. In acute myeloblastic leukaemia ML-1 cells, ATRA plus granulocyte-macrophage colony-stimulating factor suppressed activities of CDK2 and CDK4 without affecting expression of these two CDKs, which correlated with granulocytic differentiation of ML-1 cells. Activities of CDK2 and CDK4 were slightly higher 1 day after addition of ATRA followed by significant inhibition by day 2, which lasted up to day 6 when cells had accumulated in the G1 phase of the cell cycle and were induced to differentiation along the granulocytic pathway. Downregulation of CDK activity is due to an increase in CDK2-bound p27 and CDK4-bound p18 (46). In several other leukaemia cell lines, ATRA alone is sufficient to induce CKIs. For instance, in U937 cells, p21 mRNA and p27 proteins are enhanced after 24- to 48-h treatment with ATRA. Upregulation of CKIs is correlated with G1 arrest and monocyte/macrophage differentiation, as indicated by expression of differentiation-specific surface marker CD11c (47). p27 has also been found to increase in AML HL-60 and APL NB-4 cells treated with ATRA. In these studies, mechanisms for upregulation of p21 and p27 by ATRA are different. ATRA-activated p21 is regulated at the transcriptional level, whereas upregulation of p27 is a result of increased p27 stability (47). Skp2 is an enzyme that can target p27 for degradation. Cells treated with ATRA exhibit elevation in expression of p27 and marked ubiquitination of Skp2, suggesting that degradation of Skp2 promotes elevation of p27. In contrast, overexpression of Skp2 prevents accumulation of p27 and produces resistance to ATRA-induced G1 arrest (48). The close link between enhanced CKIs (p27 and p21) and growth inhibition/differentiation has also been demonstrated in human non-leukaemic cells. In NB SMS-KCNR cells, ATRA induced an increase in expression of p27 and G1 cyclin/CDK bound p27. Concordant with the increase of p27, G1 CDK activities were significantly reduced (49). In bone marrow

mesenchymal stem cells, upregulation of p27 and p16 is linked to growth inhibition, but not to osteoblastic differentiation (50). In neuronal P19 cells and a subclone of the NB cell line, SH-N, RA (ATRA) upregulates p21 and induces neuronal differentiation (51,52). Overexpression of p21 induces neuronal differentiation of NB cells in the absence of ATRA (52). Several studies have demonstrated that not only quantity of p27 but also phosphorylation at Ser10 residue of p27 is required for ATRA-induced growth arrest of ovarian carcinoma cells, as an A10-p27 mutant that cannot be phosphorylated at Ser10 of p27 has abolished ATRA-induced biological effects (53,54).

Cyclin-dependent kinases and cyclins

Activity of CDKs is regulated not only by CKIs but also by level of the CDK and amounts of bound cyclins. A number of studies suggest that ATRA-induced G1 arrest and myeloid differentiation of leukaemic cells are linked to downregulation of CDKs and cyclins. CDKs and cyclins targeted by ATRA are likely to be cell type-dependent. In U937 cells, ATRA downregulates cyclin A, cyclin G, cyclin D3 and cyclin E after 24–48 h of ATRA exposure (47). As cyclin A and cyclin E are regulatory units of CDK2, and cyclin D3 is an active determinant of CDK4 and CDK6, downregulation of these cyclins clearly contributes to reduced activities of CDK2, CDK4 and CDK6. Reduced CDK6 during myeloid differentiation has also been linked to upregulation of microRNAs (miR-29a and miR-142-3p) that occurs after ATRA stimulation. In AML THP-1 and APL NB4 cells, it has been demonstrated that CDK6 is a target of miR-29a. Upregulation of miR29a by ATRA reduced levels and activity of CDK6 (55). In mammalian cells, cyclin E-CDK2 plays a key role in cell cycle transition from G1 into S phase. A significant reduction in cyclin E levels has been observed in leukaemic U937, HL-60, NB-4 (47) and THP-1 cells (56). The earliest reduction of cyclin E mRNA was observed within 6h of ATRA treatment in THP-1 cells, suggesting that ATRA-induced reduction of cyclin E, and, perhaps, other cyclins, is regulated at the transcriptional level. Downregulation of cyclin E by ATRA in these myeloid leukaemia cells is consistent with the previous observation that inhibition of cyclin E by retinoic acid suppresses transformation of immortalized human bronchial epithelial cells (57). Although a number of studies has demonstrated that expression of G1 CDKs was not affected during ATRA-induced G1 arrest and terminal differentiation of human myeloid leukaemic cells, one recent study provides evidence that ATRA (0.1–5 μ M) can significantly inhibit expression of CDK2, CDK4 and CDK6 in NB4 and

HL-60 cells (58). Downregulation of CDK2 is a result of post-translational modification by the ubiquitin–proteasome in response to ATRA treatment. In contrast, complete proteasome inhibition suppresses ATRA-induced differentiation and cell cycle arrest in NB4 cells.

It had been generally believed that CDK1 (cdc2) controls G2-M transition only in mammalian and many other higher eukaryotic cells. However, a number of studies has shown that CDK1 not only promotes G2-M transition but is also capable of regulating G1 progress and G1-S transition by association with multiple interphase cyclins (59). In a recent study, CDK1 has been demonstrated to regulate ATRA-mediated cell cycle arrest and cell differentiation (60). In ATRA-induced differentiation of U937 cells, CDK1 was found to translocate into the nucleus where it interacted with RAR γ and formed a reciprocal regulatory circuit; these influenced function and protein stability of each other and regulated levels of p27 protein.

Rb protein

Rb protein, product of the retinoblastoma tumour suppressor gene, is a downstream target of CDK. Phosphorylation level of pRb oscillates in a cell cycle- or cyclin-dependent manner and kinase assays have demonstrated CDK induction of pRb phosphorylation *in vitro* (61). Thus, pRb acts as a signal transducer connecting the cell cycle ‘clock’ to transcriptional machinery, which is achieved by interactions with E2F transcription factors (62). Dephosphorylated pRb is an active form that interacts with E2F and inhibits DNA transcription. In contrast, phosphorylated pRb is not able to bind to E2F, resulting in activation of E2F-dependent transcription. Two reports first demonstrated that dephosphorylation of pRb is linked to ATRA-induced myeloid differentiation (63,64). Subsequently, Juan *et al.* showed that ATRA regulates pRb at both transcriptional and post-translational levels, inducing dephosphorylated pRb but also upregulating total pRb in HL-60 cells (65). Increase in pRb levels make it possible to produce large amounts of dephosphorylated pRb. This dual regulation of pRb by ATRA has also been observed in THP-1 cells in which both Rb mRNA and pRb were elevated, while proportion of hyperphosphorylated pRb was markedly reduced (56). Mechanisms responsible for Rb-mediated growth inhibition and differentiation in cells treated with ATRA are not fully understood. It has been reported that pRb is able to interact with HDAC complex containing co-repressors, and suppresses transcription of E2F target genes followed by cell cycle arrest, in G1 phase. In APL NB-4 cells, ATRA disassociates PML-RAR α /RXR

complexes and promotes co-localization of PML and pRb, leading to pRb-mediated transcriptional repression. In contrast, in absence of ATRA PML-RAR α blocks, interaction between pRb and HDAC inhibits pRb-mediated transcriptional repression, inducing accumulation of leukaemia cells (66). It appears that pRb has a different role in neuronal P19 cells. The P19 cell line was isolated and established from a mouse teratocarcinoma. In these cells, two serine sites (601 and 773) on pRb were strongly phosphorylated in ATRA-induced neuronal differentiation, which correlates with enhanced CDK4 activity. These results suggest that ATRA activates CDK4, then CDK4 phosphorylates pRb, followed by transcriptional activation (67). However, it is not clear whether phosphorylation of pRb plays a primary role in ATRA-induced differentiation of the neuronal cells, as there is no evidence to show direct correlation between phosphorylation of pRb and cell differentiation. Most likely, phosphorylation or dephosphorylation of pRb by ATRA or other molecule(s) is dependent on cell type, which is common in many biological instances. For example, TGF β phosphorylates pRb in Schwann cells and dephosphorylates pRb in epithelial cells (68).

c-Myc

A further important player in cell cycle control is c-Myc. c-Myc is a transcription factor that binds DNA at specific sites and activates gene transcription. It is now known that deregulated expression of c-Myc plays an important role in human cancer development. Downregulation of c-Myc by ATRA during myeloid differentiation has been observed in several leukaemia cell lines (47,69–71). A significant reduction in c-Myc has been observed in U937 cells treated with ATRA for 12 h. Inhibition was time-dependent with maximal effect being observed at 72 h, which is consistent with a proliferation/differentiation transition process. As downregulation of c-Myc precedes G1/G0 arrest and changes in CDKs and p27 levels, it is possible that its inhibition by ATRA downregulates CDKs and upregulates p27, which leads to arrest of cell proliferation and induction of differentiation (47). Studies on APL NB4 cells have provided further mechanisms linked to function of c-Myc in ATRA-treated cells. Thrombospondin-1 (TSP-1) protein is a member of the thrombospondin family, it plays multiple roles and is involved in angiogenesis, inflammation and cancer inhibition (72). Xu *et al.* found that ATRA treatment markedly increased TSP-1 levels and inhibited c-Myc expression in NB4 APL leukaemic cells, which was proposed to be caused by dramatically reduced c-Myc recruitment to the TSP-1 promoter (69). These data suggest that the anti-cancer effect of ATRA

is through induction of TSP-1 expression by reducing its transcriptional repressor, c-Myc. A further molecule involved in the c-Myc pathway is tetradecanoyl phorbol-13-acetate inducible sequence 21 (TIS21). TIS21 is a tumour repressor and is implicated in a variety of biological processes. In AML HL-60 cells, TIS21 downregulates c-Myc mRNA and reduces stability of c-Myc protein by increasing its phosphorylation at Ser62 and Tyr58 residues by activation of Erk1/2 and inhibition of PI3K/Akt during ATRA-induced differentiation (71). TIS21 also negatively regulates expression of cyclin E and CDK4 (73).

c-Myc stimulates transcription by forming a hetero-complex with Max (c-Myc/Max) then binding to DNA, resulting in dual functions in cell proliferation control. Studies from Uribealga *et al.* (74,75) have shown that c-Myc/Max can directly interact with RAR α , causing either differentiation or proliferation, depending on phosphorylation status of c-Myc. Unphosphorylated c-Myc-RAR α represses expression of RAR targets required for differentiation, thereby promoting cell proliferation, whereas phosphorylation of c-Myc by Pak2 kinase, induced by ATRA, activates transcription of those same genes to stimulate differentiation.

In contrast to observations on myeloid leukaemia cells, ATRA produces stimulatory proliferation in normal T cells by upregulating cyclin D3, cyclin E and cyclin A and downregulating p27. These biological activities are achieved by releasing IL-2 (76).

Cell cycle regulatory molecules and vitamin D3

CDK inhibitors

Vitamin D3 has pleiotropic biological activities including its roles in anti-proliferation and induction of differentiation. Involvement of cell cycle regulatory molecules in vitamin D3-induced growth inhibition and cell differentiation in human myeloid leukaemia cells has been demonstrated in many studies. Liu *et al.* (77) made the first clear demonstration of p21 as a 1,25(OH) $_2$ D3 target gene. In that report, expression of a p21 clone was detected in a cDNA library prepared from leukaemic U937 cells treated with 1,25(OH) $_2$ D3, but not in control cells. Direct evidence for interaction between VDR and p21 includes the finding that the p21 promoter contains a VDR binding site (VDRE). In addition, induction of p21 mRNA by 1,25(OH) $_2$ D3 was rapid and was in a VDR-dependent and p53-independent manner. Finally, transient overexpression of p21 in U937 cells in the absence of 1,25(OH) $_2$ D3 led to cell-surface expression of monocyte/macrophage-specific markers, CD14 and CD11b (77). Recent studies show that binding of VDR

to the *p21* gene in U937 cells promotes acetylation of histone H3 (78). A further cell line often used for studying differentiation is HL-60. Phenotypically, both U937 and HL-60 are blast cells; however, U937 is a neoplastic derivative of committed progenitors of monocytes and HL-60 is a derivative of granulocyte progenitors, but can be induced to differentiate into granulocytes or monocytes/macrophages, depending on type of inducer used. In studies performed by Seol *et al.* (79), exposure of HL-60 cells to EB1089, a vitamin D₃ analogue, cause G1 arrest and elevated levels of p21. Immunoprecipitation has detected elevated levels of p21-CDK2 and p21-CDK6 complexes, suggesting that upregulated p21 is responsible for reduced activities of CDK2 and CDK6. However, p53-dependent p21 is unlikely to have a role in differentiation-associated G1 arrest in AML cells, as transfection of p53 in HL60 cells (p53 null) enhances expression of p21, but has been insufficient to induce G1 arrest (80), which is consistent with the previous finding in U937 cells (77). Another important observation in the study of anti-proliferative activity of vitamin D₃ was identification of p27 as a downstream effector of vitamin D₃ signalling. Wang *et al.* detected G1 block in HL-60 cells treated with 1,25(OH)₂D₃ was significant at 48 h and reached its maximum at 72 h. In association with G1 arrest, p27 was markedly elevated, whereas p21 was only transiently higher by 48 h, suggesting close correlation of upregulated p27 and vitamin D₃-induced G1 arrest (81). In agreement with the findings in myeloid leukaemia cells described above, anti-proliferative effect of vitamin D₃ by induction of p21 and p27 has also been found in a variety of other malignant cells, including those of osteosarcoma (82), breast cancer (83), colon adenocarcinoma (84) and prostate carcinoma (85). In prostate cancer cell line ALVA-31, 1,25(OH)₂D₃ induces elevation in both p21 mRNA and protein levels and causes growth inhibition. In contrast, transfection of the cells with a p21 antisense construct abolishes 1,25(OH)₂D₃-induced growth inhibition (85). It is most likely that induction of p21 by 1,25(OH)₂D₃ receptor is through direct interaction by activating *p21* gene expression, as it has been demonstrated that *p21* gene contains a consensus VDRE that allows VDR binding. However, several studies have provided evidence that 1,25(OH)₂D₃ may activate p21 *via* other pathways. For example, in HL-60 cells, inhibition of Akt and activation of Raf/MEK/ERK MAPK pathway by vitamin D₃ may be critical for upregulation of p21, because ectopic overexpression of Akt inhibits MAPK signalling, downregulates p21 and inhibits cell differentiation (86). In LNCaP prostate cancer cells, induction of p21 by 1,25(OH)₂D₃ is mediated by insulin-like growth factor binding protein-3 (IGFBP-3). Adding

1,25-(OH)₂D₃ to LNCaP cells causes an approximate 3-fold upregulation of IGFBP-3 at mRNA and protein levels and cell growth inhibition. Conversely, IGFBP-3 antisense oligonucleotides or antibodies abolish growth inhibitory actions of 1,25-(OH)₂D₃. In addition, LNCaP cells treated with IGFBP-3 showed approximately 2-fold elevated expression of p21. Finally, adding an IGFBP-3 neutralizing antibody completely prevented the 1,25-(OH)₂D₃-induced upregulation of p21 (87). These data suggest that 1,25(OH)₂D₃-induced growth inhibition is mediated by IGFBP-3. A further important CKI in the KIP/CIP family is p27. Addition of 1,25(OH)₂D₃ to U937 cells induces rapid upregulation of p27 (77,86,88,89) and ectopic overexpression of p27 results in induction of expression of monocyte/macrophage-specific markers in the absence of the hormone (77,88). In some of these studies, although 1,25(OH)₂D₃ also up-regulated expression of INK family members, p15, p16 and p18 (77), forced expression of p15, p18 (88) and p16 (90) was not able to induce myeloid differentiation of U937 cells, suggesting that INK family molecules may not be involved in differentiation, but may be involved in proliferation arrest. Taken together, vitamin D₃ uses both CIP/KIP and INK inhibitors to magnify its efficiency in anti-proliferation and facilitate subsequent differentiation induction. It is not clear how vitamin D₃ upregulates p27, as there is no evidence indicating that p27 contains a VDRE. A recent report has demonstrate that vitamin D₃ upregulates p27 by downregulation of microRNAs181 (miR181) (86). This study provided the first evidence that 3'-UTR of p27 has a miR181 binding site. Exposure of HL60 and U937 cells to 1,25(OH)₂D₃ suppresses expression of miR181. In contrast, transfection of pre-miR181 abolishes vitamin D₃-induced p27 and monocytic differentiation in both HL60 and U937 cells. Mechanisms responsible for upregulation of p27 by vitamin D₃ appear to be cell type-dependent. In human prostate cancer cell line LN-CaP, 1,25-(OH)₂D₃ has no effect on p27 mRNA levels or rate of p27 protein synthesis. Conversely, 1,25(OH)₂D₃ reduces phosphorylation of p27, reduces levels of Skp, an enzyme targeting phosphorylated p27 for degradation, and interferes with CDK2 nuclear localization. All these factors contribute to upregulation of p27 by vitamin D₃ (91). Based on the data described above, p27 is shown to be regulated by vitamin D₃ at both transcriptional and post-translational levels, which is cell type-dependent. However, importance of p27 in control of cell proliferation and differentiation has been challenged by several studies. In one report, insulin-like growth factor I (IGF-I) promoted vitamin D₃-induced macrophage differentiation of HL-60 cells, as indicated by elevated expression of CD11b, CD14 and macrophage-specific esterase,

alpha-naphthyl acetate esterase, as early as 24 h following initiation of terminal differentiation. Interestingly, early expression of CD11b (24 h) was simultaneously accompanied by downregulation of p27, indicating that accumulation of p27 was not required for initiation of differentiation and early (48 h) differentiation processes of HL-60 cells (92). In prostate cancer LNCaP cells, p27 appears not to be important for vitamin D₃-mediated biological activities. Although 1,25-(OH)₂D₃ upregulated expression of p27 and depletion of p27 has led to more rapid LNCaP cell proliferation in the absence of the hormone, depletion of p27 did not prevent 1,25-(OH)₂D₃-induced growth inhibition of cells (93).

Cyclin-dependent kinases and cyclins

As described above concerning ATRA, downregulation of CDK activity can be due to elevated CKI binding, dissociation from cyclins and reduced expression of CDK. In many cases, reduced CDK activity by vitamin D₃ is due to upregulated p21 (79,83,85) or p27 (86,91,94), or both p21 and p27 (84,88), complexed with CDKs (mainly CDK2 and CDK6). However, downregulation of expression of CDKs has also been observed in a variety of leukaemia cells treated with vitamin D₃. For example, 1,25B1089, a vitamin D₃ analogue, causes significant reduction in level of CDK2 and CDK6. Interestingly, although CDK2, CDK4 and CDK6 all control G1 progression, only CDK2 and CDK6 are downregulated by 1, 25(OH)₂D₃, while expression of CDK4 is not affected (79,84) or may even be increased (79). Mechanisms for downregulation of CDK2 and CDK6 and upregulation of CDK4 by vitamin D₃ metabolites or analogues are not fully understood. Studies on NIH 3T3 cells have shown that cyclin D3 is able to interact with vitamin D₃ receptor and regulate its transcriptional activity. This effect is counterbalanced by overexpression of CDK4 and CDK6 (95).

Rb protein family

Juan *et al.* (65) have specifically analysed phosphorylation status and level of pRb in leukaemic HL-60 cells during proliferation and differentiation. One of the interesting findings is that not only phosphorylation level of pRb but also expression of total pRb was linked to cell cycle status. HL-60 cells in G₀/G₁ phase induced either by retinoic acid or 1,25(OH)₂D₃ had increased levels of both total pRb and hypophosphorylated pRb compared to cells in G₁ without inducers. Elevated hypophosphorylation of pRb has also been observed in APL NB-4 cells treated with a combination of 1,25(OH)₂D₃ and aurano-fin, a lipophilic gold compound, that has been used

to treat rheumatoid arthritis (96). These data suggest that irreversible cell cycle arrest with terminal differentiation of HL-60 cells, by inducers, requires both elevated total and phosphorylated pRb, which appears different from mechanisms responsible for transforming growth factor-beta (TGFβ)-induced G₁ arrest in human myeloid leukaemia cells (97). Requirement of dephosphorylation and upregulation of pRb for growth inhibition and cell differentiation have been further confirmed by using 1, 25(OH)₂D₃ and several vitamin D₃ analogues (98,99). In these studies, anti-proliferative activity of vitamin D₃ correlated with extent of hypophosphorylation of pRb. Although low concentration of vitamin D₃ is insufficient to induce any detectable cell cycle arrest, expression of differentiation marker, CD14 was significantly higher (98,99). Kinetics of upregulation of pRb (mRNA and protein) induced by 1,25(OH)₂D₃ paralleled expression of CD14. Wang *et al.* (86) demonstrated that dephosphorylation and upregulation of pRb are regulated by vitamin D₃-induced MAPK signalling, resulting in Rb binding to transcription factor E2F1 and subsequent differentiation. Conversely, knockdown of Rb by siRNA prevented vitamin D₃-induced differentiation.

The pRb family has three members: pRb, p107 and p130. It has been long recognized that pRb is a tumour suppressor, whereas p107 and p130 are not. *Rb* gene was originally found to be mutated in patients with hereditary retinoblastoma and subsequently in various other cancer patients, whereas *p107* and *p130* genes appear to be less frequently mutated in human cancers. However, studies from several laboratories have shown that p130 and p107 also are actively involved in E2F-dependent transcriptional activation. For example, major E2F complexes in quiescent fibroblasts are E2F4–p130. As cells enter the cell cycle, E2F4–p130 is replaced by E2F4–p107 and E2F4–pRb (100,101). In some types of cells, including those of human myeloid leukaemia, pRb is not detectable in promoters containing E2F-responsive sites in cycling cells, but is associated with E2F4–p130 or E2F4–p107 during G₀/G₁ phase (97). Studies from Verlinden *et al.* (102,103) have shown that p130, but not pRb, play an important role in vitamin D₃-induced growth inhibition, but not in differentiation of U937 cells; in bone forming cells, anti-proliferative effect of 1,25(OH)₂D₃ is completely dependent on presence of p107 and p130, as 1,25(OH)₂D₃ fails to repress E2F target genes and loses the anti-proliferative effect in p107- and p130-depleted cells, but not in pRb-knockout cells.

Involvement of cell cycle regulatory molecules in induction of cell differentiation by vitamin D₃ is not limited to leukaemia cells. Changes in expression of several CDKs, CDK inhibitors and phosphorylation of pRb induced by vitamin D or its analogues have also

been observed in BxPC-3, a human pancreatic carcinoma cell line, MART-10 and a breast cancer cell line, MCF-7 (104).

Summary

Leukaemic transformation results in excess of immature cells with reduced ability to differentiate. Thus, induction of differentiation has been a major strategy for development of treatment for this cancer. ATRA, a vitamin A derivative, and 1,25(OH)₂D₃, an active metabolite of vitamin D₃, have been used as anti-proliferation and differentiation induction therapy in APL and non-APL leukaemia cells over the past number of decades. Involvement of cell cycle regulatory molecules in ATRA and vitamin D₃ signalling pathways has been clearly demonstrated in two ways. First, it has been observed that ATRA- and vitamin D₃-induced differentiation of leukaemia cells usually is accompanied by downregulation of CDK 2 and CDK6 activities and elevated expression of several CKIs, especially p21 and p27. Downregulation of CDKs is a result of increased CKIs bound to the CDKs. Although the INK family CKIs (p15, p16, p18) are also upregulated in ATRA- and vitamin D₃-treated cells, most likely, these inhibitors are involved in growth inhibition, but not in differentiation induction. It has been demonstrated that the p21 promoter contains a VDR binding site and p27 is a target of several micro RNA molecules that are negatively regulated by vitamin D₃. Leukaemia cells treated with ATRA and vitamin D₃ frequently also have low levels of c-Myc, CDK2, CDK 6, and cyclin E and high levels of pRb. As downregulation of c-Myc precedes G1/G0 arrest and changes in CDKs and p27 levels, it is possible that inhibition of c-Myc by ATRA downregulates CDKs and upregulates p27, which lead to arrest of cell proliferation and induction of cell differentiation. Cyclin E is a regulatory subunit of CDK2 whose activity is critical for G1/S transition of the cell cycle. Downregulation of cyclin E, as well as p21, contributes to inhibition of CDK2 activity. Although both ATRA and vitamin D₃ downregulate G1 CDKs, especially CDK2 and CDK6, inhibition of cyclin E expression has only been observed in ATRA-treated, but not vitamin D₃-treated cells.

Second, ATRA and vitamin D₃ exert their functions via post-translational modifications of some cell cycle regulatory molecules. Biological functions of ATRA and 1,25(OH)₂D₃ are known to be mediated by their corresponding nuclear receptors (RARs and RXRs or VDR and RXRs) that belong to type II superfamily of nuclear hormone receptors. Upon ligand binding, these receptors interact with RARE or VDRE and trigger formation of coactivator complexes for transcriptional activation.

Recent studies have demonstrated that CAK is able to interact with RAR α , and inhibition of CAK by ATRA causes hypophosphorylation of PML-RAR α , leading to cell growth arrest and differentiation of APL cells. Most likely, elevated p27 induced by ATRA and vitamin D₃ is involved in both transcriptional regulation and post-translational modification. In addition, it has been demonstrated that hypophosphorylation of pRb can promote leukaemia cell differentiation.

Although we have accumulated much knowledge on the role of cell cycle regulatory molecules in ATRA- and 1,25(OH)₂D₃-induced differentiation of leukaemia cells, a number of questions remain to be answered in future studies. For example, in ATRA- and vitamin D₃-induced downregulation or upregulation of expression of several CDKs, cyclin E, CKIs, cMyc and pRb, pathways still are unclear and remain to be defined in detail. It is apparent that p21 contains a VDR binding site, however, it is not known whether p21 has an RAR binding site. For several leukaemia cell lines, ATRA usually induces granulocyte differentiation, whereas vitamin D₃ induces differentiation of cells into monocytes/macrophages. It is unclear which cell cycle regulatory molecule(s) may play critical roles in determining differentiation of AML cells into either granulocytes or monocytes/macrophages.

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