Differentiation induction in acute promyelocytic leukemia

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Inductie van differentiatie in acute promyelocyten leukemie

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Contents

CHAPTER 1

General Introduction

Part of this introduction has been published in:

Marleen C. Breems-de Ridder, Bob Löwenberg and Joop H. Jansen

Retinoic acid receptor fusion proteins: Friend or Foe

Molecular and Cellular Endocrinology 165, 1-6, 2000

1.1 Hematopoiesis

Hematopoiesis or blood cell formation is a continuous process in which maturing hematopoietic cells with a limited life span are formed. The formation of all different blood cell lineages originates from a small population of pluripotent stem cells that reside in the bone marrow [1]. Progenitor cells that are committed to a certain lineage of differentiation orginate from these pluripotent stem cells. Hematopoiesis is regulated by a network of cytokines and hematopoietic growth factors (HGF) (Figure 1.1). The HGFs are produced locally by stromal cells, mature blood cells, endothelial cells or specialized cells in organs such as lungs, liver and kidney. The levels of HGFs are elevated in response to extracellular stimuli, such as infection or bleeding, when a rapid rise of specific blood cell types is necessary. HGFs exert their effect by binding to their corresponding receptors expressed on the membrane of their target cells. Ligand binding results in the activation of downstream signaling pathways. A cascade of phosphorylation events is involved in signal transduction. In one pathway, the JAK (janus kinase) family of protein tyrosine kinases are tyrosine phosphorylated and in turn activate a family of latent cytoplasmic transcription factors, called STAT (Signal Transduction and Activation of Transcription) proteins [88,90]. Following their activation, these STAT proteins are assembled into complexes which then translocate to the nucleus and activate target genes by interaction with specific DNA sequences [29]. Another major HGF receptor signal transduction pathway includes proteins that belong to the Ras family (Figure 1.2). Signaling molecules like Shc and Grb2 function as adaptor proteins in this pathway by linking phosphorylated receptors to downstream effectors. Grb2 binds to the activated receptor, and to Sos (Son of sevenless) which after translocation to the plasma membrane activates Ras triggering phosphorylation of Raf. The products of Raf, a serine tyrosine kinase and mitogen activated protein kinases (MAPK) transmit signals for futher transmission to the nucleus [8]. In the nucleus, activation of transcription factors by phosphorylation or other mechanisms results in activation of genes involved in cellular proliferation and differentiation (Figure 1.2). Apart from affecting transcription, activated Ras results in cyclin D1 activation and stimulates p27kip1 degradation via Rho [30,44]. Both events positively influence cell cycle entry (Figure 1.2).

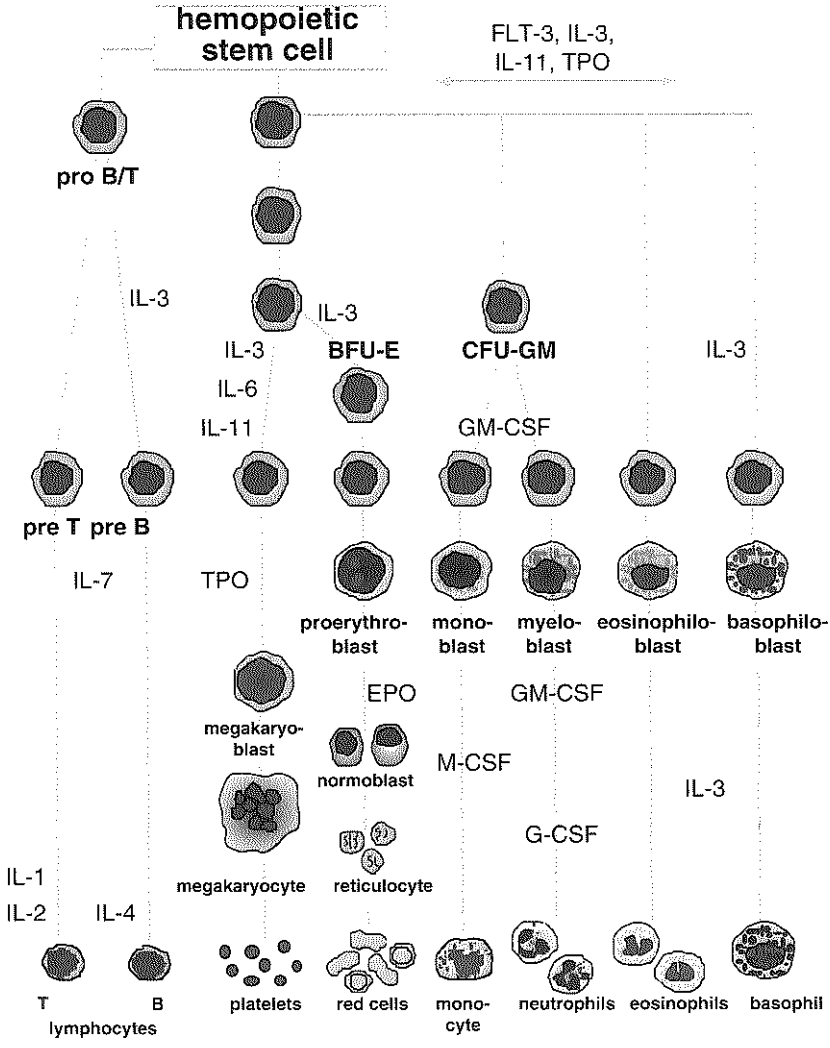

Figure 1.1. Schematic representation of hematopoietic stem cell differentiation

Mature blood cells orginate from pluripotent stem cells. This process is regulated by various hematopoietic growth factors (IL: interleukin; SCF: stem cell factor; G-CSF: granulocyte colony-stimulating factor; FLT-3: fetal-liver tyrosine kinase-3; GM-CSF: granulocyte-macrophage colony-stimulating factor; EPO: erythropoietin; TPO: thrombopoietin; M-CSF: macrophage colony-stimulating factor). The lineage-specific burst- and colony forming units (BFUs and CFUs) are indicated (GEMM: granulocyte-erythroid-monocyte-megakaryocyte; E: erythroid; GM: granulocyte-monocyte; Meg: megakaryocyte; Eo: eosinophil).

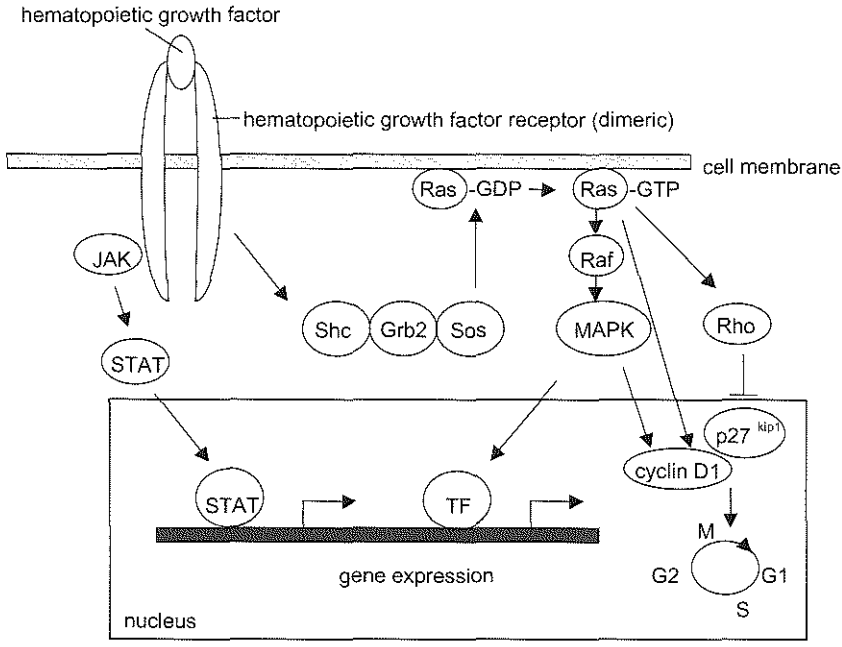

Figure 1.2. Control of hematopoiesis by growth factors

The hematopoietic growth factors (HGF) exert their effect by two major signaling pathways. HGF binding results in receptor dimerization and activation of JAK tyrosine kinases. JAKs may directly activate STAT family transcription factors, which subsequently translocate to the nucleus and regulate gene expression. SH2-adaptor protein Shc may bind to activated receptors and is tyrosine-phosphorylated. Phosphorylated Shc interacts via its SH2 domain with Grb2, which in turn binds to Sos. This complex then modulates Ras, leading to activation of the serine/threonine/tyrosine phosphorylation cascade and ultimately induction of transcription. In addition, Ras induces cyclin D1 expression and stimulates $p27^{kip1}$ degradation promoting cell cycle entry.

1.2 Transcription factors involved in hematopoiesis

Specific lineages do not appear to be controled by a unique transcription factor but rather by specific combinations of factors each of which may occur in a number of different lineages [73]. For instance, it has been demonstrated that transcription factors such as GATA-1 and NF-E2 are involved in erythroid differentiation. Similary,

E2A and TCF are implicated in lymphoid lineage and the C/EBPα (CCAAT/enhancer binding protein α), C/EBPε and Ets (E26 specific) family members are crucial in myeloid development [20,21,77,86,89,92]. One mechanism by which transcription factors drive differentiation into a certain lineage is the upregulation of receptors for terminal differentiation factors. For example, myeloid transcription factors as C/EBPα and PU.1, a member of the Ets family, may affect the myeloid lineage by regulating expression of multiple CSF (colony-stimulating factor) receptors such as macrophage colony-stimulating factor (M-CSF) receptor, the granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor and the granulocyte colony-stimulating factor (G-CSF) receptor. Various promoters of myeloid specific genes (for example G-CSF and GM-CSF receptor) have a functional PU.1 binding site upstream of the transcription start site [92]. PU.1 contacts proteins of the basal transcription machinery to activate gene expression. Figure 1.3 depicts a model of induction of myeloid differentiation by these transcription factors. The transcription factor PU.1 is expressed at low levels in the pluripotent stem cells, as are specific growth factor receptors (GM-CSF-R). Under the direction of signals that are yet to be defined (or possibly by a stochastic process), transcription factors are expressed which leads to upregulation of specific growth factor receptors such as the G-CSF-R and M-CSF-R. Further lineage-restricted differentiation is mediated by the upregulation or downregulation of differentiation genes regulated by the transcription factors. It is not yet known how C/EBP proteins are regulated during multilineage development of stem cells but they appear to be selectively expressed at high levels in neutrophilic and not monocytic or erythroid cells and may use a mechanism similar to the model for PU.1 in contributing to myeloid development [83]. In addition, studying translocation breakpoints in acute myeloid leukemia's (AML) showed the involvement of RARα (retinoic acid receptor α) (see below), AML-1, and CBF-β (core binding factor-β) in myeloid development [49,61,69,75,76]. AML-1 is a sequence-specific DNA binding protein that complexes with CBF-β to activate transcription of target genes [67]. Many of the AML-1 dependent genes have been implicated in myeloid differentiation, including interleukin-3 (IL-3), GM-CSF and M-CSF [42].

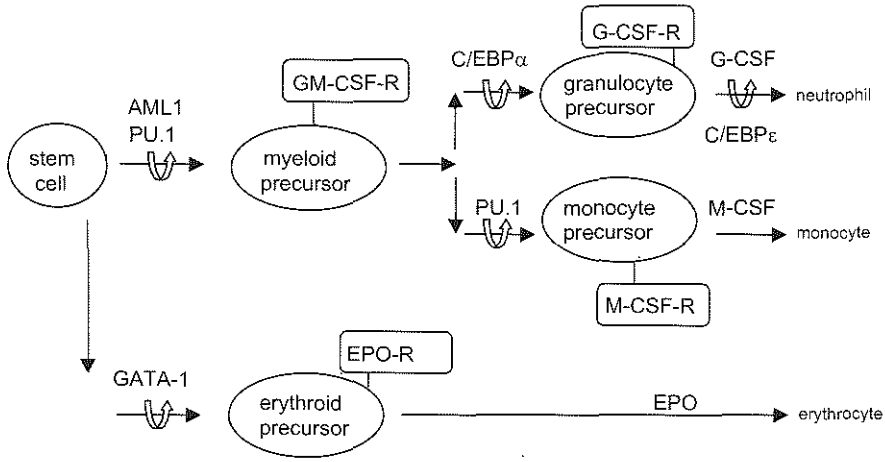

Figure 1.3. Model of induction of myeloid differentiation by specific transcription factors.

Transcription factors are expressed in stem cells. Under directions of signals that are as yet not defined, specific transcription factors as GATA-1 and PU.1 are expressed. This leads to upregulation of specific growth factor receptors, resulting in lineage-restricted myeloid differentiation. GATA-1 induces EPO-R (erythropoietin receptor) expression and directs the cell to the erytroid lineage, while enhanced PU.1 and AML1 expression result in myeloid commitment. At later stages, PU.1 directs M-CSF-R expression, regulating monocytic development. C/EBPα is crucial for early granulocytic development, while C/EBPε is essential for maturation of neutrophils.

1.3 Leukemia

Leukemia is characterized by a block in normal differentiation resulting in proliferation of immature, nonfunctional hematopoietic cells. Leukemia may be caused by genetic mutations associated with aberrant signal transduction or deregulating transcription factors that are important for hematopoiesis. For example, an internal tandem duplication of the *FLT-3* (*fetal liver tyrosine kinase-3*) gene (FLT3-ITD) resulting in constitutive activation of the FLT-3 receptor is found in 20% of the AML patients and correlates with poor prognosis [71,101]. The exact contribution to leukemogenesis

remains to be determined. *Ras*-gene mutations, the majority of which involve the *N-Ras* gene, are found in up to 14% of *de novo* AML cases [55,74,82]. Mutant *N-Ras* may also be associated with leukemia progression through aberrant signal transduction [54]. Mutations of myeloid transcription factor families like the CBF family and RARs may contribute to leukemogenesis because of altered function of these factors. In addition, chromosome translocations may also compromise the function of the involved partner proteins (Table 1.1) [63,64].

Table 1.1. The French-American-British (FAB) classification of AML and (cyto)genetic abnormalities [63,64].

FAB Subtype	Name (% in AML)	Associated translocations and rearrangements (% of cases)[1]	Genes Involved
M0	Acute myeloblastic leukemia with minimal differentiation (3%)	inv(3) (q21;q26), t(3;3) (q21;q26) (1%)	EVI1
M1	Acute myeloblastic leukemia without maturation (15-20%)		
M2	Acute myeloblastic leukemia with maturation (25-30%)	t(8;21) (q22;q22) (40%), t(6;9) (q23;q34) (1%)	AML1-ETO, DEK-CAN
M3	Acute promyelocytic leukemia (5-10%)	t(15;17) (q22;q21) (98%) t(11;17) (q23;q21), t(5;17) (q35;q21), t(17;17) (q13;q21), t(11;17) (q11;q21) (1%)	PML-RARα, PLZF-RARα, NPM-RARα NuMA-RARα, STAT5b-RARα
M4	Acute myelomonocytic leukemia (20%)	11q23 (20%), and t(3;3) (3%), t(6;9) (1%)	MLL, DEK-CAN, EVI1
M4E0	Acute myelomonocytic leukemia with abnormal eosinophils (5-10%)	inv(16) (p13;q22), t(16;16) (80%)	CBFβ-MYH11
M5	Acute monocytic leukemia (2-9%)	11q23 (20%), t(8;16) (p11;p13) (2%)	MLL, MOZ-CBP
M6	Erythroleukemia (3-5%)		
M7	Acute megakaryocytic leukemia (3-12%)	t(1;22) (p13;q13) (5%)	

[1]% indicates frequencies of specific (cyto)genetic abnormalities within FAB subtypes of AML.

1.4 Classification of acute leukemia

Acute leukemia can be divided into: acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). In acute leukemia immature myeloblasts or lymphoblasts are increased (more than 30% blasts) in the bone marrow. The distinction between AML and ALL is based on morphology, cytochemistry and immunological methods. AML is further subdivided based on morphological criteria according to the French-American-British (FAB) classification (Table 1.1) [3,4,5,6,9]. ALL is subdivided on a morphological basis into L1 (blast cells small, uniform high nuclear to cytoplasmic ratio), L2 (blast cells larger, heterogenous, lower nuclear to cytoplasmic ratio) and L3 (vascuolated blasts, basophilic cytoplasm) but more frequently according to immunophenotype (pro-B, precursor-B, B-cell, precursor-T, thymic).

1.5 Acute promyelocytic leukemia

Acute promyelocytic leukemia (APL, FAB-classification AML-M3) accounts for 5-10% of all acute myeloid leukemias. In this disease the leukemic cells are blocked at the promyelocytic stage of development and fail to differentiate into mature, nondividing granulocytes [87]. APL is characterized by chromosomal translocations that lead to the fusion of the *retinoic acid receptor* α (*RAR*α) gene to various partner genes. RARs are ligand-dependent transcription factors that bind to DNA and directly regulate the expression of target genes. In APL, the RARα-fusion proteins contribute to leukemic transformation by dominant interference with the expression of the retinoic acid receptor target genes and probably also by compromising the function of the RARα-partner genes. In the last decade, it has become clear that the malignant cells can be forced to overcome the block of differentiation by the administration of pharmacological doses of the RAR ligand all-*trans* retinoic acid (ATRA) [45,46], thus exploiting the residual functionality of the mutated proteins. This has proven to be of clinical use: where treatment with chemotherapy induces durable disease free survival in 50-60% of the cases, the combination of ATRA and chemotherapy improves durable disease-free survival to up to 80% [15,17,22,25,28,32,65,91,97].

This treatment constitutes the first generally accepted form of leukemia therapy that is based on the induction of differentiation of the malignant cells.

1.6 The role of retinoic acid receptors in hematopoiesis

a. Wild-type retinoic acid receptors

Retinoic acid receptors are important in the regulation of growth and differentiation of epithelial tissues, embryonic and central nervous system development and hematopoiesis [52]. Retinoids mediate their effect by two classes of nuclear receptor proteins, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), that each consist of three isotypes (α, β, and γ) encoded in separate genes [16,57,93]. Upon dimerization with RXR, RARs can bind to specific enhancer sequences in the DNA, so-called retinoic acid response elements (RAREs), resulting in transcriptional activation of target genes in the presence of ligand [57,60,80,102]. *RARα* and *RARγ* null mutant mice show poor viability, growth deficiency and male sterility. *RAR α/γ*, *α/β*, *β/γ* and *RAR/RXRα* double mutants exhibit a dramatically reduced viability [51,52,53].

The role of RARs in hematopoiesis has been studied by several groups. Retinoic acid is not capable of inducing *in vitro* hematopoietic colony formation from progenitor cells, but it modulates the growth of precursor cells in culture in the presence of hematopoietic growth factors. In cultures of unfractionated or CD34+ purified bone marrow cells, retinoic acid inhibits the growth of erythroid (BFU-E) and monocytic (CFU-M) colony forming cells. In contrast, the growth of granulocytic (CFU-G) colony forming cells is enhanced upon retinoic acid stimulation [11,31]. These effects may be explained by contrasting stimulative and inhibitory effects on the different lineage-commited precursor cells, or by the induction of granulocytic differentiation in progenitor cells at the expense of monocytic and erythroid differentiation. In more immature (lin-/sca+/c-kit+) bone marrow cell fractions, the colony forming cells and the spleen colony forming cells (CFU-s) were maintained significantly better in cultures supplemented with retinoic acid [47,81]. These studies are consistent with the notion that retinoic acid prevents the differentiation of very

immature (lin-/sca+/c-kit+) progenitor cells while it enhances the granulocytic differentiation of more mature lineage-committed precursor cells.

b. Mutated retinoic acid receptors

Mutations of RARs have profound effects on hematopoiesis. Overexpression of a dominant negative truncation mutant of *RARα* (*RARα403*) in murine bone marrow cells results in a differentiation block at the promyelocytic stage and immortalization of multipotent hematopoietic progenitors [23,93,94]. In clinical APL, RARα is involved in non-random chromosome translocations in which *RARα* is fused to one of five different partner genes of RARα. These partner genes include *PML* (for *promyelocytic leukemia* gene) [15,32], *PLZF* (*promyelocytic leukemia zinc finger*) [17], *NPM* (*nucleophosmin*) [84], *NuMA* (*nuclear mitotic apparatus protein*) [100] and *STAT5b* (*signal transducer and activator of transcription-5b*) [2] (Table 1.1).

In more than 98% of the APL patients, the chromosomal rearrangement represents the fusion of *RARα* to the *PML* gene. The PML-RARα fusion protein has altered DNA-binding activity when compared to wild-type RARα. It can bind to RAREs as a heterodimer with RXR but also as a homodimer independently of RXR [48,79]. Moreover, in contrast to wild-type RARα, PML-RARα inhibits AP-1 transcriptional activity in the absence of ATRA, but it becomes a potent activator of AP-1 activity in the presence of ATRA [26]. This suggests that PML-RARα and normal RARs act on a different spectrum of target genes. In addition to different DNA-binding, PML-RARα shows altered transactivational activity. Wild-type RARs modulate transcription through interaction with cofactors. In the absence of ligand, RXR/RAR heterodimers bind corepressors like N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors). These proteins recruit SIN3 and HDAC I (histone deacetylase I) resulting in histone deacetylation which renders the chromatin inaccessible to transcriptional activators [24,33,35,59,66,68,98]. Structural studies have shown that ligand binding induces conformational changes in the corepressor binding domain of the receptor [12,85] causing the dissociation of the corepressor complex and allowing binding of coactivator proteins with histone acetylase activity. Subsequent histone acetylation leads to unwinding of the chromatin allowing gene transcription (Figure 1.4A).

Relative to wild-type RARα, PML-RARα binds retinoids with the same affinity and specificity (Kd=0.09 nmol/L and Kd=0.13 nmol/L respectively) but shows enhanced binding with corepressor proteins [7,50,72]. As a consequence, the corepressor complex is not released at physiological concentrations (10^{-9}-10^{-8} M) of ATRA and transcription of RARα target genes remains repressed. However, at pharmacological doses (10^{-7}-10^{-6} M) of ATRA the corepressors are replaced by coactivators allowing transcriptional activation (Figure 1.4B) [33,36,43,59]. This explains why high doses of ATRA can effectively induce granulocytic differentiation in APL cells and induce remissions in the majority of the patients.

The transforming properties of *PML-RARα* have been confirmed in transgenic mice that develop APL-like acute myeloid leukemia with accumulation of promyelocytic cells in the bone marrow [13,34,38]. The majority of mice develop leukemia with a late onset (> 6 months) suggesting that additional genetic mutations are required for full transformation of the cells. In addition to deregulation of RARα-target genes, interference with the function of PML may contribute to the development of APL. PML is a nuclear protein with a RING-finger motif that mediates its localization in large multiprotein nuclear structures termed PODs (PML oncogenic domains), ND-10 (nuclear domain-10) or nuclear bodies. These structures appear as dense spherical particles and are tightly associated with the nuclear matrix. Although more than 20 proteins have been shown to colocalize in these structures, their exact function remains unclear. Interestingly, PML-RARα disrupts the normal structure of nuclear bodies to a microspeckled nuclear pattern and delocalizes RXR from a nuclear diffuse towards a microspeckled pattern. After treatment with ATRA, normal nuclear bodies are reassembled and the RXR protein returns to a nuclear diffuse distribution [27,56,99]. The role of this delocalization in transformation is unclear. A possible role of PML in myeloid differentiation was found in *PML* knockout mice. *PML* -/- mice are viable and fertile but highly susceptible to certain fungal and bacterial infections [95]. In addition, myeloid cell counts were reduced while the number of bone marrow precursor cells were normal. This effect may be mediated by direct interference of PML with the expression of RARα target genes. Coimmunoprecipitation and transactivation studies suggest that PML may interact directly with the RARα protein and enhance transcription by RXR/RARα

heterodimers [103]. Interestingly, the RARα target gene *p21*, encoding a cyclin-dependent kinase inhibitor, could not be upregulated by ATRA in *PML* -/- fibroblasts [95]. Other studies have suggested a role for PML in apoptosis either directly or indirectly mediated by caspases [37,41,96]. The relevance of deregulation of normal PML function by PML-RARα for leukemic transformation remains to be resolved.

In about 1% of the APL patients a *PLZF-RARα* fusion gene is expressed [17,36]. In contrast to PML-RARα positive APL, treatment with ATRA does not induce terminal differentiation and complete remissions can not be achieved with ATRA alone in these patients [58]. PLZF is a nuclear protein that binds to DNA in a sequence-specific manner, and acts as a transcriptional repressor by recruiting corepressor proteins to the DNA [66]. Although PLZF-RARα binds ligand with approximately the same affinity (Kd=0.17 nmol/L) as wild-type RARα [7], these cells do not respond to ATRA, even at pharmacological doses due to an ATRA-insensitive binding site for corepressors in the PLZF part of the fusion protein (Figure 1.4C). HDAC inhibitors like trichostatin A (TSA) overcome this suppressive effect and synergize with ATRA to induce transcriptional activation. The transforming properties of PLZF-RARα have been confirmed in transgenic mice which develop chronic myeloid leukemia-like disease [18,39]. *RARα-PLZF* transgenic mice do not develop leukemia but *PLZF-RARα/RARα-PLZF* double transgenic mice develop leukemia with APL characteristics instead of CML. This suggests that both fusion proteins contribute to the APL phenotype [40].

Only a few patients have been described with NPM-RARα or NuMA-RARα positive APL and these leukemias were responsive to ATRA [84,100]. whereas the only described STAT5b-RARα leukemia was not [2].

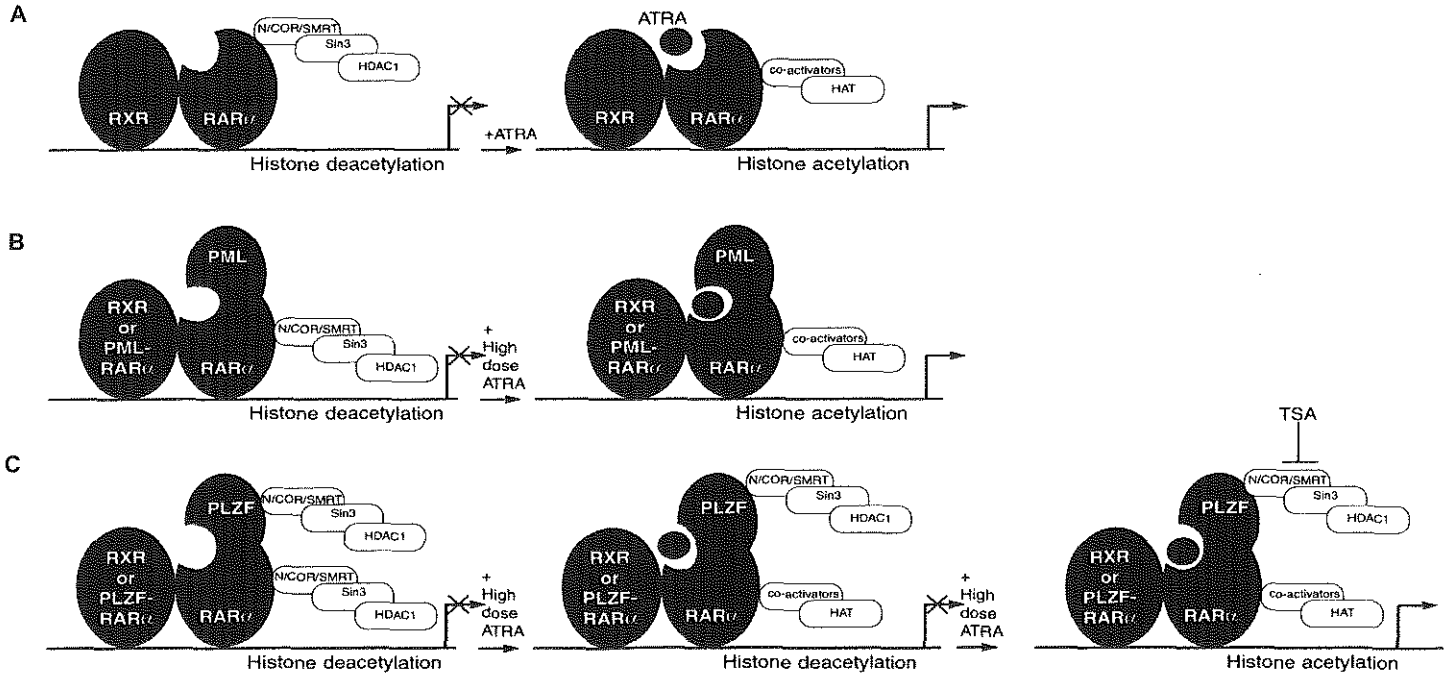

Figure 1.4. Transcriptional activation by wild-type RARα, PML-RARα and PLZF-RARα.

(A) The unliganded RXR/RARα heterodimer represses transcription by recruitment of a corepressor complex, containing proteins like N-CoR, SMRT, SIN3 and HDAC1 resulting in histone deacetylation. Upon binding of ATRA, the RXR/RARα heterodimer releases the corepressor complex and binds a coactivator complex with histone acetylase (HAT) activity resulting in transcriptional activation. (B) Comparable to wild-type receptors, the PML-RARα fusion protein interacts with the corepressor complex. In contrast to wild-type receptors, physiological doses of ATRA do not induce the release the corepressor proteins. Only in the presence of high-dose of ATRA the corepressors are replaced by coactivators allowing transcriptional activation. (C) PLZF-RARα lacks the response to high-dose ATRA due to an ATRA-insensitive binding site for corepressors in the PLZF part of the fusion protein. HDAC inhibitors like trichostatin A (TSA) overcome this suppressive effect and synergize with ATRA to induce transcriptional activation.

1.7 Relevant ATRA-response genes for APL

Although silencing of RARα target genes may be an important factor for the transforming properties of *RARα* fusion genes, it is not yet clear which target genes are critical for leukemogenesis. Various ATRA-response genes have been identified in cells that are likely to be involved in the deregulation of differentiation. C/EBPε expression is rapidly induced in APL cells by retinoic acid and a RARE was identified in the promoter. This gene is of relevance for terminal granulocytic differentiation as *C/EBPε* knock out animals lack functionally active granulocytes [19,70,78]. A second gene that is rapidly induced by retinoic acid in APL cells is $p21^{waf1/CIP1}$, a cyclin-dependent kinase inhibitor that is involved in the regulation of the activity of several cyclin-dependent kinases [10,62,95]. As for C/EBPε, a RARE was identified in the human *p21* promoter [14].

1.8 Outline of this thesis

In this thesis, studies on the effect of RARα fusion proteins on cellular differentiation and gene transcription are described. In Chapter 2, a patient with PLZF-RARα positive APL is presented. In *in vitro* and *in vivo* experiments we studied whether the combined use of ATRA and G-CSF may overcome the maturation block of the leukemic cells and induce granulocytic maturation. The therapeutic use of ATRA is frequently combined with dexamethasone. Potential interference of dexamethasone with ATRA has not been examined. In the experiments described in Chapter 3 we assessed the effects of dexamethasone on the ATRA-inhibited proliferation, differentiation induction and thrombomodulin expression in PML-RARα positive APL cells.

In Chapter 4 we present experiments showing a direct ATRA-responsive gene, *Id2*, and describe the functional role of this gene in leukemic cell lines. Finally, in Chapter 5 we found an additional direct ATRA-responsive gene, *Id1*, which belongs to the same *Id* gene family. The ATRA-induced transcriptional activation of the *Id1* promoter was examined.

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CHAPTER 2

Complete remission of t(11;17) positive acute promyelocytic leukemia induced by all-*trans* retinoic acid and G-CSF

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ABSTRACT

The combined use of retinoic acid and chemotherapy has led to an important improvement of cure rates in acute promyelocytic leukemia. Retinoic acid forces terminal maturation of the malignant cells and this application represents the first generally accepted differentiation-based therapy in leukemia. Unfortunately, similar approaches have failed in other types of hematological malignancies suggesting that the applicability is limited to this specific subgroup of patients. This has been endorsed by the notorious lack of response in acute promyelocytic leukemia bearing the variant t(11;17) translocation. Based on the reported synergistic effects of retinoic acid and the hematopoietic growth factor G-CSF, we studied maturation of t(11;17) positive leukemia cells using several combinations of retinoic acid and growth factors. In cultures with retinoic acid or G-CSF the leukemic cells did not differentiate into mature granulocytes, but striking granulocytic differentiation occurred with the combination of both agents. At relapse, the patient was treated with retinoic acid and G-CSF prior to re-induction chemotherapy. With retinoic acid and G-CSF treatment alone, complete granulocytic maturation of the leukemic cells occurred *in vivo,* followed by a complete cytogenetical and hematological remission. Bone marrow and blood became negative in FISH analysis and semi quantitative PCR showed a profound reduction of PLZF-RARα fusion transcripts. This shows that t(11;17) positive leukemia cells are not intrinsically resistant to retinoic acid, provided that the proper costimulus is given. These observations may encourage the investigation of combinations of ATRA and hematopoietic growth factors in other types of leukemia.

INTRODUCTION

In more than 95% of the cases of acute promyelocytic leukemia (APL) a balanced t(15;17)(q22;q21) chromosome translocation is present that fuses the *PML* (for *promyelocytic leukemia*) and *retinoic acid receptor-α* (*RARα*) genes[1-7]. The resulting PML-RARα fusion protein is implicated in the leukemic transformation of the cells in a dominant fashion [5-14]. APL cells respond to treatment with the vitamin A derivative all-*trans* retinoic acid (ATRA) with terminal granulocytic differentiation followed by cell

death, and treatment with ATRA alone may induce complete remissions in more than 80% of the cases [15-18]. Remissions induced with ATRA alone are short-lived, but combination of ATRA with chemotherapy has improved durable disease-free survival up to 75% [19,20]. The additive value of ATRA and chemotherapy probably reflects the disparate modes of action of maturation induction and cytotoxic treatment. Unfortunately, as yet, similar approaches have failed in other types of leukemia. Even in cases of APL bearing the variant t(11;17)(q23;q21) translocation, which *represents a fusion of the RARα gene to another gene named promyelocytic* leukemia zinc finger (*PLZF*) [21], treatment with ATRA does not induce terminal differentiation, and complete remissions cannot be achieved with ATRA alone [22,23]. Although one patient has been reported with a good response on ATRA and one course of chemotherapy [24], t(11;17) positive leukemia is generally considered to have a poor prognosis. Interestingly, the patient that responded well to therapy [24] was randomized to receive G-CSF at completion of chemotherapy, and a role for G-CSF can therefore not be excluded in this case.

In vitro studies have shown that induction of differentiation of PML-RARα positive cells by ATRA can be enhanced when G-CSF is applied as a costimulus [25,26]. The basis of this synergistic effect is not known and since treatment with ATRA alone is sufficient to induce granulocytic maturation in t(15;17) positive leukemia, the combination of ATRA and G-CSF has not been extensively examined clinically. Here, we present a patient with a t(11;17) positive acute promyelocytic leukemia in whom we evaluated whether the combined use of ATRA and G-CSF could overcome the maturation block of the leukemic cells.

METHODS

Case report

A 31-year-old male was referred with a white blood cell count (WBC) of 69 x 10^9/L, 128 x 10^9/L platelets and a Hb of 5.4 mmol/L. The bone marrow and blood contained more than 90% leukemic cells that varied morphologically from promyelocytes to metamyelocytes. Several leukemic cells contained multiple small bright red granules, sometimes together with more basophilic larger granules, other cells were hypogranulated. Auer rods were frequently observed, either as single rods or as faggots, and cells with pseudo-Pelger nuclei were present. The immunophenotype of the cells was CD13+, CD33+, myeloperoxidase+, CD14-, CD15-, CD34-, CD117-, TdT- and HLA-DR-. A diagnosis of AML-M3 was made according to the French-American-British-classification [27].Treatment with ATRA (45 mg/m^2/day) was initiated, but was discontinued at day 7 when cytogenetic analysis revealed a t(11;17)(q23;q21) chromosomal translocation that was confirmed by fluorescence in-situ hybridization (FISH). Three cycles of chemotherapy were applied according to the AML-29 protocol of the Dutch-Belgian Hematology-Oncology Group (HOVON) and the Swiss Cancer Leukemia Group (SAKK). The first cycle consisted of cytosine-arabinoside (Ara-C) (200 mg/m^2/day per continuous infusion for 7 days) and idarubicin (12 mg/m^2 bolus injection on days 5 through 7). The second cycle consisted of Ara-C (1000 mg/m^2, twice daily for 5 days) and amsacrine (120 mg/m^2/day on day 3 through 5). The third cycle consisted of etoposide (100 mg/m^2/day for 5 days) and mitoxantrone (10 mg/m^2/day for 5 days). The leukemia did not respond to the first cycle, but following the second cycle, the patient entered a complete hematological and cytogenetic remission. In addition, the bone marrow and blood became PCR negative for the PLZF-RARα fusion transcript. After the third cycle of chemotherapy, the patient remained in an unmaintained complete remission for 11 months when he presented with a medullary relapse. The bone marrow contained 20% leukemic cells, the white blood cell count was 3.7 x 10^9/L with no apparent leukemic cells in the differential count, platelets were 95 x 10^9/L and the hemoglobin value was 8.8 mmol/L. At this time, cytogenetic analysis of a bone marrow sample revealed 1 among 50 metaphases to be t(11;17)(q23;q21) positive. Interphase FISH showed 15% t(11;17) positive cells in the bone marrow, whereas the number in the peripheral blood was not above background (4%). Re-induction treatment was started with a combination of ATRA plus G-CSF following informed consent, prior to

chemotherapy.

In vitro proliferation and differentiation

At first presentation, fresh leukemic cells were obtained from the blood (containing more than 90% leukemia cells) by Ficoll-Isopaque density centrifugation (d=1.077). Cells were washed and kept at 37^0 C in a completely humidified 5% CO_2 atmosphere in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 2mM glutamine (Gibco) and 10% fetal calf serum (FCS, Gibco). For differentiation studies, cells were cultured in this medium supplemented with either 10^{-6}M ATRA (Sigma, St Louis, MO), 0.1 μg/ml G-CSF (Amgen, Thousand Oaks, CA), or a combination of ATRA and G-CSF. At several time points, cell numbers were counted and cytospin preparations were made for cytological examination.

PCR analysis

The breakpoint in the *PLZF* and *RARα* genes in the leukemic cells was determined by sequencing of a PCR fragment generated with PLZF and RARα specific primers. The breakpoint was located in the fourth intron of the *PLZF* and the second intron of the *RARα* gene. For follow-up monitoring, a more sensitive nested RT-PCR was developed both for PLZF-RARα and RARα-PLZF amplification. Reverse cDNA transcription was performed on CsCl-cushion purified RNA, and nested PCR was performed with two times 30 cycles of 1 min. At 94°C, 1 min. at 46°C and 1 min. at 72°C in 2.0 mM $MgCl_2$ buffer. PLZF-RARα transcripts were amplified with oligonucleotides 5'GGA GCC AAC TCT GGC TGG G3' and 5'CAT GTT CTT CTG GAT GCT GC3' for the first PCR and 5'TCG GAG AGC AGT GCA GCG TG3' and 5'GGC GCT GAC CCC ATA GTG GT3' for the nested PCR. For RARα-PLZF, oligonucleotides 5'GGC CAG CAA CAG CAG CTC CT3' and 5'TTT GAG AGC CGT GTG GCT G3' were used for the first PCR and 5'GGT GCC TCC CTA CGC CTT CT3'and 5'TGC GCT CTG CGC CTG GAAG C3' for the nested PCR. The sensitivity of the PLZF-RARα PCR was 1 positive cell in 10^4 negative cells, and the sensitivity of the RARα-PLZF RT-PCR was 1 positive cell in 10^5 negative cells as assessed with serial dilutions of t(11;17) leukemic cells with t(11;17) negative NB4 cells. To verify proper RNA isolation and reverse transcription, a parallel PCR was performed on each sample using primers specific for the nonrearranged RARα transcripts (5'CAG CAC CAG CTT CCA GTT AG3' and 5'GGC GCT GAC CCC ATA

GTG GT3'). PCR products were separated on 1.5% agarose gels and their identity was confirmed in Southern blots using radiolabelled oligonucleotide probes spanning the *PLZF-RARα* and *RARα-PLZF* breakpoints.

FISH analysis

The numbers of leukemic cells in sequential bone marrow and blood samples were also monitored by FISH analysis of Interphase nuclei. After incubation with biotin and digoxigenin-labelled cosmid probes of the *RARα* and *NCAM* genes (kindly provided by Dr. F. Birg, Institut Paoli-Calmettes, Marseilles, France), slides were incubated with fluorescein-isothiocyanate (FITC) and Texas red-conjugated secondary antibodies (Boehringer, Mannheim, Germany). Nuclei were visualized with 4,6 diamidino-2-phenylindole (DAPI, Sigma). The presence of the t(11;17) was visible as a fusion spot formed by the colocalization of red and green signals. The background, which represents the percentage of signal colocalization in cells without the t(11;17) translocation, was maximally 5% as determined on bone marrow and blood samples from 10 non-t(11;17) positive acute leukemia patients (mean=2.7% +/- 1.8, range =0-5%), 16 patients with MDS (mean=1.6% +/- 1.2, range=0-4%) and 5 healthy donors (mean=0.72% +/- 0.9 range =0-2%).

RESULTS

In vitro proliferation and differentiation

To test the *in vitro* response of the t(11;17) positive leukemia cells to ATRA and G-CSF, nucleated cells were isolated from the blood at first diagnosis, containing more than 90% leukemic cells. The cells were cultured in medium supplemented with G-CSF (0.1 μg/ml), ATRA (10^{-6} M) or G-CSF plus ATRA. In medium alone and in cultures with G-CSF, cell numbers doubled over a 7-day period, whereas in cultures with ATRA or ATRA plus G-CSF, no significant increase of cell numbers was observed (Figure 2.1). Cytospin preparations from the same cultures revealed that the cells incubated in medium remained promyelocytic throughout the culture period (14 days), while cells cultured with G-CSF or ATRA showed some differentiation towards metamyelocytes (Figure 2.2, Table 2.1). The limited differentiation in response to ATRA is in concordance with previous reports [22,23] and confirms the insensitivity of the t(11;17) positive leukemia cells to ATRA. Strikingly, after 1 week of culture with the combination of ATRA and G-CSF, the majority of the cells showed complete differentiation with nuclear segmentation, frequently in association with prominent Auer rods (Figure 2.2E and Table 2.1). The complete differentiation of the t(11;17) positive cells raised the question whether the combination of ATRA and G-CSF could be of clinical use in case of a relapse.

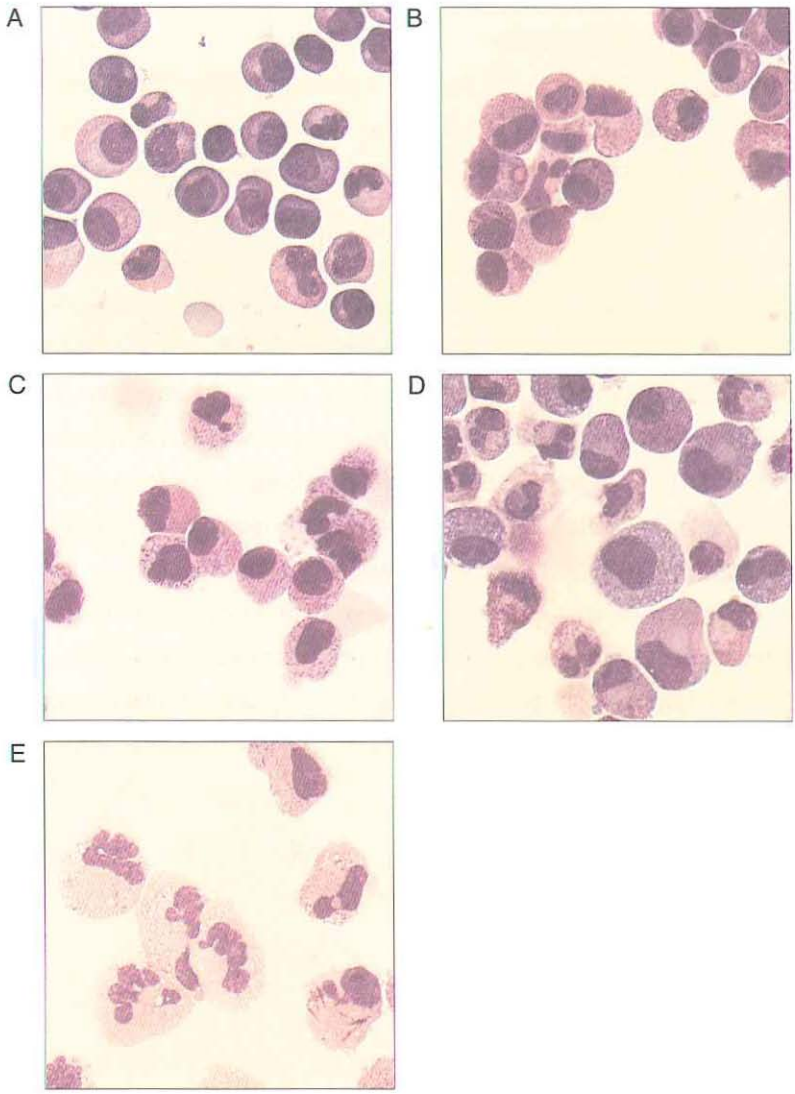


Figure 2.2. Morphology of t(11;17) positive leukemia cells cultured with G-CSF and ATRA.
Mononuclear cells, consisting of more than 90% of leukemic cells, were isolated from the blood at first diagnosis and cultured under various conditions for up to 14 days. Cytospins were made after various time intervals and stained with May-Grünwald-Giemsa. Depicted are uncultured cells (A) and cells that were grown for one week in medium (B), 10^{-6} M ATRA (C), 0.1 μg/ml G-CSF (D) and ATRA plus G-CSF (E).

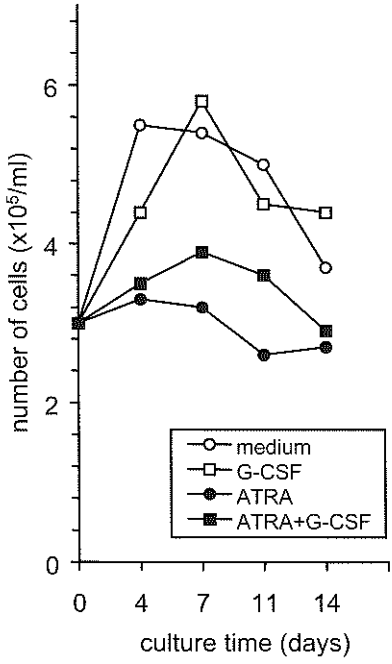


Figure 2.1. Proliferation of t(11;17) positive leukemia cells in response to G-CSF and ATRA.

Mononuclear cells, consisting of more than 90% of leukemic cells, were isolated from the peripheral blood at first diagnosis. Cells were cultured at 2 x 10^5 cells/ml with medium alone, G-CSF (0.1 μg/ml), ATRA (10^{-6} M) or with a combination of ATRA and G-CSF. At the indicated times cell numbers were counted. Values represent the mean of triplicate measurements.

Table 2.1. *In vitro* differentiation of t(11;17) positive leukemia cells at first diagnosis with ATRA and G-CSF.

	No culture	Medium	G-CSF	ATRA	ATRA+G-CSF
Lymphocytes	0.0*	2.8	5.8	2.0	2.8
Promyelocytes	**93.4**	**90.4**	**38.4**	1.0	8.4
(Meta)myelocytes	0.4	1.0	**41.8**	**84.6**	13.6
Band cells	2.4	0.2	7.4	4.0	6.2
Neutrophils	2.2	5.6	6.6	8.4	**69.2**

*Numbers indicate % of cells. After 1 week of culture of leukemic cells, cytospin slides were stained with May-Grünwald-Giemsa. Five hundred cells were differentiated for each slide. ATRA was used at 1μM, G-CSF at 0.1 μg/ml.

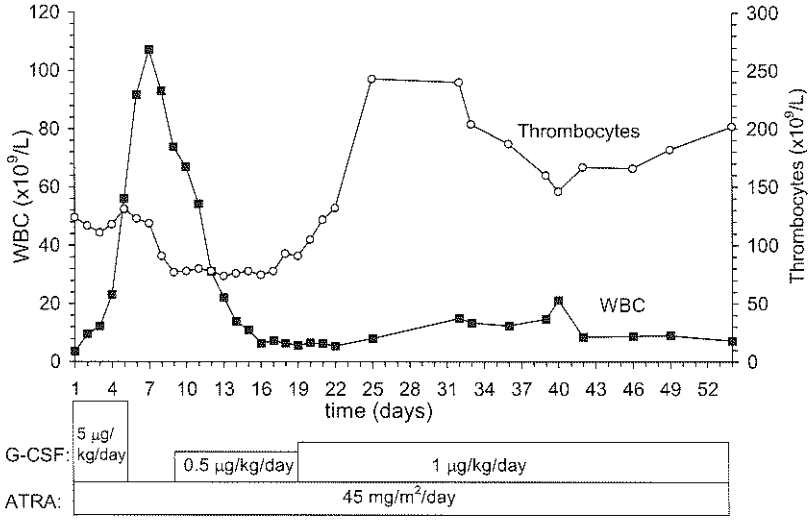
Treatment of relapse with G-CSF + ATRA

Because of the *in vitro* differentiation of the leukemic cells in response to ATRA and G-CSF, treatment with the combination of both agents was applied prior to reinduction chemotherapy at the time of a relapse at 14 months after presentation.

To evaluate a potential stimulatory effect of ATRA and G-CSF on clonogenic leukemia growth, bone marrow mononuclear cells obtained at relapse (containing 15% FISH-positive leukemia cells) were cultured in methylcellulose with titrated amounts of G-CSF (0-100 ng/ml), in the presence and absence of ATRA (10^{-6} M). In cultures with G-CSF, colony formation by the bone marrow cells was similar to the number of colonies in cultures of bone marrow cells from healthy donors. In cultures with ATRA plus G-CSF, colony numbers were considerably lower than in cultures with G-CSF alone (data not shown). Thus the addition of G-CSF and ATRA did not stimulate detectable clonogenic leukemia growth *in vitro*.

Treatment with a combination of ATRA (45 mg/m^2/day) and G-CSF (5 μg/kg/day) was started (Figure 2.3). After two days the WBC began to rise, reaching 55 x 10^9/L at day 5 (Figure 2.3A). At this time, the G-CSF treatment was interrupted, but ATRA treatment was maintained. The WBC continued to rise for two additional days, and then rapidly declined. At day 9, G-CSF treatment was restarted at a ten-fold lower dose (0.5 μg/kg/day). Cell numbers continued to decrease to below 10 x 10^9/L at day 16, and the dose of G-CSF was adjusted to 1 μg/kg/day. Subsequently, the white blood cell counts stabilized at 10-15x10^9/L. Cytological examination revealed a transient appearance of promyelocytes in the blood from day 4, which peaked at day 6 and had disappeared by day 11 (Figure 2.3B). More mature (meta)myelocytes appeared after day 5, peaked at day 7 and normalized after day 14. The number of mature granulocytes was elevated from day 4 to day 15 with peak levels around day 11 of treatment. A normal differential was seen on day 14 and beyond. Platelet counts dropped from 124 to 80 x 10^9/L between days 1 and 18, but subsequently rose to stabilize at around 200 x 10^9/L, concurrently with the disappearance of t(11;17) FISH positive cells from bone marrow and blood (Figure 2.3A, Table 2.2). The Hb gradually dropped from 8.8 mmol/L before treatment to 6.5-7.0 at day 22, and subsequently stabilized at 7.5-8.0 mmol/L from day 25 (not shown).

A


WBC (x10⁹/L)
Thrombocytes (x10⁹/L)
Thrombocytes
WBC
time (days)
G-CSF:
5 µg/ kg/day
0.5 µg/kg/day
1 µg/kg/day
ATRA:
45 mg/m²/day

B

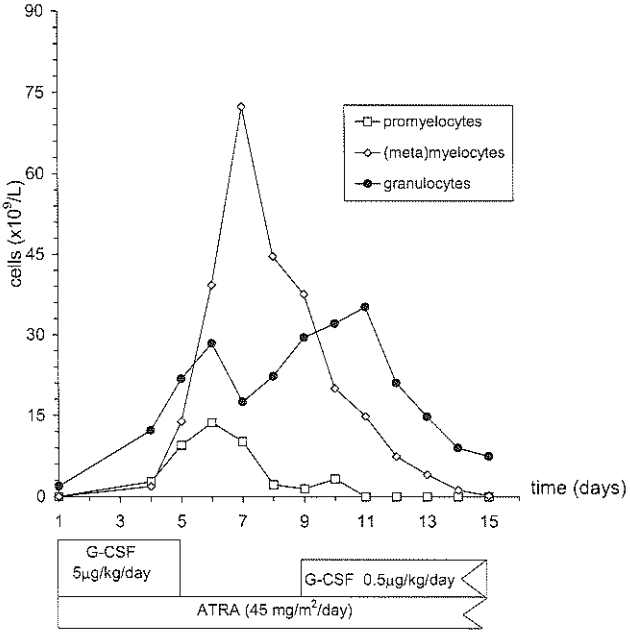

cells (x10⁹/L)
promyelocytes
(meta)myelocytes
granulocytes
time (days)
G-CSF 5µg/kg/day
G-CSF 0.5µg/kg/day
ATRA (45 mg/m²/day)

Figure 2.3. Peripheral blood counts during ATRA plus G-CSF treatment.

During ATRA and G-CSF treatment platelet and white blood cell counts (WBC) were determined (A). Cytological differentiation of peripheral blood smears was assessed daily. The percentage of promyelocytes, (meta)myelocytes, band cells and segmented granulocytes was scored. From the total white blood cell counts, the absolute numbers of cells with the various stages of differentiation was calculated (B). At the bottom, the treatment regimen is indicated.

Table 2.2. Percentage of t(11;17) FISH-positive cells in sequential bone marrow samples during ATRA and G-CSF treatment.

ATRA and G-CSF treatment (days)	% FISH positive cells
before treatment	
-14	15.0
-4	15.2
after start of treatment	
5	11,6
12	9.6
15	Negative
19	Negative
25	Negative
32	Negative
39	Negative
46	8.3
54	8.5

FISH was determined on interphase nuclei. For each value, at least 300 nuclei were assessed. Neg. indicates values below detection level (4%).

Monitoring of leukemic cells in marrow and blood during ATRA plus G-CSF treatment

At day 7, when the white blood cell count peaked, t(11;17) interphase FISH became positive in 20% of the peripheral blood cells. Since the bone marrow revealed 15% FISH positive cells before treatment (Table 2.2), and the peripheral blood values at that time were below background (4%), this suggested that the treatment with ATRA and G-CSF had mobilized both normal and malignant cells from the bone marrow to the blood.

Sequential bone marrow samples analyzed by FISH showed 12% t(11;17) positive cells at day 5 and 10% positive cells at day 12. Subsequent values at days 15 to 39 were below background. Interestingly, at day 12, FISH positivity was seen predominantly in cells with segmented nuclei (visualized by DAPI-staining), indicative of granulocytic differentiation of t(11;17) positive leukemia cells. To document this, concurrent FISH and morphological staining [28] of the same cytospin slides was done and FISH-positive cells were shown to be morphologically mature granulocytes (Figure 2.4). This provides further evidence for the *in vivo* maturation of leukemia cells.

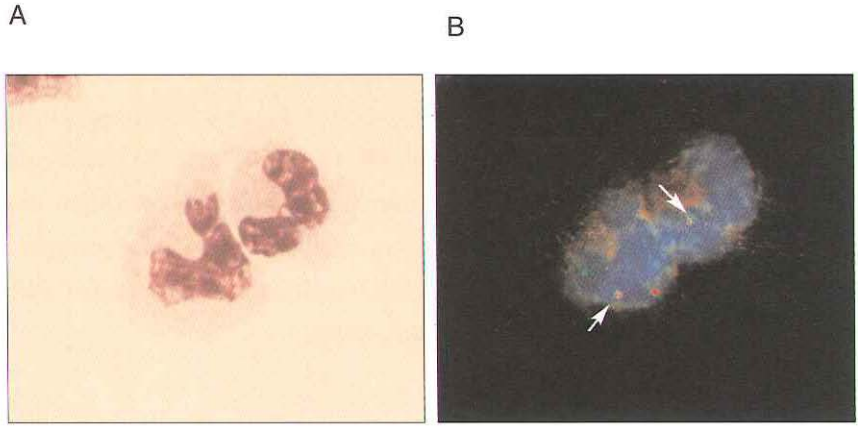


Figure 2.4. *In vivo* maturation of t(11;17) FISH positive leukemia cells.

Twelve days after initiation of ATRA and G-CSF treatment, FISH-positive cells in bone marrow and blood predominantly showed segmented nuclei (as visualized by DAPI staining) indicative of granulocytic differentiation of the leukemic cells. To establish the morphology of the FISH-positive cells, slides were stained with May-Grünwald-Giemsa (A). The same fields were photographed after hybridization of the slides with labeled FISH probes (B) to obtain dual morphological and FISH staining. The t(11;17) translocation is indicated by the colocalization of red and green signals.

Because of the limited sensitivity of FISH, residual leukemia was also monitored with semi-quantitative RT-PCR using the leukemia-specific *PLZF-RARα* fusion transcript as a target (Figure 2.5). PLZF-RARα expression before ATRA and G-CSF treatment was high in bone marrow (Figure 2.5A), and barely detectable in peripheral blood cells (Figure 2.5B). The levels of PLZF-RARα expression in bone marrow gradually dropped

and became undetectable after eight weeks of treatment (Figure 2.5A). In peripheral blood, PLZF-RARα expression initially rose concomitantly with the leukocytosis, probably due to the mobilization of leukemic cells to the blood but subsequently became negative along with the maturation and disappearance of t(11;17) FISH positive cells (Figure 2.5B) . To see whether the expression of the reverse fusion transcript followed the same pattern, we also performed RT-PCR for the RARα-PLZF transcript (Figure 2.5C and 2.5D). Similar to PLZF-RARα, the expression of RARα-PLZF in the bone marrow continued to drop throughout the treatment (Figure 2.5C) whereas the expression in the peripheral blood cells was down-regulated after an initial increment during leukocytosis (Figure 2.5D). Interestingly, both in the bone marrow and in the peripheral blood, the disappearance of RARα-PLZF transcripts went slower than PLZF-RARα suggesting that the expression level of both fusion transcripts was influenced differentially by the treatment. The cytological, FISH and RT-PCR data are all consistent with a transient phase of mobilization of normal and leukemic cells from the bone marrow to the peripheral blood, followed by maturation and disappearance of the malignant cells, compatible with a complete hematological and partial molecular remission following treatment with G-CSF plus ATRA.

Subsequent clinical course

After 46 days of treatment, reappearance of FISH positivity (4% above background) was seen in the bone marrow indicating that the response had been transient (Table 2.2). Notably, at that time, very low to undetectable PLZF-RARα and RARα-PLZF expression levels were measured (Figure 2.5). Apparently, therapy resistant leukemia cells emerged with a very low expression of both fusion transcripts. At day 54 chemotherapy was started and after allogeneic bone marrow transplantation the patient now remains in complete remission for more than 12 months, with no detectable FISH or PCR signals in bone marrow or blood.

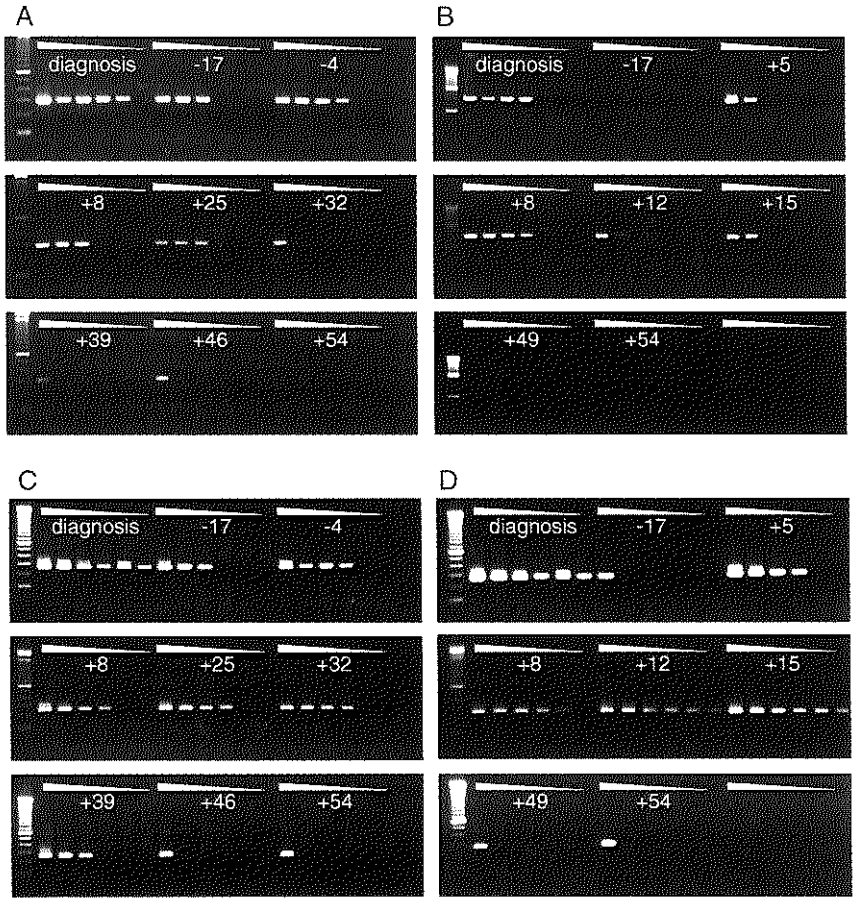


Figure 2.5. PLZF-RARα and RARα-PLZF expression in bone marrow and blood cells during ATRA and G-CSF treatment.

RNA from sequential bone marrow (A,C) and peripheral blood samples (B,D) was obtained and RT-PCR for PLZF-RARα (A, B) or RARα-PLZF (C,D) fusion transcripts was performed. Transcripts were quantified by serial, 10 fold dilutions of the patient cells in t(11;17) negative cells, and subsequent RNA-isolation and RT-PCR. The dilution at which amplification of the transcript is lost indicates the abundance of the fusion transcript. For each sample, an undiluted and five 10-fold dilutions were processed (left to right). Numbers indicate days before (negative numbers) or after the start of treatment. In addition to sequential samples taken at the time of relapse, a sample from the initial first diagnosis was analyzed. To verify proper RNA isolation and reverse transcription, a control amplification was performed on each sample using primers that are specific for unrearranged RARα transcripts (not shown). For uniformity, RNA isolation, reverse transcription and PCR was performed on all samples at the same time. The specificity of the amplification was confirmed by Southern blotting and hybridization with oligonucleotides probes spanning the respective fusion points (not shown). Data are representative of three independent experiments.

DISCUSSION

The application of retinoic acid to the treatment of t(15;17) positive acute promyelocytic leukemia has established that induction of differentiation can be a valuable mean of tumor cell eradication. The additive effect of retinoic acid and cytotoxic treatment on durable disease free survival is probably the result of the targeting of different biological processes by both forms of treatment. So far, therapeutic approaches based on maturation-induction have failed in other types of leukemia, suggesting that the applicability of this type of treatment might be limited to patients with acute promyelocytic leukemia with *PML-RARα* gene fusions.

This report shows that induction of terminal differentiation and a subsequent complete clinical and partial molecular remission may be obtained with retinoic acid in t(11;17) positive leukemia, provided that G-CSF is applied as a costimulus. In t(15;17) positive leukemia, addition of G-CSF is not required for ATRA-induced differentiation and complete remission induction. However, a role for G-CSF cannot be ruled out, as ATRA induces the expression of both G-CSF and the G-CSF receptor in these cells [25,26], which might result in autocrine stimulation.

Retinoid receptors are ligand-dependent transcription factors that directly regulate the expression of target genes by binding to their regulatory DNA-sequences. Which target genes initiate the granulocytic differentiation program in the malignant cells is not well known. Recent studies have provided a mechanism by which the PML-RARα and PLZF-RARα fusion proteins may deregulate the expression of target genes [29-32]. Unliganded retinoic acid receptors inhibit gene expression by recruiting corepressor proteins like N-CoR or SMRT and histone deacetylase to the DNA. This results in histone deacetylation and silencing of the expression of target genes. Upon ligand binding the corepressor complex is released and replaced by a coactivator protein complex with histone acetylation activity, upon which transcription is activated. The release of corepressor proteins from the PML-RARα fusion protein was shown to require higher doses of ligand when compared to the unrearranged RARα receptor, explaining why pharmacological doses are needed to induce differentiation of t(15;17) positive leukemia cells. Interestingly, retinoic acid was unable to completely release the corepressor proteins from the PLZF-RARα fusion protein due to a second binding site for corepressor proteins in the PLZF part of the fusion protein which is not sensitive to

retinoic acid. This explains the insensitivity of t(11;17) positive leukemia to retinoic acid. The synergistic action of ATRA and G-CSF reported here could be explained if activation of G-CSF receptor signaling would lead to the release of corepressor proteins from the PLZF part of the PLZF-RARα fusion protein. This hypothesis is currently being tested.

The effect of ATRA and G-CSF described in this report was significant since it was characterized by a complete hematological and cytogenetical response, a partial molecular response with normalization of bone-marrow morphology and recovery from thrombocytopenia towards normal platelet values. The response was transient, as FISH-positive cells reappeared in the bone marrow after seven weeks of treatment. In analogy, treatment of t(15;17) positive leukemia with ATRA alone does generally not render the patients PCR negative for PML-RARα and does not induce durable remissions. The observed down regulation of both the PLZF-RARα and the RARα-PLZF fusion transcripts in the reappearing leukemia suggests a selective pressure during treatment for low expression of both fusion transcripts. This might suggest that both fusion transcripts play a role in conferring the differentiation signal by ATRA and G-CSF. In addition, these results indicate that both PLZF-RARα and RARα-PLZF were dispensable for the transformed phenotype of the reappearing leukemia cells, possibly due to extra genetic alterations in the resistant cells. The relapse within seven weeks suggests that a shorter period of ATRA plus G-CSF treatment should be given before chemotherapy is started, or that ATRA and G-CSF should be applied concomitantly with the chemotherapy. Although this approach should be confirmed in other t(11;17) positive leukemia patients, this report might warrant the investigation of combinations of ATRA with hematopoietic growth factors in other types of leukemia.

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CHAPTER 3

Dexamethasone does not counteract the response of acute promyelocytic leukaemia cells to all-*trans* retinoic acid

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SUMMARY

Retinoic acid syndrome is a serious condition that may complicate the treatment of acute promyelocytic leukaemia patients. This syndrome may be treated effectively with high-dose corticosteroid therapy and, as a result, many patients with acute promyelocytic leukaemia receive dexamethasone at some point during treatment. We investigated whether dexamethasone would also antagonize the beneficial effects of retinoic acid. In t(15;17)-positive NB4 cells, dexamethasone did not affect the retinoic acid induced differentiation, normalization of PML-nuclear bodies or the induction of thrombomodulin mRNA. Finally, dexamethasone did not inhibit the anti-proliferative effect of retinoic acid but rather showed anti-proliferative activity itself.

INTRODUCTION

Acute promyelocytic leukaemia (APL, FAB-classification AML-M3) represents 5-10% of the cases of acute myelogenous leukaemia in adults and is characterized by a t(15;17) translocation [9]. As a result, the leukaemic cells express a mutant chimaeric PML-RARα fusion protein. Combination treatment with chemotherapy and all-*trans* retinoic acid (ATRA) currently results in long-term disease free survival in up to 75% of the cases [7]. The response to ATRA involves the terminal differentiation of the leukaemic cells. The retinoic acid (RA) syndrome is a serious complication of ATRA treatment and may occur in 15% of patients with varying severity [2,4]. Early recognition of RA syndrome and prompt treatment with corticosteroids should diminish the incidence of ATRA-induced death to <5% [1,2,11]. In this study we investigated whether the beneficial effects of ATRA were also counteracted by dexamethasone, especially since the biological activity of both substances is mediated through binding to receptors belonging to the same superfamily of nuclear hormone receptors.

MATERIALS AND METHODS

Proliferation and differentiation

NB4 cells [6] were treated with all-*trans* retinoic acid (Sigma, St Louis, MO., U.S.A.) or dexamethasone (Sigma). Cells were counted at different time points. Clonogenic growth was measured by plating the cells in semisolid (1.2% methylcellulose; Methocel, Stade, Germany) culture medium (Iscove's modified Dulbecco's medium (IMDM); Gibco). Colony formation was counted after 7 days. Differentiation was assessed on May-Grünwald-Giemsa (MGG) stained cytospin slides. In addition, the nitroblue-tetrazolium (NBT, Sigma) reduction test was performed as described [5].

Immunofluorescence microscopy

Immunofluorescence was performed using the anti-PML monoclonal antibody (PG-M3, Santa Cruz Biotechnology, Santa Cruz, Calif., U.S.A.) and a goat anti-mouse antibody conjugated to fluorescein isothiocyanate (GAM-FITC; Becton Dickinson, Mountain View, Calif., U.S.A.).

Northern blot hybridization

Total RNA was isolated from NB4 cells before and after induction with ATRA and dexamethasone. Northern blots were hybridized to radiolabelled human thrombomodulin (ATCC, no. 61349, Rockville, Md., U.S.A.), CD18 (kindly provided by Dr. D.G. Tenen, Beth Israel Hospital Boston, Mass., U.S.A.) and GAPDH (777bp HindIII-EcoRI fragment) probes.

RESULTS AND DISCUSSION

Dexamethasone does not counteract inhibition of proliferation by ATRA

The effects of dexamethasone (10^{-6} M) and ATRA (10^{-6} M) on the proliferation of NB4 cells were examined by cell counting after various intervals. Dexamethasone alone induced 30% growth retardation at day 7 when compared to cells cultured in medium. ATRA inhibited the proliferation of NB4 cells, which was not counteracted when dexamethasone was added. In a dose-response curve in a colony-forming assay we found that $5x10^{-8}$ M ATRA was sufficient to suppress proliferation, both in the presence

and absence of dexamethasone. This indicated that dexamethasone did not abrogate the inhibitory effect of ATRA on cell expansion but rather had an antiproliferative effect itself. Colony numbers in the presence of dexamethasone were consistently lower (50%) than in cultures with ATRA alone.

Dexamethasone does not abrogate induction of terminal differentiation by ATRA

We investigated whether dexamethasone would interfere with differentiation induction by ATRA in NB4 cells (Figure 3.1). After 48 hours of incubation with ATRA or ATRA plus dexamethasone the cells had a lower nucleus/cytoplasm ratio and showed nuclear segmentation. These alterations were not present in cells grown in medium nor in cells incubated with dexamethasone alone (Figure 3.1A). After 48 hours >90% of the NB4 cells cultured in ATRA or ATRA plus dexamethasone stained positive in the NBT assay, indicating identical granulocytic differentiation (Figure 3.1B).

The effect of dexamethasone on PML-body reorganization [3,12] was assessed by immunofluorescence using an anti-PML antibody. NB4 cells treated with ATRA or the combination of ATRA and dexamethasone showed reappearance of normal PML nuclear bodies which was complete after 4 days. Treatment with dexamethasone alone did not restore normal PML body distribution in NB4 cells (Figure 3.1C). Thus dexamethasone did not suppress the ATRA effect on PML-body normalization.

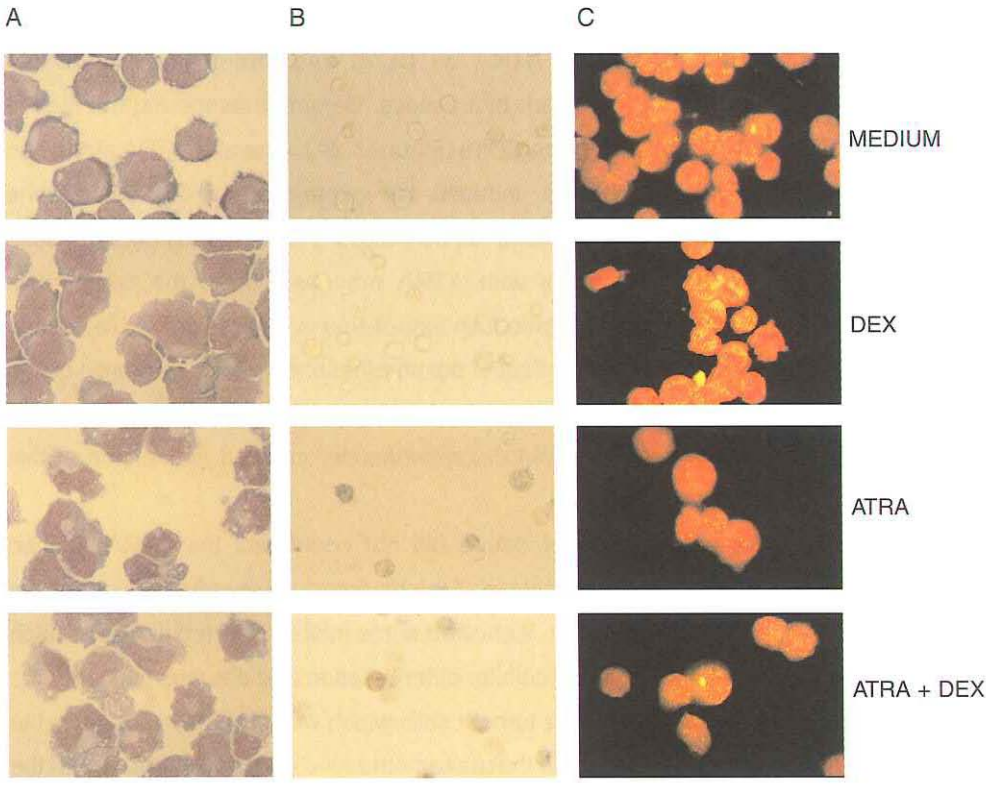

Figure 3.1. Differentiation of NB4 cells in response to ATRA and dexamethasone. NB4 cells were induced with dexamethasone (second row), ATRA (third row) or ATRA + dexamethasone (bottom row) for 48 hours. Cells cultured in medium (top row) were used as a control. Cells were stained with (A) May-Grünwald-Giemsa, (B) nitroblue tetrazolium, and (C) anti-PML antibody.

Dexamethasone does not abrogate the induction of CD18 and thrombomodulin mRNA

The effect of dexamethasone and ATRA on CD18 and thrombomodulin mRNA expression was determined in Northern blot assays. Dexamethasone did not induce expression of the differentiation antigen CD18 (Figure 3.2A), whereas ATRA alone and in combination with dexamethasone, induced the expression of the *CD18* gene indicating again that dexamethasone does not counteract the process of differentiation induction by ATRA. Since treatment with ATRA may ameliorate the coagulation problems in APL patients and thrombomodulin expression in the leukaemic blasts may be involved [10], we investigated the effect of dexamethasone on the expression of this gene. Dexamethasone alone did not induce expression of thrombomodulin (Figure 3.2B), whereas ATRA, with and without dexamethasone, induced expression of this gene.

The experiments show that dexamethasone did not counteract the ATRA-induced proliferation arrest, differentiation induction and thrombomodulin expression in the acute promyelocytic leukaemia cells. Rather, it showed some inhibition of proliferation which was not accompanied by any signs of cellular differentiation and this was similar to the direct antiproliferative effect on various tumour cells which was probably caused by the induction of apoptosis [8]. We conclude that dexamethasone does not interfere with the induction of relevant ATRA-responsive genes involved in granulocytic differentiation of acute promyelocytic leukaemia cells. Therefore, the application of dexamethasone should not compromise the therapeutic effect of ATRA.

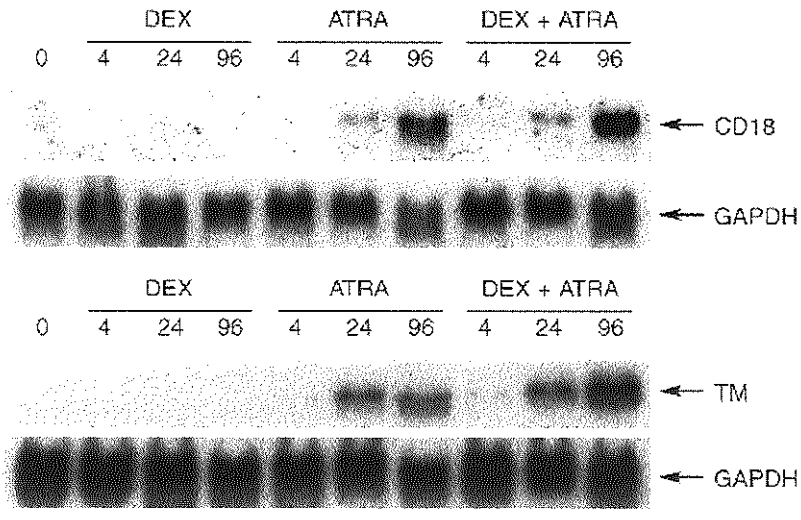

Figure 3.2. CD18 and thrombomodulin mRNA expression in response to ATRA and dexamethasone.

Northern blot analysis was performed using 10 μg of total RNA extracted from NB4 cells after exposure to ATRA, dexamethasone, and ATRA + dexamethasone for 0, 4, 24 and 96 hours. The blot was hybridized with a labelled cDNA fragment of the *CD18* gene (A) or *thrombomodulin* (B). A GAPDH probe was used as a control for equal loading of RNA.

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CHAPTER 4

Id2 is a direct target gene of retinoic acid receptors in acute promyelocytic leukemia cells

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ABSTRACT

Acute promyelocytic leukemia (APL) is characterized by a differentiation arrest through the silencing of direct retinoic acid receptor target genes caused by the PML-RARα fusion protein. The target genes that are critically involved in deregulation of the proliferative and differentiation response of the cells to retinoic acid are yet to be elucidated. Here, we report that Id2 mRNA expression is induced by retinoic acid in APL cells. Induction starts within 30 minutes and is not affected by the protein translation inhibitor cycloheximide, indicating that *Id2* is a direct target gene. To investigate the effect of Id2 on proliferation and differentiation of APL cells, an Id2 expression vector was transfected into the maturation-competent APL cell line NB4. Transfected cells showed a severely decreased proliferation, resulting in 85-90% inhibition of clonogenic growth. A possible role for Id2 during myeloid differentiation was also investigated in non-APL cells. Id2 was overexpressed in the myeloid cell line 32D which resulted in a slower growth rate accompanied by an increase of differentiated cells.

INTRODUCTION

Retinoic acid exerts a striking effect on the growth and differentiation of hematopoietic cells. The biological effects of retinoic acid are mediated through the retinoic acid receptors (RARs) and the retinoic X receptors (RXRs), nuclear receptors that are members of the steroid/thyroid hormone receptor superfamily of ligand-activated transcription factors [1-3]. Retinoic acid receptors bind as heterodimers to specific enhancer sequences in the DNA, so-called retinoic acid response elements (RAREs), resulting in transcriptional activation of target genes in the presence of ligand [2,4,5,6]. Several studies have reported on the role of RARs in normal hematopoiesis. Retinoic acid modulates the growth of hematopoietic precursor cells *in vitro* in the presence of hematopoietic growth factors. It has been shown that retinoic acid has different effects on hematopoietic cells depending on their maturation state. Retinoic acid appears to prevent and/or delay the differentiation of very immature hematopoietic progenitor cells (lin-/sca+/c-kit+ bone marrow cells) [7,8]. In more mature lineage-committed precursor cells (unfractionated or CD34+

purified bone marrow cells), retinoic acid enhances the granulocytic differentiation [9,10].

The importance of RARs for hematopoiesis is illustrated by the effect of *RARα* mutations. Murine bone marrow cells overexpressing a dominant negative truncation mutant of *RARα* (*RARα403*) are blocked at the promyelocytic stage of development and do not differentiate in mature granulocytes [3,11]. Acute promyelocytic leukemia (APL) is in more than 98% of the patients characterized by a reciprocal chromosomal translocation that fuses the PML gene with the *retinoic acid receptor α* (*RARα*) gene resulting in the expression of a PML-RARα fusion protein [12-16]. PML-RARα-positive leukemia is uniquely sensitive to treatment with all-*trans* retinoic acid (ATRA), which induces terminal differentiation of the leukemic cells towards mature granulocytes with a limited life span. Application of retinoic acid in APL has clearly proven that forced differentiation of leukemic cells may translate into significantly better cure rates [17]. Although remissions induced with ATRA alone are short-lived due to the emergence of retinoic acid resistant cells, a major therapeutic contribution of retinoic acid to improve long-term disease free survival rates has been shown when it is combined with chemotherapy.

The PML-RARα chimaeric protein has several altered features when compared to normal RARα. The fusion protein is distributed in an abnormal, microspeckled pattern in the nucleus [18-20]. Moreover, it can bind to DNA as a heterodimer with RXR but also as a homodimer independently of RXR [21,22]. When compared to normal RARα, PML-RARα binds ATRA with the same affinity and specificity but shows enhanced interaction with corepressor proteins that inhibit transcriptional activation in the absence of ligand [23-25]. Pharmacological doses of ATRA are required for the fusion protein to initiate transcription of target genes [26-29]. The phenotype of transgenic mice overexpressing the fusion gene has confirmed the involvement of the PML-RARα fusion protein in leukemogenesis. These mice develop AML with promyelocytic features [30-32].

Deregulation of RARα-target genes may be an important factor for the transforming properties of the PML-RARα fusion protein. However, it is not clear which target genes are critical for leukemogenesis. To date, only a few primary ATRA responsive genes, like the *p21* $^{waf1/cip1}$ cyclin-dependent kinase inhibitor [33] and the transcription factor *CCAAT/enhancer-binding-protein-ε* (*C/EBPε*) [34], have been

reported to play a role in the regulation of differentiation and proliferation of myeloid cells. Various known and unknown transcription factors were found to be potentially interesting during ATRA-induced differentiation by differential display and subtractive PCR applied to myeloid cells treated for brief periods with ATRA. One of the identified transcription factors was Id2. Id proteins function as dominant negative regulators of a class of transcription factors, the basic helix-loop-helix proteins, which makes them interesting to investigate. In this report we identify *Id2* as a direct ATRA responsive gene in APL cells.

MATERIALS AND METHODS

Cell culture

Bone marrow mononuclear cells of three APL patients were isolated at diagnosis by lysis of erythrocytes in NH_4Cl buffer. Indirect immunostaining with a monoclonal anti-PML antibody (PG-M3, Santa Cruz Biotechnology, Santa Cruz, CA) showed a microspeckled pattern characteristic for the PML-RARα fusion protein [18-20]. Cytogenetic analysis showed that all three patients were positive for the t(15;17)(q22;q21) chromosome translocation. Patient and NB4 cells [35] were cultured in RPMI 1640 medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine (Gibco). The interleukin-3 (IL-3)-dependent murine myeloid cell line 32D [36] was maintained in RPMI 1640 medium supplemented with 10% FCS and 10 ng/ml of murine IL-3. Phoenix E. cells, kindly provided by Dr. G. P. Nolan, Stanford University Medical Center, Stanford, California, USA, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FCS. Cultures were incubated at 37°C in a 5% CO_2 completely humidified atmosphere.

Northern blotting

NB4 cells were induced with all-*trans* retinoic acid (Sigma, St Louis, MO) at a final concentration of 10^{-6} M and 32D cells were induced with human G-CSF (Amgen, Thousand Oaks, CA) at a final concentration of 100 ng/ml and harvested at various time points. Where appropriate, cycloheximide (ICN, Costa Mesa, CA) was used at a final concentration of 4 μg/ml which was added 30 minutes before the start of

induction with ATRA. Total cellular RNA was isolated by guanidinium-isothiocyanate lysis and centrifugation on a 5.7 M cesium chloride cushion. PolyA$^+$ RNA was isolated from total RNA by using a mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). 10 μg of total RNA or 1μg of PolyA$^+$ RNA was size-fractionated by 1% agarose-formaldehyde gel electrophoresis and transferred onto Hybond N$^+$ nylon membranes (Amersham, Buckinghamshire, UK). The filters were pre-hybridised and hybridized at 65°C overnight in phosphate buffer (2N NaH_2PO_4, 2N Na_2HPO_4), 7% sodium dodecyl sulphate (SDS), 1 mM EDTA pH=8.0 and 1% BSA). DNA probes were labelled with [^{32}P]α-dATP by random primed labelling (Boehringer, Mannheim, Germany). After hybridisation, the filters were washed in 0.2xSSC/ 0.2% SDS for 15 minutes at 65°C. The E2A probe was provided by Dr. S. Stegman, NKI, Amsterdam, The Netherlands and murine Id2 cDNA was a gift from Dr. X.H. Sun, NYU, New York, USA. As a control for equal loading, filters were stripped and hybridized to a 777bp HindIII-EcoRI human GAPDH fragment.

Expression vectors

The Enhanced Green Fluorescence protein (EGFP) expression construct pEGFP-C1 was purchased from Clontech Laboratories (Palo Alto, CA). From this, pCMV-C1 was made by deleting the NheI-XhoI fragment which corresponds to the EGFP fragment. pCMV-Id2 was constructed by cloning the human *Id2* coding sequence (obtained by PCR and confirmed by sequencing) into the BamHI site of pCMV-C1. In pCMV-αId2, Id2 cDNA was cloned in the anti-sense orientation. The S001-EGFP retroviral vector which contains a multiple cloning site in front of an IRES-EGFP sequence was kindly provided by Dr. G.P. Nolan, USA and Dr. H. Spits, NKI, Amsterdam, the Netherlands [55]. The S001-EGFP-Id2 was constructed by cloning the human *Id2* cDNA sequence into the BamH1 site of S001-EGFP.

Transfection of NB4 cells and flow cytometry

Cells were split to 1 x 10^5/ml 24 hours prior to transfection. At the time of transfection, 5 x 10^6 cells were washed with RPMI without FCS at room temperature. Cells were resuspended in 700 μl RPMI with 20μg pEGFP-C1 and 20μg pCMV-C1, pCMV-Id2 or pCMV-αId2 and placed in a 0.4 cm electroporation cuvette (BioRad, BioRad Laboratories, Richmond, CA). After electroporation (300V, 960μF), cuvettes were

transferred to ice for 15 minutes. The cells were then transferred to 10 ml of RPMI 1640 supplemented with 10% FCS. Transfected cells were sorted 24 hours after electroporation with a FACS Vantage flow sorter (Becton Dickinson Immunocytometry System Inc., San Jose, CA) or FACS Calibur flow cytometer (Becton Dickinson). Cells were gated on low angle (Forward Scatter, FSC) vs 90 angle (Side Scatter, SSC), EGFP expressing cells were gated in a FL1 versus FL3 biparametric graph. 7-Aminoactinomycin D (1μg/ml) (Molecular Probes, Leiden, the Netherlands) was added to the cells to select viable cells and DNAse (10 μg/ml, Euro-Biochem, Bierges, Belgium) to prevent clogging.

Colony Forming assay of NB4 cells

Clonogenic growth of FACS sorted NB4 cells was measured by plating 500 cells/ml in semisolid (1.2% methylcellulose; Methocel, Stade, Germany) culture medium (Iscove's modified Dulbecco's medium, IMDM, Gibco) supplemented with 20% fetal calf serum. Colonies (clusters containing more than 50 cells) were counted after 7 days of culture.

Preparation of retrovirus and transduction of 32D cells

The retrovirus producer cells, Phoenix E., were split to 2 x 10^6/ml 24 hours prior to transfection. Chloroquine was added to each plate 5 minutes before transfection at a final concentration of 25 μM. 10 μg DNA was transfected in the cells by the calcium phoshate coprecipitation technique. Chloroquine was removed 9 hours after transfection and fresh medium was added to the cells supplemented with 10% FCS and IL-3 (10 ng/ml). The supernatants were collected from the cells 48 hours post-transfection. 2,5 x 10^5 32D cells were repeatedly transduced with 1.5 ml supernatant on retronectin coated plates. The cells were replaced from the retronectin coated plates 48 hours afer transduction and the virus was removed by washing the cells three times with phosphate-buffered saline (PBS; Gibco). The transduced 32D cells were cultured in RPMI 1640 supplementd with 10% FCS and IL-3 (10 ng/ml) and cells were kept at 0.2 x 10^6 cells/ml. The transfection and transduction efficiencies were measured on FACS Calibur flow cytometer in two independent experiments (Becton Dickinson).

Id2 is a direct retinoic acid receptor target gene in APL cells

Basic helix-loop-helix (bHLH) transcription factors and their inhibitors, Id proteins, play crucial roles in the regulation of differentiation in various cell types. We assessed the expression of Id2 in APL cells before and after stimulation with 10^{-6} M ATRA. Total RNA was isolated from the APL positive cell line NB4, that can be induced to differentiate with ATRA. Id2 mRNA was hardly detectable in untreated cells, but levels strongly increased after exposure to ATRA (Figure 4.1). Expression was induced within 2 hours and remained elevated for at least 96 hours after stimulation (Figure 4.1A). Since Id proteins act to inhibit DNA binding by several bHLH proteins, we measured the expression of E2A, a ubiquitous bHLH protein that serves as a heterodimeric partner for many other, more tissue specific bHLH proteins. E2A was expressed in unstimulated NB4 cells but was downregulated after treatment with ATRA within 48 hours (Figure 4.1B).

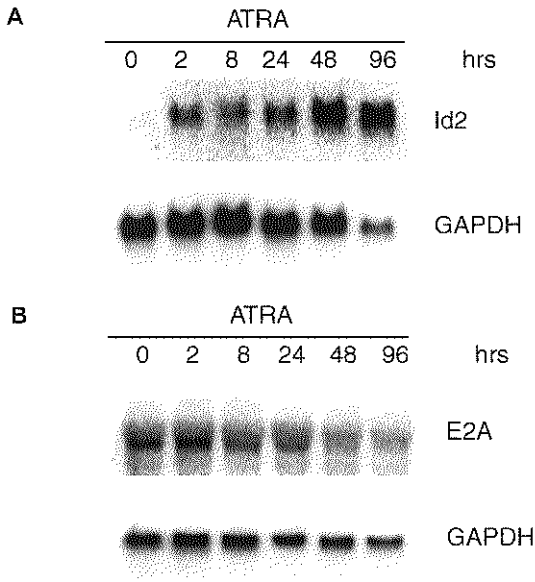

Figure 4.1. Id2 mRNA is upregulated and E2A mRNA is downregulated by ATRA in NB4 cells

Northern blot analysis was performed with total RNA from NB4 cells after exposure to 10^{-6} M ATRA for 0, 2, 8, 24, 48 and 96 hours. The blots were hybridized with Id2 (A) and E2A (B) cDNA probes. A GAPDH probe was used as a control for equal loading of RNA.

To analyze the expression in patient cells, leukemic cells were isolated from bone marrow of newly diagnosed patients with previously untreated APL and incubated in the presence or absence of ATRA. After culture for various times, cytospins were made to confirm differentiation of the cells and RNA was isolated. Id2 mRNA was undetectable in non-stimulated cells but was induced by ATRA within 1 hour, remained elevated at 24 hours and then declined to minimal levels after 6 days of incubation (Figure 4.2A). E2A mRNA was expressed in the preculture patient cells, but strongly declined after 24 hours in cells that were cultured either with or without the addition of ATRA (Figure 4.2B).

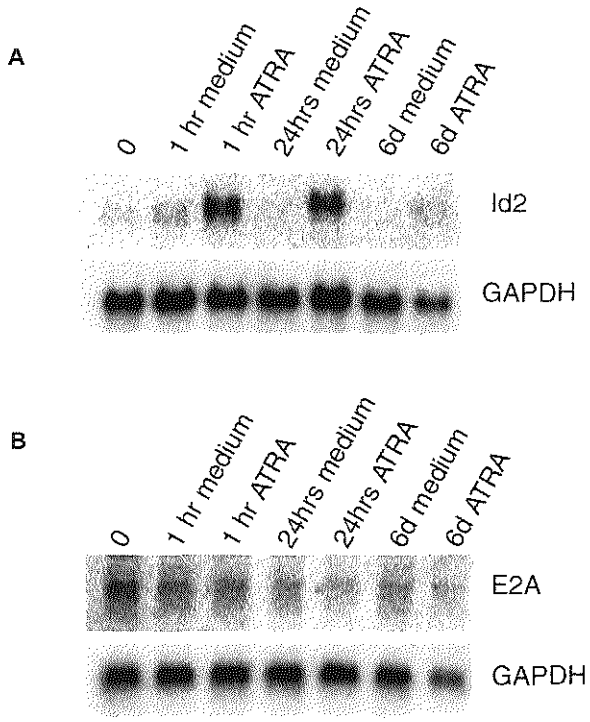

Figure 4.2. Id2 mRNA is upregulated and E2A mRNA is downregulated by ATRA in APL patient cells

Northern blot analysis was performed with total RNA derived from APL patients after cells were induced with ATRA or kept in medium. The blots were hybridized with Id2 (A) and E2A (B) cDNA probes. A GAPDH probe was used as a control for equal loading of RNA.

The induction of Id2 by ATRA was rapid raising the possibility that *Id2* might be a direct target gene of RARs. Cycloheximide was used to inhibit protein translation allowing the analysis of transcription in the absence of intermediate protein production. The induction of Id2 mRNA expression in NB4 cells by ATRA (within 30 minutes) was not affected by coincubation of the cells with cycloheximide and ATRA (Figure 4.3), thus indicating that *Id2* is a direct ATRA-responsive gene.

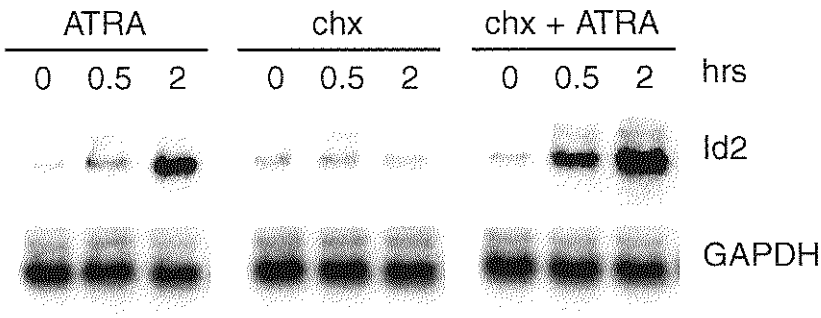

Figure 4.3. *Id2* is a direct ATRA responsive gene in APL cells

Northern blot analysis was performed with total RNA extracted from NB4 cells after exposure to ATRA, cycloheximide, and ATRA + cycloheximide for 0, 30 minutes, and 2 hours. The blot was h ybridized with a labeled *Id2* cDNA probe. A GAPDH probe was used to indicate equal loading of RNA.

Id2 inhibits clonogenic growth of NB4 cells

The biological relevance of the observed Id2 expression induction during APL cell differentiation was studied by overexpressing Id2 in NB4 cells. An Id2 (pCMV-Id2) expression construct was transfected into NB4 cells. Transfected cells were selected by co-electroporation of a green-fluorescent protein (GFP) expression vector (pEGFP-C1) followed by FACS-sort. The sorted cells consisted of more than 90% transfected cells in all experiments (not shown). The effect of Id2 overexpression was analyzed in a colony forming assay (Figure 4.4). Control cultures with cells that were transfected with pEGFP-C1 alone, or with a combination of pEGFP-C1 and the empty pCMV expression vector produced mean numbers of 78 (± 7) and 49 (± 8) colonies per 1000 plated cells, respectively. Comparable numbers (50 ± 7) were seen in cells

that were transfected with the control pCMV-αId2 plasmid containing the *Id2* coding sequence in the antisense orientation. In contrast, *Id2* transfected NB4 cells lost their colony forming capacity by 85-90%, resulting in only 7 (± 2) colonies per 1000 cells. These data indicate that Id2 has a profound suppressive effect on the proliferation of NB4 cells.

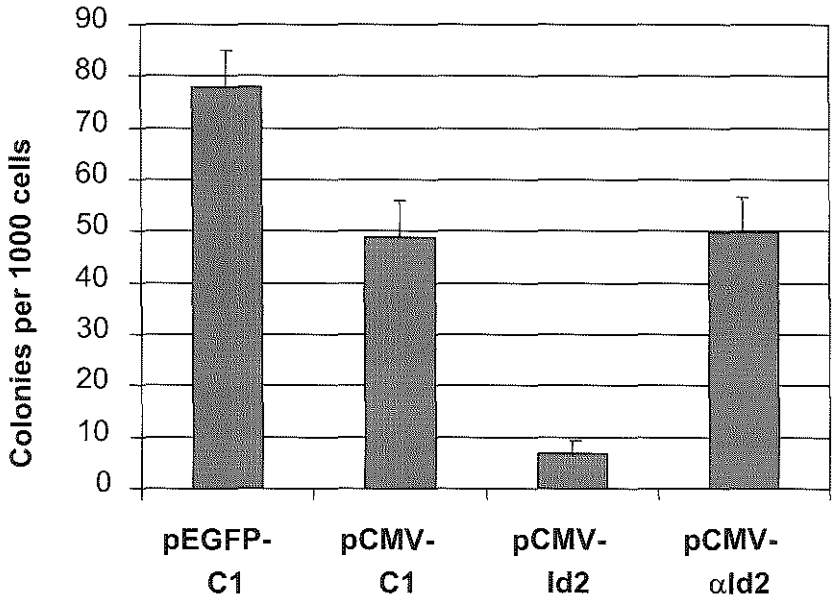

Figure 4.4. Id2 inhibits the clonogenic growth of NB4 cells

pCMV-Id2 was transfected into NB4 cells along with pEGFP-C1. pEGFP-C1, pCMV-C1 and pCMV-αId2 were transfected as controls. Colony forming assays were performed on FACS-sorted EGFP positive cells in four independent experiments. Analysis of the sorted cells for fluorescence indicated a purity of more than 90% in all experiments. Colony formation per 1000 cells was counted after 7 days. pEGFP-C1 and pCMV-C1 showed a mean (+/- SD) of 78 (± 7) and 49 (± 8) colonies per 1000 plated cells respectively. Comparable numbers were seen in the control pCMV-αId2 (50 ± 7). Overexpression of Id2 resulted in only 7 (± 2) colonies per 1000 cells.

Id2 is upregulated in 32D cells upon granulocytic differentiation induction

The expression of Id2 during myeloid differentiation was studied in the 32D-cl3 cell line [36]. This myeloid cell line is interleukin-3 (IL-3)-dependent for proliferation but also stably expresses the G-CSF (granulocyte-colony-stimulating factor) receptor. It grows in an IL-3 dependent manner, but decreases proliferative activity and terminally differentiates into neutrophilic granulocytes upon replacing the IL-3 for G-CSF over a 7 day culture period [36]. Proliferating cells cultured in IL-3 did not express Id2, but when the cells were induced to differentiate with G-CSF, Id2 mRNA levels increased (Figure 4.5). This indicates that Id2 may also be important during granulocytic differentiation induced with other stimuli than ATRA, independently of PML-RARα.

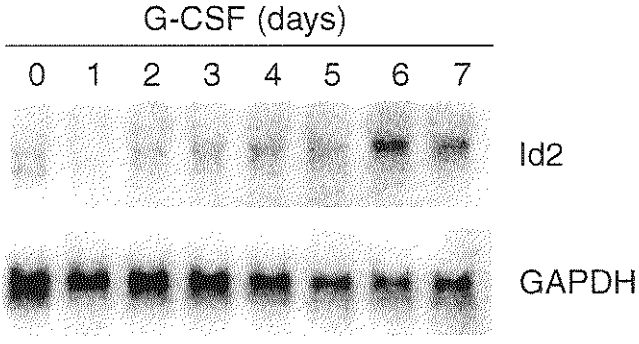

Figure 4.5. Id2 is upregulated in 32D cells upon differentiation induction with G-CSF

Northern blot analysis was performed with total RNA from 32D cells after exposure to G -CSF for 0, 1, 2, 3, 4, 5, 6 and 7 days. The blot was hybridized with an Id2 probe. A GAPDH probe was used to indicate equal loading of RNA.

Overexpression of Id2 in 32D cells decreases proliferation

The biological relevance of the G-CSF-induced Id2 expression during granulocytic differentiation was studied by overexpression of Id2 in 32D cells. Id2 was transduced using a retroviral vector, which contains the Id2 coding sequence in front of an IRES followed by EGFP (S001-EGFP-Id2). As a control, cells were transduced with the parental vector containing only the IRES-EGFP sequences (S001-EGFP). The

transduction efficiencies for Id2 were 60-70% and for the control cells 40-50%. EGFP expression was followed for 6 weeks allowing the comparison of growth rates of transduced and non-transduced cells. The percentages of transduced cells during the culture time were compared to day 0 (set at 100%) in three independent experiments (Figure 4.6). In cultures transduced with the vector control, the percentage of EGFP positive cells remained constant during the 6 weeks. The percentage Id2-transduced cells decreased with more than 90%. In addition, from cell counts during 30 days of culture, the cell doubling times of the transduced cells and non-transduced cells were calculated. EGFP-Id2-positive cells had a doubling time of 29.2 (±0.3) hrs while the EGFP-negative cells in the same culture had a doubling time of 24.8 (±0.3) hrs. In the control cultures containing S001-EGFP transduced cells, the doubling time was 25.5 (±0.5) hrs in both the EGFP-positive and negative cells. To see whether the prolonged doubling time of the Id2 expressing cells was due to enhanced differentiation or apoptosis, cells were harvested at 10 days of incubation. EGFP-positive and negative cells were separated by fluorescence activated cell sorting and stained with May-Grünwald-Giemsa and Annexin-V. A proportion of 12% (± 2) of maturing granulocytes was seen among the Id2 transduced cells as compared to 3% (± 0.2) differentiated granulocytes in control transduced cells. Percentages apoptotic cells were less than 2% in all populations. These data are compatible with the possibility that the quantitative decrease in EGFP-positive cell numbers is due to induction of cellular differentiation in the presence of Id2 overexpression.

To determine the effect of Id2 overexpression on 32D cells during G-CSF induced differentiation, Id2-EGFP-positive clones were isolated by limiting dilution after transduction. The clones were cultured in the presence of G-CSF and the number of cells were counted for 9 days (Figure 4.7). S001-EGFP-positive clones were used as controls. No difference was seen between the growth of the Id2-EGFP positive clones and the control clones. These data show that Id2 expression does not accelerate differentiation of 32D cells in the presence of G-CSF.

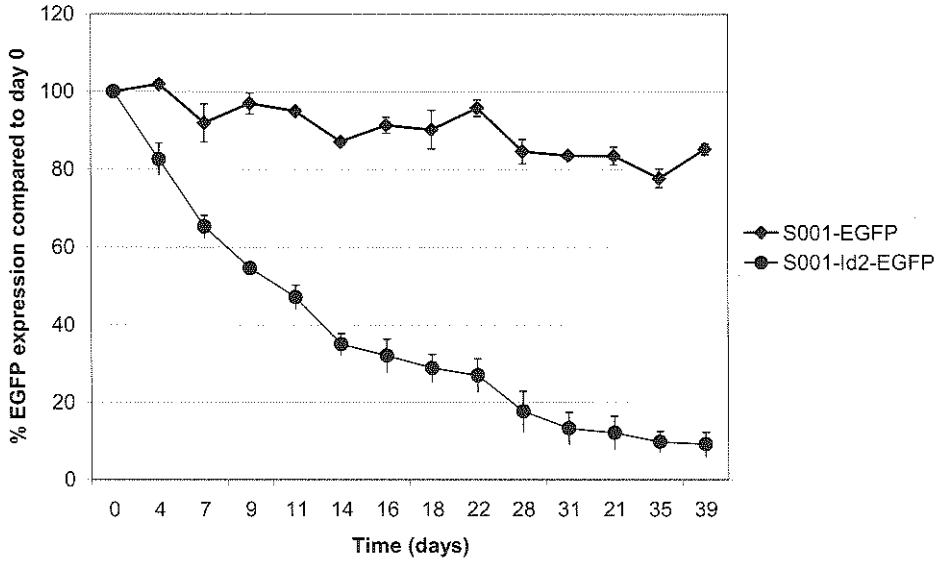

Figure 4.6. Id2 inhibits the proliferation of 32D myeloid cells in response to IL-3

S001-EGFP-Id2 and S001-EGFP (as a control) were retrovirally transduced to 32D cells. Analysis of the cells for EGFP expression was performed by FACS and showed a decrease in EGFP-positive cells of 93% in Id2 transfected cells in three independent experiments. Percentage EGFP-positive cells of the control did not change (mean values ± SD).

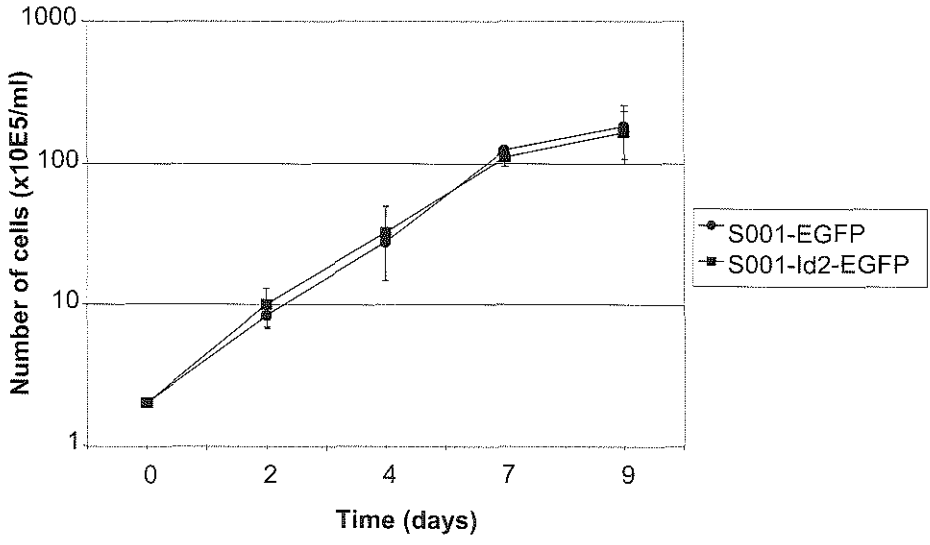

Figure 4.7. Id2 does not affect the proliferation of 32D myeloid cells in response to G-CSF
S001-EGFP-Id2 and S001-EGFP (as a control) were retrovirally transduced to 32D cells. Positive clones were isolated by limiting dilution and cultured in the presence of G-CSF. Cell numbers of two S001-EGFP-Id2 and two S001-EGFP clones were counted for 9 days. Id2 positive clones showed no effect on proliferation when compared to the control (mean values ± SD).

DISCUSSION

APL is characterized by a block in differentiation through silencing of direct ATRA responsive genes by the PML-RARα fusion protein. A few genes have been identified that are likely to be involved in the deregulation of differentiation. A target gene that is of relevance for terminal granulocytic differentiation is the transcription factor *C/EBPε*. Expression is rapidly induced in APL cells by ATRA and a RARE was identified in the promoter that is activated by RARα/RXR and PML-RARα receptors in an ATRA-dependent manner. A role for C/EBPε in granulocytic differentiation was shown in *C/EBPε* knockout animals that lack functionally active granulocytes [34,37,38]. A second gene that is rapidly induced by ATRA is $p21^{waf1/CIP1}$, a cyclin-dependent kinase inhibitor that is involved in the regulation of the activity of several cyclin-dependent kinases [33,39,40]. As for C/EBPε, a RARE was identified in the human *p21* promoter through which the gene was transactivated by RARα/RXR

receptors in a ligand-dependent manner. In addition, the p21 promoter could be transactivated by PML-RARα and ATRA [41]. In the experiments reported here, we show *Id2* as a direct ATRA responsive gene in APL cells and show that forced overexpression of Id2 leads to 85% inhibition of clonogenic growth of APL cells. A more general role for Id2 during granulocytic differentiation was suggested by the induction of this gene during G-CSF induced differentiation of 32D cells. This indicates that Id2 is also upregulated by differentiation-inducing stimuli other than ATRA. This is consistent with the results from previous studies showing that Id2 is expressed in mature myeloid cells [56]. Inhibition of proliferation as a consequence of Id2 expression was shown in 32D cells in the presence of IL-3. The biological role of Id2 during normal hematopoiesis needs to be examined further in retrovirally transduced murine primary bone marrow cells overexpressing Id2.

The *Id* (*Inhibitor of DNA-binding*) gene family consists of four members: *Id1* [45,46], *Id2* [47], *Id3* [48] and *Id4* [49,50]. They form heterodimers with either class I (ubiquitously expressed) bHLH proteins (like the *E2A* gene products E12 and E47) or class II (more tissue-specifically expressed) bHLH proteins (like MyoD and Scl/Tal-1) [42-44] but lack the amino-terminal basic region necessary for DNA binding. As a consequence, Id proteins act as dominant antagonists of bHLH proteins. The upregulation of Id2 and downregulation of E2A in APL cells suggests a dual mechanism by which bHLH proteins that are present at an immature stage of myelopoiesis, are inhibited during the initiation of differentiation. The role of E2A proteins in cell-growth regulatory pathways is illustrated by the lack of B cells in *E2A* null mice [51]. In addition, disruption of the *E2A* gene enhanced the incidence of T-cell tumors between the age of 3 to 10 months and resulted in postnatal lethality. *Id-1* knockout mice were essentially normal [52] but *E2A/Id-1* double-knockout mice survived at significant higher frequency than E2A null mice indicating that Id1 is a negative regulator of *E2A* and that the unbalanced expression of E2A in the absence of Id1 may contribute to postnatal death of the *E2A* null mice [52]. *Id1/Id3* double knockout mice were non-viable and *Id2* knockout mice showed retarded growth, neonatal lethality and indicated that Id2 has an essential role in the generation of peripheral lymphoid organs and NK cells [53,54]. In steady-state conditions, *Id2* knockout mice do not show a prominent defect in granulocytic differentiation. However, the role of Id2 in differentiation in challenged conditions like infection is not known. We show that overexpression of Id2 has an effect on granulopoiesis. This

may suggest that functional redundancy by other *Id* genes causes granulocytic differentiation in *Id2* knockout mice.

BHLH proteins have been shown to have an important role in lymphopoiesis [57]. Our data suggest also a role for bHLH proteins in myeloid differentiation. The identification of myeloid specific bHLH-partners of Id2 will provide new insight in their function during differentiation in APL cells and normal myelopoiesis.

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CHAPTER 5

Id1 is a direct ATRA-responsive gene in acute promyelocytic leukemia cells and is transactivated by a novel mechanism involving PML-RARα and NF-Y

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ABSTRACT

Acute promyelocytic leukemia (APL) cells express a PML-RARα fusion protein as a result of a t(15;17) chromosome translocation. APL is uniquely sensitive to treatment with retinoic acid, which overcomes the differentiation arrest and activates granulocytic differentiation of the leukemic cells. Here we report that similar to *Id2* in Chapter 4, *Id1* is a direct retinoic acid responsive gene in APL cells. It is rapidly induced by retinoic acid (within 30 minutes) in a cycloheximide insensitive manner. We also found a similar biological role for Id1 in myeloid differentiation. Transfection of Id1 in 32D myeloid cells inhibits the proliferative ability of the cells. To identify the mechanism underlying the induction of Id1 transcription by retinoic acid, transactivation of a 1.0 kilobase 5'flanking region of the human *Id1* gene was examined using luciferase reporter constructs. The transcriptional activity of the 1.0 kilobase construct was activated by retinoic acid mediated by the PML-RARα fusion protein but not by wild-type retinoic acid receptors. Unexpectedly, mutational analysis revealed that transactivation by PML-RARα was not mediated through a consensus retinoic acid response element. Rather, a CCAAT-box was shown to be essential for the retinoic acid-induced transcription. Gel shift assays showed that the transcription factor NF-Y binds to this CCAAT-sequence. In addition, transfection of a dominant negative NF-Y form abolished the PML-RARα-induced transactivation. These data show that PML-RARα transactivates the *Id1* gene through a novel mechanism, independently of a consensus retinoic acid response element.

INTRODUCTION

Acute promyelocytic leukemia (APL, FAB-classification AML-M3) accounts for 5-10% of all acute myeloid leukemias. In this disease the leukemic cells are blocked at the promyelocytic stage of development and fail to differentiate into mature, non-proliferative granulocytes [45]. APL is characterized by chromosomal translocations that lead to the fusion of the *retinoic acid receptor α* (*RARα*) gene to various partner genes. These partner genes include *PML* (for *promyelocytic leukemia* gene) [8,17], *PLZF* (*promyelocytic leukemia zinc finger*) [10], *NPM* (*nucleophosmin*) [44], *NuMA*

(*nuclear mitotic apparatus protein*) [54] and *STAT5b* (*signal transducer and activator of transcription-5b*) [2]. Retinoids mediate their effect by two classes of nuclear receptor proteins, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), that each consist of three isotypes (α, β, and γ) encoded in separate genes [9,27,51]. Upon dimerization with RXR, RARs can bind to specific enhancer sequences in the DNA, so-called retinoic acid response elements (RAREs), resulting in transcriptional activation of target genes in the presence of ligand [27,30,43,55]. RAREs consist of a repeated consensus sequence (A/G)G(G/T)TCA separated by 2 to 5 nucleotides. In more than 98% of the APL patients the *RARα* gene is fused to the *PML* gene resulting in the expression of a PML-RARα chimaeric protein. This type of leukemia is uniquely sensitive to treatment with all-*trans* retinoic acid (ATRA), which induces terminal granulocytic differentiation of the leukemic cells. This has proven to be of therapeutic use in patients with APL.

The PML-RARα chimaeric protein has several altered properties when compared to the unrearranged PML and RARα proteins. The DNA-binding and transactivational properties are different from normal retinoid receptors [5,48], and the fusion protein is distributed in an abnormal, microspeckled pattern in the nucleus. Both the interference of PML-RARα with the transcription of ATRA-responsive genes and with the function of PML may contribute to the development of APL. PML is a nuclear protein with a RING-finger motif and is localized in large multiprotein nuclear structures termed PODs (PML oncogenic domains), ND-10 (nuclear domain-10) or nuclear bodies. These structures appear as dense spherical particles tightly associated with the nuclear matrix. Although more than 20 proteins have been shown to colocalize in these structures, their exact function remains unclear. Interestingly, PML-RARα disrupts the normal structure of NBs to a microspeckled nuclear pattern and delocalizes RXR from a nuclear diffuse to a microspeckled pattern. After treatment with ATRA, normal nuclear bodies are reassembled and the RXR protein returns to a nuclear diffuse distribution [15,25,53].

Various ATRA-responsive genes have been identified in APL cells. However, it is not yet clear which target genes are critical for leukemogenesis. We recently identified *Id2*, *inhibitor of DNA-binding 2*, as a direct ATRA-responsive gene involved in granulocytic differentiation. In addition, C/EBPε expression is rapidly induced in APL cells by retinoic acid and a RARE was identified in the promoter. This gene is of

relevance for terminal granulocytic differentiation as *C/EBPε* knockout animals lack fully functional granulocytes [11,36,41]. A third gene that is rapidly induced by retinoic acid in APL cells is *p21*$^{waf1/CIP1}$, a cyclin-dependent kinase inhibitor [3,31,52], and the *p21* promoter is also transactivated by PML-RARα through a RARE [7]. This suggests that PML-RARα is involved in deregulation of RARα targets. Here we report that another member of the *Id* (*Inhibitor of DNA binding*) gene family, *Id1*, is a direct ATRA responsive gene. We also studied the mechanism by which PML-RARα mediates the transcription of this gene.

MATERIALS AND METHODS

Cell culture

Bone marrow mononuclear cells of APL patients were isolated at diagnosis by lysis of erythrocytes in NH4Cl buffer. Indirect immunostaining with a monoclonal anti-PML antibody (PG-M3, Santa Cruz Biotechnology, Santa Cruz, CA) showed a microspeckled pattern characteristic for the PML-RARα fusion protein [15,25,53]. Cytogenetic analysis showed that all patients were positive for the t(15;17)(q22;q21) chromosome translocation. Patient and NB4 cells [26] were cultured in RPMI 1640 medium (Gibco, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine (Gibco). The interleukin-3 (IL-3)-dependent murine myeloid cell line 32D [16] was maintained in RPMI 1640 medium supplemented with 10% FCS and 10 ng/ml of murine IL-3. Phoenix E. cells, provided by Dr. G. P. Nolan, Stanford University Medical Center, Stanford, California, USA, were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% FCS. Hep3B cells were cultured in α- modified Eagle's medium (α-MEM; Gibco) supplemented with 10% charcoal-treated-FCS. Cultures were incubated at 37°C in a 5% CO_2 completely humidified atmosphere.

Northern blotting

NB4 cells were induced with all-*trans* retinoic acid (Sigma, St Louis, MO) at a final concentration of 10^{-6} M and harvested at various time points. Where appropriate, cycloheximide (ICN, Costa Mesa, CA) was used at a final concentration of 4 μg/ml. Cycloheximide was added 30 minutes before the start of induction with ATRA. Total cellular RNA was isolated by guanidinium-isothiocyanate lysis and centrifugation on a 5.7 M cesium chloride cushion. PolyA$^+$ RNA was isolated from total RNA by using a mRNA purification kit (Pharmacia Biotech, Uppsala, Sweden). 10 μg of total RNA or 1μg of PolyA$^+$ RNA was size-fractionated by 1% agarose-formaldehyde gel electrophoresis and transferred onto Hybond N$^+$ nylon membranes (Amersham, Buckinghamshire, UK). The filters were pre-hybridized and hybridized at 65°C overnight in phosphate buffer (2N NaH_2PO_4, 2N Na_2HPO_4), 7% sodium dodecyl sulphate (SDS), 1 mM EDTA pH=8.0 and 1% BSA). DNA probes were labelled with [^{32}P]α-dATP by random primed labelling (Boehringer, Mannheim, Germany). After hybridisation, the filters were washed in 0.2xSSC/ 0.2% SDS for 15 minutes at 65°C. Northern blots were hybridized to radiolabelled human Id1 (kindly provided by Dr. S. Stegman, NKI, Amsterdam, The Netherlands) human Id-3 (kindly provided by Dr. J.D. Norton, Christie CRC Research centre, Manchester, UK) and human Id-4 (kindly provided by Dr. L. Lania, University of Naples 'Federico II', Naples, Italy). As a control for equal loading, filters were stripped and hybridized to a 777bp HindIII-EcoRI human GAPDH fragment.

Plasmids and antibodies

The S001-EGFP retroviral vector (kindly provided by Dr. G.P. Nolan, USA and Dr. H. Spits, NKI, Amsterdam, the Netherlands [48]) which contains a multiple cloning site in front of an IRES-EGFP sequence was used. The S001-EGFP-Id1 was constructed by cloning the human Id1 sequence into the BamH1 site of S001-EGFP. For transactivation studies, the RARE from the *RARβ* gene was used as described [50], the pGL3-promoter vector was purchased from Promega, Madison, WI, USA. Plasmid containing 5' human *Id1* promoter sequence was kindly provided by Dr. J. Campisi, Department of Cancer Biology and Molecular Cytogenetics Resource, Berkeley National Laboratory, University of California, USA. *Id1* promoter deletion constructs were made by cloning the individual fragments into the XhoI-HindIII sites

of pGL3-basic luciferase reporter (Promega). The vector NF-YA13m29 was kindly provided by Dr. R. Mantovani, University of Milano, Milano, Italy [32]. The C/EBPα expression vector was kindly provided by Dr. R.P. de Groot, Department of Pulmonary Diseases, University Medical Center Utrecht, The Netherlands. Anti-PML antiserum [53] was used for immunofluorescence (1: 500) and gel shifts (1 μl per reaction). Anti-NF-YB polyclonal antiserum was kindly provided by Dr. R. Mantovani, Italy, and used in gel shifts (1 μl per reaction).

Preparation of retrovirus and transduction of 32D cells

The retrovirus producer cells, Phoenix E., were split to 2 x 10^6/ml 24 hours prior to transfection. Chloroquine was added to each plate 5 minutes before transfection at a final concentration of 25 μM. 10 μg DNA was transfected in the cells by the calcium phoshate coprecipitation technique. Chloroquine was removed 9 hours after transfection and fresh medium was added to the cells supplemented with 10% FCS and IL-3 (10 ng/ml). The supernatants were collected from the cells 48 hours post-transfection. 2,5 x 10^5 32D cells were repeatedly transduced with 1.5 ml supernatant on retronectin coated plates. The cells were replaced from the retronectin coated plates 48 hours afer transduction and the virus was removed by washing the cells three times with phosphate-buffered saline (PBS; Gibco). The transduced 32D cells were cultured in RPMI 1640 supplemented with 10% FCS and IL-3 (10 ng/ml) and cells were kept at 0.2 x 10^6 cells/ml. The transfection and transduction efficiencies were measured on FACS Calibur flow cytometer (Becton Dickinson). Differentiation was assessed by May-Grünwald-Giemsa staining.

Transactivation studies

Hep3B cells were plated into 12-well plates at a density of 0.6 x 105 cells/well and cultured for 18 hours. Subsequently, cells were transfected using calcium-phosphate precipitates with 0.3 μg of the appropriate nuclear receptor expression vectors, 0.5 μg of the luciferase reporter plasmid and 0.5 μg of the RSV-β-galactosidase vector (β-gal, kindly provided by Dr. C. Berrevoets, Erasmus University of Rotterdam, the Netherlands). The total amount of DNA was normalized with pBluescript plasmid to 4 μg. Cells were washed after 24 hours and ATRA was added. Cells were lysed after 48 hours using lysisbuffer (25mM trisphosphate pH 7.8, 8mM MgCl2, 1mM DTT, 1%

Triton X-100, 15% glycerol). Luciferase activity was measured on a Biocounter M2500 luminometer (Lumac, Landgraaf, The Netherlands) using an equal volume of of a luciferine solution (1mM luciferine, 1mM ATP, 8 mM MgCl2) as a substrate. Reporter gene expression was calculated in arbitrary units, relative to β-gal expression.

Preparation of nuclear extracts and Electrophoretic Mobility-Shift Assays

Nuclear extracts were prepared as described [1]. To analyze DNA-binding, nuclear extracts (5 μg) were incubated in a total volume of 15 μl containing 1 μg of double-stranded poly(dI)-poly(dC), 10 mM Hepes, 50 mM KCl, 1mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM $MgCl_2$, 10% (wt/vol) glycerol, 300 μg of bovine serum albumin per ml, and 0.5 ng of a labeled oligonucleotide probe. The sequence of the oligonucleotides used were:

C/EBP G-CSF-R 5'-AAGGTGTTGCAATCCCCAGC-3' [47];

Id1 5'-CCGCCCATTGGCTGCTTTTGAACGT-3';

Id1 mutant 5'- TCAGAGTTCAAGGTTCTAGTCGCTGCGGC-3';

DR5-G 5'-GGGTAGGGGTCACCGAAAGGTCACTCG-3' [42];

NF-Y (CD10) 5'-ATCCCGACCAATGAGCGCACGGGGCCGGGT-3' [23].

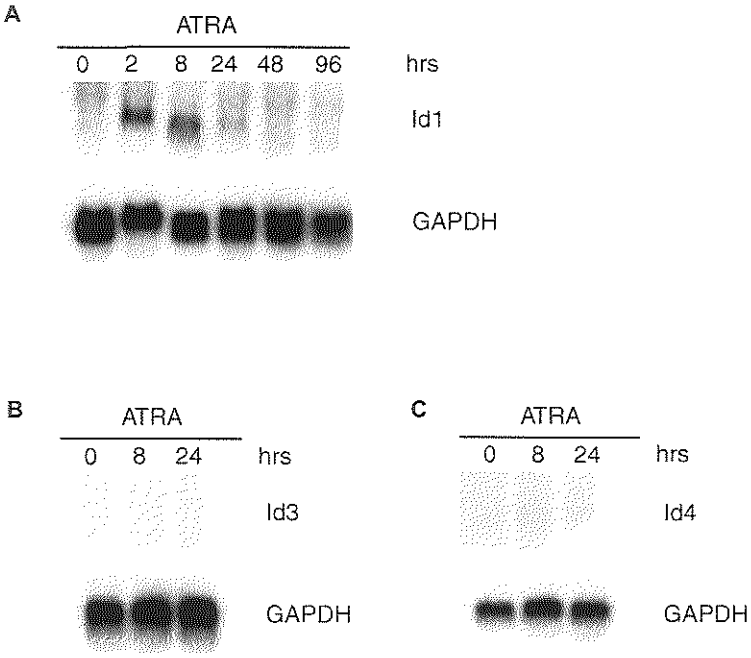
DNA-protein complexes were separated on 5% nondenaturating polyacrylamide gels in 0.5 X TBE buffer.

RESULTS

Id1 is a direct ATRA-responsive gene

We have recently identified the *Id2* gene as a direct ATRA-responsive gene in APL cells [4]. Therefore, we assessed the expression of the other *Id* gene family members in these cells before and after stimulation with ATRA (Figure 5.1). In untreated cells, Id1 mRNA was hardly detectable. Expression was induced within 2 hours after exposure to ATRA and reached a maximum at 8 hours, to return to undetectable levels at 48 hours after stimulation (Figure 5.1A). Id-3 or Id-4 mRNA could not be detected on Northern blots using total RNA (not shown), or polyA+ mRNA (Figure 5.1B and 5.1C). The expression pattern of Id1 was confirmed in RNA isolated from

primary APL cells from newly diagnosed patients. Id1 mRNA was undetectable in non-stimulated cells but was induced by ATRA within 2 hours, to decline to undetectable levels at 24 hours (Figure 5.1D). Cycloheximide was used to inhibit protein translation allowing the analysis of transcription in the absence of intermediate protein production. The rapid induction of Id1 mRNA expression in NB4 cells by ATRA (within 30 minutes) was not affected by cycloheximide (Figure 5.2) indicating that, like *Id2*, *Id1* is a direct retinoic acid target gene.



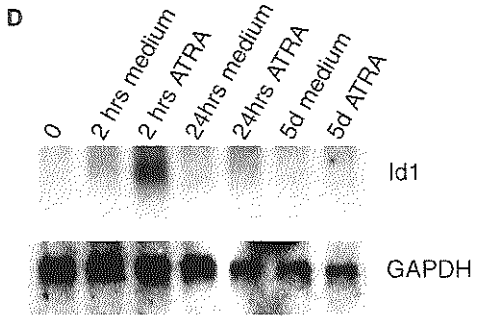


Figure 5.1. Id1 mRNA is induced by ATRA in APL cells

Northern blot analysis was performed with total RNA (A) or polyA+ RNA (B and C) extracted from NB4 cells after exposure to 10^{-6}M ATRA for 0, 2, 8, 24, 48 and 96 hours. Expression of Id1 in patient cells was performed by Northern blot analysis with total RNA derived from APL patients after cells were induced with ATRA or kept in medium. The blots were hybridized with Id1 (A and D), Id3 (B) or Id4 (C) probes. A GAPDH probe was used as a control for equal loading of RNA.

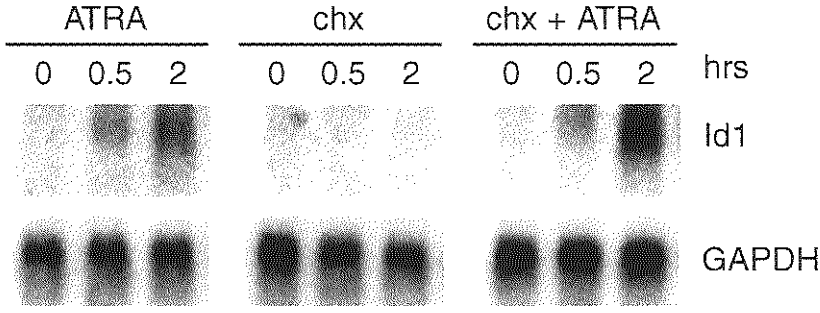


Figure 5.2. *Id1* is a direct ATRA responsive gene in APL cells

Northern blot analysis was performed with total RNA extracted from NB4 cells after exposure to ATRA, cycloheximide, and ATRA + cycloheximide for 0, 30 minutes, and 2 hours. The blot was hybridized with a labeled cDNA fragment of the *Id1* gene. A GAPDH probe was used to indicate equal loading of RNA.

Id1 plays a role in myeloid cell development

To assess the biological role of Id1 during granulocytic differentiation, Id1 was overexpressed in 32D cells by transduction using a retroviral vector which contains the *Id1* coding sequence in front of an IRES sequence followed by the EGFP coding sequence. The control cell population was transduced with the S001-EGFP vector. The growth rate of the transduced and non-transduced cells was derived from measuring the percentage of EGFP-positive cells and the absolute cell counts during 6 weeks of culture in IL-3. Figure 5.3 shows the percentage of EGFP-positive cells of three independent experiments compared to day 0 (set at 100%). The proportion of EGFP-positive cells among the control transduced cells remained constant over a 6 week period. However, in the Id1-EGFP-transduced cultures, EGFP-positive cells decreased to 26% over a 6 week period. The EGFP-Id1-positive cells showed a doubling time of 28.2 (± 0.4) hrs compared to a doubling time of 25.3 (± 0.2) hrs of the non-transduced cells. The vector control cells showed no difference in doubling time between transduced and non-transduced cells (25.5 (± 0.5) hrs). Cytological examination of the transduced cells after 10 days of culture revealed an 11% (± 4.2) of the Id1 transduced cells as mature granulocytes compared to 3% (± 0.2) of mature granulocytes in cultures of control transduced cells. Apoptotic cells (Annexin-V) were less than 2% and not different in Id1 positive and control populations. We conclude that Id1 overexpression results in some inhibition of the proliferation of 32D cells which is accompanied by enhanced maturation. To test whether Id1 would strongly accelerate the G-CSF induced differentiation, stably transduced 32D clones were selected harboring the S001-EGFP control and the S001-EGFP-Id1 vectors. When switched from IL-3 to G-CSF, no difference was observed in the timing or morphological aspects of differentiation between the Id1- and control-transduced clones (Figure 5.4). This indicates that Id1 is not a limiting factor in the cascade of signals that induce granulocytic differentiation of 32D cells in response to G-CSF.

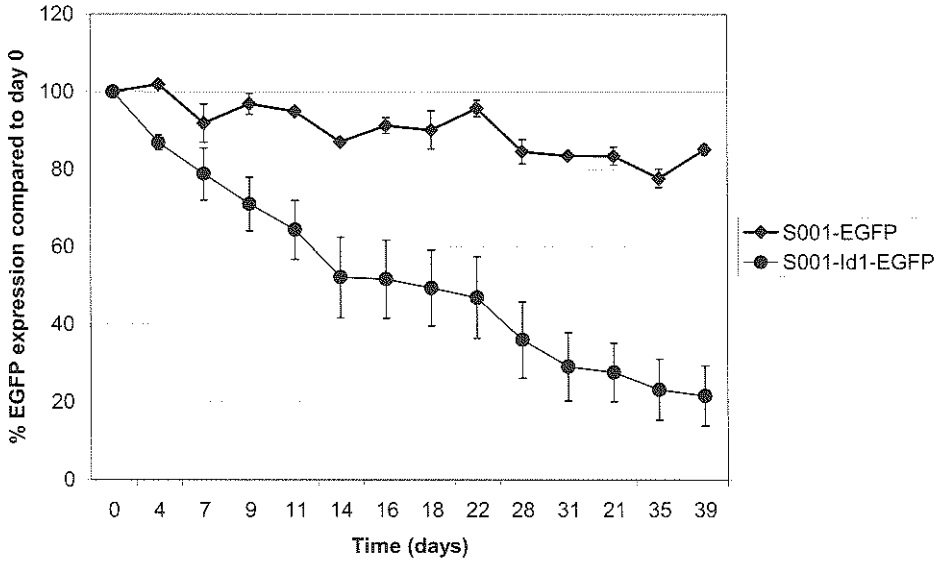


Figure 5.3. Id1 inhibits the proliferation of 32D cells

S001-EGFP-Id1 and S001-EGFP (as a control) were transfected in 32D cells. Analysis of the cells for EGFP expression was performed by FACS and showed a decrease in EGFP-positive cells to 26% in Id1 transfected cells in three independent experiments. Percentage EGFP-positive cells of the control remained constant (mean values ± SD).

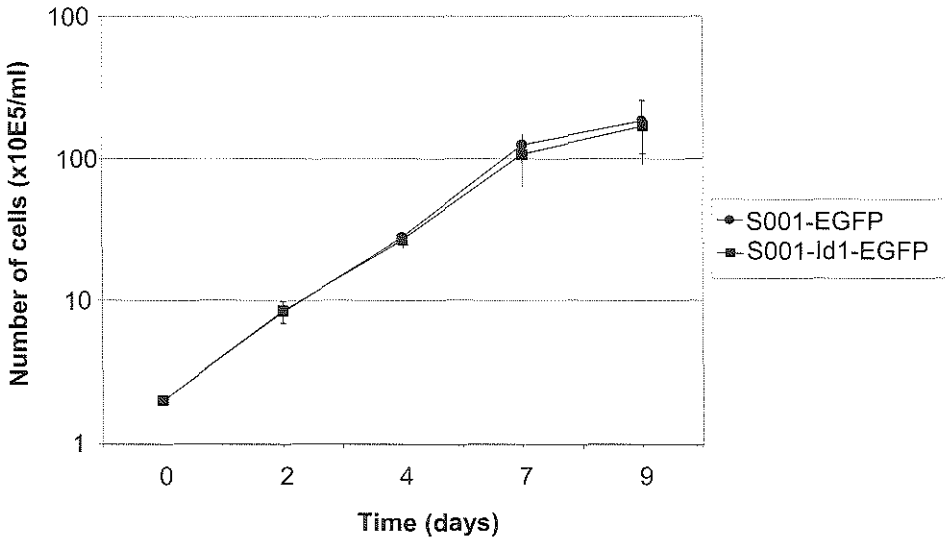


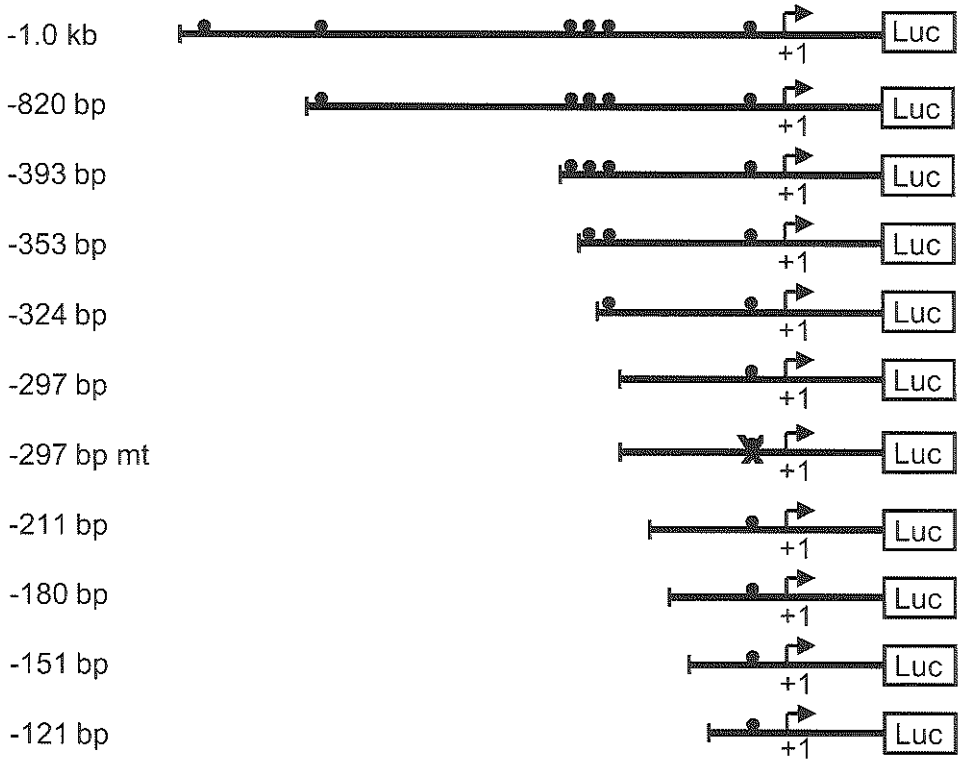
Figure 5.4. Id1 does not affect the proliferation of 32D myeloid cells in response to G-CSF
S001-EGFP-Id1 and S001-EGFP (as a control) were retrovirally transduced to 32D cells. Positive clones were isolated by limiting dilution and cultured in the presence of G-CSF. Cell numbers of two S001-EGFP-Id1 and two S001-EGFP clones were counted for 9 days. Id1 positive clones showed no effect on proliferation when compared to the control (mean values ± SD).

Transactivation of the *Id1* promoter by the fusion protein PML-RARα

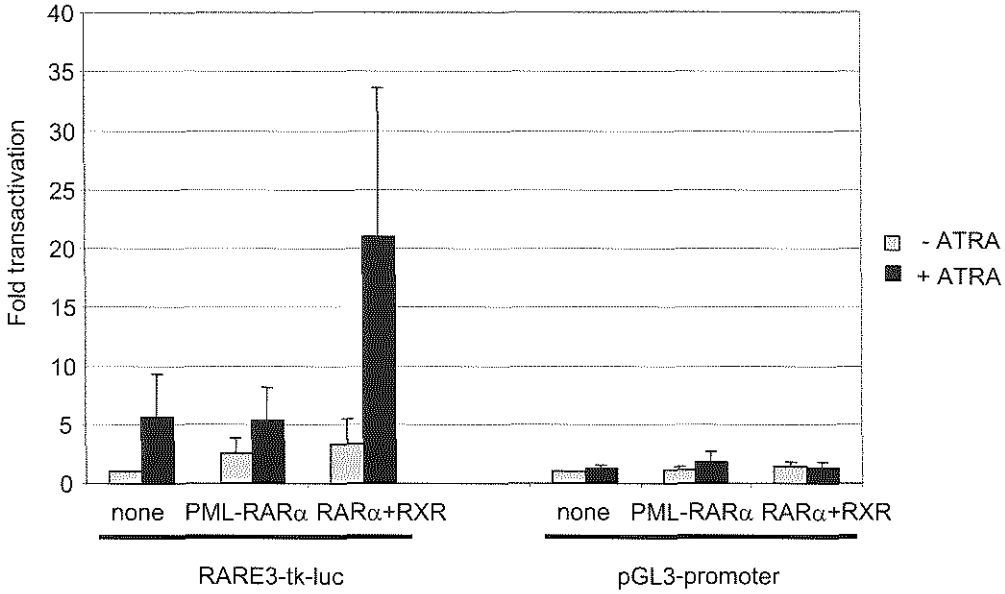
The rapid and cycloheximide insensitive Id1 mRNA induction by ATRA indicates that *Id1* is a direct ATRA-responsive gene. To localize the relevant RAREs, we analyzed a series of deletion constructs of the promoter for luciferase inducibility in response to ATRA in Hep3B cells (Figure 5.5A). As a control, a bonafide RARE (RARE3-tk-luc) was used [50]. Figure 5.5B confirms the transcriptional activation of RARE3-tk-luc construct by wild type RARα and PML-RARα. In addition, possible background signal from the vector was tested and the retinoic acid receptors did not induce transcription of the pGL3-promoter vector indicating that the pGL3-vector was not activated by these receptors. The transfection assays with the deletion constructs of the *Id1* promoter showed that ATRA-dependent transactivation of the *Id1* promoter construct was induced by the fusion protein PML-RARα but, surprisingly, not by wild-type

RARα/RXR (Table 5.1). In addition, the -297bp mt construct which contains 297 bp of the *Id1* promoter with deletion of the putative RARE was still transactivated by PML-RARα (Table 5.1) showing that ATRA induction was not lost after deletion of all putative RAREs. Moreover, the –121 bp construct lacking consensus RARE still showed a 10-fold induction by ATRA (Figure 5.5C). This suggests that another element than a RARE between –121 bp and the transcription start site conferred the ATRA-activated transcription.

A.



B.


40
35
30
25
20
15
10
5
0
Fold transactivation
- ATRA
+ ATRA
none
PML-RARα
RARα+RXR
none
PML-RARα
RARα+RXR
RARE3-tk-luc
pGL3-promoter

C.

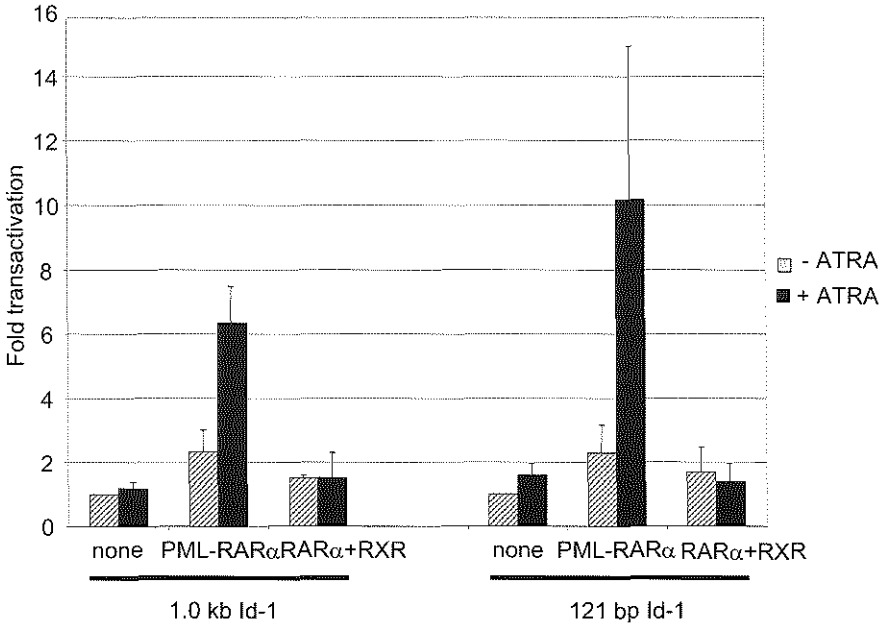

16
14
12
10
8
6
4
2
0
Fold transactivation
- ATRA
+ ATRA
none
PML-RARα
RARα+RXR
none
PML-RARα
RARα+RXR
1.0 kb Id-1
121 bp Id-1

Figure 5.5. ATRA-induced Id1 expression is mediated by the fusion protein PML-RARα and not by wild-type RARα

(A) Various *Id1* promoter deletion constructs were cloned in front of the luciferase reporter gene. The transcriptional start site is defined as +1. The -297 bp mt construct contains mutation of a putative RARE at –30 bp. ● represents potential RAREs. (B) transient transfections of Hep3B cells with RARE3-tk-luc and pGL3 promoter constructs as a positive and negative control respectively for transcriptional activation by the retinoic acid receptors. (C) transfections with 1.0 kb and 121 bp of the Id1 promoter in the presence and absence of ATRA.

Construct	No receptor		PML-RARα		RARα+RXR	
	- ATRA	+ ATRA	- ATRA	+ ATRA	- ATRA	+ ATRA
-1.0 kb	1	1.2 ± 0.1	2.3 ± 0.7	**6.3** ± 1.1	1.5 ± 0.1	1.5 ± 0.8
-820 bp	1	1.4 ± 0.1	2.4 ± 0.4	**11.1** ± 1.7	1.2 ± 0.4	1.8 ± 0.1
-393 bp	1	1.5 ± 0.4	2.2 ± 0.3	**7.7** ± 0.4	1.4 ± 0.2	1.9 ± 0.7
-353 bp	1	1.2 ± 0.4	1.3 ± 0.3	**7.8** ± 0.3	1.0 ± 0	1.6 ± 0.6
-324 bp	1	1.6 ± 0.1	1.3 ± 0.2	**6.7** ± 1.6	1.3 ± 0.5	2.2 ± 1.1
-297 bp	1	1.6 ± 0.4	2.0 ± 1.1	**8.9** ± 4.5	1.3 ± 0.6	2.0 ± 1.0
-297 bp mt	1	1.6 ± 0.5	2.0 ± 0.5	**8.1** ± 1.6	1.4 ± 0.1	1.9 ± 0.8
-211 bp	1	1.2	0.7	**3.7**	1.7	1.6
-180 bp	1	1.7	1.4	**5.4**	2	1.7
-151 bp	1	1.2	1	**2.8**	1.2	1.2
-121 bp	1	1.6 ± 0.4	2.0 ± 0.9	**9.5** ± 4.7	1.7 ± 0.8	1.4 ± 0.6

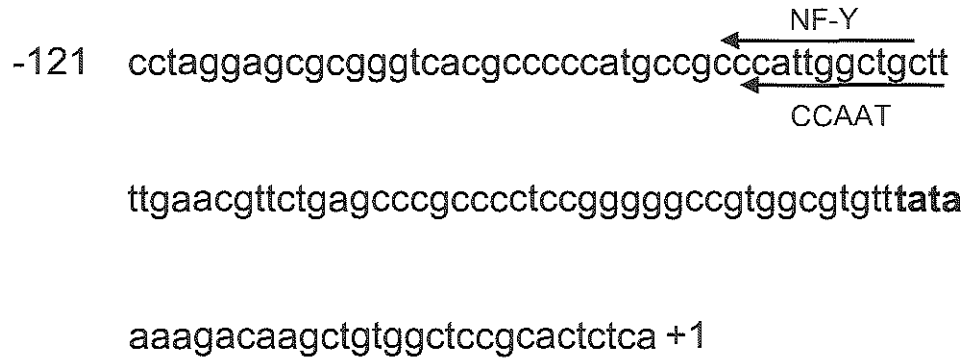
Table 5.1. Fold transactivation of the *Id1* promoter

Transient transfections in Hep3B cells of *Id1* promoter deletions in the presence and absence of ATRA and receptor. The *Id1* promoter was transiently activated by PML-RARα and not RARα/RXR.

A CCAAT-box located 80 bp upstream of the TATA-box is required for PML-RARα-induced transcriptional activation of the Id1 promoter

Inspection in the 'TFSEARCH transcription factor database' (http://pdap1.trc.rwcp.or.jp/research/db/TFSEARCH.html) of the residual 121 bp that still confered PML-RARα mediated transactivation revealed a consensus CCAAT-box 43 bp upstream of the TATA-box (Figure 5.6A). Mutation of this site was made in the context of the 1.0 kb promoter fragment. ATRA-mediated transactivation by PML-RARα was completely abolished (Figure 5.6B) indicating that this site is required for ATRA-induced transcriptional activation.

A.



B.

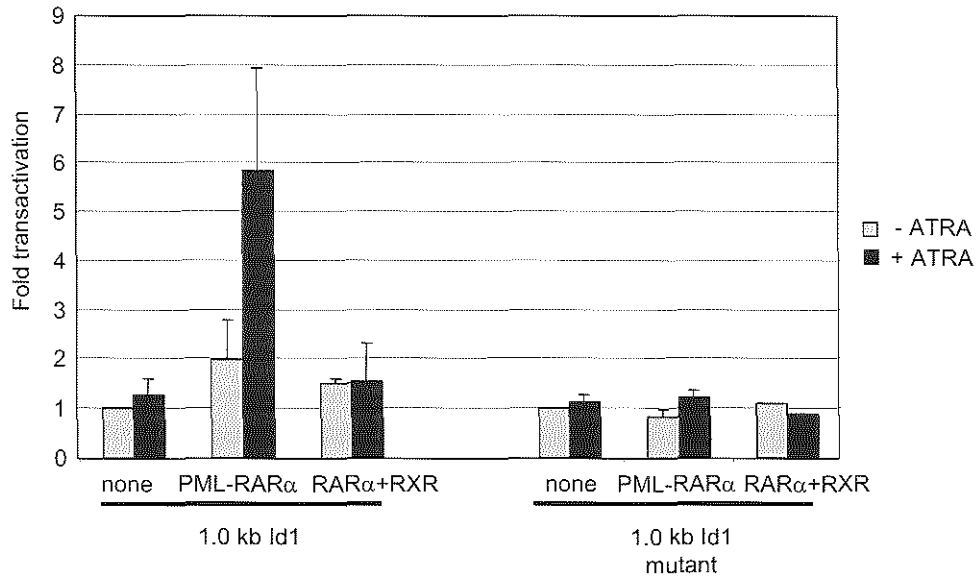


Figure 5.6. The CCAAT-box 80 bp upstream of the TATA box is involved in ATRA-induced transcriptional activation of the *Id1* promoter

(A) Sequence of the –121 bp of the *Id1* promoter containing a CCAAT-box and consensus *NF-Y* binding site. (B) transfections were carried out with the intact 1.0 kb *Id1* promoter as well as with mutated *NF-Y* binding site in the presence and absence of ATRA.

NF-Y directly regulates Id1 transcription through the CCAAT-box

Various CCAAT-binding proteins, such as NF-Y (Nuclear Factor-Y), CTF/NF-1 (CCAAT-Transcription Factor/Nuclear Factor-1), C/EBP (CCAAT-Enhancer Binding Protein) and CDP (CCAAT Displacement Protein), have been identified and characterized. In view of the role of C/EBPα during granulopoiesis, we first investigated whether C/EBPα could bind to the site involved in transcription of the Id1 promoter. Electrophoretic mobility shift assays (EMSA) were performed with COS-nuclear extracts overexpressing C/EBPα and incubated with an oligonucleotide probe containing the C/EBPα-binding site of the G-CSF-receptor promoter [47]. Figure 5.7A shows that the nuclear extracts contained C/EBPα. Shifted complexes were competed by a 10 and 100 fold excess of cold probe containing the C/EBP binding site (Figure 5.7A, lane 3 and 4) but not by a 10 and 100 fold excess of the Id1 oligo comprising the CCAAT-box of the Id1 promoter (Figure 5.7A, lanes 5 and 6). These data show that C/EBPα does not bind to this CCAAT-box in the Id1 promoter with high affinity.

A CCAAT-box is often found in NF-Y bindings sites. Therefore, the interaction of NF-Y with the CCAAT-box in the Id1 promoter was tested. In EMSAs using COS-nuclear extracts incubated with the Id1 oligo containing the CCAAT-binding site, the shifted complex bound to 100 fold excess of cold probe (Figure 5.7B, lane 2) but was not competed by a 100 fold excess of the mutant Id1 oligo containing mutations in the CCAAT-binding site (Figure 5.7B, lane 3). The shifted band was affected by the polyclonal anti-NF-YB antibody (Figure 5.7B, lane 4) and by a 100 fold excess of the oligo containing the NF-Y-binding site of the CD10 promoter [23] (Figure 5.7B, lane 5) indicating that the complex represents NF-Y. These data show that NF-Y binds to the CCAAT-box of the Id1 promoter that is involved in ATRA-induced transcriptional activation by PML-RARα. The direct interaction of PML-RARα to this site was excluded in an EMSA with nuclear extracts of cells overexpressing PML-RARα incubated with the DR5-G probe containing a PML-RARα-binding site [42] (Figure 5.7C). The Id1 oligo did not compete for PML-RARα (Figure 5.7C, lane 4) suggesting that PML-RARα does not bind to this site in the Id1 promoter.

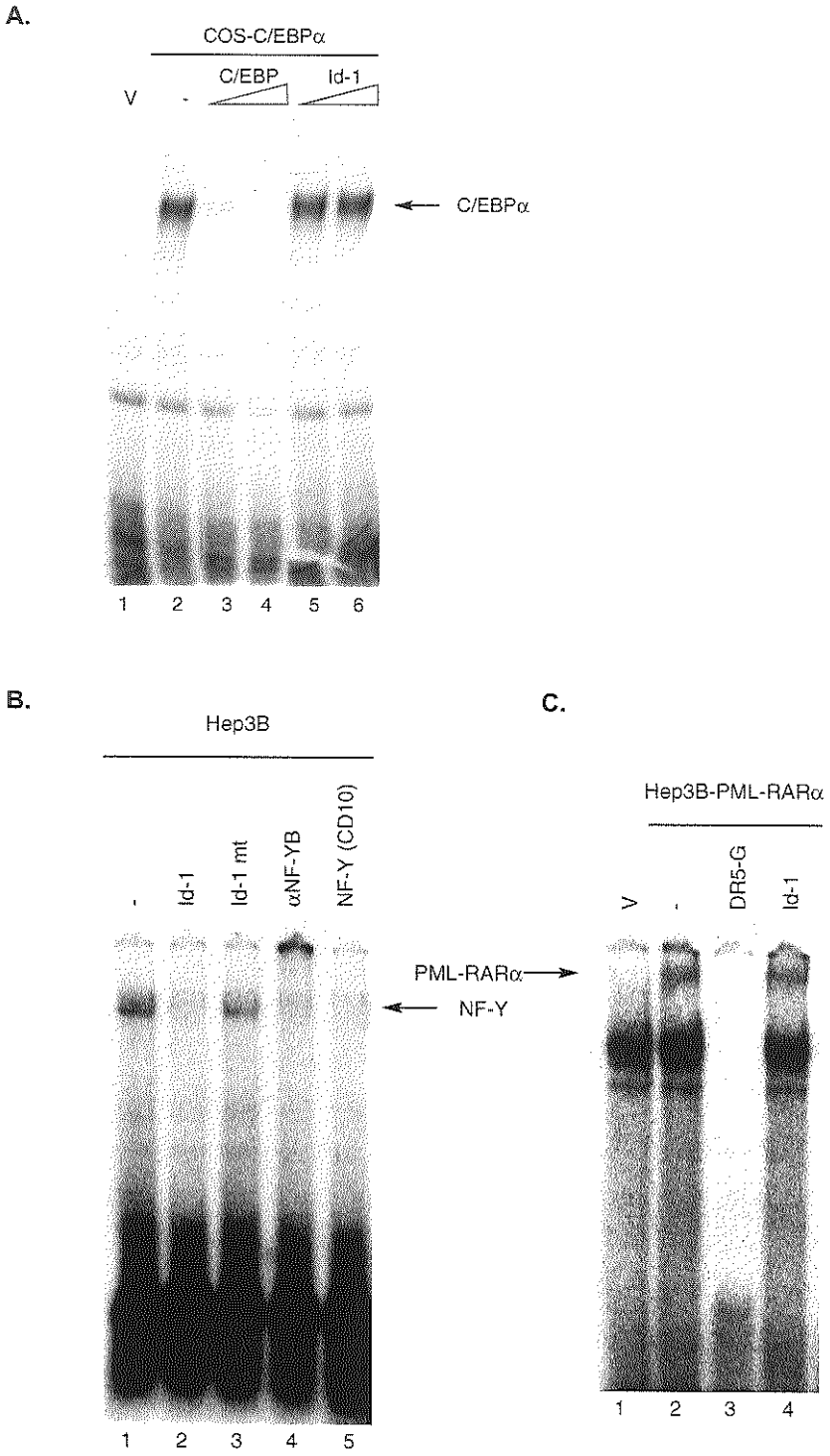

A.
COS-C/EBPα
V
-
C/EBP
Id-1
C/EBPα
1 2 3 4 5 6
B.
Hep3B
-
Id-1
Id-1 mt
αNF-YB
NF-Y (CD10)
PML-RARα
NF-Y
1 2 3 4 5
C.
Hep3B-PML-RARα
V
-
DR5-G
Id-1
1 2 3 4

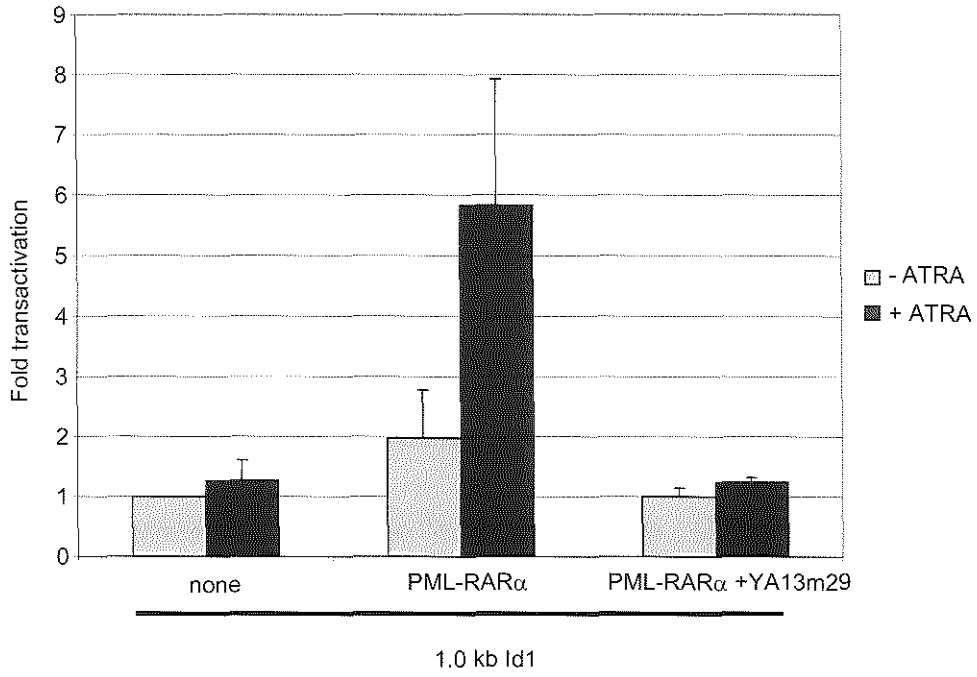
Figure 5.7. Transcription factor NF-Y and not C/EBPα binds to the *Id1* promoter

(A) COS-nuclear extracts overexpressing C/EBPα were incubated with the C/EBP-binding site from the *G-CSF-R* promoter (lanes 2-6) and analyzed in an *in vitro* gel mobility shift assay. As a control, the probe was incubated with COS-nuclear extract overexpressing vector control. To analyze whether C/EBPα binds to the *Id1* promoter, competition with wild-type C/EBP (lane 3 and 4) and Id1 (lane 5 and 6) was performed as indicated. (B) Hep3B-nuclear extracts were incubated with the Id1 probe. Specificity of the indicated band was determined by competition with wild-type Id1 oligo, mutated Id1 oligo and the CD10 oligo, and by addition of the polyclonal antibody against NF-Y. (C) Hep3B-nuclear extracts overexpressing PML-RARα were incubated with the DR5-G probe. Competition was performed with Id1 oligo.

NF-Y is essential for ATRA-induced transcriptional activation of the *Id1* promoter

The involvement of NF-Y in ATRA-induced *Id1* promoter transactivation by PML-RARα was confirmed in cotransfection experiments using a dominant negative form of the NF-YA transcription factor, NF-YA13m29 in which three amino acids in the DNA binding domain are mutated. NF-YA13m29 forms a complex with NF-YB and NF-YC, but the complex fails to bind to the DNA [32]. Figure 5.8A shows that NF-YA13m29 abolishes the ATRA-dependent transactivation of the *Id1* promoter by PML-RARα. Transfection experiments with RARE3-tk-luc, containing three tandem copies of the RARE from the *RARβ* gene, and wild-type RARα and PML-RARα in the presence and absence of dominant negative NF-Y, show that NF-Y does not directly regulate ATRA-induced transcriptional activation via RAREs (Figure 5.8B). This indicates that the involvement of NF-Y in ATRA-induced gene expression is specific for PML-RARα and not for RARα-RXR.

A.


Fold transactivation
9
8
7
6
5
4
3
2
1
0
- ATRA
+ ATRA
none
PML-RARα
PML-RARα +YA13m29
1.0 kb Id1

B.

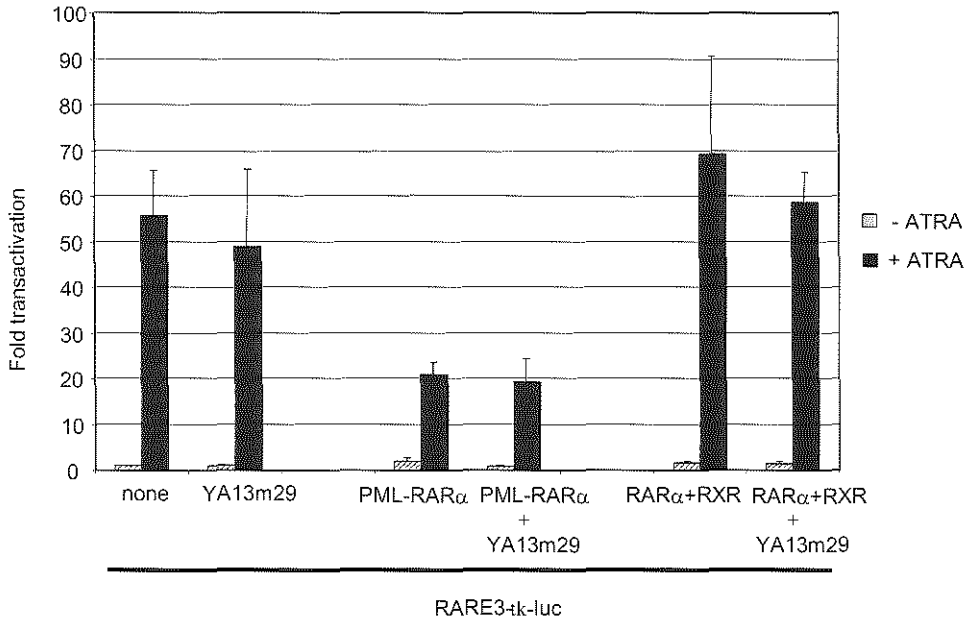

Fold transactivation
100
90
80
70
60
50
40
30
20
10
0
- ATRA
+ ATRA
none
YA13m29
PML-RARα
PML-RARα + YA13m29
RARα+RXR
RARα+RXR + YA13m29
RARE3-tk-luc

Figure 5.8. NF-Y directly regulates ATRA-induced Id1 expression

Transient transfections in Hep3B cells with 1.0 kb *Id1* promoter (A) or RARE3-tk-luc (B) and PML-RARα or RARα-RXR with and without ATRA, with and without dominant negative NF-Y (NF-YA13m29).

DISCUSSION

Various retinoic acid target genes like *Id2* [4], *C/EBPε* [11,36,41] and the growth inhibitor *p21*[3,31,52] have been identified that are likely involved in the deregulation of granulocytic differentiation in APL cells. After the identification of *Id2*, we report here that *Id1* also is a direct ATRA responsive gene in APL but *Id3* and *Id4* are not. Id proteins form heterodimers with either class I (ubiquitously expressed) bHLH proteins (like the E2A gene products E12 and E47) or class II (more tissue-specific expressed) bHLH proteins (like MyoD and Scl/Tal-1) [37,38,39] but lack the amino-terminal basic region necessary for DNA binding. As a consequence, Id proteins act as dominant antagonists of bHLH proteins. Our results suggest that bHLH proteins play a role in the differentiation of APL cells as well as in non-leukemic myeloid cells.

So far, cotransfection assays have shown that PML-RARα does not function like wild-type RARα. An aberrant PML-RARα/RXR or PML-RARα homodimer complex may occupy the DNA target sites and alter the transcription of target genes. Delocalization of RXR in APL cells could also lead to the sequestration of RXRs away from their normal site of action. Moreover, unlike normal RARα, which requires RXRs, PML-RARα can homodimerize and bind RAREs [24,42] or closely related DNA sequences. Thus, PML-RARα could deregulate ATRA target genes or even other sets of genes not normally regulated by nuclear receptors. Deregulation of the expression of ATRA responsive genes by PML-RARα may be the consequence of enhanced interaction with co-repressor proteins like N-CoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid-hormone receptors) compared to unrearranged RARα. These co-repressor proteins recruit histone deacetylases to the DNA [18,20,21,28,29] resulting in transcriptional repression of target genes [19]. The corepressor complex is not released at physiological concentrations but at pharmacological doses of ATRA the corepressors are replaced by coactivators allowing transcription.

In this study we show that the *Id1* promoter is transcriptionally activated in response to ATRA by the fusion protein PML-RARα but not by wild-type RARα/RXR. We show that transactivation by PML-RARα was not mediated through a consensus RARE. This implies that PML-RARα interferes with the transcription of Id1 by a novel mechanism. Interference of PML-RARα may be important not only during the induction of differentiation but possibly also in the transformation of the cells: the recruitment of corepressor proteins to the *Id1* regulatory sequences may lead to an abnormal silencing of this gene in the absence of ligand. Interestingly, a CCAAT-box 80 bp upstream of the TATA-box is involved in the ATRA-induced Id1 expression by PML-RARα and the ubiquitous transcription factor NF-Y binds to this site. The CCAAT-box is a widely distributed regulatory sequence. NF-Y has been originally identified as the protein binding to the major histocompatibility complex class II *Ea* promoter Y box [14]. NF-Y is a heteromeric transcription factor composed of three subunits, NF-YA, NF-YB, and NF-YC, all necessary for DNA-binding [35,46]. The close association of NF-YB and NF-YC is a prerequisite for NF-YA binding and sequence-specific DNA interaction. Transcriptional activation of *Id1* in APL requires both PML-RARα and NF-Y. The exact interaction between PML-RARα and NF-Y remains to be elucidated. Various mechanisms may be involved as shown in Figure 5.9. PML-RARα may bind directly to NF-Y and transactivate NF-Y target genes in response to ATRA (Figure 5.9A). Alternatively, PML-RARα may alter the interaction of NF-Y with other transcription factors (like Sp1) which have been shown [22] to interact with NF-Y for efficient transactivation (Figure 5.9B). Finally, an indirect interaction between PML-RARα and NF-Y may be involved (Figure 5.9C). PML-RARα mediates transcription through binding of a complex of coactivators in response to a high dose of ATRA. Recent reports describe that coactivators are also associated with NF-Y. NF-YA was shown to interact with P/CAF and NF-YB with p300 [34]. This may indicate that PML-RARα and NF-Y indirectly interact by binding to the same co-factors. Additional experiments should determine whether PML-RARα and NF-Y interact directly or indirectly.

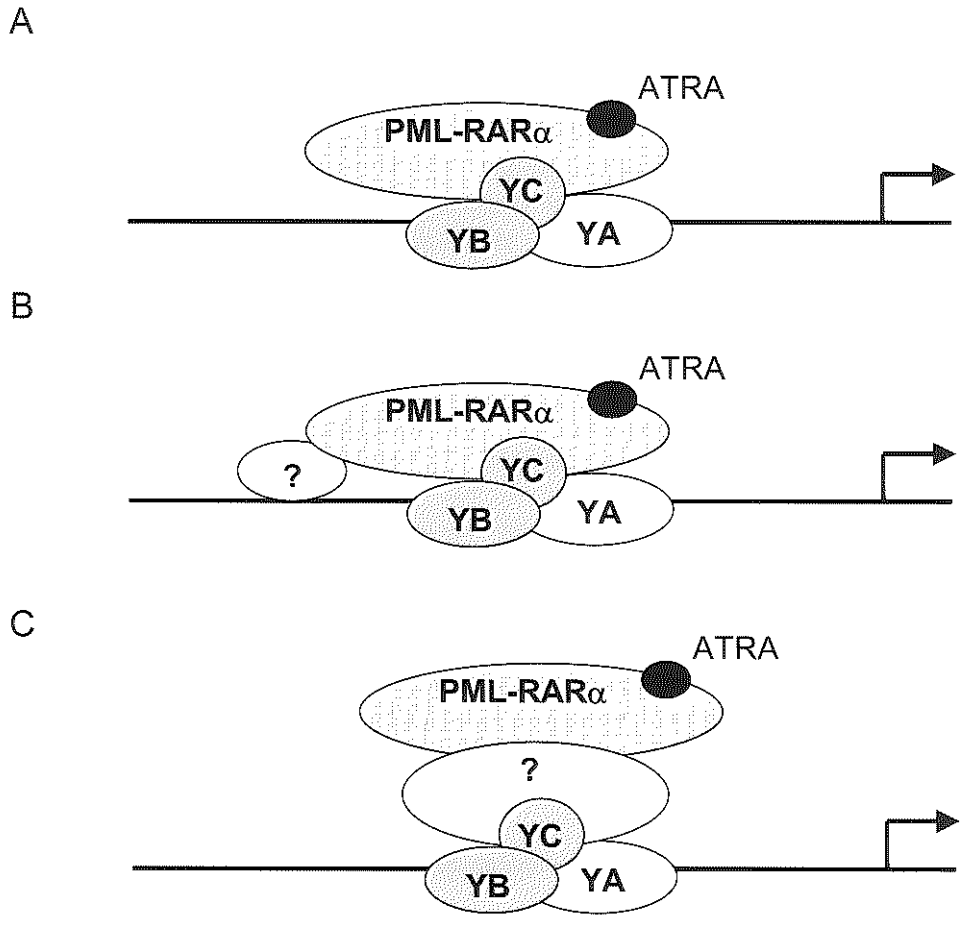


Figure 5.9. Possible mechanisms involved in transcriptional activation by PML-RARα and NF-Y

(A) PML-RARα may bind directly to NF-Y and the complex is activated in response to ATRA. (B) PML-RARα may alter the interaction of NF-Y with other transcription factors like Sp1. (C) PML-RARα and NF-Y may bind indirectly.

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CHAPTER 6

General Discussion

6.1 Introduction

In this final chapter, the perspective of differentiation induction therapy in acute promyelocytic leukemia (APL) and other types of leukemia will be discussed. In addition, as the identification of *Id1* and *Id2* as direct ATRA-responsive genes gives more insight in the complexity of APL pathogenesis (Chapters 4 and 5), we will discuss here their potential role in myelopoiesis and the contribution to terminal granulocytic differentiation in response to ATRA therapy in APL.

6.2 Can differentiation induction therapy be used in other types of leukemia?

Currently, G-CSF is clinically used to stimulate the development of myeloid precursor cells to increase the number of mature granulocytes in patients with congenital and chemotherapy-induced neutropenia. In AML, G-CSF is successfully administered to shorten neutropenia following stem cell transplantation or chemotherapy [6,18,32,35]. Since the leukemic blasts may express functionally active G-CSF-receptor [18], there was concern of inducing proliferation of these cells by G-CSF. Up to now, clinical studies do not show evidence for an increase in relapse when G-CSF is applied during the hypoplastic phase following cytotoxic therapy. G-CSF may conceivably have a therapeutic role in differentiation induction therapy in leukemia. Depending on the target cell, G-CSF may stimulate proliferation or induce differentiation. In this thesis, evidence was provided for a clinically useful role of G-CSF in combination with ATRA in differentiation induction therapy in a PLZF-RARα-positive APL patient (Chapter 2). Neither G-CSF alone nor ATRA alone had significant effects *in vitro*, but together they induced terminal differentiation of the leukemic cells. This effect was also seen *in vivo*. An attractive hypothesis for the function of G-CSF in PLZF-RARα-positive APL may be that binding of G-CSF to the G-CSF receptor triggers downstream signal transduction pathways as shown in Figure 1.2. These pathways may exert a cascade of phosphorylation events leading to dissociation of the ATRA insensitive corepressor complex from the PLZF-part of the fusion protein. As a result, coactivators may bind to the fusion protein and transcription is initiated.

Previous reports show that ATRA together with the histone deacetylase inhibitor TSA induces differentiation of transformed cells carrying the PLZF-RARα fusion protein *in vitro* [8,10]. Recently, Kitamura et al. (2000) published the differentiation response to ATRA and TSA *in vitro* in PLZF-RARα-positive APL patient cells [14]. A model which may explain the sensitivity to ATRA and TSA has been derived from molecular studies as described in Chapter 1 (Figure 1.4) [8,9,10,16].

Alteration of transcriptional activity of key transcription factors by abnormal recruitment of corepressor proteins may be a more common mechanism in leukemia. Abnormal transcriptional inhibition by corepressor proteins may not only have a role in the treatment of APL, but also in t(8;21) positive AML which expresses the AML1-ETO fusion protein [11]. This fusion protein inhibits the expression of AML1-responsive genes including *IL-3, GM-CSF, M-CSF* and *myeloperoxidase* which may contribute to a block in myeloid development of the maturing hematopoietic progenitors [24]. Repression of AML1 responsive promoters by the AML1-ETO fusion protein may be mediated through recruitment of the N-CoR/SMRT/histone deacetylase-1 repressor complex by ETO [7,20,37]. It has been shown that also in AML1/ETO positive leukemia, TSA is able to partially induce differentiation *in vitro*, probably by inhibiting histone deacetylase activity of the corepressor complex [38]. This suggests on the one hand that TSA may release corepressors. But on the other hand it also indicates that an additional factor is necessary for activating gene expression leading to differentiation of the leukemic cells. Blast cells of t(8;21) AML have been shown to promote neutrophilic differentiation following *in vitro* exposure to G-CSF [18] by activation of the STAT pathway [34]. Recently, a role for G-CSF in differentiation induction was confirmed in a case report describing a t(8;21) AML patient who achieved complete remission following G-CSF treatment [5]. It would be interesting to study whether combinations of agents, like TSA in combination with G-CSF, would be useful as differentiation induction therapy in this type of leukemia.

6.3 How do Id1 and Id2 function in normal granulopoiesis?

Id proteins are helix-loop-helix (HLH) proteins that contain a heterodimerization domain but lack a DNA-binding domain. They function as dominant negative regulators of bHLH proteins through the formation of nonfunctional Id-bHLH heterodimers (Figure 6.1). The bHLH proteins include ubiquitously expressed proteins like E2A or tissue-specific proteins like Scl/Tal-1 [26-28]. The *Id1*, *Id2*, *Id3* and *Id4* gene products are closely related in their HLH regions and show similar affinity for the ubiquitously expressed E2A protein. However, different combinations of Id proteins are expressed in most of the cell lineages. Overexpression of Id1 and Id2 in the 32D myeloid cell line inhibits the growth rate of these cells. Inhibition of proliferation was accompanied by a moderate increase of cellular differentiation (Chapters 4 and 5). Very similar biological functions for Id1 and Id2 were found in these experiments. Together with the overlap in kinetics after ATRA treatment in APL cells, this may suggest a functional redundancy of Id1 and Id2 in granulopoiesis. Studies on *Id1* or *Id2* single knockout mice do not show prominent defects in granulocytic differentiation [21,39,40]. *Id1*/*Id2* double knockout mice should be generated to determine the significance of Id1 and Id2 in steady-state and challenged granulopoiesis.

Similar or redundant biological functions of Id1 and Id2 suggests that these genes may act on the same basic HLH protein partners or proteins with similar functions. So far, bHLH proteins were implicated in lymphopoiesis. For example, constitutive expression of Id1 by targeting the pro-B cells in transgenic mice have revealed an impairment of B lymphocyte development [30]. Mutations affecting bHLH proteins are also frequently found in lymphoid leukemia as a result of recurrent chromosomal translocations. The t(1;14) in patients with T-ALL transposes the *Tal/Scl* gene to the TCR alpha/delta locus on chromosome 14 [1]. The t(7;19) in acute T cell lymphoblastic leukemia involves the *Lyl* gene that is translocated to the TCR beta locus [22], and the t(1;19) [4] that occurs in approximately 25% of the cases of childhood pre-B ALL results in the generation of a E2A-PBX fusion protein [23]. Our data suggest also a role for bHLH proteins in myeloid differentiation. The identification of myeloid specific bHLH partners of Id1 and Id2 will further elucidate the role of bHLH proteins in myelopoiesis.

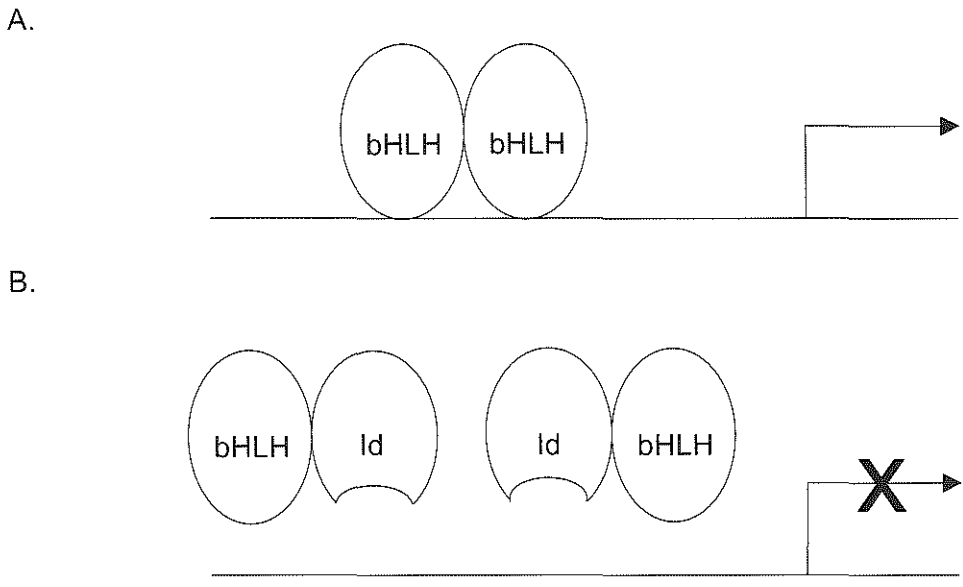

Figure 6.1. The function of Id proteins

(A) bHLH proteins bind to specific sequences of DNA in the absence of Id proteins and activate gene transcription. (B) Id proteins contain a heterodimerization domain but lack the DNA-binding domain. After heterodimerization with Id proteins, the bHLH proteins cannot activate gene transcription.

6.4 What is the role of Id1 and Id2 after ATRA therapy in APL?

The PML-RARα fusion protein transcriptionally activates the *Id1* (Chapter 5) and *Id2* promoter [Breems-de Ridder, unpublished data] in response to ATRA, and the mRNAs encoding Id1 and Id2 are upregulated within 30 minutes after induction. This suggests that both genes are involved in the initial response to ATRA treatment triggered through a PML-RARα dependent signaling pathway. Resistance to ATRA may develop in patients by mutations in the ligand binding domain of the fusion protein [12,13]. In addition, *PML-RARαm4* transgenic mice were generated expressing PML-RARα with a mutation in *RARα* designated as m4 [15]. The m4 mutation was originally identified in an ATRA resistant subclone of human APL cells, and impairs ligand binding and abrogates ligand-induced transcriptional activation

[33]. The leukemias arising in these mice were acute leukemias with promyelocytic features, and they did not differentiate in response to ATRA. This demonstrates that the ability of ATRA to cause differentiation of APL cells requires a direct effect of ATRA through the PML-RARα fusion protein. To determine the biological role of Id1 and Id2 in response to ATRA therapy in APL, it would be interesting to generate targeted mutations of *Id1* and *Id2* in PML-RARα transgenic mice.

Previously, *C/EBPε* [3,29,31] and *p21*$^{waf1/cip1}$ [2,17,36] were also identified as relevant genes involved in the deregulation of differentiation in APL. How these genes are interrelated with respect to induction of granulocytic differentiation in response to ATRA is not known yet. *C/EBPε* and *C/EBPα* knockout mice lack functionally active granulocytes, indicating that these genes are critical for granulopoiesis. Id proteins inhibit the function of bHLH transcriptional regulators that drive differentiation-linked gene expression. This means that in PML-RARα positive APL Id1 and Id2 in response to ATRA may inhibit the activity of bHLH proteins that block the expression of genes involved in differentiation (Figure 6.2). Whether this inhibition is direct or indirect, or at what stage of differentiation it occurs, is not known yet. The search for other candidate genes regulated by ATRA is necessary to understand the mechanism. Techniques such as subtractive PCR, differential display and microarray techniques applied to myeloid cells treated for brief periods with ATRA, will reveal the possible involvement of multiple additional genes in ATRA-induced differentiation. Known genes as well as several unknown sequences were identified by GenBank search. To confirm the involvement of genes in APL pathogenesis, they should be examined for their expression, their interaction with other proteins, and especially their biological function, by examining their effects on cells when they are over- and under-expressed. Current efforts towards the identification of ATRA target genes should finally reveal how the different genes are related and together induce terminal differentiation of APL cells in response to ATRA.

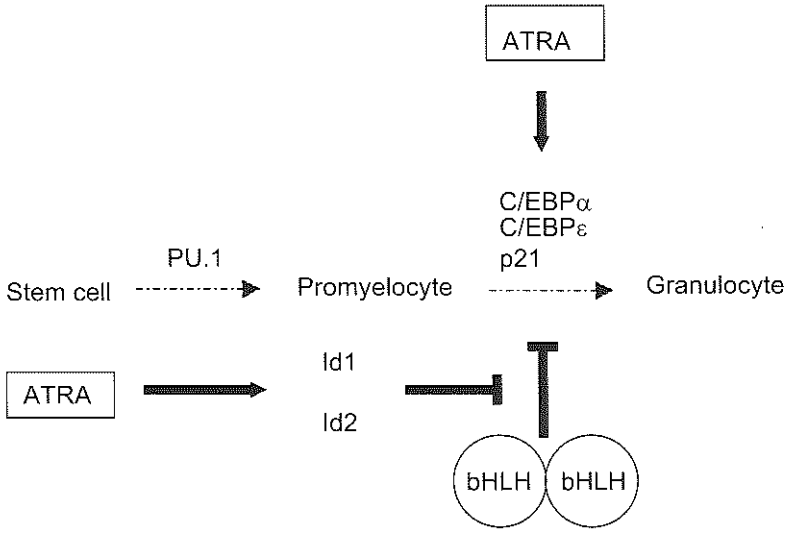

Figure 6.2. The possible role of Id1 and Id2 in granulopoiesis in response to ATRA

Id1 and Id2 are induced in response to ATRA therapy in the presence of the PML-RARα fusion protein. They inhibit the activity of bHLH proteins involved in transcriptional respression of genes involved in granulopoiesis.

6.5 References

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LIST OF ABBREVIATIONS

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ATRA	all-*trans* retinoic acid
BFU-E	burst forming unit-erythroid
CBF	core binding factor
C/EBP	CCAAT/enhancer binding protein
CFU-G	colony forming unit-granulocyte
CFU-M	colony forming unit-macrophage
CFU-S	colony forming unit-spleen
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
Eo	eosinophil
EPO	erythropoietin
FAB	French-American-British
FLT-3	fetal-liver tyrosine kinase-3
G-CSF	granulocyte colony-stimulating factor
GEMM	granulocyte-erythroid-monocyte-megakaryocyte
GM	granulocyte-monocyte
GM-CSF	granulocyte-macrophage colony-stimulating factor
HGF	hematopoietic growth factors
HDAC-1	histone deacetylase-1
Id	inhibitor of DNA binding
IL	interleukin
Jak	janus kinase
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
Meg	megakaryocyte
N-CoR	nuclear receptor co-repressor
ND-10	nuclear domain-10

PLZF	promyelocytic leukemia zinc finger
PML	promyelocytic leukemia
POD	PML oncogenic domain
RAR	retinoic acid receptor
RXR	retinoic x receptor
SCF	stem cell factor
SMRT	silencing mediator for retinoid and thyroid hormone receptors
Sos	son of sevenless
STAT	signal transduction and activation of transcription
TPO	thrombopoietin

SUMMARY

Acute promyelocytic leukemia (APL, FAB-classification AML-M3) accounts for 5-10% of all acute myeloid leukemias. In APL, the leukemic cells are blocked at the promyelocytic stage of development and fail to differentiate into mature, non-proliferative granulocytes. This disease is characterized by chromosomal translocations that lead to fusion of the *retinoic acid receptor* α (*RAR*α) gene to various partner genes. In APL, the RARα fusion proteins contribute to leukemic transformation by dominant interference with the expression of the retinoic acid receptor target genes, and probably also by compromising the function of the involved RARα partner genes. In the last decade, it has become clear that the malignant cells can be forced to overcome the block of differentiation by administration of pharmacological doses of the RAR ligand all-*trans* retinoic acid (ATRA), thus exploiting the residual functionality of the mutated proteins. This has proven to be of clinical use: where treatment with chemotherapy induces relapse free survival in 50-60% of the cases, the combination of ATRA and chemotherapy improves the durable disease-free survival to up to 80%. This treatment constitutes the first generally accepted form of leukemia therapy that is based upon induction of differentiation of the malignant cells.

In about 1% of the APL patients a *PLZF-RAR*α (*promyelocytic leukemia zinc finger*) fusion gene is expressed. In contrast to PML-RARα positive APL, which represents more than 98% of the patients, treatment with ATRA does not induce terminal differentiation, and complete remissions cannot be achieved with ATRA alone in these patients. Based on the reported synergistic effects of ATRA and the hematopoietic growth factor G-CSF with respect to differentiation induction in myeloid cells, the maturation of PLZF-RARα positive leukemia cells was studied using the combination of ATRA and G-CSF. The combined use of ATRA and G-CSF could overcome the maturation block of the leukemic cells *in vitro* and induce granulocytic maturation and complete remission *in vivo*. Bone marrow and blood became negative in FISH analysis and semi-quantitative PCR showed a profound reduction of PLZF-RARα fusion transcripts. This shows that t(11;17) positive leukemia cells are not

intrinsically resistant to retinoic acid, provided that the proper co-stimulus is given (Chapter 2).

Apart form these positive effects, ATRA-treatment may induce a serious complication known as the retinoic acid (RA) syndrome. The RA syndrome includes fever, hypotension, renal failure, respiratory distress and pericardial and pleural effusion. Early recognition of RA syndrome and prompt treatment with corticosteroids (such as the synthetic compound dexamethasone) diminishes the incidence of ATRA-induced death to <5%. Retinoids and corticosteroids are both ligands for transcription factors, implying that their effects are mediated through activation or inactivation of the transcription of their respective target genes. The question that we addressed in Chapter 3 is whether the antagonistic effect of dexamethasone on the ATRA-induced development of the RA syndrome is also accompanied by an antagonistic effect on the ATRA-induced differentiation of leukemic blasts. In *in vitro* experiments, ATRA-induced proliferation arrest, differentiation and thrombomodulin expression in the acute promyelocytic leukemia cell line NB4 were unaffected by dexamethasone. Rather, dexamethasone exerted some inhibitory effect on proliferation. We conclude that dexamethasone does not interfere with the induction of relevant ATRA-responsive genes involved in granulocytic differentiation of APL cells. Thus, it is unlikely that the application of dexamethasone would compromise the therapeutic effect of ATRA.

Deregulation of RARα-target genes may be an important factor for the transforming properties of RARα-fusion genes. However, it is not yet clear which target genes are critical in leukemogenesis and how they might interact. Chapter 4 shows the identification of the *Id2* (Inhibitor of DNA binding) gene as a direct ATRA-responsive gene. This gene belongs to a family of genes of which four members, *Id1*, *Id2*, *Id3* and *Id4* have been identified. *Id2* gene expression is rapidly induced by ATRA in a cycloheximide resistant manner in APL cells. The biological relevance of this gene in myeloid differentiation was shown by its potential to inhibit proliferation of APL cells as well as the murine myeloid cell line 32D.

Subsequently, as for *Id2*, *Id1* was identified as a direct ATRA-responsive gene. Similar biological effects were found in 32D cells upon overexpression of Id1. The observation of identical effects of Id1 and Id2 may suggest that both genes bind to the same basic helix-loop-helix partners in myeloid cells. To identify the mechanism underlying the induction of Id1 transcription by retinoic acid,

transactivation of a 1.0 kilobase 5' flanking region of the human *Id1* gene was examined using luciferase reporter constructs. The *Id1* promoter was transcriptionally activated by PML-RARα but not by the wild-type RARs. Transactivation was independent of a putative RARE. Interestingly, a CCAAT-box was shown to be required and transcription factor NF-Y binds to this site (Chapter 5). In addition, transfection of a dominant negative NF-YA form abolished the PML-RARα-induced transactivation, suggesting that NF-Y is required. Thus, the induction of Id1 and Id2 after ATRA treatment of APL cells may have profound effects on cellular proliferation and maturation. Together with their biological effect in overexpression studies, this suggests a role for Id1 and Id2 as a mediator of ATRA-induced granulocytic differentiation of APL cells. A new mechanism is involved in the ATRA-induced *Id1* transactivation that involves the fusion protein PML-RARα and not wild-type RARs. This suggests a gain of function mutation in APL in addition to the previously reported dominant negative effect of the fusion protein. The identification of the bHLH partners of Id1 and Id2 will give more insight in the role of Id1 and Id2 during the ATRA response in APL and myelopoiesis.

SAMENVATTING

Acute promyelocyten leukemie (APL, FAB-classificatie AML-M3) komt voor bij 5-10% van alle acute myeloide leukemieën. Bij APL is er een rijpingsblok van granulocyten in het stadium van de promyelocyt. Deze vorm van leukemie wordt gekarakteriseerd door een ophoping van promyelocyten in het beenmerg. Tevens is er sprake van chromosoomtranslocaties die leiden tot de fusie van het *vitamine A receptor α* (*RARα*) gen met verschillende andere genen. Door deze translocaties is het RARα gen structureel veranderd en is de functie van het RARα eiwit gestoord. Bij APL dragen de veranderde RARα fusie-eiwitten bij aan leukemische transformatie door dominant te interfereren met de expressie van RARα target-genen en mogelijk ook door verstoring van de functie van de betrokken fusie-genen. In het laatste decennium is duidelijk geworden dat de uitrijping van promyelocyten kan worden geïnduceerd door het toedienen van een hoge dosis vitamine A (ATRA). De leukemievrije overleving is verbeterd door toevoeging van ATRA aan chemotherapie van totaal 50-60% tot 80-90%. Deze behandeling is de eerste algemeen geaccepteerde vorm van leukemietherapie die gebaseerd is op inductie van differentiatie van tumorcellen.

Bij ongeveer 1% van de APL patiënten komt het zeldzame PLZF-RARα (promyelocyten leukemie zinc finger) fusie-eiwit voor. Bij deze patiënten leidt behandeling met ATRA niet tot herstel van uitrijping van de leukemische cellen. Gebaseerd op het veronderstelde effect van ATRA en de hematopoietische groeifactor G-CSF werd de uitrijping van PLZF-RARα positieve cellen bestudeerd na blootstelling aan verscheidene combinaties van ATRA en groeifactoren. Stimulering van de leukemische cellen met ATRA in combinatie met G-CSF leidde tot terminale granulocytaire uitrijping van de cellen *in vitro*. Vervolgens bleek dat behandeling van een patient met dit type PLZF-RARα leukemie met ATRA en G-CSF ook resulteerde in complete granulocytaire uitrijping van de leukemische cellen *in vivo,* en daardoor in een complete cytogenetische en hematologische remissie. Het verdwijnen van leukemie in beenmerg en bloed kon worden bevestigd met moleculaire methoden zoals FISH en semi-kwantitatieve PCR. Deze data, beschreven in Hoofstuk 2, laten zien dat t(11;17) positieve leukemie niet absoluut ongevoelig is voor ATRA mits een juiste combinatie van stimuli wordt aangeboden.

Behalve de positieve effecten van ATRA op de leukemie, blijkt dat behandeling met ATRA bij 15% van de patiënten tot ernstige bijwerkingen leidt. Een ernstige verschijningsvorm is het zogenaamde ATRA-syndroom dat zich uit in koorts, lage bloeddruk, gestoorde nierfunctie, ademhalingsmoeilijkheden en vocht achter de longen en rond het hart. Het snel herkennen van dit syndroom en zo snel mogelijk behandelen met corticosteroïden (zoals het synthetische component dexamethason) is essentieel. ATRA en corticosteroïden zijn beide liganden voor transcriptiefactoren, hetgeen impliceert dat de effecten ontstaan door activatie of inactivatie van transcriptie van target-genen. In dit proefschrift werd onderzocht of het gunstige remmende effect van dexamethason op de door ATRA-geïnduceerde bijwerkingen gepaard zou gaan met een ongunstig remmend effect op de door ATRA geïnduceerde therapeutische werking op de leukemische cellen. In de t(15;17) positieve NB4 cellijn bleek dexamethason geen effect te hebben op de door ATRA tot stand gebrachte uitrijping, normalisatie van PML-bodies en inductie van thrombomoduline mRNA. Tenslotte, dexamethason bleek het antiproliferatieve effect van ATRA op de cellen evenmin te verminderen. Deze resultaten tonen aan dat dexamethason niet ingrijpt in het gunstige effect van ATRA en de inductie van genen die betrokken zijn bij differentiatie van APL cellen. Daarom is het onwaarschijnlijk dat dexamethason het therapeutische effect van ATRA tegenwerkt (Hoofstuk 3).

Ontregeling van de genen die onder invloed staan van RARα (zogenaamde doelwit of target-genen) speelt een belangrijke rol bij de transformerende eigenschappen van RARα fusie-genen. Echter, welke target-genen een cruciale rol spelen bij leukemogenese is nog niet duidelijk. In de Hoofdstukken 4 en 5 wordt de identificatie van *Id2* en *Id1* beschreven als directe RARα target-genen. *Id2* behoort tot een familie van genen bestaande uit *Id1*, *Id2*, *Id3* en *Id4*. Het gen *Id2* werd snel geactiveerd door ATRA in APL cellen, onafhankelijk van cycloheximide, en deze activatie is dus onafhankelijk van nieuwe eiwitsynthese. De biologische relevantie van Id2 en Id1 in myeloide uitrijping werd aangetoond. Id2 remde de groei van de APL cellijn na overexpressie. Nadat in aparte experimenten het gen *Id2* in de APL cellijn, en de genen *Id1* en/of *Id2* in de andere granulocytaire cellijn 32D werden ingebracht, werd telkens een onderdrukking van de celvermeerdering gezien. Kennelijk hebben Id2 (Hoofdstuk 4) en ook Id1 (Hoofdstuk 5) een groeiremmend effect. Daarnaast werd in Hoofdstuk 5 ook het transcriptionele mechanisme achter de

ATRA-geïnduceerde expressie van Id1 bestudeerd. De *Id1* promotor wordt na ATRA toediening transcriptioneel geactiveerd door het PML-RARα fusie-eiwit maar niet door de wild-type receptor RARα, onafhankelijk van een vitamine A respons element. De transcriptionele activatie van de *Id1* promotor vindt plaats via een CCAAT sequentie waar de transcriptie factor NF-Y direct aan bindt maar het RARα fusie-eiwit niet. Dit betekent dat het fusie-eiwit PML-RARα een rol speelt bij transcriptie van ATRA respons genen, maar niet, zoals tot nu toe gerapporteerd, alleen een dominant negatief effect heeft in APL. De gevolgen hiervan behoeven nader onderzoek. De rol van Id1 en Id2 in ATRA-geïnduceerde differentiatie van APL cellen en het effect na overexpressie in 32D cellen suggereert een rol voor bHLH eiwitten in myelopoiese. In toekomstige experimenten zal het identificeren van de bHLH partners van Id1 en Id2 meer inzicht moeten geven in de rol van Id1 en Id2 na de ATRA respons in APL cellen en bij myelopoiese.

CURRICULUM VITAE

Marleen C. Breems-de Ridder (1972) begon haar academische opleiding in 1991 met de studie Medische Biologie aan de Rijksuniversiteit Utrecht. Gedurende haar studie volgde zij een onderzoeksstage van 9 maanden bij de vakgroep Fysiologische Chemie aan de Rijksuniversiteit Utrecht, onder leiding van Prof. Dr. J.S. Sussenbach. Hierna vertrok zij in 1995 voor 8 maanden naar de Verenigde Staten voor een onderzoeksstage bij de Vakgroep Celbiologie aan de University of Massachusetts Medical Center in Worcester, onder leiding van Prof. Dr. G.S. Stein. Na de voltooiing van haar academische studie in juni 1996 werd zij in het kader van een promotieonderzoek aangesteld bij het Instituut Hematologie aan de Erasmus Universiteit Rotterdam, onder leiding van Prof. Dr. B. Löwenberg en Dr. J.H. Jansen. Sinds juli 2000 is zij werkzaam als hoofd van de sectie researchverpleging en datamanagement op de Afdeling Hematologie in het Academisch Ziekenhuis Rotterdam.

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TENSLOTTE

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