Review

Mechanisms of all-*trans* retinoic acid-induced differentiation of acute promyelocytic leukemia cells

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Retinoic acids (RA) play a key role in myeloid differentiation through their agonistic nuclear receptors (RAR*a*/RXR) to modulate the expression of target genes. In acute promyelocytic leukemia (APL) cells with rearrangement of retinoic acid receptor *a* (RAR*a*) (including: PML-RAR*a*, PLZF-RAR*a*, NPM-RAR*a*, NuMA-RAR*a* or STAT5b-RAR*a*) as a result of chromosomal translocations, the RA signal pathway is disrupted and myeloid differentiation is arrested at the promyelocytic stage. Pharmacologic dosage of all-*trans* retinoic acid (ATRA) directly modulates PML-RAR*a* and its interaction with the nuclear receptor co-repressor complex, which restores the wild-type RAR*a*/RXR regulatory pathway and induces the transcriptional expression of down-stream genes. Analysing gene expression profiles in APL cells before and after ATRA treatment represents a useful approach to identify genes whose functions are involved in this new cancer treatment. A chronologically well coordinated modulation of ATRA-regulated genes has thus been revealed which seems to constitute a balanced functional network underlying decreased cellular proliferation, initiation and progression of maturation, and maintenance of cell survival before terminal differentiation.

1. Introduction

Induction of differentiation of leukemic cells has to be traced to 1971 when Friend demonstrated that murine erythroid leukemia (MEL) cells exposed to dimethyl-sulfoxide (DMSO) could differentiate to orthochromic erythroblasts (Watanabe *et al* 1988). Since then, a great number of agents have been observed to trigger the differentiation of different leukemia cell lines *in vitro,* such as hexamethylenebiacetamide (HMBA) and phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA). In 1980,

Breitman *et al* demonstrated that *in vitro*, retinoic acid (RA) isomers, including 13-cis RA and all-*trans* RA (ATRA), could induce differentiation of HL-60 cell line and fresh acute promyelocytic leukemia (APL) cells (Breitman *et al* 1980, 1981). We first showed the therapeutic effect of ATRA in newly diagnosed and refractory APL patients (Sun *et al* 1986; Huang *et al* 1987, 1988). In the majority of the reports, high complete remission (CR) rates (76–95%) were achieved in APL with ATRA treatment, a result much better than conventional chemotherapy (Warrell *et al* 1991; Asou *et al* 1997; Mandelli *et al* 1997;

Tallman *et al* 1997; Burnett *et al* 1999; Fenaux *et al* 1999). Because of its low toxicity and high efficiency, ATRA is now used as the treatment of choice in the remission induction of APL. Over the last decade, great efforts have been made on studying the mechanism of ATRA-induced APL cell differentiation, and a large number of papers were published to elucidate the possible role of ATRA in the differentiation process involving signal transduction pathways, gene expression pattern and cell cycle control, etc. It was in 1990 when Collins *et al* developed a subclone of HL-60 cell line which was resistant to the effect of ATRA, that the importance of RA receptor *a* (RAR*a*) to myeloid differentiation was underscored (Collins *et al* 1990; Robertson *et al* 1992a; Damm *et al* 1993). This subclone harbours a dominant negative mutant of RAR*a* with a truncation within the C-terminal AF-2 domain. Differentiation of these cells under the effect of ATRA could be restored through a retroviral transfection of plasmids expressing either wild-type RAR*a*, or RAR*b*, or RAR*g* (Collins *et al* 1990; Robertson *et al* 1992b). Now it has become clear that ATRA exerts its function through the regulation of a batch of genes by its nuclear receptor, which is disrupted in APL cells (Liu *et al* 1998).

2. Physiology of RA pathway

It is now well known that RA isomers are a group of active metabolites of vitamin A and their physiological effects are mediated through two families of RA receptors: RARs and retinoid X receptors (RXRs) (Chambon 1996, Perlmann and Evans 1997; Freedman 1999), RARs can bind both ATRA and 9-cis RA, whereas RXRs bind only 9-cis RA with high affinity. There are 3 members in each receptor family, encoded by different genes, namely RAR*a*, *b*, and *g*, and RXR*a*, *b*, and *g*. Each member has a number of isoforms because of the alternative use of the promoters of the gene. Both RARs and RXRs share homology with other members of nuclear hormone receptor superfamily, such as steroid hormone receptors, vitamin D receptors (VDRs), and thyroid hormone receptors (THRs). As hormone-inducible transcription factors, these receptors bear six domains (A through F): A/B for ligand-independent transactivation, C with two zinc fingers for DNA binding, D which is a hinge region and E responsible for dimerization with RXR, ligand binding, ligand-dependent transactivation and association with co-repressor (CoR) or co-activator (CoA) complex. The function of F domain is still unknown. RXR can form a heterodimer not only with RAR, but also with other members of the nuclear receptor family, including some "orphan" receptors (receptors without known ligand). The RAR/RXR heterodimer is the active form of retinoic acid receptor, which binds to specific DNA sequences designated as the RA response element (RARE) in the promo-tor regions of target genes, and regulates the transcriptional expression of these genes. Because of the great multiplicity of receptors resulting from heterodimerization between distinct RARs and RXRs and from the differential use of isoforms of each family member, extremely diverse biological responses can be generated.

Recently, new progress with regard to the molecular regulation of RA signaling has been made thanks to the discovery of CoR and CoA complexes (Perlmann and Evans 1997; Freedman 1999), CoR is composed of at least three types of proteins, the nuclear receptor CoR (N-CoR) or silencing mediator of retinoid and thyroid receptor (SMRT), mSin3A or mSin3B, and histone deacetylase (HDAC). CoA is also a multi-protein complex, containing CREB binding protein (CBP)/adenoviral-E1A associated protein p300 (P300), P300/CBP associated factor (P/CAF), P300/CBP interaction protein (P/CIP), also called activator of thyroid hormone and retinoid (ACTR) and the nuclear receptors coactivator-1 (NcoA-1) or steroid hormone receptor coactivator-1 (SRC-1) or NcoA-2. All the CoA components possess histone acetylase (HAT) activity. In the absence of the ligand, RAR/RXR is associated with CoR, which keeps the chromatin structure of the target genes in a repressed status through deacetylation of histones. Upon binding of RA, CoR is dissociated from RAR/RXR and CoA will bind to the receptor heterodimer. CoA can not only open the chromatin structure by acetylating nucleosomes but also recruit the basic transcription complex to the target genes so that their transcriptional expression is activated.

The physiologic importance of RA regulatory pathway in vertebrate ontogenesis has been demonstrated in "knock-out" animal models. Mouse double mutants for RAR*a* and RAR*b*, RAR*b* and RAR*g* or RAR*a* and RAR*g* all died either in fetal life or shortly after birth, with a variety of congenital abnormalities similar to those reported in vitamin A-deficient foetuses (Lohnes *et al* 1994; Mendelson *et al* 1994). RA regulatory pathway has also been shown to play a central role in the development of hematopoiesis. Suppression of the endogenous RAR activity by a dominant negative RAR construct could block neutrophil differentiation at the promyelocyte stage in mouse FDCP mix A4 cell model (Tsai and Collins 1993).

3. Leukemogenesis of APL

Over the last decade, molecular studies have identified the fusion of the RAR*a* gene to partner genes as the major event in APL pathogenesis (Chen *et al* 1996; Warrell *et al* 1993; Grignani *et al* 1994; Melnick and Licht 1999). Several chromosomal translocations have thus been observed and all of those abnormalities are related to the *RARa* gene (table 1).

3.1 *APL with t(15; 17) (q22; q21) and PML-RARa*

In the classical translocation, *RARa* gene is fused with *PML* gene to form *PML-RARa* chimeric gene, which is expressed in all APL patients with t(15; 17). The reciprocal fusion, RARa-PML, is expressed in only 60 ~ 70% of the patients and therefore is not considered as a key player in leukemogenesis (de Thé *et al* 1991; Kakizuka *et al* 1991). A large body of evidence shows that PML-RARa encodes a chimeric receptor which interacts with the wild-type RARa, RXR and PML expressed by the normal alleles present in the same cell. The interaction of mutant receptor with those wild-type proteins disrupts the functions of the latter (Chen *et al* 1996; Grignani *et al* 1994; Melnick and Licht 1999). PML-RARa heterodimerizes with RXR via E domain of RARa moiety so that this essential partner of RARa is sequestered (Perez *et al* 1993). As a result, the RARa/RXR pathway necessary to the granulocytic differentiation is abrogated. Moreover, PML-RARa forms homodimer through the coiled-coil motif of PML and competes with RARa for binding to RARE of target genes. Different from RARa/RXR heterodimer, PML-RARa homodimer interacts on CoR with a much higher affinity, which mediates the transcriptional repression under physiological concentration of RA and blocks the cell differentiation programme (Lin *et al* 1998; Grignani *et al* 1998). However, the blockage of the RA signal transduction pathway is not sufficient for the onset of APL, since the transgenic mice of RARa with an E domain mutation which inhibits endogenous RAR activity in a dominant negative manner showed normal phenotype, while transgenic mice of both normal PML-RARa and PML-RARa with mutation in the same site of RARa portion as described before developed APL. Interestingly, the former showed sensitivity to ATRA but the latter were resistant to the drug. This indicates that the PML portion of PML-RARa is also important for the leukemogenesis (Kogan *et al* 2000). PML is a nuclear protein which contains, from N-terminal to C-terminal, a proline rich sequence, a cystein rich region [including three zinc-finger-like structures, namely, a RING (really interesting new gene) domain and two B box zinc fingers] responsible for nuclear body localization, a coiled-coil region for homo/heterodimerization, a nuclear localization signal (NLS) and a serine/proline rich domain. Topologically, PML is a major component of an organelle called nuclear body or PML oncogenic domains (PODs), which are present as large nuclear speckles 10 to 20 in number in most of investigated cells (Dyck *et al* 1994; Koken *et al* 1994). The major functional domains, i.e. cystein rich region and coiled-coil domain are retained in PML-RARa fusion gene of either "long" or "short" isoforms caused by distinct chromosomal 15 breaks within PML locus (Chen *et al* 1992). PML-RARa can form heterodimer with wild-type PML and thereby disrupts POD speckles to form hundreds of micropunctates (Dyck *et al* 1994; Koken *et al* 1994). Since PML functions as a growth inhibitor and apoptosis agonist (Wang Z G *et al* 1998a, b) and PML is necessary for the expression of some of the ATRA-induced genes, the dominant negative effect of PML-RARa on PML may allow the cells to acquire growth advantage, apoptosis resistance and differentiation arrest.

3.2 *Variant fusion genes in APL and their biological significance*

In 1992, a variant chromosomal translocation t(11; 17) (q23; q21) was identified in a Chinese patient diagnosed

Table 1. Characteristics of 5 subtypes of APL.

Chromosomal aberration	t(15; 17) (q22; q21)	t(11; 17) (q23; q21)	t(11; 17) (q13; q21)	t(5; 17) dup (q35; q21)	(17) (q21·3q23)
Frequency	> 95%	1 ~ 2%	Rare	Rare	Rare
Fusion gene	PML-RARa 100%	PLZF-RARa 100%	NuMA-RARa Yes	NPM-RARa Yes	STAT5b-RARa Yes
	RARa-PML 60 ~ 70%	RARa-PLZF 100%	RARa-NuMA No	RARa-NPM Yes	RARa-STAT5b No
Nuclear localization	In 100 micro-speckles, may be localized in cytoplasm	Localized in micro-speckles	Microspeckle pattern	Sheet like aggregation	Microspeckle pattern, may be localized in cytoplasm
Transgenic models in animal	Several models one closest to human APL	Chronic myeloid leukemia like phenotype	From typical APL to chronic myeloid leukemia	Not known	Not known
Response to ATRA	Good	No, may respond to ATRA1 + G-CSF	Good	Yes	No
Chemo	Good	No, may respond to chemo + ATRA			

as APL (Chen *et al* 1993). Since then, at least 8 confirmed APL cases with t(11; 17) (q23; q21) were reported in the literature. Morphologically, this variant type of APL shows some differences from that of t(15; 17) APL, with predominance of cells containing regular nuclei, either with many granules or more rarely with few granules, and an increased number of Pelger-like cells (Sainty *et al* 1998). Another feature of t(11; 17) (q23; q21) leukemia is its resistance to ATRA. For these two reasons, t(11; 17) (q23; q21) leukemia has been recently considered by some authors as a distinct clinical syndrome (Melnick and Licht 1999). Molecular cloning of the t(11; 17) (q23; q21) translocation has revealed that a gene named promyelocytic leukemia zinc finger (PLZF) normally located on chromosome 11q23 is translocated with RAR*a*. PLZF is a member of a zinc finger protein family termed zinc finger protein with interaction domain (ZID), characterized by the presence of the poxvirus and zinc finger (POZ) or broad complex, tramtrack, bric a brac (BTB) domain at the N-terminal and 9 *Krüpple*-like C_2H_2 zinc fingers at the C-terminal. The fusion between PLZF and RAR*a* results in the formation of both PLZF-RAR*a* and RAR*a*-PLZF chimeric genes in almost all patients investigated, suggesting that both may play a role in leukemogenesis (Melnick and Licht 1999). PLZF-RAR*a* contains the POZ/BTB and the first two zinc fingers of PLZF fused to B-F domains of RAR*a* while RAR*a*-PLZF is formed by the A domain of RAR*a* and the third to ninth zinc fingers from PLZF. Functional analysis has revealed that PLZF-RAR*a* heterodimerizes with RXR and PLZF and therefore may block both RAR*a*/RXR and PLZF regulatory pathways (Dong *et al* 1996). Due to the POZ/BTB interaction, PLZF-RAR*a* can also form homodimer which shows slightly different RARE binding behaviours than PML-RAR*a* (Dong *et al* 1996). More importantly, compared to PML-RAR*a*, PLZF-RAR*a* has a much tighter association with CoR. In fact, PLZF-RAR*a* has two binding sites for CoR, one on the E domain of RAR*a* and another within the POZ/BTB. Though the first binding site can be regulated by ATRA, the second one is insensitive to the modulation of ligand. Hence, the chromatin structure remains in repressed status even under pharmacological concentration of ATRA. This biochemical particularity may underlie the clinical resistance to ATRA in patients with expression of PLZF-RAR*a* (Perez 1993; Lin *et al* 1998; Guidez *et al* 1998; Cheng *et al* 1999). On the other hand, some studies on RAR*a*-PLZF have suggested that it may promote the Cycline A gene expression (Yeyati *et al* 1999) and may block the G-CSF pathway (Hoang T, personal communication).

Over the last few years, other variant chromosomal translocations with resultant fusion genes were reported in a few APL patients, including the t(5; 17) (q35; q21) with nucleophosmin (NPM)-RAR*a* fusion (Redner *et al* 1996),

the t(11; 17) (q13; q21) with fusion nuclear mitotic apparatus (NuMA)-RAR*a* fusion (Wells *et al* 1997) and the STAT5b-RAR*a* fusion due to a duplication of the 7q21·3-q23, (Arnould *et al* 1999). Of note, leukemic cells from patients with t(5; 17) (q35; q21)/NPM-RAR*a* and t(11; 17) (q13; q21)/NuMA-RAR*a* were sensitive to ATRA whereas those from the patient with STAT5b-RAR*a* failed to respond to ATRA (Yeyati *et al* 1999; Redner *et al* 1996; Arnould *et al* 1999). The variant chromosomal translocations, though found in very few cases, may be of biological significance, since they provide new models for understanding the leukemogenesis and the ATRA differentiation therapy. Table 1 shows the major clinical and biological characteristics of APL with different fusion genes.

3.3 *Leukemogenic power of chimeric RA receptors*

The definitive evidence for the transforming ability of APL fusion genes came from the transgenic animal models. PML-RAR*a* driven by either hMRP8 or hCG promoters induced an APL-like leukemia in mice around 1 year after birth (Brown *et al* 1997; He *et al* 1998; Chen S J, Meng X Q and Cheng G X, unpublished data). The APL-like cells from the mice could be transplanted into mice of the same strain to reproduce the same leukemia model. Like human APL with PML-RAR*a*, CR could be achieved by ATRA in transgenic mice or transplanted mice. On the contrary, transgenic mice with hCG-PLZF-RAR*a* developed leukemia much earlier in life, about 3 ~ 12 months after birth (Cheng *et al* 1999; He *et al* 1998). These leukemias resembled chronic myeloid leukemia with increased number of promyelocytes in bone marrow and were insensitive to ATRA. NPM-RAR*a* transgenic mice could develop either typical APL or CML-like leukemia phenotype one year after birth. Cells from these leukemia mice seemed to respond to the differentiation-inducing effect of ATRA (Cheng *et al* 1999).

4.   Mechanisms underlying ATRA differentiation therapy

4.1 *Modulation of PODs and chimeric RA receptors*

An important finding in understanding the action of ATRA was that the treatment of APL cells *in vitro* or *in vivo* with the drug induces relocalization of the PML and restores the normal structure of POD (Dyck *et al* 1994; Koken; *et al* 1994). Next, it was reported that the binding of ATRA to receptors could cause a degradation of PML-RAR*a* (Raelson *et al* 1996; Yoshida *et al* 1996; Nervi *et al* 1998). This degradation seems to be biphasic,

with a rapid decrease of fusion protein within 1 h, and a second step after 12 h characterized by the appearance of a 90 kDa cleavage product. Both the proteasome-ubiquitin system and the caspase system have been suggested to be involved in the degradation of PML-RAR*a*. A recent study demonstrated more clearly two proteolysis pathways for RA receptors: one is the caspase-mediated cleavage of fusion protein, and the second is a proteosome-dependent degradation of both PML-RAR*a* and wild-type RAR*a* as well as RXR*a* (Zhu *et al* 1999). The catabolism of RAR*a* seems to require heterodimerization with RXR*a*, DNA binding and the ligand-dependent activation domain. However, the significance of PML-RAR*a*/RAR*a* degradation in ATRA-induced APL cell differentiation remains controversial (Nervi *et al* 1998).

4.2 *Modulation of interaction of the receptor with CoR or CoA*

Many studies have suggested that the modulation of CoR-binding of the receptors plays a key role in differentiation by modulating the structure of chromatin. It is well established that at physiological concentration (0·01 μM), ATRA can dissociate CoR from wild-type RAR*a*/RXR*a* and recruit CoA for transcriptional activation. PML-RAR*a* and NPM-RAR*a* are less sensitive to the ligand-induced modulation. However, pharmacological concentrations (0·1 ~ 1 μM) of ATRA result in the release of CoR from these fusion receptors and subsequently the recruitment of CoA, and convert PML-RAR*a* and NPM-RAR*a* from transcription repressor to transcription activator (Lin *et al* 1998; Grignani *et al* 1998; Guidez *et al* 1998; Cheng *et al* 1999; So *et al* 2000). It is worth noting that even in the presence of 10 μM ATRA, PLZF-RAR*a* remains associated with CoR, because of the insensitivity of POZ/BTB-CoR interaction to ATRA modulation. These important findings explain why APL patients with PML-RAR*a* and NPM-RAR*a* respond well to ATRA differentiation therapy whereas patients with PLZF-RAR*a* are insensitive to the drug. Interestingly, HDAC inhibitor trichostatin A (TSA) or sodium butyrate can overcome the transcriptional repression by POZ/BTB-CoR association. Two recent clinical reports showed that CR could be obtained in PLZF-RAR*a* patients with combined use of ATRA and either sodium phenylbutyrate, an inhibitor of HDAC, or G-CSF, which has also been shown to inhibit HDAC activity (Warrell *et al* 1998; Janson *et al* 1999).

4.3 *Gene expression profile modulated by ATRA*

Although the interaction between ATRA and the aberrant as well as wild-type RA receptor-CoR/CoA complexes

has been largely elucidated, the molecular events downstream of RA receptor complexes remained obscure. It is well known that ATRA-induced differentiation is accompanied by degradation of PML/RAR*a*, cell cycle arrest at G1 phase and inhibition of cell growth. After treatment with ATRA, the malignant cells are able to differentiate towards mature granulocytes, thus regaining most functions of their normal counterparts. Unlike chemotherapy, ATRA can induce a high rate of CR in APL patients without leading to hemostatic disorders. However, some side effects may occur in the course of treatment, such as the RA syndrome. Long term use of ATRA in most patients may also induce ATRA-resistance. All these phenomena should be related to the modulation of gene expression by ATRA. Over the past few years, through the effort of several groups including our own, a number of ATRA-modulated genes have been identified, providing some clues to the understanding of the mechanism of ATRA-induced differentiation (Mao *et al* 1996; Duprez *et al* 1997; Yu *et al* 1997; Tamayo *et al* 1999). More recently, by applying techniques allowing relatively large-scale transcriptional expression analysis, such as cDNA array, differential display-PCR (DD-PCR) and suppression subtractive hybridization (SSH), we screened the expression pattern of a large number of genes modulated in APL cell line NB4 treated with ATRA. A list of 169 genes has been presented, including 100 genes up-regulated and 69 genes down-regulated (Liu *et al* 1998; 2000). The expression has been shown to be induced within 12 h of ATRA treatment in half of the up-regulated genes, and over 24–72 h in the other half. In contrast, over 90% of the down-regulated genes have their expression levels decreased within 8 h of treatment. The time course of the regulated gene expression patterns is associated with the differentiation status of NB4 cells. By analysing the functional information of RA-modulated genes from both literature and our own work, a picture of well-coordinated gene expression network has emerged, which may reflect an elegant and intricate cellular programme for the commitment to differentiation.

For example, it is well established that a few transcription factors play important roles in RA signal pathway. These transcription factors are much likely to be direct targets of RA receptors and are induced at the very beginning of the ATRA treatment. In fact, RA receptors themselves may be considered as such transcription factors, since they are up-regulated shortly after the application of ATRA. RARE has been found in the promoters of the RARs. Over-expression of RARs could overcome the dominant negative effects of PML-RAR*a* and lead APL cells to neutrophil differentiation (Gudas 1994; Leroy *et al* 1991; Chomienne *et al* 1991). Another transcription factor induced by ATRA is CEBPε, whose expression is accompanied by granulocytic differentiation and its over-

expression could lead U937 cells to mature granulocytes (Morosetti *et al* 1997; Park *et al* 1997). HOX genes are expressed in myeloid cell lines in a coordinated, dynamic manner. Enforced expression of the HOX genes or disruption of their expression has been shown to be associated with altered myeloid growth and differentiation. Some HOX genes such as HOX-A1 have been confirmed as ATRA direct target genes (Magli *et al* 1991; Allen and Adams 1993; Ogura and Evans 1995; Nakamura *et al* 1996).

Altered transcriptional expression after ATRA treatment has also been shown for those genes involved in proliferation arrest and cell cycle exit (Liu *et al* 1996; Kenneth and Harris 1997; Steinman *et al* 1998). For example, genes known to be able to suppress proliferation and cell cycle progress, such as P21WAF1/CIP1, P19INK4D, GADD153, LIMK, BTG1 and Src-like adaptor protein, are up-regulated. In concert with this, a number of genes favouring DNA synthesis/repair and G1-S/G2-M transition, including c-MYC, c-MYB, NFkB, GATA2, XRCC1, P55CDC, cyclin B1 and cyclin A, are down regulated at early time of ATRA treatment.

As mentioned earlier, the degradation of PML-RAR*a* has been shown to be associated with the protein modulation of ubiquitin/proteasome system. Interestingly, three genes involved in this system, UAE1, UCE and SUMO-1, are up regulated just at the time of PML-RAR*a* degradation and restoration of PODs (Müller *et al* 1998). In some systems, the degradation of PML-RAR*a* and the release of PML have been reported to lead the cells to apoptosis (Wang Z G *et al* 1998a, b). It is however worth noting that in ATRA-treated APL cells, although PML-RAR*a* is degradated 12–24 h after application of ATRA, no apoptosis occurs until terminal differentiation. According to the results of the gene expression analysis, the balance between apoptosis and differentiation could be maintained through the induction of several apoptosis antagonists such as Bcl-2 related (A1), GADD153, DAD1 and FGR protein-tyrosine kinase, and the inhibition of the expression of some apoptosis agonists such as ICH-1L and FAST kinase.

Genes participating in several important signal transduction pathways, including JAKs/STAT, cAMP/PKA, PKC, calmodulin and some of their down-stream genes have been found up-regulated. On the other hand, genes belonging to three mitogen-activated protein kinase families, namely P38, ERK3 and JNK, are shown to be down-regulated, suggesting that MAPK/SAPK pathways could be transcriptionally modulated in favour of growth arrest and granulocytic differentiation.

Finally, modulation of some of the genes may explain the clinical features of ATRA treatment. For example, the induced expression of some neutrophil function-related genes, such as MCP-1, defensin, and X-CGD, may reflect biological activities of the terminally differentiated granulocytes. On the other hand, the up-regulation of

some cytokines, integrins and their receptors, such as IL-1, IL-8, G-CSF receptor, CD11b, CD18 and ICAM-1, may be required not only for granulocytic differentiation, but also responsible, at least to some extent, for the development of RA syndrome. The restoration of the balance of hemostasis could be ascribed to the induced expression of thrombomodulin and urokinase plasminogen activator receptor, and the inhibited expression of tissue factor by ATRA. The induction of CRABPII and dioxin-inducible cytochrome P450 may represent a feedback mechanism of the cells under ATRA treatment. It is possible that long term use of ATRA treatment may cause a sustained expression of these genes at high level and thereby render the cells resistant to ATRA.

5. Perspectives

So far, the selective differentiation therapy has been successful only in APL. Some questions can thus be raised: what can we learn from the APL model? Can the concept of differentiation therapy be extended to other leukemias and solid tumours to benefit more patients?

We believe that the major lesson we have learned from the ATRA treatment of APL is the targeting of the oncoprotein PML-RAR*a* by the drug (Chen *et al* 1996; Melnick and Licht 1999). With the rapid development of human genome project and cancer research, it is possible that the genetic abnormalities in major leukemia and solid tumour types will be identified at the beginning of the 21st century. Therefore, if the discovery of ATRA was mainly based on empirical clinical experience, the "targeting" therapy will be developed much more on the basis of drug design or screening according to the specific genetic defect in different human cancers. The "targeting" approach together with the more rational use of relatively non-specific chemotherapy may significantly improve the outcome of cancer patients.

Another inspiration that we may draw from APL model is that the basic research on protein-protein interaction and protein-DNA interaction in some disease gene-associated common pathways may lead to the discovery of important new targets for therapeutic agents (Melnick and Licht 1999). PML-RAR*a* is associated with CoR/CoA and thus linked to one of the key parts of the cellular transcriptional regulatory machinery, the control of histone deacetylation/acetylation. Therefore, the ATRA differentiation therapy can be considered, after all, a therapy based on reprogramming the transcriptional repression/activation. As a matter of fact, the fusion gene products in some other forms of malignant hemopathies have also been found to be in abnormal interaction with CoR/CoA, such as AML-1-ETO (or MTG-8) in AML-M2b with t(8; 21) (q22; q22) (Wang J X *et al* 1998, 1999), TEL-AML1 in ALL with t(12; 21) (p13; q22) (Guidez *et al*

1999) and involvement of LAZ/BCL6 (a gene homologous to PLZF) in diffuse large cell lymphoma (Dhordain *et al* 1997). ETO, TEL and LAZ/BCL6 have all been found to possess CoR binding motif. In some instances, even the genes encoding proteins with HDAC (fusions between MOZ on 8p11 and distinct partners in AML-M4 and M5) or HAT [MLL-CBP in t(11; 16) (q23; p13·3) or MLL-P300 in t(11; 22) (q23; q13)] activities were directly involved in chromosomal translocations (Ida *et al* 1997; Rowley *et al* 1997; Carapeti *et al* 1998). Recently, several groups have shown that combined use of HDAC inhibitors and G-CSF could induce differentiation of t(8; 21) leukemia cells *in vitro* (Wang *et al* 1999; Kosugi *et al* 1999). It is hoped that the research on inhibitors of HDAC (and may be also activators of HAT) may open new perspectives for the cancer therapy.

A third important issue is the combination of differentiation/apoptosis inducers and other drugs. ATRA in combination with chemotherapy has been confirmed to give better clinical results than either ATRA or chemotherapy alone. Another approach can be to combine differentiation inducers such as HDAC inhibitor or G-CSF with ATRA, as proved in the PLZF-RAR*a* model. It is well recognized that targets to multiple proteins at different levels of the same pathway by different agents may generate synergistic effect on disease treatment. As previously mentioned, ATRA in association with cAMP, or RXR specific agonist combined with cAMP could overcome the differentiation arrest of APL subclones which cannot be corrected by ATRA alone. On the other hand, 13-cis RA with IFN*a* could produce high response rates for patients with squamous cell carcinomas of the head, neck and cervix (Smith *et al* 1992). Therefore, combination of differentiation inducers for distinct but crosslinked pathways may also be tried in cancer treatment.

In conclusion, differentiation therapy successfully applied in APL may represent one of the most promising approaches for cancer therapy in the new century and is certainly worth continuing endeavour.

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