MOLECULAR TARGETING OF RETINOIC ACID RECEPTOR ACTIVITIES IN AML

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All-trans-retinoic acid (ATRA) is derived from vitamin A whose dietary intake is essential for the development and proper function of all vertebrate organisms. At a cellular level, ATRA can regulate multiple processes ranging from stem cell self renewal to apoptosis. ATRA signalling appears to be inhibited in a variety of cancers, including acute myeloid leukaemias (AMLs) and in acute promyelocytic leukaemia (APL) pharmacological doses of ATRA lead to leukaemic cell differentiation. The effects of ATRA on gene expression are mediated through nuclear retinoic acid receptors (RARs) which belong to a structurally conserved super-family of steroid/thyroid hormone receptors. RARs and other nuclear receptors contain several structurally and functionally conserved regions that include ligand and DNA binding domains, transcription activating regions, dimerisation domain and regions of interaction with various co-regulators. In order to bind to their DNA target sites and activate transcription RARs need to heterodimerise with RXRs, which are receptors for 9-cis-RA or a variety of synthetic compounds called retinoids. RXRs are common heterodimerisation partners for a large number of nuclear receptors, including thyroid hormone (TR) and vitamin D3 (VDR) receptors, allowing for cross-talk between different signalling pathways. RXR heterodimers, such as RAR/RXR or VDR/RXR bind to directly repeated DNA hexamers that are identical in sequence. The specificity of such response elements for a given heterodimer is determined through spacing between the two repeats. The most optimal spacing in the RAR response elements (RAREs) is either 2 or 5 nucleotides, and for TR and VDR is 4 and 3, respectively. When unliganded RAR/RXR heterodimer recruits nuclear receptor co-repressor/histone deacetylase (HDAC) complexes and can actively silence gene expression by deacetylation of N-terminal lysine residues in core histone tails, thus promoting formation of condensed and transcriptionally inactive chromatin. Upon ligand binding the confirmation of RAR ligand binding domain changes causing release of the co-repressor complex and facilitating association of co-activator proteins with histone acetyltransferase (HAT) activities. These proteins acetylate histones in the vicinity of RARE leading to decondensation of chromatin and transcriptional derepression. Underlying genetic abnormality in APL is the translocation between PML and RARα genes, which leads to expression of the PML-RARα fusion oncoprotein. In a small proportion of APL patients variant RARα gene translocations have been characterised including the second most common fusion with the PLZF gene that we have identified. The discovery of the two leukaemogenic RARα fusion oncoprotein facilitated for us studies of molecular mechanisms of APL through comparing functions of PML-RARα and PLZF-RARα. The results of these studies laid a foundation for our more recent work on mechanisms of transcriptional repression and retinoid signalling in AML.

From studies of PML and PLZF-RARα, as well as of other leukaemogenic fusion oncoproteins (TEL-AML1 and AML-ETO, for example), a common mechanism emerged (see Figure) where a key transcriptional activator is rendered constitutive repressor by acquisition of co-repressor/HDAC binding domains from the fused sequences encoded by the translocation partner gene. Important therapeutic implications of these findings were that agents capable of inhibiting enzymatic components of the co-repressor complexes could potentially revert the differentiation blocks imposed by such fusion proteins. For example, HDAC inhibitor NaButyrate and trichostatin A enhanced ATRA mediated differentiation of APL cells and cells derived from PLZF-RARα transgenic mice.

An important question which has been stimulated by results from studies of APL and we have been continuing to address is whether successful use of differentiation therapy could be extended to non-APL-AMLs. Our studies addressing this issue stem in part also from comparative analyses of ATRA signalling in leukaemic cells and in non-leukaemic multiprotein myeloid progenitor cell line FDCPmixA4.

To understand better the role of retinoids in myelopoiesis we have examined expression of the retinoid receptor genes (RARs and RXRs) during differentiation of FDCPmixA4 murine progenitor cells. The major receptor expressed in undifferentiated A4 cells was RARβ (primarily the RARβ1 isoform). Following induction of myelomonocytic differentiation with G- and GM-CSF a dramatic increase in RARα expression (particularly the RARα2 isoform) was seen. In contrast, expression of both RARα isoforms was rapidly extinguished upon induction of erythroid differentiation with EPO. A modest induction of RXRα expression was seen, particularly during differentiation in the myelomonocytic lineage. Low expression levels of RARγ2 and RXRβ remained unchanged, irrespective of differentiation pathway. Consistent with the gene expression patterns, RARα agonists and antagonists stimulated myelomonocytic and erythroid differentiation of FDCPmixA4 cells, respectively. Taken together, these results suggest that erythropoiesis and granulopoiesis require diminished and enhanced RARα activities, respectively, which at physiological ATRA concentrations may be accomplished by reciprocal effects of EPO and myelomonocytic growth factors on its expression. Data showing that ATRA, which positively regulates RARα2 expression, can exert inhibitory effects on erythroid differentiation corrobates this hypothesis. Subsequently, we have extended these studies and demonstrated that myelomonocytic growth factors (G-CSF and/or GM-CSF) potentiate differentiation effects of ATRA in different AML cell lines as well as primary cells from patients with myeloid leukaemia. The ligand dependent activities of endogenous and transiently expressed retinoic acid receptor α (RARα) isoforms can be potentiated by G/GM-CSF in U-937 cells and correlate with increased expression of ATRA inducible RARα2 isoform. Specific inhibitors of MEK-1/2 or p53 mitogen activated protein (MAP) kinase diminish the ATRA as well as ATRA and G/GM-CSF induced activation of the RARα proteins and decreased the differentiation-induced decline in cell numbers. These results demonstrate that acting, at least in part, via the MAP kinase pathways, myelomonocytic growth factors enhance ATRA dependent activation of the RARα isoforms as well as maturation of myeloid leukaemia cells. These results suggest that combinational use of these agents may be effective in differentiation therapy of AML. We have also observed that compared to normal human peripheral blood CD116+/CD33− cells AML cells express very little RARα2 isoform. In cells that do not respond to ATRA, there is also a lack of induction of RARα2 expression. Treatment of ATRA non-responsive cells with the demethylating agent 5-Aza-2-deoxycytidine (decitabine) restores RARα2 expression and differentiation.
response to ATRA in different AML subtypes, suggesting abnormal epigenetic changes as a possible mechanism underlying silencing of RARα2 and contributing to a differentiation deficit in these cells. We compared the methylation status of ATRA-inducible RARα2 and constitutive RARα1 promoters in 5 human AML cell lines and in normal human CD11b+/CD33+ leukocytes (NLs) by sequencing their respective bisulfite modified CpG islands. Whereas the CpG-island of the RARα2 promoter was completely methylated in all AML samples, it was not methylated in NLs nor in a control pre-B cell line, NALM-6. The methylation status of the RARα2 promoter directly correlates with the RARα2 expression level, which was shown by real-time RT-PCR to be 100-1000 fold higher in NLs or NALM-6 cells than in AML cell lines. Expression of RARα2 in human primary AML cells was found to be as low as in AML cell lines. In contrast, RARα1 promoter was not methylated in any of the samples and RARα1 expression levels were similar in AML cells and NLs. We have previously shown that stimulation of AML cell differentiation by ATRA and G/GM-CSF (GFs) is associated with a 10-fold induction of RARα2 expression through direct effects on receptor activity. We now show that pre- or co-treatment with decitabine results in up to 100 fold further increase in RARα2 expression levels, rendering the RARα2 mRNA levels similar to these observed in NLs. This synergism in RARα2 induction is paralleled by marked enhancement of leukemic cell differentiation. These results highlight the potential therapeutic use of epigenetic modifiers like decitabine, which may relieve the repression of aberrantly silenced genes required for cellular maturation and amplify the effects of differentiation inducers like ATRA and GFs in AML.

References


Acute Promyelocytic Leukemia

MOLECULAR TARGETING OF FUSION PROTEINS/COREPRESSORS INTERACTIONS: EFFECTS ON DIFFERENTIATION RESPONSE AND GENE-SPECIFIC CHROMATIN ALTERATIONS

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Introduction

The Acute mieloid leukemia (AML) fusion proteins AML1/ETO and PML-RARα function as transcriptional repressors. This molecular activity stems from one primary pathogenetic event. Due to their ability to generate oligomers, the AML1/ETO and PML-RARα fusion proteins engage to the promoter of RARs or AML1 target genes an abnormally stable protein complex including co-repressor N-CoR/SMRT, Sin3A and histone deacetylases (HDACs). HDACs deacetylate histones and secondarily enroll DNA methyltransferases. The overall effect is hypacyetylation of histones and promoter DNA methyla-

tion that rearrange chromatin structure hampering transcrip-
tion of crucial myeloid differentiation genes. In normal

and AML1/ETO proteins have been mapped within well defined interaction domains (ID). Peptides representative of co-repressors IDs are able to competitively displace co-repressors from nuclear receptors and induce conformational changes that are similar to those caused by the complete co-repressor protein. Thus, targeting fusion protein/co-repressors interaction in leukemia cells can be achieved by the expression of these peptides. These protein fragments may also be a prototype of novel therapeutic strategy acting on chromatin structure on specific target genes.

Results and Discussion

To verify whether this approach could be effective in leukemia cells, we took advantage of well established in vitro models. Since the PML-RARα and the AML1/ETO proteins are similar in the mechanism of their transcriptional repression function, we employed used for both these proteins. We employed retroviral vectors to stably express in cell lines short N-CoR protein sequences derived from the regions that interact with the PML-RARα and the AML1/ETO fusion proteins. We expressed these domains in the APL cell line NB4 and its RA-resistant mutants NB4R4, the myeloid cell lines HL60 and U937 and its derivative PR9 that has inducible expression of PML-RARα. We also expressed AML1/ETO – N-CoR/SMRT interaction region in the cell line SKNO-1, that carries the AML1/ETO fusion gene. We performed co-immunoprecipitation experiments to show that the expression of these sequences competes for the fusion protein – co-repressors contact and blocks the interactions between the leukemia fusion proteins and both the co-repressors N-CoR and SMRT. Thus, the transcriptional repression complex is disrupted and HDACs are not recruited to the promoters of target genes.

Accordingly, the histone acetylation level on the RARβ promoter is increased.

To prove that the block of co-repressor interaction significantly modified the activity of the fusion proteins, we studied the expression of fusion proteins targets genes in the cell lines that expressed the N-CoR fragments. We chose genes that are significant for the regulation of myeloid differentiation (RARα2 and GM-CSF receptor as PML-RARα targets and GM-CSF receptor and p14ARF as AML1/ETO targets). The expression of these target genes, which are repressed by these fusion proteins is increased in the cells with the N-CoR fragments. We studied the expression of the fusion protein, the co-repressors N-CoR and SMRT and of the HDAC1 and 3, the proteins that participate in the repressor complexes. These proteins were unmodified, with the specific exception of PML-RARα. The alteration of PML-RARα-N-CoR/SMRT connections triggers the degradation of the fusion protein. The expression was restored by inhibitors of the proteasomal enzymes, indicating that PML-RARα degradation is mostly mediated by the proteasome. These molecular findings had a biological counterpart. The N-CoR fragments restored the differentiation response to vitamin D3 and retinoic acid in cells that carry the chimeric leukemia genes AML1/ETO or PML-RARα and that were previously resistant to these inducers.

Protein sequences can be expressed in target cells by gene transfer or direct protein transfer by means of protein transduction domains (PTD). PTDs were identified within known proteins, such as HIV TAT or Drosophila Antenna-pedia and are able to mediate adsorption to the cell mem-

brane and subsequent intracellular penetration of the pro-
tein. PTDs are a promising novel approach to protein therapy since they can be used in vivo. Thus, the N-CoR protein fragments described above can be produced in bacteria, purified and directly transduced into target cells by virtue of a HIV-TAT PTD. These peptides proved to be biologically effective since the cells became responsive to differentiation induction.

Taken together these data show that overexpression of the N-CoR fragments can revert the block of differentiation response in leukemia cell lines. Moreover these findings indicate that the chromatin modifications induced by the fusion proteins on target genes promoters are necessary to generate the differentiation block. Our data have implications regarding the role of fusion proteins in the construction of the leukemia phenotype, a critical issue in the selection of targets for molecular therapy. Transgenic animal models indicate that fusion proteins activity is not sufficient to cause differentiation block. However, fusion proteins block differentiation more effectively in murine bone marrow transduction-transplantation models and is highly efficiently in cell lines. We showed that, although fusion protein function may not be sufficient to block myeloid differentiation, it is necessary. Thus, full malignant features in leukemia require fusion protein activity. This phenomenon implies that the elimination of fusion proteins function may prevent the leukemia phenotype. Thus, fusion proteins are an important target for molecular therapy of leukemia. Our data establish the basis for a targeted treatment approach to
leukemia, based on its molecular pathogenesis. Improvements of protein transfer efficiency or the development of small interfering molecules that act on protein interactions may render this strategy applicable in human therapy.

References


EPIGENETIC TARGETS OF RETINOIC ACID ACTION IN ACUTE PROMYELOCYTIC LEUKEMIA

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Correct gene expression is modulated by epigenetic mechanisms including DNA methylation, chromatin modification (acetylation and methylation of histones) and expression of small regulatory RNAs (of recent identification). These events are essential during development and for the maintenance of tissue- and cell-type specific functions. Indeed, epigenetic alterations of DNA and chromatin are linked to tumorigenesis by causing a disregulated expression of genes regulating proper cell function, differentiation and proliferation. Aberrant gene silencing is often associated with methylation of promoter CpG dinucleotides and/or deacetylation of the N-terminal tails of nucleosomal histone H3 and H4 by histone deacetylases (HDACs). Interestingly, histone deacetylation often occurs within chromatin areas enriched of specific methylated histone residues (e.g. lysine 9 in histone H3), while histone acetylation, by histone acetyltransferases (HAT), and methylation of lysine 4 in histone H3 are associated with gene activation. A link between gene silencing, induced by DNA methylation, and the regulation of chromatin structure by histone acetylation has been demonstrated, since DNA methylationtransferases (DnMT1 and DnMT3) and methyl-CpG binding domain proteins (MBDs) have been found present in chromatin remodeling complexes containing HDACs. In addition, deregulation of HATs, HDACs, DNMTs or MBDS results in abnormal transcriptional regulation of target genes that are relevant to the transformation process. Therefore, heritable changes in gene expression due to epigenetic modifications of chromatin by HATs, HDACs, DNA methyltransferases offer a mechanism by which upstream signaling pathways can converge to common targets associated with normal development and neoplasia. Interestingly, in contrast to genetic cancer causes, all these aberrant epigenetic changes in cells can be reversed by drugs targeting chromatin regulators. This highlights the chromatin regulators as novel targets for the development of anti-neoplastic drugs, which might induce lasting remissions and spare the short and long term toxicity associated with high dose chemotherapy.

Transcriptional repression of retinoic acid (RA) signaling pathway due to the an aberrant recruitment of histone deacetylases (HDACs) and DNA methyltransferases by the acute promyelocytic leukemia (APL)-associated RARα-fusion proteins on RA-target genes is the molecular event underlying the block at the promyelocytic stage of myeloid differentiation and leukemogenesis in APL. Paradoxically, APL is also the most striking clinical success of a RA-based differentiation therapy in human neoplasia, and has became the molecular paradigm for therapeutic approaches utilizing differentiating agents. Indeed, pharmacological doses of RA can release the HDAC repressor complex and recruit the multigene histone acetyltransferases activation complex on RA target genes, resulting in terminal differentiation of PML-RARα-positive APL blasts, which account for more than 90% of APL. Thus, differentiation induction should be regarded as a promising therapeutic approach of leukemias.

Treatment with RA reprograms APL blasts to a non-leukemic phenotype also by inducing an early and coordin-
nated decrease of DNMT1, DNMT3a and DNMT3b expression/activity in RA-responsive APL cell lines and primary blasts from APL patients. This correlates with de-methylation of the promoter/exon-1 regions on its target gene RARβ2 in RA-responsive APL blasts cultured in vitro, and in blood samples from APL patients undergoing treatment with RA and chemotherapy. Of note, that: i) DNMT3b mRNA expression, which shows the earliest and largest decrease during normal myeloid differentiation was mainly affected by RA treatment in the RA-responsive APL blasts in vitro and in vivo; ii) down-regulation of DNMT expression/activity, of their occupancy at RARE sites and exon-1 on RARβ2 gene and of the methylation status of the RARβ2 promoter/exon-1 are not measurable in the RA-resistant NB4-MR4 cells following RA treatment; iii) nanomolar concentrations of a selective RARα agonist (AM580) also modulated DNMT expression, and its effect is fully abolished by a RARα selective antagonist (Ro41-5253).

Together, these results suggest a direct involvement of RA binding to either a functional PML-RARα or endogenous wild type RARα in the down-regulation of DNMT expression in APL. Indeed, a decreased expression of DNMT1, DNMT3a and DNMT3b and granulocytic differentiation is also induced by the RARα agonist AM580 in HL-60 cells, a myeloid leukemia cell line morphologically and biochemically very similar to the APL blast lacking the t(15;17) but expressing the functional RARα. Therefore, down-regulation of DNMT expression might be a more general mechanism of RA action in cells. In regard of general mechanisms of RA action in cells, our recent findings indicate that RA effect in myeloid differentiation also occurs through the processing of specific RNA transcripts. By using the DNMT and HDAC inhibitors in the presence or in the absence of RA we found that all these agents are active in synergize potentiating the effect of RA on RA-responsive promoter activities, endogenous RA target genes (RARα and Type II TGase) and myeloid differentiation in either RA-responsive or RA-resistant APL blasts. Thus, the reversion of both DNA methylation and histone deacetylation status imposed by PML-RARα at RA-target promoters may represent a key step for RA to trigger terminal differentiation of malignant cells and induction of disease remission in APL patients.

These evidences suggest a scenario where the accessibility at specific DNA-binding sites might represent the key event for generating a chromatin code coupled to specific differentiation decision in leukemic blasts. Targeting of specific chromatin remodeling activities may represent a therapeutic strategy potentially applicable also to RA-resistant APL patients and to AML in which an aberrant transcriptional repression underlies gene silencing, maturation arrest, and leukemogenesis.

References