C04b

COMPARATIVE EVOLUTION OF HEMOSTASIS IN ACUTE PROMYELOCYTIC LEUKEMIA AT ONSET AND IN RELAPSE

Colita A,[1] Lupu A,[1] Avram S,[1] Uscatescu V,[2] Angelescu S,[1] Barbu D,[1] Ostroveanu D,[2] Colita AC,[2] Mut Popescu D[1]

[1]Coltea Clinical Hospital/UMF Carol Davila, Bucharest; [2]Fundeni Clinical Institute, Bucharest, Romania

APL is a distinct subtype of acute myeloid leukemia characterized by the severe alteration of hemostasis at the onset, that is accentuated after the introduction of chemotherapy. Modern treatment with retinoic acid has permitted a better management of APL related coagulopathy. This study evaluates the hemoststic parametres in 31 APL patients with relapse. Clinical examination showed a greater incidence of severe hemorrhagic syndrome at onset compared with the moment of relapse. Biological data showed superior values of hemoglobin, circulating leukemia cells and platelets at relapse compared with the initial data. At the same time, coagulation parameters (fibrinogen, APTT, AP, TQ, TMF, FDP) where less altered at relapse than at onset. On the other hand, the incidence of severe hemorrhagic complications is greater during the treatment of relapse with 25,8% deaths caused by cerebral hemorrhage. Coagulation data showed the predominence of severe coagulopathy and a slower normalization of coagulation parameters and platelets at relapse. This data suggest the possible action of different mechanisms in the pathogenesis of APL related coagulopathy at onset and during relapse, that might by an explanation for the severe course of coagulopathy at relapse.

WORKSHOP II
IN VITRO AND IN VIVO MODELS

RETINOIC ACID-DEPENDENT GENE TRANSACTIVATION BY PML-RAR· WITHOUT DIRECT DNA-BINDING

van Wageningen S, Nigten J, Breems-de Ridder MC, Nikoloski G, Erpelinck-Verschueren CAJ, Löwenberg B, de Witte T, van der Reijden BA, Jansen JH

Dept Hematology Erasmus Univerity Rotterdam and UMCN St Radboud Nijmegen, The Netherlands

Liganded PML-RARα induces ID1 and ID2 expression

In a search for PML-RARα target genes, we found that retinoic acid rapidly induces ID1 and ID2 expression, both in the acute promyelocytic leukemia (APL) cell line NB4 as well as in primary APL patient cells. ID proteins act as negative regulators of basic-helix-loop-helix transcription factors, and are implicated in the regulation of various differentiation processes, including hematopoiesis. To test the relevance of this upregulation, ID1 and ID2 were overexpressed in NB4 cells using retroviral vectors. Overexpression inhibited proliferation and induced a G0/G1 accumulation, indicating that ID1 and ID2 may be biologically important for the retinoic acid response in APL cells.

PML-RARα transactivates ID1 and ID2 without direct DNA-binding

As ID1 and ID2 were induced within 30 minutes after stimulation with retinoic acid, we studied wether these genes are direct retinoic acid targets. ID1 and ID2 mRNAs were rapidly upregulated, both in the presence and absence of cycloheximide, suggesting that these genes were directly transactivated without intermediate protein expression. We cloned the 5' upstream *ID1* and *ID2* promoters into a luciferase construct for transactivation studies. Surprisingly, we found that the promoters were transactivated by PML-RARα in a retinoic acid dependent manner, but not by unrearranged RARα/RXR. To identify the regulatory DNA sequences through which the induction by PML-RARα was mediated, several deletion mutants were constructed. Deletion mutants that lacked all (even remotely) consensus retinoic acid response elements were still transactivated by PML-RARα . To test whether PML-RARα might transactivate the ID1 and ID2 promoters without directly binding to the DNA, we used a PML-RARα construct in which the DNA binding domain was deleted (PML-RAR/ΔR). While we confirmed that PML-RAR/ΔR was unable to transactivate a promoter containing a well defined retinoic acid response element (β-RAR promoter), it could still transactivate the ID1 and ID2 promoters. This indicated that transactivation by PML-RARα occurred without direct binding of PML-RARα to the DNA. When the coiled-coil domain of PML-RARα was deleted (PML-RAR/ΔCC) transactivation was abolished. As the coiled-coil domain is involved in protein-protein interactions, this suggested that the transactivation of these genes is dependent on homodimerisation and/or interaction with other protein(s) mediated through the PML-part of the chimera. Further deletion and point mutants identified the site in the ID1 promoter that was necessary for PML-RARα mediated transactivation, showing that a CCAAT box (binding site for the transcription factor NFY) and two adjacent GC boxes (binding sites for SP1) were essential for transactivation. SP1 and NF-Y are rather ubiquitously expressed transcription factors that work in concert on many different promoters. NF-Y is a trimeric protein complex consisting of three subunits (NF-YA, NF-YB and NF-YC). Assembly of all three subunits is necessary for

DNA-binding and transactivation. When a dominant-negative form of NF-YA (NFYAm29) was used, transactivation of the ID1 and the ID2 promoter by PML-RARα was abolished. To test wether PML-RAR could be recruited to the DNA through binding to either SP1 or one of the NFY subunits, we performed GST-pull down experiments. These showed that PML-RARα may bind to SP1, whereas no direct interaction with any of the NFY subunits was observed.

No repression of ID1 transcription in the absence of ligand

The effect of PML-RARα on the endogenous ID1 and ID2 genes was tested using a U937 cell line that is stably transfected with a Zn^{2+}-inducible PML-RARα expression cassette (U937-PR9, provided by Dr. Pelicci). In PML-RARα expressing U937 cells, ATRA strongly induced ID1 and ID2 mRNA and protein expression, contrary to U937 cells lacking PML-RARα. In the absence of ligand, DNA-bound PML-RARα recruits corepressors to the DNA resulting in silencing of the target gene. We tested whether PML-RARα expression would lead to active repression of the *ID1* and *ID2* genes. Induction of PML-RARα in U937 cells did not result in further down-regulation of the low levels ID1 or ID2 mRNA expression, measured by quantitative RT-PCR. In addition, when a large panel of different types of AML samples was tested for ID1 and ID2 expression, the AML-M3 cases did not stand out as a group with exceptionally low expression compared to other FAB-types. Together, this suggests that PML-RARα does not actively repress *ID1* or *ID2* gene expression in the absence of ligand. Therefore, the stimulatory effect of PML-RAR on ID1 and ID2 through this DNA-binding-independent mechanism may be involved in the induction of differentiation of APL cells upon exposure to retinoic acid, rather than in the transformation of the cells

In Summary

Our data implicate that PML-RARα may interfere with the transcription of two classes of genes. The first class concerns genes that are regular targets of retinoid receptors, the second class consists of genes that are normally not regulated by retinoid receptors, but become retinoic acid responsive in the presence of the PML-RARα fusion protein.

TARGETING P73-P53AIP1 APOPTOTIC PATHWAY IN ACUTE PROMYELOCYTIC LEUKEMIA CELLS

Lunghi P,[1] Costanzo A,[2] Salvatore L,[1] Noguera N,[3] Tabilio A,[4] Lo Coco F,[3] Levrero M,[5] Bonati A[1]

[1]Department of Clinical Sciences, Section of Hemato-Oncology, University of Parma; [2]Department of Dermatology, University of Rome "Tor Vergata"; [3]Department of Biopathology, Section of Hematology, University of Rome "Tor Vergata"; [4]Department of Clinical and Experimental Medicine, Section of Hematology and Clinical Immunology, University of Perugia; [5]Laboratory of Gene Expression, Fondazione Andrea Cesalpino, University of Rome "La Sapienza", Italy

Summary

In this study we report that acute promyelocytic leukemia (APL) cells exploit the Ras-MAPK activation pathway to phosphorylate at Ser112 and to inactivate the pro-apoptotic protein Bad, delaying arsenic trioxide (ATO)-induced apoptosis. Both in APL cell line NB4 and in APL primary blasts, the inhibition of ERK1/2 and Bad phosphorylation by MEK1 inhibitors enhanced apoptosis in ATO-treated cells. We isolated an arsenic-resistant NB4 subline (NB4-As^R) which showed stronger ERK1/2 activity (2.7 fold increase) and Bad phosphorylation (2.4 fold increase) compared to parental NB4 cells in response to ATO treatment. Upon ATO exposure, both NB4 and NB4-As^R cell lines doubled protein levels of the death antagonist Bcl-xL but the amount of free Bcl-xL that did not heterodimerize with Bad was 1.8 fold greater in NB4-As^R than in the parental line. MEK1 inhibitors dephosphorylated Bad and inhibited the ATO-induced increase of Bcl-xL, overcoming ATO resistance in NB4-As^R. We then studied the anti-leukemic mechanisms of MEK1 inhibitor + ATO by investigating p73-p53AIP1 apoptotic pathway. Combined treatment results in the induction of the p53AIP1 (p53-regulated Apoptosis-Inducing Protein 1) gene in NB4. We investigated the possible role of p73, a p53 paralog that has been shown to regulate several p53-target genes including p21, Bax and p53AIP1. We found that MEK1 inhibitors reduce the levels of dominant negative ΔN-p73 proteins and promote the accumulation of endogenous p73α through its transcriptional activation and its tyrosine phosphorylation, resulting in p21 up-regulation and significant cell growth inhibition. ATO reduces ΔN-p73 levels and promotes a p300-mediated acetylation of endogenous p73, thus favouring cell cycle arrest and apoptosis. Finally, the combined treatment with MEK1 inhibitors and ATO enhances the affinity of phospho-acetylated p73 for the p53AIP1 promoter *in vivo*, as determined by chromatin immunoprecipitation experiments, leading to p53AIP1 up-regulation and increased apoptosis. Together, these findings support a model in which the p73-p53AIP1 apoptotic pathway is potentiated by Bad dephosphorylation through the increased capacity of Bad, after MEK1 inhibition, to associate with Bcl-xL and Bcl-2. Dephosphorylated Bad displaces Bcl-2 from p53AIP1 and heterodimerizes with Bcl-2 (and with Bcl-xL), thereby blocking their anti-apoptotic function.

Introduction

Arsenic Trioxide (ATO) is considered the treatment of choice for patients with relapsed acute promyelocytic leukemia (APL), particularly in patients exposed to all-*trans* retinoic acid (ATRA) within the prior 12 months.[1] The mechanisms of action of ATO have been investigated both *in vivo* and *in vitro*: at low doses (0.1-0.25 μM) ATO induces a partial differentiation in APL cells through degradation of PML-RARα, while at higher doses (0.5-2 μM) this agent inhibits cell growth and induces apoptosis through both PML-RARα dependent and independent mechanisms with

caspase activation of neoplastic cells.[2] According to recent laboratory studies, the blast cells of most acute myelogenous leukemias (AML) including APL show constitutive activation of extracellular signal-regulated kinases 1/2 (ERKs 1/2) as well as of the kinases immediately upstream of ERK, known as mitogen-activated protein (MAP)/ERK kinases (MEKs).[3,4] Furthermore, we and others have demonstrated that down-modulation of MEK1 phosphorylation inhibits the proliferation and induces apoptosis of primary AML blasts.[3, 4] In this study, we aimed at investigating whether the combination of ATO with agents that block the phosphorylation of MEK1 can potentiate the anti-leukemic action of ATO in APL, and can restore ATO sensitivity in ATO resistant NB4 leukemic cell lines. The results of our experiments confirmed this hypothesis. We than investigated the mechanisms of the anti-leukemic synergism of ATO+MEK1 inhibitor combination. Interestingly, we have demonstrated that both Bad dephosphorylation and p53 paralog p73 activation cooperate in inducing apoptosis of the leukemic cells.

Results and Discussion

MEK1 inhibition enhances ATO-mediated apoptosis both in NB4 and in NB4-As^R cells, and in fresh APL primary blasts

We studied the effect of MEK1 inhibitor, ATO, or both agents in combination on apoptosis in NB4, in NB4 ATO resistant (NB4-As^R) cell line produced in our laboratory and in fresh APL primary blasts from patients. After 72 h of treatment we observed that the combination of MEK1 inhibitors (PD 98059 40 μM or PD 184352 1 μM) with Arsenic Trioxide (ATO 1 μM) strikingly increased the percentage of sub-G_1 apoptotic cells induced by ATO alone in both parental and NB4-As^R cells (Figure 1a).

Changes in mitochondrial transmembrane potential ($\Delta\Psi_m$) are a critical step in cells undergoing apoptosis, regardless of the death signal. Therefore we compared $\Delta\Psi_m$ in ATO or PD+ATO treated cells by using a mitochondrion-specific dye, MitoLight, which displays a green to red spectral shift proportional to $D\Psi_m$. The combined treatment with PD184352 and ATO strikingly potentiated loss of $\Delta\Psi_m$ induced by ATO in both cell lines (Figure 1b) indicating that the dual treatment affects the mitochondria apoptotic pathway.

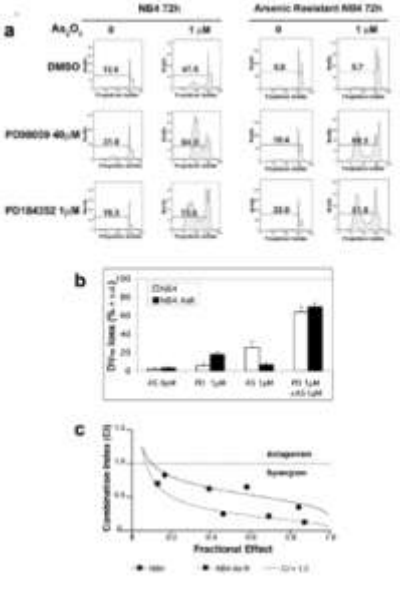

Figure 1..

Similar results were observed with PD 98059 (*data not shown*). Finally, to characterize the pharmacologic interactions between PD184352 and ATO more rigorously and over a range of drugs concentrations, median dose effect analysis was used. When apoptosis by sub-G_1 DNA content or annexin V/PI staining was measured after 48 h of drugs exposure, combination index (CI) values considerably less than 1.0 were obtained in arsenic-resistant and to a lesser extent in the parental NB4 cells, corresponding to highly synergistic interactions in both NB4-As^R cells and parental cells (Figure 1c). Thus, these agents appear to synergize for the induction of apoptosis primarily in arsenic resistant but also in parental NB4 cells. We next studied the apoptotic effect of the combined treatment in fresh APL primary blasts. Interestingly, the percentage of sub-G_1 apoptotic cells in patients (cases # 1, # 2 and # 3) treated for 48 h with PD98059 40 μM and ATO at concentrations of 1 or 2 μM - concentrations that are usually achieved in the plasma of ATO-treated APL patients[5]- was greater (2 or 3 fold more) than that found after treatment with ATO 1 or 2 μM alone (Figure 1D). The treatment with PD98059 alone showed a low increase of cell death in patient # 2, while a stronger apoptosis induction was observed in patients # 1 and # 3 (relapse) (Figure 1D).

Abrogation of MEK1 expression by siRNA sensitizes NB4-As^R cells to ATO treatment

To determine the contribution of MEK-ERK pathway activation in mediating ATO resistance, the MEK1 mRNA was selectively knocked-down by means of specific double-stranded RNA oligonucleotides (siRNA). Transfection of MEK1 siRNA but not the non-specific control siRNA, led to decrease MEK1 in NB4-As^R without affecting the levels of the unrelated protein actin (Figure 2a). When NB4-As^R were treated with ATO an increased PARP fragmentation and an important shift in green fluorescence, indicating loss of $\Delta\Psi_m$, was observed in cells transfected with MEK1 siRNA relative to cells transfected with control siRNA (Figure 2a and b). These findings indicate that the MEK-ERK pathway is determinant in the development of ATO resistance.

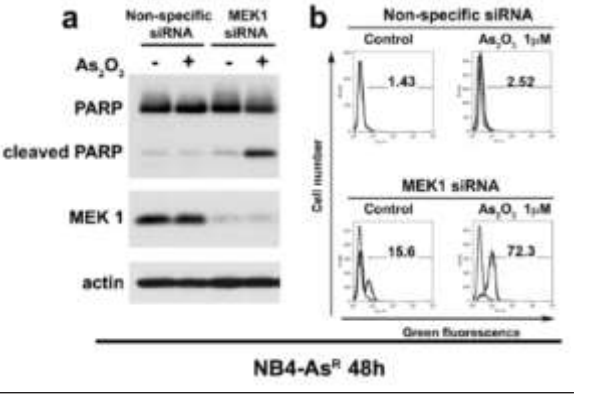

Figure 2..

ATO triggers dissociation of Bcl-xL and Bcl-2 from Bad in APL cells

We proceeded to investigate whether the phosphorylation status of Bad affected its capacity to associate with the death antagonists Bcl-xL and Bcl-2 in NB4-As^R and parental NB4 cell lines. To this end, we examined the constitutive and ATO-induced protein-protein interactions of Bad pathway components using coimmunoprecipitation. Preliminary studies confirmed that the Bad antibody effectively precipitated Bad (*data not shown*). In untreated NB4-As^R and

NB4 cells, we observed a significant binding of Bcl-xL and Bcl-2 proteins to Bad immunoprecipitates indicating the presence of endogenous Bad/Bcl-xL or Bad/Bcl-2 heterodimers (Figure 3). The levels of Bcl-xL and Bcl-2 that coimmunoprecipitated with Bad were comparable in both cell lines (Figure 3). After treatment with ATO, the binding of Bcl-xL and Bcl-2 to endogenous Bad sharply decreased in both cell lines (Figure 3).

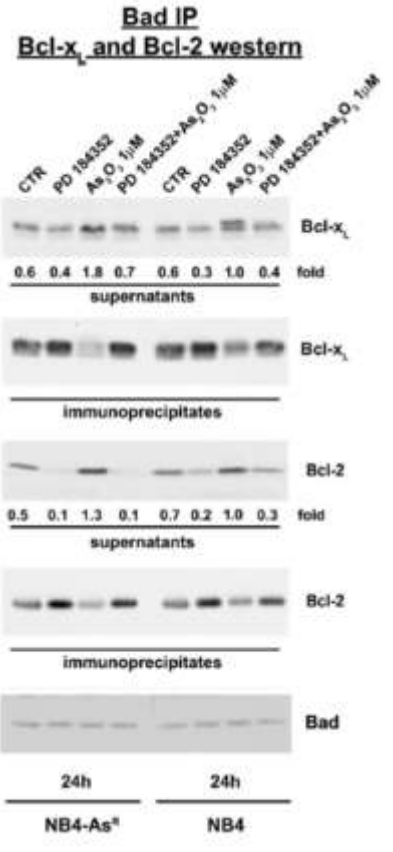

Figure 3..

Interestingly, after ATO treatment, we observed a reduced amount of Bad/Bcl-xL and Bad/Bcl-2 heterodimers in NB4-As^R compared to NB4 (Figure 3); this behavior correlated well with the different phosphorylation status of Bad in these two lines treated with ATO (Figure 4). In order to examine the amounts of free Bcl-xL and Bcl-2 that did not coimmunoprecipitate with Bad, the supernatants after Bad immunoprecipitation were Western blotted for Bcl-xL and Bcl-2. The amount of free Bcl-xL in untreated NB4-AsR or NB4 cells was comparable, but after ATO treatment we observed a stronger increment of free Bcl-xL in NB4-AsR compared to parental NB4 (3 versus 1.7 fold increase compared to the control respectively) (Figure 3). Interestingly, even if the total amount of Bcl-xL was comparable in both cell lines (Figure 4), free Bcl-xL was nearly doubled in NB4-As^R compared to NB4 in ATO-treated cells (Figure 3). In untreated cells the amount of free Bcl-2 was more evident in NB4 than in NB4-As^R. As per free Bcl-xL, a stronger increment of free Bcl-2 was observed in NB4-As^R compared to NB4 (2.6 versus 1.4 compared to the control) after ATO treatment; the amount of free Bcl-2 was higher in NB4-As^R. The treatment with MEK-1 inhibitors was able to down-modulate ERK 1/2 and Bad phosphorylation (Figure 4) leading to a strong increment of Bcl-xL and Bcl-2 protein that immunoprecipitated with Bad in both cell lines (Figure 3).

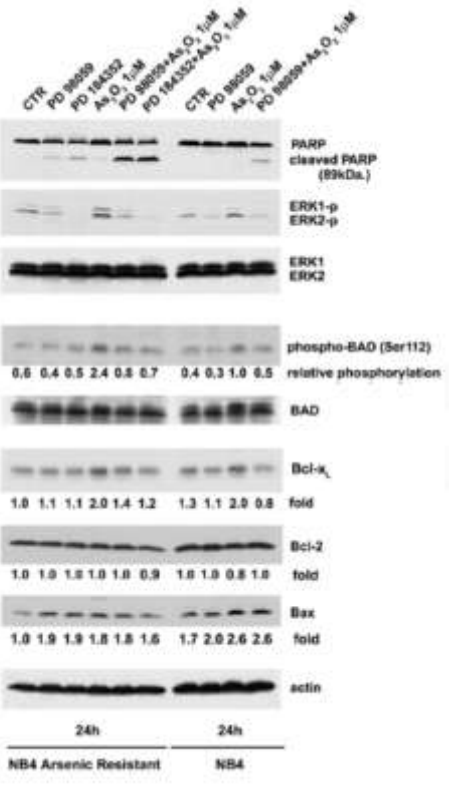

Figure 4..

The rationale to investigate p73-p53AIP1 pathway

Despite the well documented clinical efficacy of ATO the precise mechanisms regulating arsenic-dependent induction of apoptosis have not been elucidated and several molecular targets have been proposed. (reviewed in[6]) Whereas the role of p53 in stress responses is well established, recent advances strongly support a pivotal role for the p53 paralogue p73[6] in the execution of drug-induced cell death and chemosensitivity of cancer cells in both p53 wild type and p53 null tumors. (reviewed in[6]) Multiple TA (transactivation competent, pro-apoptotic and anti-proliferative) p73 COOH-terminal splicing isoforms (α,β,γ,δ,ε,ζ) exist. In addition, dominant negative (ΔN) variants are expressed from a second promoter, which lack the amino-terminal transactivation domain, act as trans-repressors of p53 and p73-dependent transcription and possess anti-apoptotic and pro-proliferative potential. ΔNp73 inhibits both TAp73 and p53-induced apoptosis. (reviewed in[6]) Enhanced expression of ΔNp73, rather than inactivating mutations within the TP73 gene, has been associated with tumor development.[7] The ability of TAp73 proteins to induce cell cycle arrest and apoptosis in cells exposed to anti-cancer drugs rely on their ability to activate a number of p53-responsive elements (p53-RE) containing target genes. p53AIP1 (p53-regulated Apoptosis-Inducing Protein 1), whose expression is induced by p53 and p73 under apoptotic conditions has been recognized as a primary effector gene of wild type p53 and TAp73-induced apoptosis.[8,9]

Taken these values we have considered of great interest to investigate whether the p73-p53AIP1 pathway is involved in the pro-apoptotic mechanisms induced by ATO+MEK1 inhibition combination. We studied the promyelocytic leukemia NB4 cell line whose p53 protein has lost the ability to bind and activate its target genes. Treatment with

ATO and MEK1 inhibitor activates the p73-p53AIP1 apoptotic pathway in NB4 leukemic cells. We found that MEK1 inhibitors reduce the levels of dominant negative ΔN-p73 proteins and promote the accumulation of endogenous p73α through its transcriptional activation and its tyrosine phosphorylation, resulting in p21 up-regulation and significant cell growth inhibition.
ATO reduces ΔN-p73 levels and promotes a p300-mediated acetylation of endogenous p73, thus favouring cell cycle arrest and apoptosis. Finally, the combined treatment with MEK1 inhibitors and ATO enhances the affinity of phospho-acetylated p73 for the p53AIP1 promoter *in vivo*, as determined by chromatin immunoprecipitation experiments, leading to p53AIP1 up-regulation and increased apoptosis (Figure 5).

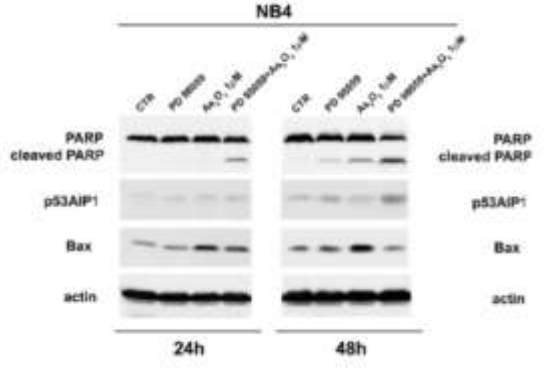

Figure 5..

Our results are extensively reported in.[10] Together, these findings support a model in which the p73-p53AIP1 apoptotic pathway is potentiated by Bad dephosphorylation through the increased capacity of Bad, after MEK 1 inhibition, to associate with Bcl-xL and Bcl-2 thereby blocking theirs antiapoptotic functions.

Figure 6 summarizes that MEK1 inhibitor+ATO treatment promotes the up-regulation of p53AIP1 protein via p73 activation by acetylation and phosphorylation. Furthermore p73-P53AIP1 apoptotic pathway is strengthened by Bad dephosphorylation induced by MEK1 inhibitor: dephosphorylated Bad displaces Bcl-2 from p53AIP1[11] and heterodimerizes with Bcl-2 (and with Bcl-xL), thereby blocking their anti-apoptotic function. Bcl-xL (not represented) has a behaviour similar to Bcl-2.

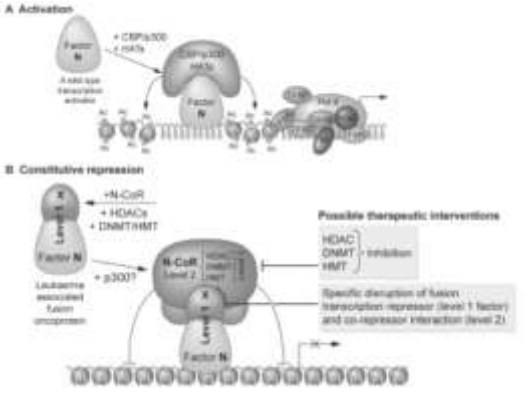

Figure 6..

Material and Methods

Leukemia cells were isolated and enriched as described.[12] ATO was purchased from Sigma (St Louis, MO); PD184352, a potent and highly selective MEK1/2 inhibitor[3,4] was kindly provided to us by Dr J.S. Sebolt-Leopold (Cancer Molecular Sciences, Pfizer Global Research & Development, Ann Arbor, MI). PD98059 was provided by Cell Signaling Technology, Beverly, MA.

Cell lysis, *in vitro* treatment, apoptosis assays, immunoblotting, transfection and statistical analysis were carried out as previously described.[10,12]

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C05

SHORT CHAIN FATTY ACID -HDAC INHIBITORS INDUCE SPECIFIC CHROMATIN MODIFICATION AND RELEASE GENE SILENCING IN CBF/AML CELLS

Santini V, Barbetti V[1] Gozzini A, Ferrari G, Dello Sbarba P,[1] Rovida E[1]

Department of Hematology and [1]Department of Pathology and Experimental Oncology , University of Florence, Firenze, Italy

Acute myeloid leukaemia (AML) characterized by the presence of AML1/ETO fusion protein, or by other rearrangements involving core binding factors, share a common leukemogenic mechanism, determining transcriptional repression of target genes important for myeloid maturation. The oncogenic fusion proteins determine the permanent recruitment to the bound portion of DNA of protein complexes containing HDACs, DNMTs and co-repressor molecules. We demonstrated that butyrates are able as single agents to restore histone acetylation and to reinduce gene expression allowing granulocytic maturation in AML1/ETO pos Kasumi-1 cell line, as well as in primary CBF-AML blasts. Core histone proteins can be acetylated by histone acetyl transferases (HAT) or methylated by histone methyltransferases (HMT) at N-terminal lysines, at different residues which are specifically associated to the transcriptional state of the nearby chromatin. Transcriptionally active chromatin is characterized by histones with acetylated lysine tails, and methylated lysine 4 of histone H3, whereas di-methylated lysine 9 of H3 and deacetylated lysines in H4 and H3 seem associated with transcriptional silence . We investigated the acetylation and di-methylation pattern of lysine residues of histone H4 and H3 in our AML1/ETO pos cell model, prior and after exposure to butyrate as HDAC inhibitor. Cells were lysed after 6-12-72 and 96 hrs of culture and whole cell lysate was analysed by SDS-PAGE electrophoresis and Western blot, with antibodies specific to different lysine residues of the two histones. K5 and K16 residues of histone H4 were not acetylated in the absence of butyrate and their acetylation strongly increased after 6 h of butyrate administration. K8 and K12 residues showed a basal acetylation which was slightly enhanced by the treatment with the short chain fatty acid HDAC inhibitor. Lysine methylation of histone H3 was also evaluated. Butyrates determined a significant reduction of di-methylated K9 H3. Di-methylated K4 H3 was, on the other hand, unmodified after butyrate administration. These histone epigenetic modifications were paralleled by an increase of p21/cip1 protein expression and a decrease of p27/kip1 protein expression. Appearance of a conspicuous amount of p57/kip2, an as yet uncharacterised oncosuppressor, after 72-96 h treatment with butyrates was also observed. Moreover, CEBP family expression and phosphorylation were induced. In parallel, AML1/ETO mRNA and protein expression was markedly reduced after D1 exposure. Our findings are consistent with the hypothesis of a specific direct activity of HDAC inhibitors in removing the corepressor complex which causes the block of transcription in CBF/AMLs.

C06

ROLE OF EPIGENETIC MODIFICATIONS IN ACUTE PROMYELOCYTIC LEUKEMIA

Villa R, Di Croce L

Center for Genomic Regulation, Passeig Maritim Barcelona, Spain

Many human cancers are characterized by alterations in the balance of DNA methylation. In cancer cells, a large part of the genome undergoes dramatic hypomethylation, which is often linked to genome instability, concomitantly with regional gain of methylated sequences at sites usually unmethylated (CpG islands). The major outcome of promoter hypermethylation appears to be long-term silencing of the associated gene. Silencing of tumor suppressors due to such a mechanism can provide a growth advantage to cancer cells. The ability of the PML-RARα fusion protein to block hematopoietic differentiation and to induce acute promyelocytic leukemia is based on aberrant gene repression. Mechanistically, PML-RARα inactivates its target genes by recruiting histone deacetylase (HDAC) and DNA methyltransferase activities to the promoters.

We show that MBD1 is required for silencing the PML-RARα target promoter, RARα. Following PML-RARα-induced promoter hypermethylation, MBD1 is recruited to and remains associated with the silenced RARα promoter. Mutations in the MBD and TRD domains of MBD1 restore RARα transcriptional activity. We provide evidence that HDAC3 is a common interactor for both PML-RARα and MBD1. Chromatin immunoprecipitation analysis revealed that MBD1 association to PML-RARα target genes is not confined to the promoter region but instead is spread over the locus.

Retroviral expression of dominant negative mutants of MBD1 in hematopoietic precursors compromised the ability of PML-RARα to block their differentiation and thus restored cell differentiation. Together these results identify MBD1 as a critical mediator of PML-RARα-induced gene silencing subsequent to promoter hypermethylation. Further characterization of the PML-RARα co-repressor complex, which establishes and allows spreading of the silenced state, will provide insight into crosstalk among the different epigenetic layers as well as into the molecular pathology of leukemia.

C07

SYNERGISM BETWEEN α TOCOPHEROL AND ARSENIC TRIOXIDE IN ACUTE PROMYELOCYTIC LEUKEMIA TREATMENT. ANALYSIS IN THE TRANSGENIC MOLDEL HCG-PML-RARα

Abreu e Lima RS, Scheucher PS, Saggioro FP, Garcia AB, Pintao MCT, Santana BAA, Teixeira HLG, Paneppuci RA, Falcão RP, Rego EM

Medical School of Ribeirao Preto, Ribeirao Preto, Brazil

Background. α tocopherol succinate (VES) is a vitamin E analog with antiproliferative activity against several cancer cell types. VES has strong redox activity and has been shown to inhibit protein kinase C (PKC) and target Akt and JNK pathways, and thus activating the intrinsic cell death mediators of caspase-9 and -3. Likewise, As_2O_3 [an agent that has successfully been employed in the treatment of patients with acute promyelocytic leukemia (APL)] was shown to induce apoptosis through activation of APAF-1, caspases-3 and -9 and cleavage of Bid. Unfortunately, As_2O_3 treatment is unavailable to most APL patients in Latin America. Aims: a) to determine if VES is effective against t(15;17)/APL; b) to study its mechanism of action and c) to characterize if there is synergism between VES and As_2O_3.

Methods. We used a syngenic transplantation model of APL: blasts from hCG-PML-RARα transgenic mice (TM) were IV injected in irradiated non-transgenic littermates. Massive infiltration of bone marrow (BM), spleen and liver was detected by 21st day. Recipient mice were treated with: VES (50 UI/g/d) (n=15), As_2O_3 (2.5 μg/g/d) (n=15), VES+ As_2O_3 (n= 15) at the same doses, or vehicle (Control; n=15) starting from day 4 for 21 days. Molecular remission was

determined by RT-PCR for PML-RARα. Another group of twelve mice was treated with VES or DMSO for 6 days, and the percentage of apoptotic CD117+ leukemic cells in spleen and liver was determined by flow cytometry. Differentiation was evaluated morphologically on Leishman stained BM cytospin preparations after 72h of treatment with VES. Furthermore, we compared the gene expression profile of BM cells obtained from leukemic mice treated with VES or DMSO (n=6, per group) using nylon microarrays representing 598 genes.

Results. The mean survival time in the Control group was of only 25,6 days (95% C.I. = 20,9±30,3), whereas in the VES it was 160,40 (95%CI = 134,2±185,6); in the As_2O_3 of 162,1 (95% CI = 137,97±186,3) and in the VES+ As_2O_3 of 163 (95% CI = 139,93±186,1). Compared to controls, all treatments significantly prolonged survival. Molecular remission was attained in 86,5% of mice in the VES arm, 80% in the As_2O_3, and 86,5% in VES+As_2O_3. No significant organ toxicity was found by histopathological analyses. The mean percentage of CD117+ apoptotic cells in VES treated mice was significantly higher (41,17±5% versus 62,11±4%, in spleen $p<0,03$; and 94,65±1% versus 12,94±11,95%, in liver, $p<0,01$). No significant difference in the number of mature granulocytic cells was detected. Data mining from our microarray results revealed the higher expression of Mitogen Activated Protein Kinase 3, Protein Kinase C δ and BCL2 antagonist Killer-1 genes in VES treated samples. In conclusion, our results demonstrate that VES induces prolonged remissions and that it activates of signaling pathways leading to apoptosis. Moreover, VES in combination with As_2O_3 was well tolerated and effective. Therefore, our experimental data suggest that VES may be an alternative to As_2O_3 for APL treatment.

C08

EPIGENETIC SILENCING OF THE RETINOIC ACID SIGNALLING PATHWAY IN ACUTE MYELOID LEUKEMIAS

Fazi F, Gelmetti V, Zardo G, Ciolfi A, Travaglini L, Di Cesare S, Cimino G, Lo Coco F, Pelicci PG, Nervi C

Department of Histology & Medical Embryology and of Cellular Biotechnology and Hematology, University of Rome La Sapienza; San Raffaele Bio-medical Science Park of Rome, Department of Biopathology, University of Rome Tor Vergata; European Institute of Oncology, Milan, Italy

AML1/ETO, the fusion product of the acute myeloid leukaemia (AML) chromosomal translocation t(8;21), promotes leukemogenesis by recruiting histone deacetylase activities (HDAC) on critical genes involved in myeloid differentiation. In the case of the acute promyelocytic leukaemia (APL), the PML-RARα fusion product aberrantly recruits both histone deacetylase (HDAC) and DNA methyltransferase (DNMT1-3a) activities on the retinoic acid (RA)-target promoter RARβ2, resulting in its transcriptional silencing and block of myeloid differentiation. Using Southern blot analysis of genomic DNA and methylation- specific PCR (MSP) we found that the RARβ2 promoter region, which includes the RA responsive element (RARE) and transcription start site, is methylated in samples from 7/9 AML-M2, 9/10 AML-M4, while the region located in the 5 portion of the exon 1 of RARβ2 is methylated in 9/9 AML-M2, 8/10 AML-M4. Neither of these RARβ2 regions was found methylated in CD34+ normal hemopoietic precursors. Accordingly, RARβ expression was detectable in normal CD34+ cells but not in any of the 24 AML cases analysed. We therefore analysed whether the expression of AML1-ETO into hematopoietic progenitors induces repression of RA-signaling pathway by affecting the methylation status at RA-target genes. By using cell lines carrying an endogenous AML1/ETO (Kasumi and SKNO) and myeloid cell lines stably transfected with an HA tagged AML1/ETO (U937-AE) we found that the AML1/ETO: i) binds and modulates the activity of a RARβ2 gene promoter; ii) recruits DNMTs and MBDs on genes that are target of both RAR and AML1; iv) induces hypermethylation of the promoter/exon1 region of RARβ2 gene. Finally, silencing of AML1-ETO mRNA restores the differentiation response of SKNO cells to RA. In summary, our results suggest that the AML1/ETO fusion protein, which is present in non-APL AML blasts, induce repression of RA-signaling pathway, as the APL-associated PML-RARα. Moreover, this repression occurs in a HDAC/DNMTs dependent manner that affects the acetylation/methylation status of a RA-target gene. This indicates that common mechanism of leukemogenesis is shared by the two most frequent fusion proteins in AMLs.

C09

TARGETING OF TELOMERASE BY RETINOIDS IN ACUTE PROMYELOCYTIC LEUKEMIA AND NON ACUTE PROMYELOCYTIC LEUKEMIA

Ségal-Bendirdjian E, Hillion J, Tarkanyi I, Pendino F, Dudognon C, Rousselot PH, Delaunay J, Mourah S, Podgorniak M-P, Lafon I, Chomienne C, Dombret H, Lanotte M, Aradi J

Département d'Hématologie, Hôpital Saint-Louis, Paris, France; Dep of Biochem. and Mol. Biol., University of Debrecen, Debrecen, Hungary

Acute promyelocytic leukemia (APL) is efficiently treated with a cell differentiation inducer, all-trans retinoic acid (ATRA). However, a significant percentage of patients still develops resistance to this treatment. Recently, arsenic trioxide (As_2O_3), alone or in combination with ATRA, has been identified as an alternative therapy in patients with both ATRA-sensitive and ATRA-resistant APL. Molecular investigations have provided theoretical support to this synergism in that both ATRA and As_2O_3 induce modulation and /or degradation of PML-RARα oncoprotein through distinct pathways. Recently, we have shown that long-term treatment with all-trans retinoic acid, represses hTERT in differentiation-resistant APL cell lines, leading to telomere shortening and death. This signaling requires the co-activation of the retinoic acid receptor α (RARα) and the retinoic X receptor (RXR). We have also demonstrated *in vitro* that the success ATRA/As_2O_3 treatment in APL pathology can be explained, at least in part, by a synergistic effect of these two drugs in triggering downregulation of telomerase efficient enough to cause telomere shortening and subsequent cell death in several ATRA-maturation resistant APL cells. The synergism between these two agents highlights important aspects of their integration into the clinical protocols. First, that such long-term low-dose combinational treatments could lead to a favourable outcome in APL patients, with the enhanced benefit of minimal toxicity. Second, the fact that altered regulation of hTERT expression in cancer cells could rescue them from death, clearly indicates that eradication of residual cells will likely require pharmacological treatment targeting telomerase activity. These results reinforce the notion that the anti-telomerase strategy, based on a combination of active agents, should now be considered and evaluated not only in APL but also in other malignancies. *In vitro* treatments performed on samples from patients suffering from APL and non APL leukemia are shown. The results support the previous *in vitro* studies on cell lines.

C10

INHIBITION OF P38MAPK-DEPENDENT PHOSPHORYLATION AND DEGRADATION OF SRC-3/AIB1 MODULATES RARα-MEDIATED TRANSCRIPTION AND FAVOURS GRANULOCYTIC MATURATION OF ACUTE PROMYELOCYTIC LEUKEMIA CELL LINES

Giannì M, Parrella E, Raska I, Gaudon C,[1] Rochette-Egly C,[1] Garattini G

Laboratorio di Biologia Molecolare, Istituto di Ricerche Farmacologiche Mario Negri, Milano, Italy; [1]Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ULP, UMR 7104, Illkirch, France

The transcriptional activity of RARs and RXRs is modulated by phosphorylation processes involving the receptors and specific co-regulator proteins. Among the large number of co-activators that have been identified, the SRC/p160 family and p300/CBP stand out. These co-activators are phosphorylated in response to several signaling pathways. Phosphorylation was shown to regulate co-activator activity through the control of their interaction with nuclear receptors or other co-regulators. In addition, like RARs, co-activators are targets for the ubiquitin-proteasome pathway. Transactivation experiments performed on COS cells show, that during ligand-dependent activation of RARα, the p160 co-activator SRC-3 is phosphorylated by p38MAPK and subsequently degraded by the proteasome. The co-transfection of a dominant-negative p38MAPK construct demonstrates that this biphasic process is correlated to an attenuation of RARα-mediated transcription. In fact, inhibition of p38MAPK enhances the interaction RARα-SRC-3 and this correlates with increased RARα-mediated transcription. Moreover, since inhibition of p38MAPK blocks also the degradation of SRC-3, the phosphorylation of this protein is also a permissive signal for proteasome-mediated degradation. In COS cells, RA-induced SRC-3 phosphorylation and degradation occur only within the context of RARα and possibly PML-RARα complexes, suggesting RAR isotype specificity. Myeloid leukemia HL60 and NB4 express SRC-3 and the protein is degraded in the presence of RA treatment. Degradation of SRC-3 is blocked by the p38 inhibitor, PD169316. Combined treatment of NB4 or HL60 with PD169316 and RA increases granulocytic maturation of these cells and increases the expression of several markers associated with granulocytic maturation (cEBP-β, STAT1). PD169316 exerts its effects essentially on SRC-3 since a specific siRNA directed against SRC-3 not only decreased SRC-3 expression but also attenuates NBT reducing activity and depresses RA target gene activation. Since RA is used in the treatment of acute promyelocytic leukaemia (APL) and several other malignancies, the combined use of pharmacological inhibitors of p38MAPK may have clinical potential. In this context, it is worth underscoring that inhibition of p38MAPK can also relieve the RA resistance of NB4-LR2 and NB4-007/6 cell lines.

POSTER SESSION I
BIOLOGY AND CHARACTERIZATION STUDIES

P01

THE PROTECTIVE EFFECT OF GANGLIOSIDE ON HUMAN CORTICAL NEURON TREATED BY ARSENIC TRIOXIDE

Zhou J,[1] Meng R,[2] Li L,[1] Jia J,[2] Yang B[1]

[1]The first hospital of Habin Medical University; [2]Xuanwu Hospital, The Capital University of Medical Sciences, China

Background. In the present paper, we report on the results obtained about the tolerable difference to arsenic trioxide between leukemia cells and normal axoneuron. Looking for the specific protective agent of nerve cell might provide a safe and feasible warrant of As_2O_3 in central nervous system leukemia treatment, and might offer a method of arsenic poisoning relevant nervous lesion.

Objective. Study on the protective effect and the possible mechanisms of ganglioside on cortical neuron treated by arsenic trioxide.

Methods. Five groups of human cortical neuron incubated *in vitro* were enrolled in this trial, the control, the 5 μ-mol/L As_2O_3 group, and the 5 μ-mol/L As_2O_3+ganglioside 50,100,200 μg/L groups. Cytosolic calcium$[Ca^{2+}]^i$ of cortical neurons was labeled by fluorescent probe Fluo-3/AM, the changes of $[Ca^{2+}]^i$ were monitored by laser confocal microscopy in real time, the activation of protein kinase C on these changes were assayed by Phosphorus radioisotope assay, the percentage of apoptosis was analyzed by flow cytometry.

Results. The $[Ca^{2+}]^i$ of cortical neurons began to rising at the 3th min after 5 μ-mol/L As_2O_3 treatment, and in $As_2O_3^+$ ganglioside group, which was began to rising at the 9th min after incubated *in vitro*, and the $[Ca^{2+}]^i$ increasing degree was not remarkable than that in the As_2O_3 group. After incubated *in vitro* in 27 min, the $[Ca^{2+}]^i$ of cortical neurons in As_2O_3 group reached 301±27 nmol/ L, while, in As_2O_3 ganglioside group, the $[Ca^{2+}]^i$ was only 188±20 nmol/ L. The PKC activities of cortical neurons, after incubated *in vitro* for 24h, were 435.5±18.1 on cell membrane and 339.5±11.2 in cell plasma nmol/min protein in As_2O_3 group and 159±20.4 on cell membrane and 19±21.5 in cell plasma nmol/min protein in As_2O_3+gangliosied 200 μg/L group. The apoptosis percentages of cortical neurons were 78.5% in $As_2O_3$3 group, and 13.5% in As_2O_3+gangliosied 200 μg/L group.

Conclusions. Ganglioside inhibited the increase of $[Ca^{2+}]^i$, the PKC activity, and the apoptosis of cortical neurons treated by arsenic trioxide concentration-dependently, and the target preventive points of ganglioside was the cell membrane of cortical neuron which the As_2O_3 interfered initially.

P02

STUDY ON THE TOLERANT DIFFERENCE BETWEEN HUMAN CORTICAL NEURONS AND ACUTE PROMYELOCYTIC LEUKEMIA LEUKEMIC CELLS TO ARSENIC TRIOXIDE

Zhou J,[1] Meng R,[2] Ji X,[2] Jia J,[2] Yang B[1]

[1]The First Hospital of Harbin Medical University; [2]Xuanwu Hospital, The Capital University of Medical Sciences, China

Background. Arsenic trioxide (As_2O_3) is a very effective therapeutic agent for acute promyelocytic leukemia (APL), especially in cases resistant to conventional chemotherapy. It is also effective in other leukemia subtypes, malignant