Oncogenes and tumor suppressors in the molecular pathogenesis of acute promyelocytic leukemia

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Received 30 January 2001; Accepted 30 January 2001

Acute promyelocytic leukemia (APL) is associated with reciprocal chromosomal translocations always involving the retinoic acid receptor α (RAR) gene on chromosome 17 and variable partner genes (X genes) on distinct chromosomes. RARα fuses to the PML gene in the vast majority of APL cases, and in a few cases to the PLZF, NPM, NuMA and Stat5b genes, respectively, leading to the generation of RARα-X and X-RARα fusion genes. Both fusion proteins can exert oncogenic functions through their ability to interfere with the activities of X and RARα proteins. Here, it will be discussed in detail how an extensive biochemical analysis as well as a systematic in vivo genetic approach in the mouse has allowed the definition of the multiple oncogenic activities of PML-RARα, and how it has become apparent that this oncoprotein is able to impair RARα at the transcription level and the tumor suppressive function of the PML protein.

INTRODUCTION

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (the AML-M3 subtype according to the French–American–British classification) that accounts for >10% of all AMLs and affects approximately 3000 individuals every year in the US alone (1–3 and references therein), and is therefore a relatively rare form of cancer. Nevertheless, this leukemia has become a unique paradigm for its distinctive biological, molecular and clinical features:

(i) APL is caused by the clonal expansion of tumor cells with promyelocytic morphological characteristics, a feature that gives the leukemia its name. Promyelocytes are present in normal bone marrow and are myeloid progenitor cells from which the granulocytic cells circulating in the peripheral blood are constantly generated. The accumulation of promyelocytic blasts in all organs of APL patients has suggested since the very early stages of APL research that the disease may be the consequence of a block in myeloid differentiation, which makes this leukemia a unique model system for the study of the molecular mechanisms controlling normal and aberrant hemoipoiesis.

(ii) The second feature of APL is molecular and karyological. In almost 100% of cases, APL blasts harbor specific reciprocal chromosomal translocations that always involve chromosome 17. In almost 99% of APL patients the translocation observed is a reciprocal and balanced translocation between chromosomes 15 and 17, t(15;17)(q22q11.2–12), which has become the hallmark of the disease. It is interesting to note that from a historical point of view this chromosomal translocation represents the second evidence ever obtained that human cancer indeed has a genetic basis (4). In view of this molecular homogeneity, the molecular cloning of the APL-specific translocation was a long sought achievement and, a decade ago, a major goal for many laboratories.

(iii) The last feature, which makes APL a paradigm from a therapeutic stand-point, is that APL blasts have been proven to be exquisitely sensitive to the differentiating action of retinoic acid (RA). From this perspective APL has become the paradigm for therapeutic approaches for cancer utilizing differentiating agents (differentiation therapy). This therapeutic approach is conceptually novel in that, it does not involve chemical or physical agents to eradicate the tumor by ‘killing’ the neoplastic cells, but rather re-programs these cells to differentiate normally. However, although effective, treatment of APL patients with RA alone induces disease remission transiently and relapse is inevitable if remission is not consolidated with chemotherapy. Based on these findings, the work of many laboratories has focused on understanding why APL cells respond to RA to start with, why RA cannot completely eradicate the neoplastic clone and how to potentiate the effects of RA, thus sparing the patient conventional chemotherapy.

Almost 10 years ago the t(15;17) translocation of APL was molecularly cloned and the two genes involved at the breakpoint identified (5–7). Surprisingly, and paradoxically, the breakpoints were found to lie within the retinoic acid receptor α (RARα) gene on 17 and a new gene, originally termed myl and subsequently PML (for promyelocytic leukemia gene) on chromosome 15 (8–11). At the time the translocation was cloned, RARα was, and possibly still is, one of the most well characterized transcription factors (12). Its involvement in the t(15;17) translocation of APL made this leukemia one of the first convincing examples of aberrant transcription in cancer pathogenesis. However, the involvement of RARα also raised a paradox, in that it was very surprising to find the nuclear receptor for the ligand, which is therapeutically effective in the
treatment of the disease, involved in the APL translocation. Based on the rearrangement of RARα one would have anticipated that RA does not function in APL. This contradictory observation has also been the subject of intense research in the last few years, and has led to the proposal of a transcriptional model for APL pathogenesis with exciting and straightforward therapeutic implications.

Finally, APL has also become a compelling and reassuring example of the power of modern molecular medicine in conquering cancer. Suffice to say, in the 1960s APL survival rates at MSKCC were, on average, 1–2 weeks from diagnosis depending on the quality of care (B. Clarkson, personal communication). Nowadays, in view of the progress made in understanding the molecular basis of this disease and also because of the advent of specific and effective therapeutic strategies, this disease is now regarded as curable, with success rates in ~70–90% of APL cases (1–3).

Here, the most recent progress made in elucidating the molecular genetics of APL and the mechanisms of action of the PML–RARα fusion protein, and also how this new understanding has allowed the development of novel therapeutic strategies, will be reviewed.

THE MOLECULAR GENETICS OF APL

As mentioned previously, the vast majority of APL patients harbor a chromosomal translocation that involves chromosomes 15 and 17. However, in a few cases, the translocation involves chromosome 11 instead of 15, and the promyelocytic leukemia zinc finger (PLZF) gene. In rare cases the translocation involves chromosomes 5, 11 and 17 and the nucleophosmin (NPM), NuMA and Stat5b genes, respectively (1,2 and references therein; 13). The X–RARα and RARα–X fusion genes (whereby X indicates the various RARα partner genes) generated by the reciprocal translocation in APL encode for structurally different X–RARα and RARα–X products, co-expressed in the leukemic blast, that differ in their X portions, but are identical in their RARα portion, and can therefore be considered as RARα mutants. APL associated with the various translocations results in diseases with similar features but with one very important exception: APL associated with chromosomal translocations between the RARα and the PLZF genes (PLZF–RARα) and possibly (STAT5b–RARα) show a distinctly worse prognosis with poor response to chemotherapy and little or no response to treatment with RA, thus defining a new APL syndrome (13,14). The various partners of RARα are structurally diverse. It may therefore appear that the disruption of RAR function is the major and only cause of APL. Indeed, the X–RARα protein is able to affect the function of RARα at multiple levels, as will be discussed in the following paragraphs. However, the various X–RARα fusion molecules are always able to form heterodimeric complexes with the respective X protein. As a consequence, these fusion products have the potential ability to interfere, at least in theory, with both X and RARα pathways.

THE MULTIPLE ONCOGENIC FUNCTION OF PML–RARα

Both in vitro analysis in cell lines and in vivo analysis in transgenic mice have defined the functional importance and the oncogenic role of the X–RARα fusion protein. Transgenic mice in which PML–RARα was expressed in the myeloid/ promyelocytic compartment develop leukemia, with APL features, but only after a long pre-leukemic phase, conclusively demonstrating that PML–RARα is critical, albeit not sufficient, in causing this leukemia (15–18). The transgenic approach in the mouse also demonstrated that RARα–X proteins are not sufficient but do play a crucial role in leukemogenesis, in acting both as tumor modifiers and tumor metamorphosers (19,20). As an example, we have shown that while PML–RARα transgenic mice develop leukemia with APL features, PLZF–RARα transgenic mice develop myeloid leukemias that completely lack the distinctive differentiation block at the promyelocytic stage, which characterizes human APL, thus resembling human chronic myelogenous leukemias (CML). However, double transgenic mice that co-express RARα–PLZF and PLZF–RARα develop leukemia with APL features (20). Thus, RARα–PLZF is capable of metabolizing a CML-like phenotype in an APL-like phenotype. On the other end RARα–PML can accelerate leukemia onset in PML–RARα transgenic mice (19).

In summary, the in vivo analysis in the mouse strongly supports the notion that a key oncogenic event in APL pathogenesis is the expression of the X–RARα fusion protein while the activity of RARα–X cooperates with it, not only in concurring to full-blown leukemogenesis, but in determining the critical feature of the leukemia in its native form.

A detailed analysis of the biological activity of PML–RARα soon rendered obvious that this molecule exerted, both in vitro in transgenic mice and in vitro in hematopoietic cell lines, multiple oncogenic functions such as the ability: (i) to block myeloid differentiation (15–18,21); (ii) to confer a survival advantage and possibly a proliferative advantage to the expressing cell (21,22); and (iii) to render the cells genomically unstable (23,24) (Fig. 1). How PML–RARα could exert so many diverse oncogenic activities at the molecular level remains a puzzling question. Progress in elucidating the molecular functions of PML–RARα will be discussed in the subsequent paragraphs.
PML–RARα IS A DOMINANT NEGATIVE RARα MUTANT

RARs are members of the superfamily of nuclear hormone receptors that act as RA-inducible transcriptional activators in their heterodimeric form with retinoid-X-receptors (RXRs), a second class of nuclear retinoid receptors (12 and references therein). In the absence of RA, however, RAR/RXR heterodimers can repress transcription through histone deacetylation by recruiting nuclear receptor corepressors (N-CoR or SMRT), Sin3A or Sin3B, which in turn form complexes with histone deacetylases (HDACs), thereby resulting in nucleosome assembly and transcriptional repression (25 and references therein). The presence of RA at physiological concentrations induces an allosteric change in the receptor, leading to the dissociation of the corepressors complex and the recruitment of transcriptional co-activators to the RAR/RXR complex. This results in the activation of gene expression, which in turn may induce terminal differentiation and growth arrest of cells of various histological origins, including normal myeloid hemopoietic cells (see following paragraphs; 12,26,27 and references therein). PML–RARα can bind to retinoic acid response elements (RARE) and, through the RARα DNA binding domain, can form multimeric complexes with RXRs (1,2 and references therein). Furthermore, PML–RARα binds RA with the same affinity as RARα (28). Therefore, the fusion protein can potentially interfere with the RARα pathway at multiple levels. However, the molecular mechanisms by which PML–RARα would be leukemogenic at physiological doses of RA, and permissive for the differentiating ability of RA at pharmacological doses, remained unexplained until recently. It was also unclear whether APL was caused by the aberrant RA-dependent transactivation of gene expression by PML–RARα proteins since, in this case, APL should always be exacerbated by RA, whereas on the contrary, RA was extremely effective in APL cases harboring PML–RARα. Recently, the aberrant transcriptional activity of the X–RARα fusion proteins has been molecularly defined in a unified model that provides a transcriptional basis for both the molecular pathogenesis of APL and the differential response to RA in APL (16,29,30). Others and we have found that at physiological concentration of the ligand, the various X–RARα proteins act as potent transcriptional repressors in view of an increased and aberrant affinity for nuclear corepressors and HDACs (16,29,30). At a pharmacological dose of RA, whereas PLZF–RARα, via the PLZF moiety, renders the leukemic cells unresponsive to RA through RA-insensitive corepressor/HDAC interactions, the PML–RARα–corepressor complex is resolved and the fusion protein is able to directly mediate trans-activation of RARα target genes (16,29,30). Indeed, HDAC inhibitors (HDACIs) such as trichostatin A (TSA) could overcome the transcriptional repressive activity of PML–RARα and PLZF–RARα (16,29,30 and our unpublished data). HDACIs could also overcome the unresponsiveness of PLZF–RARα leukemic cells to RA (16). It has also been reported recently that the repressive ability of PML–RARα may strictly depend on its dimerization ability through the PML moiety and that the PML–RARα homodimer could compete with RARα for RXR binding (31–33).

In summary, PML–RARα certainly affects the RARα pathway at multiple levels. However, as will be discussed below, PML–RARα can also impair the function of PML. This PML–RARα activity could be as relevant for APL pathogenesis as its ability to interfere with the RARα pathway and introduces a second level of complexity in the analysis of the molecular mechanisms underlying APL pathogenesis.

PML–RARα IS A DOMINANT NEGATIVE PML MUTANT

As soon as the first anti-PML antibody was obtained, it became apparent that PML–RARα can act as a dominant negative PML mutant. In fact, PML was typically found concentrated in discrete nuclear speckles, termed nuclear bodies (NBs; also called Kremer bodies, ND10 or POD, for PML oncogenic domains; 34 and references therein). Between 10 and 30 of these macromolecular structures are found in the nucleus of each cell, although their number and size change during the cell cycle and upon the introduction of various stimuli, such as interferon (9 and references therein). PML colocalizes in the NB with multiple proteins such as SUMO-1, Sp100, Sp140, CREB-binding protein (CBP), DAXX, RB and p53 (34 and references therein). In APL, PML–RARα physically interacts with PML, disrupts the PML–NB in a dominant negative manner and induces the delocalization of PML as well as the other NB component into aberrant nuclear subdomains, thus potentially impairing PML function (35–37) (Fig. 2). While the PML/PML–RARα interaction provided a straightforward explanation for the delocalization of PML from the NBs, until very recently it was unclear how PML–RARα would be able to induce an aberrant localization of all the other NB components. By studying primary Pml−/− cells in which Pml was inactivated by homologous recombination, it could be demonstrated that PML is essential for the proper formation and stability of the NB since in Pml−/− cells, irrespective of the histological origin, all the other NB components no longer accumulate in
NB and display aberrant localization patterns (38). This provided a direct explanation for the reason why PML–RARα through PML can lead to the disruption of the NB and in turn implied that PML–RARα could affect the NB-dependent functions of the various NB components. The fact that PML–RARα could simultaneously affect both PML and RARα functions made it imperative to elucidate the normal function of PML and how this relates to APL pathogenesis.

**PML–RARα BLOCKS DIFFERENTIATION OF MYELOID HEMATOPOIETIC PROGENITORS**

As mentioned previously, PML–RARα can cause a block in myeloid differentiation. This notion is supported by *in vitro* experiments in leukemic cell lines such as U937. These cells would normally differentiate in response to RA. The RA differentiating activity in U937 is abrogated as a consequence of PML–RARα expression (21). Not only do PML–RARα transgenic mice develop an APL-like leukemia *in vivo*, but these leukemias are preceded by a long pre-leukemic phase characterized by a block of differentiation at the promyelocytic stage that results in the slow and progressive accumulation of promyelocytes in the bone marrow and spleen of these transgenic mice (15–18). RA modulates important developmental processes as well as myeloid differentiation in adulthood (12,26,27). Thus, it was logical to hypothesize that PML–RARα could impair myeloid differentiation by blocking RA–RARα responses in a dominant negative fashion. However, while there is little doubt that this is a critical molecular event for APL leukemogenesis, since the various X–RARα fusion proteins appear to be oncogenic *in vivo* in transgenic mice despite distinct X moieties (15–18,39), it is still bewildering in vivo and in vitro that the various NB components. The fact that PML–RARα in transgenic mice results in embryonic lethality (1). Furthermore, ubiquitous expression of PML–RARα in bone marrow cultures (15,16), the cell cycle profile of PML–RARα promyelocytes prior to leukemia onset has not yet been characterized in detail. Nevertheless, it is tempting to speculate that the dominant negative action of PML–RARα on PML could result in a shortening of the transition through the cell cycle. In fact Pml–/– cells also have an increased proliferative potential in view of a faster transition through the G1 phase of the cell cycle (41 and our unpublished data). The RA–RARα/RXRα and the vitamin D/vitamin D receptor (VDR) pathways can also negatively control cell proliferation (12,26,27,44 and references therein). Thus, PML–RARα can lend a proliferative advantage to the neoplastic cells through the blockade of these pathways. Indeed, PML–RARα can render the cells unresponsive to RA and vitamin D both *in vitro* and in vivo (21,44).

While the role of PML–RARα in cell cycle control is still unclear, it has been shown conclusively that the fusion protein renders myeloid hematopoietic progenitors, as well as leukemic cells, resistant to multiple apoptotic stimuli (21,22). In particular, we could demonstrate that bone marrow progenitors from transgenic mice harboring the fusion protein are protected from apoptotic stimuli such as Fas, tumor necrosis factor (TNF), and interferons (IFNs) prior to leukemia occurrence (22). The inactivation of PML also results in protection from the aforementioned apoptotic stimuli, amongst others (22). Thus, it is logical to propose that PML–RARα could lend a survival advantage to the leukemic cells, at least in part, through its ability to impair the function of PML (Fig. 3). In this respect, it was recently reported that PML can act as a positive regulator of p53-dependent as well as p53-independent pathways for apoptosis (Fig. 3) (45). We found that PML physically interacts with p53 both *in vitro* and *in vivo*. PML is required for proper acetylation of p53 upon γ-irradiation, p53-dependent DNA damage-induced apoptosis, p53 DNA binding...
Figure 3. Consequence of the PML–RARα interference in PML function. PML is essential for the induction of apoptosis and growth arrest upon multiple cellular stresses. PML mediates both p53-dependent and p53-independent apoptotic responses. PML–RARα can block these PML pro-apoptotic activities. Although a thorough cell cycle analysis of hemopoietic cells harboring PML–RARα has not yet been performed, it is logical to propose that the fusion oncoprotein could also lend a proliferative advantage to the APL blasts through its ability to impair the PML function.

Figure 4. Roles of PML–RARα and PML in the p53 tumor suppressive pathway. PML is required for proper acetylation of p53 upon DNA damage and oncogenic transformation (45,46). Acetylation of p53 may occur in the NB where p53 is found to colocalize with PML and the acetyltransferase CBP (45,46). Acetylation of p53 results, in turn, in its transcriptional activation. These processes may be antagonized by PML–RARα as they depend on the presence and the proper formation of the PML–NB. The blockade of p53 function in APL may explain why p53 is rarely mutated in this form of cancer.

ability and the induction of p53 target genes such as RAX upon γ-irradiation are impaired in Pml–/– thymocytes (Fig. 4) (45). PML is also essential for p53-induced senescence upon oncogenic transformation (46). This is also in agreement with the surprising observation that in APL, unlike other acute myeloid leukemia subtypes, p53-inactivating mutations are extremely rare since p53 function in APL could be defective as a result of impaired PML function (47). However, PML is also involved in p53-independent pathways such as those that control Fas-induced apoptosis in activated splenocytes (Fig. 4) (48 and references therein). In this respect, we recently reported that Daxx, an important molecule for the transduction of the Fas pro-apoptotic stimulus, accumulates in the PML–NB at the position where PML and Daxx physically interact (48 and references therein). In the absence of PML, Daxx acquires a dispersed nuclear pattern, and activation-induced cell death of splenocytes is profoundly impaired. Furthermore, Pml inactivation results in the complete abrogation of the Daxx pro-apoptotic ability. In APL cells Daxx is delocalized from the NB. Following treatment with RA, which induces disease remission in APL, Daxx relocates to the PML–NBs. These results indicate that PML and Daxx cooperate in a novel NB-dependent p53-independent pathway for apoptosis.

Based on these findings it can be hypothesized that PML antagonizes leukemogenesis by the PML–RARα oncoprotein. This may be facilitated in APL by the reduction to heterozygosity of the normal PML allele. Indeed, the progressive reduction of the dose of Pml resulted in a dramatic increase in the incidence of leukemia, and in an acceleration of leukemia onset in PML–RARα TM. In hemopoietic cells from PML–RARα TM, Pml inactivation resulted in impaired response to differentiating agents such as RA and vitamin D₃ as well as in a marked survival advantage upon pro-apoptotic stimuli (44). Thus, in vivo, PML acts as a tumor suppressor by rendering the cells resistant to pro-apoptotic and differentiating stimuli and it is haploinsufficient in antagonizing the leukemogenic potential of PML–RARα. This also suggests that the reduction of PML to heterozygosity as a consequence of its involvement in the t(15;17) translocation may be a critical event in APL pathogenesis.

PML–RARα: A GENOME DESTABILIZER?

Leukemic cells from PML–RARα transgenic mouse display numerous recurrent chromosomal abnormalities (24). A provocative interpretation of these data suggests that PML–RARα favors the accumulation of these genetic lesions by rendering the cells genomically unstable. Indeed, numerous proteins involved in the maintenance of genomic stability accumulate in the PML–NB, including the Bloom syndrome DNA helicase BLM (49). BLM is in fact delocalized in APL cells as well as in Pml–/– cells (23). Furthermore, in Pml–/– cells, as in Bloom cells, the frequency of sister chromatid exchange is greatly augmented, which suggests that the PML-dependent localization of BLM in the PML–NB is required for its normal function (23). Additional proteins important for the maintenance of genomic stability, such as Nibrin/p95 (the protein mutated in Nijmegen breakage syndrome) (50), MRE11 (50), topoisomerase IIβ (also a BLM-interacting protein) (51), are also found in the PML–NB. Thus, PML–RARα could favor genomic instability and the accumulation of additional genetic events selected by the leukemic phenotype through its ability to target the PML–NB.

IMPLICATION FOR THERAPY

In vivo analysis in the mouse has revealed that PML–RARα is a key oncogenic event on which the leukemic cells depend in order to proliferate and thrive, even if additional genetic events do ultimately coexist with it in the leukemic blasts (52). This observation has important therapeutic implications in that drugs that directly target the activity and/or stability of the PML–RARα fusion protein may be extremely effective in t(15;17) APL. Indeed, both RA and arsenic trioxide, another powerful weapon for the treatment of this disease (53), are capable of inducing the proteolytic degradation of the PML–RARα fusion protein (1,2,52 and references therein). RA, as mentioned above, can also overcome the aberrant transcriptional repressive capacity of PML–RARα. RA has already been
ACKNOWLEDGEMENTS

I would like to thank all the past and present members of the Molecular and Developmental Biology (MADB) laboratory at the Memorial Sloan-Kettering Cancer Center who have or are working on APL and related subjects: Austin Changou, Jose Costoya, Maria Barna, Mantu Bhaumik, Laurent Delva, Mirella Gaboli, Domenica Gandini, Marco Giorgio, Ailan Guo, Carmela Gurrieri, Nicola Hanwe, Li-Zhen He, Sundee Kalantry, Letizia Longo, Taha Merghoub, Francesco Piazza, Daniela Peruzzi, Eduardo Rego, Roberta Rivi, Simona Ronchetti, Davide Ruggero, Paolo Salomoni, Carla Tribioli, Zhu-Gang Wang, Hui Zhang and Sue Zhong. P.P.P. is a scholar of the Lymphoma and Leukemia Society. This work is supported by the NCI, the De Witt Wallace Foundation for the Memorial Sloan-Kettering Cancer Center, the Mouse Model of Human Cancer Consortium (MMHCC) and NIH grants to P.P.P.

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