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Molecular Pathways

Therapy-induced PML/RARA Proteolysis and Acute Promyelocytic Leukemia Cure

Rihab Nasr,1 Valérie Lallemand-Breitenbach,2 Jun Zhu,2,3 Marie-Claude Guillemin,2 and Hugues de Thé2

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number of processes, including stress response and apoptosis, and have been recently implicated in normal or cancer stem cell maintenance (14, 15).

The oncogenic properties of PML/RARA have been associated with several features. PML/RARA is a DNA-binding transcription factor that may bind not only to consensus RARE, but also to novel binding sites, in which the two AGGTCA half-sites can be in any orientation or spacing (16–18). This characteristic is due to the existence in PML of a powerful dimerization domain, which allows formation of stable PML/RARA homodimers. Such homodimer formation also enhances corepressor recruitment, leading to transcriptional repression and silencing of target genes (19, 20). It is assumed that the differentiation block is the consequence of the transcriptional inhibition of key developmental regulators of myeloid differentiation (1), as in many other myeloid leukemia in which translocations turn master transcriptional activators into dominant repressor oncogenes (21). Recent studies have shown that the differentiation block and the transcriptional repression induced by PML/RARA involve not only RARA homodimerization (22), but also PML sumoylation, RXR binding, and recruitment of the polycomb complex (23–27). Other studies have shown that PML/RARA may be tethered onto DNA through specific interactions with other transcription factors, identifying yet another way for the fusion to alter gene expression (28). Apart from transcriptional regulation, PML/RARA also disrupts PML nuclear bodies (29–32), which regulate stem cell self-renewal (14, 15, 33, 34), raising the tantalizing prospect that other molecular mechanisms than transcriptional repression may be implicated in APL leukemogenesis.

**Therapeutic effects of retinoic acid in APL.** Before the 1980s, chemotherapy was the only available treatment for APL. Anthracyclines induce a complete remission (CR) rate of between 50 and 90%, but unfortunately this was followed by a very high rate of relapse (3). Then later, RA was discovered and has marked a major advance in APL treatment, as it dramatically increases the clinical remission (4). However, with RA as a single agent, the great majority of patients will also experience a relapse within a few months. The cloning of PML/RARA allowed the detection of minimal residual disease, which, despite clinically complete remissions, was never abolished by RA therapy (35). It was subsequently shown that the combination of RA with conventional chemotherapy greatly increases cure (4): RA combined with anthracycline-based chemotherapy can achieve CR in 90 to 95% of patients with APL and overall 5-year disease-free survival in up to 75% of patients (6).

At the cellular level, RA induces terminal differentiation of APL cells along the granulocytic lineage. RA binds PML/RARA through the ligand-binding domain of RARA, triggering a conformational change that releases transcriptional corepressors and recruits the coactivators, leading to transcriptional activation of targets and differentiation. RA also induces the recruitment of the proteasome that degrades PML/RARA. Arsenic enhances sumoylation of PML/RARA on Lys 160, which modulates Daxx binding (and thus repression), but also triggers SUMO-dependent polyubiquitination proteasome-dependent degradation of PML and PML/RARA. This will modulate Daxx binding to PML/RARA, leading to the release of transcriptional repression. C. PML/RARA degradation is responsible for the eradication of APL LIC. RA and As also induce PML/RARA transcriptional activation that leads to the differentiation of promyelocytes. However, only loss of LIC self-renewal is correlated to the cure from APL.

**Fig. 1.** APL pathogenesis and mechanisms of action of the curable combination RA-As. A, APL is characterized by the expression of PML/RARA that confers self-renewal properties to committed cells and blocks promyelocyte differentiation. B, in APL cells, PML/RARA heterodimerize with RXR and recruit corepressors onto master genes that control promyelocyte differentiation. RA releases corepressors and recruits the coactivators, leading to transcriptional activation of targets and differentiation. RA also induces the recruitment of the proteasome that degrades PML/RARA. Arsenic enhances sumoylation of PML/RARA on Lys 160, which modulates Daxx binding (and thus repression), but also triggers SUMO-dependent polyubiquitination proteasome-dependent degradation of PML and PML/RARA. This will modulate Daxx binding to PML/RARA, leading to the release of transcriptional repression. C. PML/RARA degradation is responsible for the eradication of APL LIC. RA and As also induce PML/RARA transcriptional activation that leads to the differentiation of promyelocytes. However, only loss of LIC self-renewal is correlated to the cure from APL.
and recruits transcriptional co-activators, allowing the RARA moiety of the fusion to activate transcription of its target genes and to differentiate cells expressing PML/RARA. It was first believed that the therapeutic effect of RA stems from its ability to reverse repression of critical target genes required for myeloid differentiation, thereby inducing granulocytic differentiation. RA-treatment of APL indeed constitutes the first, and only, example of differentiation therapy (36). RA binding also allows the direct recruitment of the proteasome to the ligand-activated transcriptional activation domain AF2 of RARA, leading to PML/RARA degradation (Fig. 1B; refs. 37, 38). A RA-induced PML/RARA degradation by a specific cleavage by caspases was also reported (39). Because RA directly targets the PML/RARA oncoprotein for activation and/or degradation, it also constitutes one of the first examples of oncogene-targeted treatments (4, 40, 41). However, the relative contributions of transcriptional activation, APL cell differentiation, and PML/RARA degradation to the therapeutic effect of RA have remained controversial.

**Therapeutic effects of arsenic trioxide in APL.** Arsenic trioxide (As) is one of the oldest drugs known to man (42). After its reintroduction in 1990s, it proved to be of great benefit to the treatment of APL patients. Several studies have shown that As has improved the clinical outcome of refractory or relapsed APL. It was shown to induce a CR in 85 to 90% of patients with both newly diagnosed and relapsed APL (43–46). Today, As is effective and approved for treatment of relapsed or refractory APL cases resistant to RA.

At the cellular level, arsenic induces apoptosis at high concentrations and promotes partial differentiation at lower levels (47, 48). PML/RARA protein is also targeted by As (48–51). However, in contrast to RA that targets the RARA moiety of the fusion, arsenic targets its PML moiety (40, 41). The molecular basis of this specific induction of proteolysis was only recently discovered. As treatment promotes small ubiquitin-related modifier (SUMO) conjugation of PML at amino acid K160 (52). Although in the basal state K160 sumoylation is required for the binding of the corepressor DAXX to PML/RARA (24, 53), poly-sumoylation of PML triggers its poly-ubiquitination by the SUMO-dependent ubiquitin ligase RNF-4 (Fig. 1B; ref. 54–56). Because of this As-induced PML/RARA degradation, As was identified as the second oncogene-targeted therapy in APL (40, 41).

**Mice get in the game.** The expression of PML/RARA suffices to induce APL in mice (57, 58). Several studies have investigated in mice the mechanisms through which PML/RARA triggers APL leukemogenesis. Interestingly, these have yielded conclusions somehow at variance to the studies done in cell-lines. In particular, whereas RARA dimerization seemed not only pivotal but sufficient to trigger differentiation arrest in cell-lines (59, 60), it quite inefficiently promotes leukemia in vivo (22, 61). In contrast, the essential role of specific post-translational modifications, such as sumoylation of the PML moiety of PML/RARA or RXR binding, were primarily achieved in mice (23, 24). Mice also played a role to implicate immune response against PML/RARA in the eradication of the disease (62, 63), and in characterizing in situ the cellular effects of therapeutic agents. In particular, these studies have shown that RA and arsenic both promoted significant apoptosis in vivo (52, 58). These models have also shown that PLZF/RARA leukemias are completely resistant to As (64). Unexpectedly, they terminally differentiate in response to RA (65), but this does not suffice to clear the disease.

Finally, these mouse models have had a significant role in developing therapeutic combinations for APL and in elucidating their actual mechanisms of action. Because RA and As target the PML/RARA oncoprotein for degradation through different sites and via distinct pathways, it could be envisioned that they should be synergistic. Indeed, whereas studies in cell-lines were contradictory, those done in APL mouse models have all shown synergistic effects of both drugs in prolonging survival and even eradicating the leukemia (64, 66–68).

**How is RA-As synergy accomplished?** In mice, the combination of RA and As causes a rapid disappearance of APL cells and cures leukemia in several models. Yet, RA and As do not synergize to induce differentiation (47, 48, 50), but they do cooperate to induce PML/RARA degradation (Fig. 1B; refs. 40, 41, 65). Recent work from several groups has shown that the apparent contradiction between the observed RA-As antagonism for differentiation and dramatic synergy for APL cure could be clarified by a careful examination of the heterogeneity of APL cells. Cancer stem cells (CSC) or leukemia initiating cells (LIC) are a minority of tumor cells that are able to self-renew, but usually do not cycle (69). Because of their inefficient targeting by many treatments (notably chemotherapy), LIC are believed to drive many clinical relapses. Several studies have shown that these cells have a very peculiar biology and are regulated by a small number of highly conserved pathways. In addition to the differentiation block, PML/RARA confers immortalization and self-renewal properties to APL leukemic cells (Fig. 1A; refs. 70, 71), raising the possibility that these two events may be controlled by a different gene network. That not all APL cells are identical is well shown by both the requirement for a few hundred cells to efficiently propagate APL in mice, but also by the small fraction (1%) of clonogenic cells in ex vivo transduced primary hematopoietic progenitors (24, 71). Such asymmetry raised the possibility that differentiation block and progenitor immortalization may be uncoupled, in particular during therapy response.

Several recent studies have compared the fate of LIC upon ex vivo or in vivo treatment with RA or arsenic (65, 72–74). Unexpectedly, it was shown that whereas RA could clear some LIC, previously overlooked issues of doses were critical in this process. Indeed, in vivo, a very wide range of RA concentrations suffice to initiate similar levels of differentiation. In contrast, LIC clearance is strictly observed with high concentrations and is sharply dose-dependant (65). Full blast differentiation is indeed observed with much lower RA concentration than those required for remissions in vivo. Similarly, relapses appear in patients with decreased RA plasma concentrations, but that still trigger differentiation (75). This, and other evidence, has suggested that RA-induced LIC clearance was the consequence of PML/RARA catabolism, whereas induction of differentiation likely reflected reversal of PML/RARA-dependent transcriptional repression (73). Interestingly, the few patients that were actually cured with RA alone received a liposomal form, which allowed very high and prolonged plasma concentrations (76). Arsenic alone is only a modest inducer of differentiation. Yet, it is a very potent inducer of PML/RARA degradation. Thus, putting the major emphasis on degradation naturally explains the dramatic synergy between arsenic and RA. Because of distinct mechanisms of action and different target sites, no cross-resistance between RA and As is expected (40, 41). Thus, rapid proteasome-dependent degradation of PML/RARA by curative RA-As combination is
responsible for rapid disappearance of LIC and consequently cures of the disease (Fig. 1C).

We have also shown that the RA-induced, proteasome-mediated, PML/RARA degradation is modulated by a cyclic AMP-triggered PML/RARA phosphorylation at Ser369 (77). Cyclic AMP signaling triggers the dissociation of corepressors from RARA (78) and enhances its ability to be degraded by RA (65). Interestingly, this allows arsenic to become a potent inducer of dissociation (42, 79, 80). This phosphorylation sharply enhances RA-induced APL regression, PML/RARA transactivation and degradation, and LIC clearance (16, 65, 79), identifying a third clinically achievable oncogene-targeted therapy. Patients currently resistant to RA and/or arsenic, may thus benefit from phosphodiesterase inhibitors. In fact, the use of theophylline, an inhibitor of cAMP degradation, was beneficial in the treatment of a resistant APL patient (79).

Clinical-translational Advances

Synergy between RA and As in APL patients. As a follow-up to mice studies, several clinical trials were done to see if this therapeutic synergy could be translated into treatment strategies for APL patients. These studies have all shown a striking similarity in APL patients with regard to disease eradication by RA-As (7, 81–83). Patients treated with a combination of RA and arsenic obtained a quicker clinical response, had a more rapid and complete clearance of leukemic cells, and had a significantly longer period of relapse-free survival. Recent trials in de novo patients have shown that APL patients may benefit from the early use of the combination of RA-As, for both remission induction and consolidation-maintenance. LIC eradication in patients is reflected by cure and long-term follow-up of RA-As-treated patients is now available and highly supportive (81). Although larger clinical trials would further strengthen these results, the well-controlled side effects and the minimal toxicity associated with this combination therapy supports the use of this combination as a frontline therapy for newly diagnosed APL. Remarkably, a major benefit for the RA-As combination even without any chemotherapy in the treatment of APL patients was also recently reported (5, 7), suggesting that APL may be curable without any DNA-damaging therapies.

Could the APL model be generalized? Triggering oncogene degradation could be a general therapeutic strategy in cancers in which well-defined oncogenes control the LIC maintenance. This approach could be particularly promising in leukemias or sarcomas caused by fusion proteins. As PML/RARA, they offer the unique opportunity to destabilize the protein through cooperative pathways targeting each of its moieties. In fact, in solid tumors such as breast cancers, degradation of key transcriptional activators such as the estrogen receptor, have been of clinical use (84). Targeting PML itself, out of the context of PML/RARA targeting could also be an interesting target. Indeed, PML seems to be a critical contributor of normal or cancer stem cell quiescence (14, 15, 85).

Identifying the molecular characteristics of LIC and their oncogene-dependency should be an essential step to design new therapies. In APL, this has been facilitated by the identification of PML/RARA and the prior clinical efficacy of RA and arsenic. Although other molecular events cooperate to allow emergence of the full leukemic phenotype, PML/RARA targeting is sufficient to clear LIC. In solid tumors multiple signaling pathways need to be altered for epithelial tumors to develop. Whether, as in APL, inactivation of one single of the early affected pathways will be sufficient for tumor collapse remains a key issue.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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