Induction of Differentiation and Apoptosis—
A Possible Strategy in the Treatment of Adult Acute Myelogenous Leukemia

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ABSTRACT

A differentiation block with accumulation of immature myeloid cells characterizes acute myelogenous leukemia (AML). However, native AML cells often show some morphological signs of differentiation that allow a classification into different subsets, and further differentiation may be induced by exposure to various soluble mediators, e.g., all-trans-retinoic acid (ATRA) and several cytokines. Combination therapy with ATRA and chemotherapy should now be regarded as the standard treatment for the acute promyelocytic leukemia variant of AML. Several agents can induce leukemic cell differentiation for other AML subtypes, although these effects differ between patients. Differentiation may then be associated with induction of apoptosis, and differentiation-inducing therapy may therefore become useful in combination with intensive chemotherapy to increase the susceptibility of AML blasts to drug-induced apoptosis. However, it should be emphasized that differentiation and apoptosis can occur as separate events with different regulation in AML cells, and future studies in AML should therefore focus on: A) the identification of new agents with more predictable effects on differentiation and apoptosis; B) the use of clinical and laboratory parameters to define new subsets of AML patients in which differentiation/apoptosis induction has a predictable and beneficial effect, and C) further characterization of how AML blast sensitivity to drug-induced apoptosis is modulated by differentiation induction.

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INTRODUCTION

Acute myelogenous leukemia (AML) is characterized by a neoplastic proliferation of myeloid cells [1-8]. The malignant cells have a differentiation block that results in an accumulation of immature cells, and AML can thus be diagnosed: A) if at least 30% of nucleated cells in the bone marrow are myeloblasts (or alternatively 20% [8]); or B) in the case of bone marrow showing erythroid predominance, if at least 30% of nonerythroid cells are myeloblasts, or C) if the characteristic signs of hypergranular promyelocytic leukemia (acute promyelocytic leukemia [APL]) are present [1, 2].

Cases of AML can be subclassified on the basis of morphology, cytochemistry, immunological markers and/or cytogenetics [1-5]. According to the widely accepted French-American-British (FAB) classification, AML can be divided into the following subclasses based on the differentiation of the malignant cells [1-5]: AML-M0 and -M1 show minimal differentiation; AML-M2 includes a minor maturing granulocytic component, whereas AML-M3 (APL) has a dominating accumulation of promyelocytes; AML-M4 and -M5 show myelomonocytic differentiation; AML-M6 has an erythroid predominance; and AML-M7 is the acute megakaryoblastic leukemia. Native AML blasts may also on rare occasions show basophilic or eosinophilic differentiation [6, 7]. These morphological criteria are also incorporated in the recently published World Health Organization classification of myeloid neoplasms [8]. For a subset of AML patients, the leukemia blasts even show functional evidence of differentiation and are capable of antibody-dependent attachment and internalization (phagocytosis) of bacteria as well as zymosane particles [9].

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AML treatment usually includes intensive chemotherapy administered as A) induction treatment that aims to bring the patient into complete hematological remission, and B) consolidation therapy that aims to eradicate residual disease and prevent AML relapse [5]. Consolidation therapy with intensive chemotherapy alone or in combination with autologous stem cell transplantation is associated with a relatively high risk of AML relapse and an overall long-term AML-free survival of less than 50%, whereas consolidation with autologous transplantation has a lower relapse risk but a higher treatment-related mortality [5]. The addition of differentiation induction therapy with all-trans-retinoic acid (ATRA) is now regarded as mandatory in the treatment of APL [10-12], and the use of differentiation induction as a therapeutic approach with low treatment-related morbidity and mortality is also considered for other AML patients.

**Differentiation Induction in the Treatment of APL**

Several excellent reviews of the pathogenesis, diagnosis and treatment of APL have recently been published [10-14]. This AML subtype is characterized by the expansion of malignant myeloid cells blocked at the promyelocyte stage of differentiation, and reciprocal chromosomal translocations that involve the retinoic acid receptor α (RARα) gene on chromosome 17q21 [10, 12, 13]. The RARα most commonly fuses to the PML gene on chromosome 15q22 [10-14].

The retinoids exert important effects on cell development, proliferation and differentiation, and their biological effects are mediated by the RAR and the retinoid X receptors (RXRs) [10-14]. ATRA belongs to the retinoid family of vitamin A derivatives and can only activate the RARs [10-14]. The molecular mechanisms for ATRA’s antileukemic effect in APL are complex and include ligand binding to PML-RARα with degradation of fusion proteins and altered transcription regulation [12]. The presence of ATRA increases the fraction of differentiated cells with functional characteristics of normal neutrophils, increases cytokine secretion, induces a mature membrane molecule phenotype, inhibits leukemia cell proliferation and finally induces apoptosis [14-18]. ATRA is now regarded as mandatory in the induction therapy of APL, whereas it is usually not included in consolidation therapy [10-14, 19, 20]. Recent studies also suggest that APL patients will benefit from additional maintenance treatment that probably should include ATRA [20-22]. When APL treatment is based on these principles, an overall two-year event-free survival exceeding 75% has been described [21]. However, the effects of ATRA seem to differ in the various subsets of APL patients, and patients with the uncommon t(11;17) have a worse prognosis and little or no effect of ATRA therapy [10, 12, 13].

**Differentiation Induction in AML Cells with Non-APL Phenotype**

**Cytokine Effects on AML Blast Differentiation in Vitro**

Although the effects of various cytokines on AML cell (native blasts and AML cell lines) proliferation and viability have been extensively studied, relatively few studies have examined effects of single cytokines, cytokine combinations or cytokines-vitamin-D₃ on differentiation of native AML blasts [23-38]. In addition, many of these studies are relatively small, and the patients are often heterogeneous with regard to prognostic factors and FAB classification. However, the following conclusions are justified based on the representative studies summarized in Table 1: A) AML blasts can be induced to differentiate in several myeloid directions, and the same differentiation response can often be induced by different cytokines [23-31]; B) a certain cytokine or cytokine combination usually induces differentiation only for a subset of patients, and the direction of differentiation often varies between patients [24, 26]; C) differentiation induction can be independent of the effects on blast proliferation, and D) the direction of differentiation often shows no correlation with FAB classification (i.e., previous signs of differentiation) [24, 26-28]. Thus, in contrast to the predictable effects of differentiation induction in APL, the effects in other AML subsets are difficult to predict in individual patients. The same conclusion was also drawn in a clinical study of interleukin 3 (IL-3) therapy in AML [31]. Future investigations of differentiation induction should therefore focus on A) the identification of new agents/combinations/procedures with more predictable effects, and B) the identification of patient subsets in which the effects are predictable and likely to be clinically beneficial.

Several recent studies have demonstrated that native AML cells can be induced to differentiate into a dendritic cell phenotype with increased antigen-presenting capacity [33-37]. Such cells may be used in immunotherapy to enhance autologous antileukemic reactivity. However, this approach is based on differentiation induction without apoptosis and will not be further discussed in this review.

**Molecular Mechanisms Involved in Differentiation Induction**

Our knowledge about molecular mechanisms involved in AML blast differentiation is at present fragmentary, and we will only briefly mention some of the intracellular pathways that seem to be involved. First, studies of differentiation induced by phorbol esters and tumor necrosis factor (TNF) mutants suggest that differentiation can be signaled through activation of protein kinase C [38-40], whereas TNF-mutant-induced apoptosis is signaled through a different pathway
involving the IL-1β-converting enzyme [39]. Second, several tumor-suppressor proteins (p53 wild-type, pRb, p130/Rb2) also seem to be involved in the regulation of differentiation of AML cells [41]. Third, vitamin D3-responsive genes are involved in ATRA-induced differentiation of AML-M2 cells into a monocytic phenotype, whereas this effect cannot be detected in AML-M0/M1 cells [42]. These observations are consistent with the hypothesis that regulation of differentiation in AML blasts may involve several intracellular pathways and can be independent of the regulation of apoptosis.

The translocation t(8;21) is associated with the AML-M2 phenotype and gives rise to the AML1-ETO fusion molecule. Expression of the fusion protein seems to inhibit cytokine-dependent differentiation of immature progenitors into mature neutrophils, but the inhibition can be reversed by overexpression of normal AML1 that functions in cooperation with p300 and CREB-binding protein [43, 44]. On the other hand, the AML1-ETO molecule retains the ability of ETO to form stable complexes with nuclear receptor corepressor/SMRT and histone deacetylase (HDAC), and this seems to be essential for the ability of AML1-ETO to repress transcription and inhibit differentiation [45-47]. Although the differentiation inhibition induced by AML1-ETO seems important in leukemogenesis, ribozymes targeted against the fusion transcript induce apoptosis without previous differentiation [48]. Thus, this is another example that differentiation and apoptosis can be separate events in AML cells even when the same regulatory mechanisms are involved.

The translocation t(15;17) is detected in AML-M3/APL and gives rise to the PML-RARα fusion protein that associates with the HDAC complex [49]. HDAC inhibitors will then potentiate retinoid-induced differentiation in APL cells, and HDAC inhibition may even make ATRA-resistant APL cells with t(11;17) sensitive to ATRA-mediated differentiation induction [49]. Thus, intracellular pathways involving the HDAC complex seem to be involved in the differentiation block both in AML-M2 patients with t(8;21) and AML-M3 patients with t(15;17).

### Table 1. Differentiation induction in human AML cells cultured in vitro in the presence of soluble mediators; a summary of the results from representative studies

<table>
<thead>
<tr>
<th>Direction of differentiation</th>
<th>Soluble mediators used</th>
<th>Detection of differentiation in native AML blasts</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophil granulocyte</td>
<td>Stem cell factor (SCF) or IL-3</td>
<td>Induction of CD15 expression and promyelocyte-myelocyte morphology in CD34+ AML-M1/M2 blasts</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>IL-3, G-CSF, or GM-CSF</td>
<td>Increased proportions of mature granulocytes for some patients, no correlation between differentiation induction and FAB class</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>Interferon (IFN)-γ, TNF-α, Vit-D₃, or retinoic acid</td>
<td>Increased expression of CD15 and CD33 in subsets of patients together with decreased colony formation in clonogenic assay; these effects were caused by single agents and/or combinations of mediators</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>SCF</td>
<td>Differentiation into myelocyte- and metamyelocyte-like leukemic cells with disappearance of CD34 and HLA-DR expression for a subset of patients</td>
<td>[27]</td>
</tr>
<tr>
<td>Eosinophil granulocyte</td>
<td>IL-5</td>
<td>Induction of either pure or mixed leukemic eosinophilic colonies, no correlation with FAB classification</td>
<td>[28]</td>
</tr>
<tr>
<td>Basophilic granulocyte</td>
<td>SCF</td>
<td>Differentiation into cells with segmented nuclei and basophilic/metachromatic granules for a small minority of patients</td>
<td>[27]</td>
</tr>
<tr>
<td>Monocyte</td>
<td>IFN-γ, TNF-α, Vit-D₃, or retinoic acid</td>
<td>Increased membrane expression of the monocye marker CD14 in subsets of patients caused by single agents or combinations of mediators</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>Induction of monocytic morphology with increased phagocytic capacity and expression of CD11b and CD14</td>
<td>[29]</td>
</tr>
<tr>
<td></td>
<td>IL-3, GM-CSF, G-CSF, or M-CSF</td>
<td>Increased number of AML cells with monocye/macrophage morphology</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td>SCF</td>
<td>Induction of a macrophage-like morphology and expression of CD13, CD14, and HLA-class II in a minority of patients</td>
<td>[27]</td>
</tr>
<tr>
<td>Leukemia inhibitory factor</td>
<td></td>
<td>Expression of the Wilms’ tumor suppression gene together with monocye differentiation in the M1 AML cell line</td>
<td>[32]</td>
</tr>
<tr>
<td>Dendritic cell</td>
<td>GM-CSF+IL-4 + either TNF-α or CD40 ligand; GM-CSF+TNF-α+SCF+IL-6; cytokine combinations utilizing Flt3L or IL-3 + TNF-α</td>
<td>Morphological and functional characteristics consistent with dendritic cell phenotype (upregulation of adhesion, costimulatory and antigen-presenting molecules; down-regulation of monocye marker CD14 and upregulated CD83; increased IL-12 release; induction of autologous antileukemic T cell reactivity)</td>
<td>[33-37]</td>
</tr>
<tr>
<td>Megakaryocytic differentiation</td>
<td>Thrombopoietin + IL-3 or SCF</td>
<td>Increased expression of platelet-specific antigens in the Mo7E AML cell line</td>
<td>[30]</td>
</tr>
<tr>
<td>Erythroid differentiation</td>
<td>Erythropoietin</td>
<td>Further erythroid differentiation for patients with erythroleukemia</td>
<td>[24]</td>
</tr>
</tbody>
</table>
Candidate Drugs for Differentiation Induction in Clinical Therapy

ATRA and Vitamin D$_3$, Analogues

Recent in vitro studies suggest that ATRA and vitamin D$_3$ can induce differentiation not only in APL cells, but also in AML-M2 blasts [42]. However, clinical studies have concluded that APL, ATRA, and vitamin D$_3$ are not potent enough to provide clinical benefit in non-APL patients when used at doses that can be tolerated by patients [50]. However, a recent study described that although addition of ATRA to chemotherapy did not improve patient outcome, in vitro evidence for response to ATRA was detected in 25% of the patients [51]. In vitro studies have also demonstrated that the differentiation induction effects of ATRA, vitamin D$_3$, and/or vitamin D$_3$-derivatives can be enhanced by other agents, including the drug clofibrate acid that has been used without serious side effects in hyperlipemic patients [52]. Thus, combination treatment with chemotherapy, ATRA and ATRA-potentiating agents may become useful in AML.

Arsenic Derivatives

In vitro studies have shown that certain arsenic derivatives are effective against APL cells, and a recent clinical study demonstrated that AsO$_3$ should be regarded as a promising therapeutic agent with limited toxicity [53, 54]. This agent seems to act as a differentiation and apoptosis inducer, and the results suggest a possible role of arsenic derivatives in consolidation and/or maintenance therapy of APL [53]. However, experimental studies suggest that AsO$_3$ may become useful even in other AML subsets, because it can also induce apoptosis in non-APL cell lines [55].

Cytotoxic Drugs

Anticancer agents (e.g., cytarabine, daunorubicin, 6-thioguanine) can induce differentiation in AML cell lines and in native AML blasts [56-59], and combinations including Ara-C/6-thioguanine/retinoic acid/hexamethylene/dimethylformamide seem to induce AML blast differentiation even for a majority of patients [57-59]. This effect is observed at lower concentrations than are required for drug-mediated killing, and it probably involves drug-induced alterations in the cytokine responsiveness of AML cells [56].

Altered Histone Acetylation

Acetylation and deacetylation of histones are regarded as important for transcription activation and repression, respectively. The HDAC complex seems to be involved in the pathogenesis of AML (see above), and the therapeutic principle of histone deacetylase inhibition may therefore become useful in future AML therapy. Histone deacetylase inhibitors can induce differentiation in native AML blasts for a subset of patients, and they also cause a synergistic enhancement of ATRA-induced differentiation [49, 60, 61]. These effects show no correlation with previous signs of differentiation (i.e., FAB classification). Butyrates are another group of drugs that seems to induce gene expression via histone hyperacetylation, and monosaccharide butyrate derivatives can also induce differentiation and apoptosis in native AML blasts for a subset of patients [62].

Hexamethylene bisacetamide (HMBA) is classified as a hybrid polar compound, and this agent can induce differentiation and apoptosis in malignant cells [63-65]. HMBA is not suitable for clinical therapy due to dose-limiting toxicity [64], but the second generation of hybrid polar compounds seems to include agents that are much more potent inducers, and in contrast to HMBA these new agents are also potent inhibitors of HDAC activity [65].

High-Dose Methylprednisolone

Both in vitro and in vivo studies suggest that high-dose methylprednisolone (30 mg/kg/day) can induce differentiation of AML cells to mature granulocytes that subsequently die from apoptosis [66-68]. However, this treatment has been tried only in a few patients, and one should be very careful with the interpretation of these results.

Metal Chelators

In vitro exposure to the metal chelator dithizone will induce differentiation and apoptosis in the AML cell line ML-1 [69]. Although this drug is probably not suitable for clinical use due to its side effects, the results suggest that this new therapeutic approach may become useful.

The Possible Prognostic Impact of Apoptosis Regulation in AML

Clinical Evidence

The molecules and pathways involved in apoptosis can be classified into triggers of apoptosis (e.g., chemotherapy, radiation, CD95 ligation), modulators (e.g., BCL-2 family members and their interacting proteins, Mcl-1), effectors (e.g., caspases 1-13) and cleavage substrates [70]. Several studies suggest that regulation of apoptosis is important for the outcome after intensive chemotherapy in AML. First, relapse AML cells have elevated levels of the regulator Mcl-1 [71], and intracellular levels of the effector molecules caspase 2 and 3 seem to be predictors of survival in AML [72]. Several previous studies have also suggested that the levels of the antiapoptotic mediator Bcl-2 are important for treatment outcome in AML [73-76], but a recent report described that the prognostic impact of Bcl-2 is dependent on cytogenetics.
High Bcl-2 level was then an adverse prognostic factor for patients with favorable and intermediate prognosis cytogenetics, whereas in patients with unfavorable cytogenetics, low Bcl-2 levels were associated with shorter remission duration [77]. This last study thus supports the hypothesis that AML patients are heterogeneous with regard to apoptosis regulation in the leukemic cells (see above).

A possible mechanism for ATRA on bcl-2 seems to be mediated by different mechanisms, including regulation of transcription, altered phosphorylation and decreased bcl-2 stability [84, 91]. The effects of ATRA on sensitivity in AML blasts has been studied in AML cell lines. For certain cell lines ATRA can induce differentiation and reduce intracellular bcl-2 levels without altering the susceptibility to drug-induced apoptosis [86, 89], for other cell lines an increased chemosensitivity seems to depend on G2/M cell-cycle arrest rather than the differentiation status or bcl-2 levels [86] and, for a last group of cell lines, ATRA seems to increase chemosensitivity by downregulation of bcl-2 [83, 84]. ATRA can also downregulate bcl-2 expression in native AML blasts for a subset of patients independent of their FAB classification [90]. These effects of ATRA on bcl-2 seem to be mediated by different mechanisms, including regulation of transcription, altered phosphorylation and decreased bcl-2 stability [84, 91]. The effects of ATRA on sensitivity in AML blasts has been studied in AML cell lines. For certain cell lines ATRA can induce differentiation and reduce intracellular bcl-2 levels without altering the susceptibility to drug-induced apoptosis [86, 89], for other cell lines an increased chemosensitivity seems to depend on G2/M cell-cycle arrest rather than the differentiation status or bcl-2 levels [86] and, for a last group of cell lines, ATRA seems to increase chemosensitivity by downregulation of bcl-2 [83, 84]. ATRA can also downregulate bcl-2 expression in native AML blasts for a subset of patients independent of their FAB classification [90]. These effects of ATRA on bcl-2 seem to be mediated by different mechanisms, including regulation of transcription, altered phosphorylation and decreased bcl-2 stability [84, 91]. The effects of ATRA on chemosensitivity can be further modulated by vitamin K analogues; many of these nontoxic analogues seem to induce apoptosis and enhance effects of ATRA via mechanisms involving downregulation of bcl-2 and upregulation of Bax expression with concomitant activation of caspase-3 [92].

Cyclic AMP (cAMP) is important for regulation of apoptosis, and cAMP-dependent kinase has been shown to induce apoptosis in AML cell lines [93]. Bcl-2 is able to block this cAMP-induced apoptosis, but cAMP may then instead result in differentiation induction [94]. This is an additional example that induction of differentiation and apoptosis can be separate events (see above).

Expression of Tumor-Suppressor Genes

The two tumor-suppressor genes p53 and retinoblastoma gene seem to be important as prognostic factors in AML. The p53 gene is an important regulator of apoptosis in AML blasts [95]. Recent studies suggest that certain conformational variants of p53 that occur either by mutation or the action of hematopoietic growth factors, permit AML blast survival and are associated with a bad prognosis [96]. Decreased expression of the retinoblastoma gene is also associated with an increased risk of leukemia resistance or relapse [97].

The p53 activity is modulated by different post-translational mechanisms. A recent study reported that the tumor suppressor PML regulates acetylation of p53, an event necessary for its biological function [98]. Altered acetylation of p53 may then be important in APL (AML-M3) cells where the PML gene is involved in the t(15;17) translocation with the formation of a fusion protein. Furthermore, MDM2 is a negative regulator of p53, and the expression of both p53 and MDM2 is associated with the differentiation status and increased in
AML blasts with a myelomonocytic phenotype (FAB-types M4/M5) [99]. However, it is not known whether the expression or function of these tumor-suppressor molecules can be altered by differentiation induction therapy.

CONCLUDING REMARKS

At present differentiation induction therapy (and thereby induction of apoptosis) is used only in the treatment of the AML-M3/APL subset. Although in vitro studies have demonstrated that leukemia blasts derived from other AML patients may also be induced to differentiate, these effects are difficult to predict in individual patients. Furthermore, differentiation and apoptosis may occur as separate events in AML cells. Future studies of differentiation induction in AML should therefore try to identify: A) new agents or combinations of agents with more predictable effects on differentiation and apoptosis; B) subsets of patients that are likely to benefit from differentiation/apoptosis induction, and C) cytotoxic drugs that can be used in combination with differentiation induction therapy.

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