Preclinical modeling of cytosine arabinoside response in Mll-Enl translocator mouse leukemias

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Abstract

Mouse models of human cancer are a potential preclinical setting for drug testing and for development of methods for delivery of macromolecular drugs to tumors. We have assessed a mouse model of leukemia caused by Mll-Enl protein fusion as a preclinical situation in which myeloid-lineage leukemia results from de novo occurrence of chromosomal translocations between Mll and Enl genes. Here, we show that the mouse leukemias respond to cytosine arabinoside, a frontline treatment for human leukemia. The observations show that the myeloid cells are susceptible to the drug and the mice undergo a remission that comprises a reduction of the myeloid population of cells and recovery of the lymphoid population. This translocator model should therefore prove useful for future drug assessments against the recurrent mixed-lineage leukemia–associated translocations.

Introduction

Mouse models of human cancers are important for developing rational therapeutic strategies and for preclinical drug testing. In addition, new molecular-based therapeutics require special preclinical testing as delivery of these macromolecules to tumors presents a continuing major challenge. Mouse preclinical models have been criticized because some compounds that seemed to be therapeutic when tested in xenograft models failed to be efficient in humans. In fact, the number of anticancer drugs that fail in the clinic far outweighs those considered effective in preclinical trials. This suggests that the models used for testing potential anticancer therapeutics are imperfect and that the selection procedure for progression of molecules into the clinic requires improvement. The development of more faithful genetically engineered mouse models promises to address some of these discrepancies. These should allow a more biologically appropriate model for testing novel anticancer therapeutic agents. These new models could potentially be useful to test drug efficacy as well as variables of delivery, toxicity, specificity, and effectiveness of the new drug before use in clinical settings.

Many human tumors have reciprocal chromosomal translocations as an underlying cause. Among the most frequent target of translocation in human leukemias is the mixed-lineage leukemia (MLL) gene (3). MLL gene abnormalities are found in both childhood (~10% of all pediatric leukemias) and adult leukemias (~5% of acute leukemias; ref. 4). Around 5% to 10% of all MLL-associated leukemias are therapy related as a side effect of treatment with topoisomerase II targeting drugs or of other treatments including radiotherapy. The many MLL fusion partners that have been identified to date (>50; ref. 6) represent a structurally heterogeneous group of proteins. The most frequent partners are AF4, AP9, and ENL resulting from t(4;11), t(9;11), or t(11;19).

We have generated previously a translocator mouse model in which de novo Mll-Enl reciprocal chromosomal translocations occur conditionally by means of Cre-loxP-mediated recombination (7) and these translocator mice develop leukemias. In one line of mice, the Cre recombinase expression was governed by the Lmo2 promoter, allowing Cre expression in pluripotent hematopoietic stem cells. This model resulted in a myeloproliferative-like acute myeloid leukemia (8) with a rapid onset and high penetrance of leukemogenesis. This translocator model provides a possible preclinical model for novel drug testing or for assessing untried combinations for current drugs. To assess the predictive value and validate the Mll; Enl; Lmo2-Cre translocator mouse model as a preclinical model, leukemic mice were treated with cytarabine (cytosine arabinoside or Ara-C), one of the most important compounds used in chemotherapy regimes of patients with acute myeloid leukemia (9), including MLL translocation-associated leukemias. Mll; Enl; Lmo2-Cre translocator mice treated with Ara-C showed a reduction of myeloblasts (leukemic cells) in blood and spleen, with restoration of lymphocyte counts to normal levels. Furthermore, a reduction in the size of the neoplastic organs to normal levels was observed. Thus, as in most patients with acute myeloid leukemia, these translocator mice achieve a remission of the disease in response to treatment with...
Ara-C. These results highlight the faithfulness of this model to the human MLL-ENL myeloid leukemia and support its use as a new model for preclinical studies.

**Materials and Methods**

**Translocator Mouse Strains**

Mouse lines carrying loxP sites inserted in Mll and Enl introns have been described (7, 10). Mice expressing Cre recombinase under the control of Lmo2 (11) were bred with those homozygous for both Mll and Enl loxP alleles (mice carrying Mll and Enl with loxP sites with or without a Lmo2-Cre allele are designated as Mll; Enl; Lmo2-Cre or Mll; Enl, respectively).

**Cytotoxicity Assay in Cell Cultures**

Cell lines from tumors of Mll; Enl; Lmo2-Cre mice were described previously (7). For cytotoxicity assays, 5 x 10^5 cells were plated (in triplicate) in 24-well plates and incubated in growth medium containing 0 to 1 μmol/L cytarabine (Ara-C; David Bull Laboratories) for 6, 24, and 48 h. After incubation, viable cell numbers were calculated by adding 20 μL cell culture to 20 μL trypan blue (0.4%, w/v), after which viable cells were counted based on the presence (dead cells) or exclusion (live cells) of the dye. Cell counts were conducted in duplicate. Results are shown as mean ± SD.

**In vivo Treatment of Translocators with Ara-C**

From ages 3 to 4 weeks, Mll; Enl; Lmo2-Cre or control mice had blood samples taken every 2 days as required. Blood samples were obtained before administration of Ara-C. Leukemia was monitored by increase in circulating leukemic (granulocyte) cells in blood and considered positive when granulocyte counts exceeded 6 x 10^3 cells/mm^3. Leukemic mice were injected i.p. with doses of Ara-C ranging from 0 to 100 mg/kg (resuspended in 100 μL sterile PBS) for 5 consecutive days unless otherwise stated. Blood samples were collected every 2 days and analyzed in a Scil vet abc counter (Scil Animal Care Technologies). Blood smears were stained with May-Grünwald-Giemsa stain. The low-dose experiments (30 mg/kg) consisted of two rounds of 4 consecutive days of injection followed by a 3-day injection-free interval.

At the termination point of the experiments, mice were culled and relevant tissues were removed. Whole animals and the removed tissues were weighed. Flow cytometric analysis was conducted to determine the surface protein expression phenotype of the tumors using a FACSCalibur

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**Figure 1.** Cytotoxicity assays with Ara-C of Mll; Enl; Lmo2-Cre cell cultures. Mll; Enl; Lmo2-Cre mice with leukemia were donors for cell lines made from myeloid cells from the infiltrated spleens. Dose-response and time-response effect of Ara-C in Mll; Enl; Lmo2-Cre cell cultures. A, 5 x 10^5 cells were plated in 24-well plates (in triplicate) and incubated in the presence of 0 (x), 0.1 (∅), 0.3 (●), 0.6 (□), and 1 (○) μmol/L Ara-C for 6, 24, and 48 h. After incubation, cells were harvested and viable cell numbers were calculated by trypan blue exclusion. Cell counts were obtained in duplicate. Mean ± SD. *, P < 0.05; **, P < 0.01. B, dose-response curve of Mll; Enl; Lmo2-Cre to Ara-C at 24 h. 5 x 10^5 cells were plated in 24-well plates (in triplicate) and incubated in the presence of 0 to 300 nmol/L Ara-C. After 24 h, cells were harvested and viable cell numbers were calculated by trypan blue exclusion. Exclusion assays were conducted in duplicate. Mean ± SD. The IC50 (50% inhibitory concentration) for the Mll; Enl; Lmo2-Cre cell line (A) was 56 nmol/L. Results are compared with a primary spleen cell culture (○) as a control.

**Figure 2.** Ara-C induces apoptosis in Mll; Enl; Lmo2-Cre cell culture. A, 5 x 10^5 myeloid cells (from the infiltrated spleens of Mll; Enl; Lmo2-Cre leukemic mice) were incubated in 24-well plates (in triplicate) with or without 300 nmol/L Ara-C for 24 h before assay for apoptosis by flow cytometry analysis of Annexin V-FITC binding. Data show FITC signal and propidium iodide (PI) uptake levels. B, DNA fragmentation assay. After 24-h incubation with Ara-C ranging from 0 to 100 nmol/L, 5 x 10^6 cells were harvested and DNA was prepared. Samples were subjected to electrophoresis in a 1.8% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator.
with fluorescent antibodies. For this analysis, cell suspensions (100 μL) from spleen were incubated with the appropriate antibodies for 1 h on ice, washed with PBS, and analyzed using FACSCalibur. The antibodies used (PharMingen; BD Biosciences) were PE-labeled anti-Mac-1(CD11b), FITC-labeled anti-Gr-1(Ly-6G), PE-labeled anti-CD4(L3T4), FITC-labeled anti-CD8a, PE-labeled anti-Thy1.2, and FITC-labeled anti-B220(CD45R). The appropriate isotype controls were used for each antibody (Ig2a for anti-B220, anti-Thy1.2, and anti-CD4 and Ig2b for anti-Mac-1, anti-Gr-1, and anti-CD8a).

**Results**

**Sensitivity of Mll; Enl; Lmo2-Cre Cells to Ara-C In vitro**

To evaluate if the Mll; Enl; Lmo2-Cre translocator mice would be responsive to Ara-C treatment, we analyzed in vitro cytotoxicity assays. Mll; Enl; Lmo2-Cre cell cultures were established from splenic infiltrates of myeloid leukemia cells (7). These cells were incubated in growth medium containing varying amounts of Ara-C for 6, 24, or 48 h. After treatment, viable cell numbers were calculated using trypan blue exclusion. Figure 1A shows the time-response curve of Mll; Enl; Lmo2-Cre cells when exposed to increasing concentrations of Ara-C. A reduction of cell viability to 65% was observed as early as 6 h of culture in the presence of 1 μmol/L Ara-C. Even when incubated with 0.1 μmol/L Ara-C, there was an almost complete depletion of viable cells after 48 h of treatment. Figure 1B shows the dose-response curve of Mll; Enl; Lmo2-Cre cells to Ara-C treatment at 24 h. The IC<sub>50</sub> (half-maximal inhibitory concentration) for the Mll; Enl; Lmo2-Cre cell line was calculated as 56 nmol/L. Similar results were obtained with primary spleen
cultures and parallel to those seen for Ara-C treatment of established cell lines (12).

The mechanism of cell death induced by Ara-C is apoptosis (13). This was confirmed as the killing mechanism for the Mll-Enl translocator cells. Cultures of cells were grown in the presence or absence of 300 nmol/L Ara-C for 24 h, and flow cytometry detection of externalized phosphatidylserine was done using Annexin V-FITC binding as a measure of cells dying by apoptosis (Fig. 2A). A population of 30% of apoptotic cells binds the Annexin V

Figure 4. Phenotypic disruption by Ara-C treatment of Mll; Enl; Lmo2-Cre leukemic mice. Mll; Enl; Lmo2-Cre (leukemic) or Mll; Enl mice were injected with 0 (vehicle only), 3.5, 30, or 100 mg/kg Ara-C in two rounds of 4 consecutive days of injection separated by a 3-day injection-free interval. Granulocyte blood levels were determined before treatment (day 0) and at days 3, 7, 10, and 14 after treatment by Coulter counting. Leukemia was considered to be present when granulocyte counts exceeded $6 \times 10^9$ cells/mm$^3$. A, quantification of granulocyte levels in the blood are shown for untreated or Ara-C-treated Mll; Enl; Lmo2-Cre mice. Untreated (○); treated with 3.5 mg/kg (□), 30 mg/kg (*) or 100 mg/kg (●) Ara-C. B, quantification of granulocyte levels in the blood for untreated or Ara-C-treated Mll; Enl mice. Untreated (●); treated with 30 mg/kg (□) or 100 mg/kg (●) Ara-C. C, decrease in myeloid (Mac-1/Gr-1 positive) tumor cells in response to treatment with Ara-C. Flow cytometric analysis of surface antigen expression (either Mac-1 + Gr-1, Thy1.2 + B220, or CD4 + CD8) of spleen cells of mice after two rounds of treatment with Ara-C (30 or 100 mg/kg for Mll; Enl; Lmo2-Cre mice or only 100 mg/kg for Mll; Enl only mice).
Ara-C Can Achieve Tumor Remission in Mll; Enl; Lmo2-Cre Leukemic Mice

The effects of Ara-C on leukemia in vivo was tested using the Mll-Enl translocator mice. Litters were selected from crosses between homozygous Mll; Enl and heterozygous Lmo2-Cre mice giving a mixture of pups with either Mll; Enl; Lmo2-Cre or Mll; Enl genotypes. Before drug treatment, blood leukocyte levels were determined in the Mll; Enl; Lmo2-Cre and Mll; Enl mice starting 2 weeks after birth and following leukemia by blood granulocyte levels.

Sensitivity of leukemic Mll; Enl; Lmo2-Cre mice to Ara-C was tested with a high-dose regimen. Leukemic mice (age 3-4 weeks) were injected i.p. with PBS as vehicle (three mice) or 100 mg/kg Ara-C for 5 consecutive days (five mice). Blood samples were collected every 3 days and analyzed in the Coulter counter. Figure 3A shows an example for each group. A decrease in granulocyte levels was observed in the blood immediately after treatment with 100 mg/kg Ara-C (Fig. 3A). By day 3 of the experiment, granulocyte blood levels achieved normal (nonleukemic) values of \( \sim 1.2 \times 10^9 \) to \( 1.5 \times 10^9 \) cells/mm\(^3\). After treatment, granulocyte blood levels remained within normal levels for the experimental period (reflected by the death of the control leukemic mouse that had received vehicle only). The dynamics of the blood leukemic cells is shown in Fig. 3B, comparing leukocyte counts and smears of an Mll; Enl and Mll; Enl; Lmo2-Cre mice with and without 100 mg/kg Ara-C treatment. The leukemic cells dominate in untreated mice, resulting in death within \( \sim 2 \) weeks, whereas Ara-C treatment causes leukemia remission over the allowed period of treatment.

An alternative experimental protocol was investigated in which two consecutive rounds of Ara-C treatment were given, using 4 days of injection followed by a 3-day injection-free interval, and subsequently by a further 4 days of dosing. In this approach, different Ara-C concentrations ranging from 3.5 to 100 mg/kg were assessed. As seen in Fig. 4A and B, lower doses (3.5 mg/kg) were not sufficient to deplete completely granulocyte levels in blood, and when treatment was withdrawn, most of the mice relapsed. However, higher doses (30 and 100 mg/kg) successfully decreased leukemic cells in blood to normal levels, sustained through the time of leukemia progression of untreated or 3.5 mg/kg treated mice.

Ara-C Specificity Causes Neoplastic Tumor Regression

The specific consequences for the leukocyte subpopulations were assessed using flow cytometry (Fig. 4C). As the Mll; Enl; Lmo2-Cre translocator mice show splenomegaly as a feature of the leukemia, at the termination point of the experiments, mice were culled and spleens were removed and weighed. Ara-C treatment achieved a reduction of spleen size (neoplastic tissue) to normal values (Supplementary Fig. S1). Further, spleen cells were prepared as single-cell suspension for flow cytometric analysis (Fig. 4C). Myeloid (Mac-1/Gr-1)–positive cells dramatically decreased in response to Ara-C treatment, whereas B cells (B220 positive) or T cells (Thy1, CD4, or CD8 positive) appear approximately normal after the Ara-C regimen. However, completely normal levels of granulocytes could not be sustained in this experimental protocol, suggesting that a maintenance therapy would be needed to achieve long-term remission and to avoid relapse. This is not surprising because Ara-C treatment, as well as most of the currently used therapeutics, may be effective at killing most leukemic cells but not capable of eliminating all of them. In these regards, this mouse model behaves similarly to human patients, most of whom, without any additional post-remission therapy, have a high probability of relapse (9).

Discussion

Ideally, mouse models of human cancer should have short tumor latency and high penetrance, be an authentic replication of the corresponding human tumor, and have spontaneous occurrence due to the presence of a single alteration in the mouse genome. Models in which most or all of a cohort develop tumors in a short time provide a cost-effective preclinical model. The Mll; Enl; Lmo2-Cre translocator model fulfills most of these criteria because all mice of the Mll; Enl; Lmo2-Cre genotype die within 120 days and leukemia (blood granulocytes) can be observed as early as 3 to 4 weeks (7).

We have shown that the Mll; Enl; Lmo2-Cre translocator model responds to Ara-C chemotherapy similarly to humans. As in most patients with acute myeloid leukemia, mice achieve remission characterized by cyto reduction of blast cells in blood and spleen, as well as a reduction in size of the neoplastic organ (in this case, the spleen). However, without additional postremission therapy, most of the mice relapsed as is also the case with most human patients. These results highlight the faithfulness of this model to the human MLL-ENL myeloid leukemia and support its usage as a new tool in preclinical studies. Practical improvements to the model could be introduced to increase the cost-effectiveness of the model even further. One of these options would be to include a luciferase reporter in cells that have a translocation to allow bioluminescent imaging in vivo to follow tumor response to therapy. A further feature of value would be a conditional form of Cre expression. We have developed an Lmo2-CreERT2 strain of mice and these will allow a synchronized activation of translocations in a Mll; Enl; Lmo2-CreERT2 strain. Breeding these mice with Cre-dependent expression of luciferase will give a fully conditional, image-able model for MLL-associated leukemias.

4 Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
5 R. Pannel and T.H. Rabitts, unpublished data.
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References

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