GATA1, Cytidine Deaminase, and the High Cure Rate of Down Syndrome Children With Acute Megakaryocytic Leukemia

Yubin Ge, Mark L. Stout, Dana A. Tatman, Tanya L. Jensen, Steven A. Buck, Ronald L. Thomas, Yaddanapudi Ravindranath, Larry H. Matherly, Jeffrey W. Taub

Down syndrome children with acute megakaryocytic leukemia (AMkL) have higher cure rates than non-Down syndrome acute myeloid leukemia (AML) patients treated with cytosine arabinoside (ara-C). Megakaryoblasts from Down syndrome AML patients are more sensitive in vitro to ara-C than cells from non-Down syndrome AML patients. Somatic mutations in the GATA1 transcription factor have been detected exclusively and almost uniformly in Down syndrome AMkL patients, suggesting a potential linkage to the chemotherapy sensitivity of Down syndrome megakaryoblasts. Stable transfection of wild-type GATA1 cDNA into the Down syndrome AMkL cell line CMK resulted in decreased (8- to 17-fold) ara-C sensitivity and a threefold-lower generation of the active ara-C metabolite ara-CTP compared with that for mock-transfected CMK cells. High intracellular levels of uridine arabinoside (ara-U) (an inactive ara-C catabolite generated by cytidine deaminase) and cytidine deaminase transcripts were detected in GATA1-transfected CMK sublines, whereas no ara-U was detected in mock-transfected cells. Cytidine deaminase transcripts were a median 5.1-fold (P = .002) lower in Down syndrome megakaryoblasts (n = 16) than in blast cells from non-Down syndrome patients (n = 56). These results suggest that GATA1 transcriptionally upregulates cytidine deaminase and that the presence or absence of GATA1 mutations in AML blasts likely confers differences in ara-C sensitivities due to effects on cytidine deaminase gene expression, which, in turn, contributes to the high cure rate of Down syndrome AMkL patients. [J Natl Cancer Inst 2005;97:226–31]

Since an initial report in 1992, studies of the Pediatric Oncology Group, Children’s Cancer Group, and other pediatric oncology cooperative groups have consistently reported that, after treatment with cytosine arabinoside (ara-C)-based protocols, Down syndrome children with acute myeloid leukemia (AML) have substantially higher event-free survival rates (80%–100%) and lower relapse rates (<15%) than non-Down syndrome AML patients (1–8). These findings, and the high incidence of AML in Down syndrome children, highlight the unique biologic features of leukemia associated with trisomy 21 and the localization of genes to chromosome 21 that contribute to both leukemogenesis and anticancer drug sensitivity (9).

We previously reported that, in vitro, blast cells from Down syndrome children generate higher levels of the active intracellular ara-C metabolite ara-CTP and are more sensitive to ara-C than blast cells from non-Down syndrome AML patients (10). This altered metabolism and increased ara-C sensitivity of Down syndrome blasts are likely important factors contributing to the high event-free survival rates of Down syndrome AML patients. This is linked, in part, to increased expression of cytathionine-β-synthase, the gene for which localizes to 21q22.3 (10,11). Additional mechanisms may also contribute to the high event-free survival rates of Down syndrome AML patients, including an association with the acute megakaryocytic leukemia (AMKL; M7) phenotype, which represents the predominant AML subtype in Down syndrome children (12).

Recently, Wechsler et al. (13) reported that all six Down syndrome AMkL patients analyzed had acquired somatic mutations in exon 2 of the GATA1 gene. The GATA1 gene, the most specific genetic marker linked to Down syndrome AMkL, is located to chromosome X and encodes a zinc-finger transcription factor that is essential for normal erythroid and megakaryocytic differentiation. Subsequent reports described the near-uniform detection of somatic mutations in the same exon of the GATA1 gene in individuals with Down syndrome AMkL and the transient myeloproliferative disorder but not in any individuals with non-Down syndrome AML or in those with Down syndrome non-AMKL (14–18). These somatic mutations, which include insertions, deletions, missense, nonsense, and splice site mutations at the exon 2/intron boundary and have the net effect of introducing stop codons, result in the synthesis of a smaller 40-kDa GATA1 protein from a downstream initiation site. The 40-kDa GATA1 protein is distinguishable from the full-length 50-kDa GATA1 protein and has reduced transactivation capacity (13), potentially contributing to the uncontrolled proliferation of megakaryocytes. An additional possibility is that the presence of GATA1 mutations in Down syndrome megakaryoblasts results in the differential regulation of target genes that contribute to the high event-free survival rates of this unique group of patients.

One such target may be cytidine deaminase, which deaminates ara-C and the related nucleoside agent gemcitabine to the inactive metabolites uridine arabinoside (ara-U) and difluorodeoxyuridine, respectively (19). We previously reported that expression of cytidine deaminase was decreased in a small sample of Down syndrome megakaryoblasts compared with that for non-Down syndrome AML blasts, which may contribute to the increased ara-C sensitivity among Down syndrome patients (20). Moreover, several GATA1 elements were identified in a downstream intronic enhancer sequence.
The introduction of stop codons and the synthesis of a smaller GATA1 protein (40-kDa) from a downstream initiation site. The smaller GATA1 protein has a reduced transactivation capacity compared with that of the full-length 50-kDa protein. The cytidine deaminase gene consists of a CDA's (short-form) intronic promoter that acts as an enhancer to the CDA's (long-form) promoter, which may drive the transcription of the cytidine deaminase gene (20). Based on the presence of several GATA1 binding sites in the CDA's promoter, decreased cytidine deaminase enhancer activity, which results from the synthesis of the transcriptionally less active (40-kDa) GATA1 protein, may account for the lower cytidine deaminase expression observed in Down syndrome megakaryoblasts. Decreased cytidine deaminase expression in Down syndrome megakaryoblasts results in greater ara-C phosphorylation and higher ara-CTP levels compared with that in non-Down syndrome blast cells.

We hypothesized that variations in the extent of the transactivation of cytidine deaminase by wild-type GATA1 may confer differences in cytidine deaminase activity and ara-C sensitivity (Fig. 1). To directly assess the relationship between ara-C and GATA1, the clinically relevant Down syndrome AMKL cell line CMK was stably transfected with the full-length GATA1 cDNA. Although wild-type CMK cells express only the 40-kDa GATA1 protein, two stably transfected CMK clones (designated CMK-GATA1#6 and CMK-GATA1#13) expressed the 50-kDa GATA1 protein by western blot analysis (Fig. 2, A). Cytidine deaminase transcripts were detected by real-time polymerase chain reaction (PCR) in the GATA1-transfected CMK cells but not in the mock-transfected cell line (Fig. 2, B).

In vitro drug sensitivities of the wild-type and mock- and GATA1-transfected CMK cells were examined by growth inhibition assays with a panel of chemotherapy agents to determine whether patterns of sensitivity would be altered accompanying the presence of the full-length GATA1 protein. The CMK transfectants expressing the full-length GATA1 protein were ~8- to 17-fold less sensitive to ara-C and ~15- to 25-fold less sensitive to gemcitabine than the wild-type and mock-transfected CMK cells (Fig. 2, C and D; Table 1). The GATA1-transfected cells also showed slightly decreased daunorubicin (~twofold) sensitivity and slightly increased 6-mercaptopurine (~twofold) sensitivity compared with the wild-type and mock-transfected CMK cells (Table 1). There were no differences in sensitivities to 2-chlorodeoxyadenosine, methotrexate, or cisplatin between the wild-type and GATA1-transfected clones, suggesting that the effects of GATA1 expression were relatively specific to ara-C and gemcitabine, and, to a lesser extent, daunorubicin and 6-mercaptopurine. The results with ara-C and gemcitabine are consistent with the finding of increased
To further explore whether the relationship between GATA1 and sensitivity to ara-C and gemcitabine involves the cytidine deaminase catabolism pathway, the two stable GATA1 clones were incubated in vitro with 5 μM [3H]ara-C for 3 hours and the 3H-metabolite levels were measured over time. In this series of experiments, intracellular accumulations of [3H]ara-CTP were approximately three-fold lower in the GATA1-transfected cells than in the wild-type and mock-transfected CMK cells (Fig. 3A). This was accompanied by high levels of both intracellular and extracellular ara-U in the GATA1 transfectants, whereas none of the GATA1-transfected cells showed changes in the sensitivity to this drug.

Table 1. In vitro drug sensitivities in clinically relevant Down syndrome AMkL cell lines*

<table>
<thead>
<tr>
<th>Drug</th>
<th>CMK Wild-type</th>
<th>CMK-Mock</th>
<th>CMK-GATA1#6</th>
<th>CMK-GATA1#13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ara-C</td>
<td>5.2 (2.3 to 8.1)</td>
<td>4.7 (3.9 to 5.4)</td>
<td>42.3 (26.1 to 58.4)</td>
<td>80.4 (67.7 to 93)</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>5.0 (1.3 to 8.8)</td>
<td>5.1 (3.2 to 6.9)</td>
<td>74.6 (39.8 to 109)</td>
<td>125 (44.2 to 206)</td>
</tr>
<tr>
<td>Daunorubicin</td>
<td>9.6 (7.6 to 11.7)</td>
<td>12.4 (8.6 to 16.2)</td>
<td>26.9 (19.9 to 34.9)</td>
<td>25.6 (19.7 to 29.4)</td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>962 (737 to 1188)</td>
<td>720 (592 to 847)</td>
<td>463 (377 to 550)</td>
<td>301 (138 to 463)</td>
</tr>
</tbody>
</table>

*The data represent the mean values of a single replicate of 3–5 independent experiments.

To further explore whether the relationship between GATA1 and sensitivity to ara-C and gemcitabine involves the cytidine deaminase catabolism pathway, the two stable GATA1 clones were incubated in vitro with 5 μM [3H]ara-C for 3 hours and the 3H-metabolite levels were measured over time. In this series of experiments, intracellular accumulations of [3H]ara-CTP were approximately three-fold lower in the GATA1-transfected cells than in the wild-type and mock-transfected CMK cells (Fig. 3A). This was accompanied by high levels of both intracellular and extracellular ara-U in the GATA1 transfectants, whereas none of the GATA1-transfected cells showed changes in the sensitivity to this drug.
was detected in the wild-type and mock-transfected CMK cells (Fig. 3, A and B). These results directly implicate increased cytidine deaminase expression and the increased generation of ara-U in the GATA1 transfectants as contributing to decreased ara-CTP generation.

Cytidine deaminase is secreted into the media by cultured cells, whereupon it rapidly converts extracellular ara-C to ara-U, even in the absence of cells (20). To confirm the presence of cytidine deaminase in the media of the GATA1 transfectants, the cells were incubated in complete media without [3H]ara-C for 3 hours. After the cells were removed by centrifugation, 1 mL of the cell-free supernatant was incubated with 5 µM [3H]ara-C and the accumulation of [3H]ara-U was monitored by high-performance liquid chromatography over time (Fig. 3, C). In conditioned media from both GATA1-transfected clones, ara-U levels increased linearly over time, whereas ara-U was not detected in conditioned media from wild-type and mock-transfected CMK cells (Fig. 3, C). The conversion of ara-C to ara-U was completely abolished by the addition of 100 mM tetrahydrouridine (data not shown), a specific inhibitor of cytidine deaminase that has been used clinically with ara-C in the treatment of adults with relapsed leukemia (21).

To confirm the relationship between GATA1 and ara-C metabolism and sensitivity in clinical AML samples, GATA1 gene status and both GATA1 and cytidine deaminase transcripts were analyzed in blast cells from 16 newly diagnosed Down syndrome (including four transient myeloproliferative disorder and 12 AMKL) patients and 56 non-Down syndrome pediatric AML patients. All of the Down syndrome samples were confirmed to be of the AMKL phenotype by flow cytometry analysis, whereas the non-Down syndrome samples displayed a range of French-American-British (FAB) phenotypes. GATA1 exon 2 mutations were identified in 14 of 16 Down syndrome megakaryoblast samples (mutations were not detected in two of four transient myeloproliferative disorder samples, possibly due to the sensitivity of the assay), confirming the relationship between GATA1 mutations and the AMKL phenotype. However, no mutations were detected in the non-Down syndrome blast cells, which included nine AMKL samples. The net effect of the GATA1 mutation in each of the Down syndrome samples was the introduction
of a stop codon either before or after methionine 84 that results in the synthesis of the 40-kDa GATA1 protein (13).

Cytidine deaminase transcripts, measured by real-time PCR and normalized to 18S RNA levels, were a median 5.1-fold lower in Down syndrome megakaryoblasts (n = 16; interquartile range = 0.06) than those in non-Down syndrome AML blast cells (n = 56; interquartile range = 0.33). This difference was statistically significant (P = .002; Mann-Whitney U test) (Fig. 4, A). Cytidine deaminase transcripts statistically significantly correlated with in vitro ara-C sensitivities, measured by the MTT assay, for the entire AML cohort (r = 0.34; P = .006; Spearman rank correlation) (Fig. 4, B). For two primary AML samples with sufficient cells for incubations with \[^3H\]ara-C (5 µM; 3 hours), ara-U levels detected in the media were approxi-
mately 2.5-fold lower in the Down syndrome AML sample (DS-A12) than those in the non-Down syndrome AML sample (non-DS-A49) (Fig. 4, C). Although decreased extracellular ara-U in the media was accompanied by a ~35% decrease in the level of intracellular ara-CTP in the non-Down syndrome samples, intracellular ara-U was not detected (data not shown), possibly reflecting the low cytocrit and small intra- to extracellular volumes of the clinical AML specimens.

Thus, mutations of the GATA1 gene in Down syndrome transient myeloproliferative disorder and AMKL patients that generate a transcriptionally less ac-
tive GATA1 protein can be envisaged to result in reduced cytidine deaminase enhancer activity, decreased overall cytidine deaminase expression, and decreased net

---

**Fig. 4.** Analysis of cytidine deaminase (CDA) transcripts and cytosine arabinoside (ara-C) metabolites in acute myeloid leukemia (AML) samples from patients with or without Down syndrome. A) Cytidine deaminase transcripts were measured by reverse-transcription polymerase chain reaction (RT-PCR). Down syndrome megakaryoblasts and non-Down syndrome AML cells were obtained after informed consent was obtained from the Children’s Hospital of Michigan Leukemia Cell Bank and from the Pediatric Oncology Group 9421 study as previously described (10). The study was approved by the Wayne State University Human Investigation Committee. Following isolation of total RNA from AML blast cells, first-strand cDNAs were synthesized from 1 µg of total RNA and 100-ng aliquots of the cDNAs were used for each PCR reaction. Cytidine deaminase transcripts and 18S RNA levels were quantified by using a Light-
Cycler real-time PCR machine (Roche). Blast cells were screened for the presence of GATA1 exon 2 mutations in genomic DNAs by using forward (GATA1 exon2/F, 5’-GAGGGGAAAGGAGGAGGAGCAGGTG-3’) and reverse (GATA1 exon2/R, 5’-CACCAGCCATGCCACAGCCACTCAATG-3’) flanking primers. Amplicons were sequenced directly and after subcloning in pGEM-T-Easy vector (Promega, Madison, WI). Sequence compar-
isons and translation analyses were performed using programs available from the National Center for Biotechnology Information, Bethesda, MD. B) Correlation between cytidine deaminase transcripts with in vitro sensitivities to ara-C measured by the MTT assay for 10 Down syndrome and 53 non-Down syndrome AML samples is shown (10). C) Extracellular ara-U in two clinical AML samples, one from a patient with Down syndrome (DS-A12) and one from a patient without Down syndrome (non-DS-A49) was measured. After incubating the clinical AML cells (2 × 10^7) with 5 µM \[^3H\]ara-C for 3 hours, 50-μL of each incubation medium was assayed by high-performance liquid chromatography for the presence of extracellular \[^3H\]ara-U. D) Levels of cyti-
dine deaminase and GATA1 transcripts measured by real-time PCR were correlated in non-Down syndrome AML blast cells.
conversion of ara-C to ara-U (Fig. 1). Interestingly, within the non-Down syndrome AML group alone, there was a statistically significant correlation between cytidine deaminase and GATA1 transcript levels (r = 0.35, P = .004; Spearman rank correlation), further suggesting a close relationship between GATA1 and cytidine deaminase expression (Fig. 4, D).

The analysis of Down syndrome leukemia patients has highlighted the role of chromosome 21 in both leukemogenesis and the metabolism of chemotherapy drugs. The unique pattern of clinical response to therapy of Down syndrome AML patients, particularly those with the AMKL phenotype, is likely multifactorial and, as suggested by our study, may be linked to the uniform detection of GATA1 mutations in Down syndrome AMKL patients. The linkage between GATA1 and cytidine deaminase expression, and the observed effect on the sensitivity of leukemia cells to ara-C, likely represents an important contributing factor to the high event-free survival rates of Down syndrome AML patients, in addition to the established relationship to overexpression of the cystathionine-β-synthase gene (10, 11).

REFERENCES


Notes

This study was supported by a grant from the National Cancer Institute (RO1 CA92308), the Leukemia and Lymphoma Society, The Children’s Research Center of Michigan, BPCT Golf Charity, The Elana Fund, and Justin’s Gift Charity. J.W.T. is a Scholar in Clinical Research of the National Down Syndrome Society’s Charles J. Epstein Research Award and the Children’s Research Center of Michigan Research Award. Manuscript received June 11, 2004; revised October 19, 2004; accepted November 23, 2004.