ABSTRACT

Myeloblasts from Down syndrome (DS) children with acute myeloid leukemia (AML) are significantly more sensitive in vitro to 1-β-D-arabinofuranosylcytosine (ara-C) and generate higher 1-β-D-arabinofuranosylcytosine 5'-triphosphate (ara-CTP) than non-DS AML myeloblasts. Semi-quantitative reverse transcription-PCR analyses demonstrated that transcripts for cytidine deaminase (CDA) were 2.9-fold lower in DS than for non-DS myeloblasts. In contrast, transcripts of cystathionine-β-synthase and deoxyctydin kinase were a median 12.5- and 2.6-fold higher in DS compared with non-DS myeloblasts. The ratio of deoxycytidylidy kinase/CDA transcripts significantly correlated with ara-C sensitivities and ara-CTP generation. In clinically relevant AML cell line models, high cystathionine-β-synthase transcripts and deoxyctydin kinase were a median 12.5- and 2.6-fold higher in DS compared with non-DS myeloblasts. Overexpression of CDA in non-DS myeloblasts was associated with a 100-fold decreased ara-C sensitivity and 40-fold decreased ara-CTP generation. Transfection of GATA1 into the THP-1 cell line stimulated the CDA promoter acting as an enhancer. The presence of several GATA1 binding sites in the CDA promoter regions of selected genes may result in altered gene transcription in cells containing mutated GATA1 genes (i.e., DS megakaryoblasts).

INTRODUCTION

1-β-D-arabinofuranosylcytosine (ara-C), a deoxycytidine analogue, is a member of the group of arabinos containing nucleosides that were originally isolated from the sponge Cryptotheca crypta and have shown widespread activity in lymphomatosuppressive malignancies (1). For acute myeloid leukemia (AML), ara-C comprises the essential component during both induction and consolidation therapy in both intermediate and high-dose schedules (2).

Interestingly, Down syndrome (DS) children with AML have significantly higher event-free survival rates (80–100%; relapse rates <15%) compared with non-DS children with AML (40–45%) treated with ara-C-based therapy (3). Increased ara-C sensitivity of DS myeloblasts has been confirmed by in vitro drug sensitivity assays, and DS myeloblasts generate significantly higher levels of the active intracellular ara-C metabolite, 1-β-D-arabinofuranosylcytosine 5'-triphosphate (ara-CTP), compared with myeloblasts from non-DS children (4).

The increased ara-C sensitivity of DS myeloblasts may reflect the altered expression of multiple genes encoding enzymes involved in ara-C metabolism. Ara-C is a prodrug that must be converted to its triphosphate derivative (ara-CTP) to exert its cytotoxic effects (5, 6). After intracellular transport by an energy-dependent uptake mechanism, ara-C is converted to its active form, ara-CTP, through a sequential action of deoxycytidylidy kinase (dCK), deoxycytidylidy kinase, and nucleoside diphosphate kinase (Fig. 1; Refs. 5, 6). dCK is generally considered the rate-limiting step leading to ara-CTP formation (5, 6). It has been shown that transfection of the dCK cDNA into dCK-deficient tumor cell lines restores in vitro sensitivities to ara-C (7, 8).

Of course, any of a number of other cellular enzymes can significantly alter patterns of ara-C sensitivity or resistance. For instance, we previously reported that the chromosome 21-localized gene, cystathionine-β-synthase (CBS; E.C. 4.2.1.22), can significantly modulate ara-C activity via its interaction with the reduced folate/S-adenosylmethionine pathways. When CBS-null CCRF-CEM cells were transfected with the CBS cDNA, ara-CTP generation and ara-C sensitivity were greatly enhanced (9). Interestingly, CBS transcripts were also significantly higher in DS compared to non-DS myeloblasts (4). Moreover, increased in vitro ara-C sensitivities of DS myeloblasts compared with non-DS myeloblasts were accompanied by slightly elevated dCK transcripts and higher accumulations of the active intracellular ara-C metabolite, ara-CTP, after in vitro incubations with [3H]ara-C (4).

Cytidine deaminase (CDA; EC 3.5.4.5; gene localized to 1p35-36.2) is a salvage pathway enzyme that catalyzes the hydrolytic deamination of cytidine and deoxycytidine to the corresponding uracil nucleosides (5, 6). Ara-C is subject to deamination by CDA resulting in the inactive product, 1-β-D-arabinofuranosyluracil (ara-U). Several studies have suggested an important role for increased levels of CDA in the development of resistance to ara-C (10–12).

Recently, mutations in the erythroid and megakaryocytic zinc-finger transcription factor, GATA1, have been detected in essentially all cases of DS transient myeloproliferative disorder and acute megakaryocytic leukemia (AML) and not detected in non-DS leukemia cases (13–21). The presence of GATA1 binding sites in the promoter regions of selected genes may result in altered gene transcription in cells containing mutated GATA1 genes (i.e., DS megakaryoblasts).

In this study, we expand our previous studies to additionally ex-
plore the molecular bases of chemotherapy sensitivity of DS AML patients by examining the expression of these key ara-C metabolism genes in DS and non-DS myeloblast specimens. These studies were also extended to include an in vitro analysis of ara-C cellular pharmacology in a unique series of DS and non-DS AML cell lines that closely resemble primary specimens obtained from patients. We also explore the possibility that GATA1 mutations may account for the differential expression of ara-C metabolizing genes between DS and non-DS AML cases.

MATERIALS AND METHODS

Patient Specimens. Myeloblasts were obtained from newly diagnosed pediatric DS and non-DS AML patients as previously described (4). Chemicals. [5-3H]-Cytosine-β-thiogalactoside (25Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Unlabeled ara-C, ara-U, and ara-G were obtained from Sigma Chemical Co. (St. Louis, MO). Tetrahydrofolate reductase inhibitors, including methotrexate, were purchased from Genosys Biotechnologies, Inc. (The Woodlands, TX). Bovine serum albumin, Ficoll-Paque, and fetal bovine serum were obtained from Amicon (Billerica, MA). 

Cell Culture. The DS AML cell line, CMK, and the non-DS AML cell line, CMS, were cultured as described previously (22). THP-1, HL60, and U937 leukemia cell lines and the human HT1080 fibrosarcoma cell line were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 containing 10% heat-inactivated calf serum (Hy clone Labs, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere at 37°C in the presence of 5% CO2/95% air. D. Mel-2 cells were purchased from Invitrogen (Carlsbad, CA) and maintained as described previously (34).

Reverse Transcription-PCR (RT-PCR) Analysis of Transcripts of Genes Related to Ara-C Metabolism. Total RNA was isolated from 5–10 × 10^6 patient myeloblasts or cultured cells using TRIzol Reagent (Molecular Research Center, Cincinnati, OH). CBS, dCK, and CDA transcripts were assayed from the same cDNA mixtures using the oligonucleotide primer pairs summarized in Table 1.

The RT-PCR analyses of CBS in clinical specimens were performed in triplicate as described previously (4). Similar methods were used for the detection of dCK and CDA transcripts. The linear responses of our RT-PCR assays of gene transcripts (CBS, CDA, and dCK) were established to ensure that the PCR cycle numbers were in the linear range and that there was a linear correlation between the cDNA amount and the level of transcripts determined by fluorescent imaging as described above. An example of this analysis for CBS transcripts in sample A39 is shown in Fig. 2A.

In Vitro Drug Cytotoxicity Assays. In vitro cytotoxicities of DS and non-DS leukemia cell lines were determined as described previously (22). The IC50s were calculated as the concentration of drug necessary to inhibit 50% growth compared with control cells cultured in the absence of drug.

Ara-C Incubations and Measurement of Ara-C-TP. Incubation of leukemia cells with 5 μM [3H]ara-C and measurements of intracellular and extracellular [3H]metabolites were performed as described previously (4).

Rapid Amplification of 5′-cDNA Ends (5′-RACE). cDNAs were prepared from 1 μg of total RNA from THP-1 cells using PowerScript reverse transcriptase and 5′-CDS poly-A and SMART II A primers (Clontech). Dilutions of each cDNA were used in primary and nested secondary PCR amplification reactions with SMART RACE kit sense primers (Universal Primer Mix A and Nested Universal Primer A) and gene specific antisense primers (CDA735/low (5′-caccaggaagaagagagg-3′) and CDA530/low (5′-agtggcactttggtcactc-3′), respectively). The primary PCR conditions were 94°C for 7 min (1 cycle), 94°C for 2 s and 70°C for 3 min (7 cycles), and 94°C for 2 s and 67°C for 3 min (35 cycles), followed by 72°C for 7 min (1 cycle). For the secondary PCR, the conditions were 94°C for 7 min (1 cycle), 94°C for 2 s and 70°C for 3 min (7 cycles), and 94°C for 2 s and 67°C for 3 min (20 cycles), followed by 72°C for 7 min (1 cycle). Secondary PCR products were ligated into pGEM T-Easy (Promega), and ligations were transformed into competent JM109 cells. Plasmid DNAs were extracted from bacterial cultures using the Qiagen Miniprep Spin kit. 5′-RACE inserts were sequenced using gene-specific or universal primers.

Bacterial Expression of CDA Isoforms. CDA short-form (CDA/sf) cDNA was reverse transcribed and PCR amplified with CDA1F/His A. Amplicons were digested with XhoI and HincII and ligated into pGEM T-Easy (Promega), and ligations were transformed into competent JM109 cells. Plasmid DNAs were extracted from bacterial cultures using the Qiagen Miniprep Spin kit. 5′-RACE inserts were sequenced using gene-specific or universal primers.

Table 1: Summary of transcript sequences, reverse transcription-PCR conditions and PCR product sizes for cystathionine-β-synthase (CBS), deoxycytidine kinase (dCK), and cytidine deaminase (CDA)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Annealing temperature (°C)</th>
<th>Cycle number</th>
<th>Product size (bp)</th>
</tr>
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<tr>
<td>CBS</td>
<td>5′-TGCCCTCCTGTGCCACATCACCA-3′</td>
<td>65</td>
<td>32</td>
<td>817</td>
</tr>
<tr>
<td></td>
<td>5′-CGGCTTCTTATGTTGTGCCACAC-3′</td>
<td>60</td>
<td>32</td>
<td>771</td>
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<tr>
<td>dCK</td>
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<td>64</td>
<td>35</td>
<td>498</td>
</tr>
<tr>
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<td>64</td>
<td>35</td>
<td>329</td>
</tr>
<tr>
<td>CDAf</td>
<td>5′-AGCTCTCTCTGGCCCTGGCTGTC-3′</td>
<td>64</td>
<td>35</td>
<td>498</td>
</tr>
<tr>
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<td>64</td>
<td>35</td>
<td>329</td>
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</tbody>
</table>

Fig. 1. Schematic representation of 1-β-D-arabinofuranosylcytosine (ara-C) metabolism in acute myelogenous leukemia cells. The rate-limiting enzyme of ara-C phosphorylation, deoxycytidine kinase (dCK), is under negative feedback regulatory control by dCTP and is stimulated by dTTP. Ara-C and ara-C monophosphate (ara-CMP) are deaminated by cytidine deaminase (CDA) and deoxycytidine deaminase (dCMPDA), respectively. Cystathionine-β-synthase (CBS), via its interaction with the reduced folate/methionine pathways, can potentially influence ara-C chemosensitivity as follows: (a) decreased S-adenosylmethionine pools could reduce synthesis of dTTP and dCTP; (b) decreased dCTP pools would result in greater incorporation of ara-C into DNA; (c) decreased dTTP (an allosteric inhibitor of dCMP deaminase) pools would result in greater incorporation of ara-C into DNA; (d) decreased dTTP pools would stimulate the rate-limiting enzyme of ara-C phosphorylation; and (e) decreased S-adenosylmethionine pools could reduce synthesis of dTMP and dTTP; (f) decreased dCTP pools would increase dCK activity generating higher ara-CTP levels and result in decreased competition with ara-C for incorporation into DNA.

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RESULTS

RT-PCR Analysis of Ara-C Metabolizing Enzyme Transcripts in DS and Non-DS Myeloblasts. Transcript levels of the ara-C metabolizing genes, CBS, dCK, and CDA in myeloblasts from pediatric patients [DS AML (n = 11); non-DS AML (n = 33)], were assayed by semiquantitative RT-PCR and normalized to 18S RNA (Fig. 2, B and C). Our initial goal was to expand upon our previous studies of CBS expression patterns and to determine whether global differences in the expression of these assorted genes exist between DS and non-DS specimens that might possibly contribute to the dramatically increased ara-C drug sensitivities for DS myeloblasts compared with myeloblasts from non-DS children (4).

To further validate our previously published results, new PCR primers were designed to detect CBS and dCK transcripts (4). Striking differences were observed in the expression of the CBS, dCK, and CDA genes between the DS and non-DS groups. Thus, CBS and dCK transcripts were significantly increased in DS AML compared with non-DS samples (median values of 12.5- and 2.6-fold, P = 0.0001 and P = 0.0025, respectively), similar to our previous findings of significant correlations with in vitro ara-C sensitivities by (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and ara-CTP generation, after in vitro incubations with 5 μM [3H]ara-C (4). Notably, CDA transcripts were consistently decreased in the DS myeloblasts (median, 2.7-fold) compared with the non-DS group (P = 0.001). The ratio of dCK/CDA transcripts showed a marked and highly significant 4.8-fold (P = 0.0001) difference between the DS and non-DS AML groups (Table 2). Although CDA transcripts correlated with [3H]ara-CTP generation (r = −0.498, P = 0.015), the dCK/CDA ratio showed the most significant correlations with both ara-CTP generation (r = 0.504, P = 0.0136) and ara-C sensitivity (r = −0.475, P = 0.0018; Fig. 3). There was no apparent correlation of CDA transcripts or ara-C sensitivity with French-American-British subtypes among the non-DS AML group.

<table>
<thead>
<tr>
<th>Genes</th>
<th>DS</th>
<th>Non-DS</th>
<th>Fold difference</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cystathionine-β-synthase</td>
<td>0.387</td>
<td>0.031</td>
<td>12.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Deoxycytidine kinase</td>
<td>0.213</td>
<td>0.082</td>
<td>2.6</td>
<td>0.0025</td>
</tr>
<tr>
<td>Cytidine deaminase</td>
<td>0.069</td>
<td>0.188</td>
<td>2.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Deoxycytidine kinase/cytidine deaminase</td>
<td>1.92</td>
<td>0.40</td>
<td>4.8</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

* Data are shown for median transcript levels determined by reverse transcription-PCR assay as shown in Figure 2 and as described in “Materials and Methods.” Statistical significance between the differences in transcript levels for DS and non-DS patient groups was determined by the nonparametric Mann-Whitney two-sample test.
A unique series of DS AML (CMK) and non-DS AML (CMS and THP-1) cell line models exhibited gene expression profiles similar to our patient specimens, including elevated CBS (CMK and THP-1) and CDA (THP-1) transcripts (Fig. 4A). However, no significant differences were detected for dCK between the lines. These cell line models were used to better examine the relationships between differences in CBS and/or CDA and patterns of ara-C drug sensitivity and ara-CTP generation. For CMK cells, overexpression of CBS was found to be accompanied by 10-fold decreased ara-C IC₅₀ compared with CMS cells (Fig. 4B; Ref. 22). THP-1 cells also expressed elevated CBS and CDA transcripts compared with CMS cells. However, the effects of increased CBS expression leading to increased ara-CTP generation appeared to be effectively negated by dramatically elevated expression of CDA (Fig. 4A) because THP-1 cells were 100-fold less sensitive to ara-C than CMK cells (Fig. 4B).

Secretion of CDA Protein to the Media by CMS and THP-1 Cells. To examine the effect of CDA on ara-C metabolism, CMK, CMS, and THP-1 cells were incubated with 5 μM [³H]ara-C. An unexpected finding during the [³H]ara-C incubation experiments with CMS and THP-1 cells was that a large fraction of the [³H]ara-C in the incubation media was converted to [³H]ara-U. For CMS cells, this was particularly surprising because CDA transcripts were not detected by primary RT-PCR amplification, although they were detected in a nested RT-PCR reaction. No ara-U was detected in the incubation media with CMK cells, consistent with the lack of CDA transcripts in this line. Incubation of [³H]ara-C in complete media containing 10% fetal bovine serum without cells resulted in no conversion to [³H]ara-U, indicating that the accumulation of ara-U was caused by cellular metabolism rather than serum factors.

To better identify the origin of the extracellular ara-U, THP-1, and CMS cells were incubated in complete media for 3 h without [³H]ara-C. The cells were then removed by centrifugation, and 1 ml of the cell-free supernatant was incubated with 5 μM [³H]ara-C for 3 h in the presence of varying concentrations of the CDA-specific inhibitor, THU, followed by high-performance liquid chromatography analysis of ara-C metabolites. In preconditioned media from THP-1 cells, nearly all of the [³H]ara-C was converted to [³H]ara-U; for the CMS-preconditioned media, nearly equal amounts of [³H]ara-C and [³H]ara-U were detected (Fig. 5). For both cell lines, the conversion of ara-C to ara-U was inhibited by THU in a dose-dependent manner, suggesting that CDA was secreted into the media. These results suggest that the CDA protein concentration in the media for CMS

Cell Line Models of DS and Non-DS AML. A unique series of DS AML (CMK) and non-DS AML (CMS and THP-1) cell line models exhibited gene expression profiles similar to our patient specimens, including elevated CBS (CMK and THP-1) and CDA (THP-1) transcripts (Fig. 4A). However, no significant differences were detected for dCK between the lines. These cell line models were used to better examine the relationships between differences in CBS and/or CDA and patterns of ara-C drug sensitivity and ara-CTP generation. For CMK cells, overexpression of CBS was found to be accompanied by 10-fold decreased ara-C IC₅₀ compared with CMS cells (Fig. 4B; Ref. 22). THP-1 cells also expressed elevated CBS and CDA transcripts compared with CMS cells. However, the effects of increased CBS expression leading to increased ara-CTP generation appeared to be effectively negated by dramatically elevated expression of CDA (Fig. 4A) because THP-1 cells were 100-fold less sensitive to ara-C than CMK cells (Fig. 4B).
cells was much lower than that for THP-1 cells and that no CDA was present in the media for CMK cells.

Effect of CDA on Ara-C Metabolism. To examine the effect of CDA activity on ara-C metabolism, time course analyses of intracellular and extracellular (media) ara-C metabolites in CMK, CMS, and THP-1 cells were performed. For THP-1 cells, essentially all of the extracellular [3H]ara-C was converted to [3H]ara-U after a 30-min incubation with 5 μM drug (Fig. 6F). Only 20% of extracellular [3H]ara-C was converted into [3H]ara-U after a 30-min incubation with CMS cells (Fig. 6D). For CMK cells, no [3H]ara-U was detected in the media even after a 3-h incubation (Fig. 6B).

The intracellular generation of [3H]ara-CTP in CMK cells increased linearly from time 0 to 3 h (Fig. 6A). For CMS cells, the kinetics of [3H]ara-CTP generation was similar from 0 to 2 h, then plateaued, likely because of the exhausted pool of extracellular ara-C (Fig. 6C). Low intracellular levels of [3H]ara-C and [3H]ara-U (~10-fold lower compared with [3H]ara-CTP) were also detected in CMS cells. In contrast, the generation of [3H]ara-CTP in THP-1 cells was maximal after 30 min, and its level was 40–50-fold lower than that of intracellular [3H]ara-U (Fig. 6E). Thus, the conversion of extracellular ara-C to ara-U appears to be a major mechanism accounting for the decreased in vitro generation of [3H]ara-CTP in THP-1 cells.

The effects of THU treatment on in vitro ara-CTP generation and ara-C sensitivity in CMS and THP-1 cells were assessed. Incubation of cells with [3H]ara-C in the presence of 50 μM THU resulted in an approximate 4-fold greater generation of ara-CTP and ara-C sensitivity in THP-1 cells, whereas there was no significant change for CMS cells treated with THU (data not shown).

Identification of a Modified CDA Transcript in AML Cell Lines and Myeloblasts. The differential expression of CDA in DS and non-DS myeloblasts led us to begin to analyze the transcriptional regulation of the CDA gene. Accordingly, 5′-RACE assays were performed in THP-1 cells to determine the transcriptional start site. Surprisingly, two different types of CDA transcripts were found: (a) full-length CDA/lf transcripts (3 clones) with variable length 5′-UTRs 60–179 bp upstream of the translational start (based on the sequence under GenBank accession no. XM_010650); and (b) CDA/lf transcripts (15 clones), which included the complete sequences of exons 2, 3, and 4 that, in all but one case, were juxtaposed to 2–30 bp from intron 1 (Fig. 7A). Both the CDA/lf and CDA/lf transcripts were confirmed by RT-PCR assays in patient myeloblasts (data not shown) and AML and non-AML cell lines (Fig. 7B).

Identification of a Novel Promoter Activity for CDA/lf Transcripts. A schematic representation of the redefined structure of the CDA gene, including the origin of the CDA/lf transcript and the putative CDA/lf polypeptide, is shown in Fig. 7C. The CDA/lf transcripts seemed unlikely to arise from alternate splicing of the CDA/lf transcript because the 5′ ends for this form included sequence from intron 1 of the CDA gene. A translation initiation consensus sequence (GACATGC) was identified at positions 268–274 downstream of the CDS/lf translational start that, should initiation occur, would result in a ~6 kDa polypeptide with amino acid sequence identical to the full-length CDA/lf protein (data not shown).

Interestingly, a surprisingly strong promoter activity was localized to the 5′ proximal region to the CDA/lf initiation sites (positions 125284–126820 in GenBank accession no. AL391357) by transient transfections into HT1080 cells. When normalized for transfection efficiencies with Renilla luciferase, promoter activity for the CDA/lf promoter construct (pBS-1451/+86) exceeded that for the pBL-40/+400 CDA/lf promoter construct by a factor of 300 (Fig. 7D).

Stimulation of pBS-1451/+442+L-40/+400 by GATA1 in D. Mel-2 Cells. The disproportion between CDA/lf transcript levels and promoter activity led us to examine for activator elements that could stimulate the weak CDA promoter. The upstream sequence of the CDA/lf promoter (identified by 5′-RACE as described above), contains three potential GATA1 binding sites and may act as an enhancer of the CDA/lf promoter; the occurrence of mutated GATA1 genes in
We previously reported a significant correlation between CBS transcripts and ara-C drug sensitivity and ara-CTP generation (4). Increased CBS expression and enzyme activity could enhance the metabolism of ara-C to ara-CTP via effects on folate metabolism, decreased generation of dTTP, and, ultimately, decreased dCTP, a feedback inhibitor of dCK required for activation of ara-C.

In this study, we assessed the role of CDA in ara-C catabolism and chemosensitivity of AML myeloblasts to ara-C. CDA transcripts were a median 2.7-fold higher in non-DS compared with DS myeloblasts, and the dCK/CDA ratio significantly correlated with both ara-CTP generation and in vitro ara-C sensitivity. These results suggest that increased CBS and dCK expression and decreased CDA expression in DS compared with non-DS myeloblasts contribute to increased ara-CTP generation and ara-C sensitivity of DS myeloblasts.

CDA catalyzes the inactivation of ara-C to ara-U. Constitutive overexpression of the CDA gene in murine fibroblasts and hematopoietic cells confers resistance to ara-C and the drug sensitivity can be completely restored using a CDA-specific inhibitor, THU (10–12). The clinical relevance of a high CDA activity level as a major cause of ara-C resistance in AML patients has been emphasized by several studies (26–29). Thus, overexpression of CDA in non-DS myeloblasts may contribute to the increased ara-C resistance compared with DS myeloblasts. The results of our study suggest that altered intracellular metabolism of ara-C, resulting in greater ara-CTP generation, in DS myeloblasts is accompanied by decreased catabolism of ara-C to ara-U because of low CDA levels. Previous studies demonstrated that patients identified as slow ara-C deaminators with a low plasma ara-U/ara-C ratio had a tendency toward a positive response (complete + partial response) compared with fast deaminators (30). Future studies should examine whether there are differences in plasma ara-C pharmacokinetics between DS and non-DS AML patients, which may be another factor contributing to the extremely high EFS rates of DS AML patients.

In this study, we used unique DS AML (CMK) and non-DS AML (THP-1) cell line models to further examine the effects of CDA expression/activity on ara-CTP generation and ara-C sensitivity. This was based on the finding of higher CDA levels in THP-1 cells compared with CMK cells, similar to the gene expression profiles in clinical DS and non-DS AML specimens. The DS cell line, CMK, generated 40-fold higher levels of ara-CTP, resulting in 100-fold greater ara-C sensitivity (Figs. 4 and 6). Unexpectedly, we noted that CDA was secreted into the media by the CMS and THP-1 cell lines, which was confirmed by coinocubations with THU (Fig. 5). Although no CDA transcripts were detected in CMS cell lines by primary RT-PCR, transcripts were detected at low levels by nested PCR (Fig. 4A).

The secretion of the CDA enzyme into the incubation media can cause rapid depletion of extracellular ara-C (Fig. 6F), resulting in markedly decreased ara-CPT generation. This likely accounts for the observation that ara-CPT generation in THP-1 cells remained unchanged after 30 min during the time course experiments (Fig. 6E). However, ara-CPT generation in CMS cells was apparently not affected because of a significantly lower extracellular CDA activity. Addition of 50 μM THU during the incubation with THP-1 cells resulted in 4-fold higher levels of ara-CTP accompanying a 4-fold increased ara-C drug sensitivity (data not shown). In [3H]ara-C incubations with patient myeloblasts (data not shown), ara-U was likewise detected in the media, albeit at somewhat reduced levels (3–18% of total label). Collectively, these results demonstrate that CDA can play a significant role in ara-C resistance in AML cells. Similar findings of elevated extracellular CDA in cultured leukemia cells were reported by Braess et al. (31).

What may be the basis for the differential expression of the CDA gene between DS and non-DS AML cases? Using 5′-RACE analysis
of THP-1 cells, an unexpected finding was the identification of two different CDA transcripts—a long CDA/lf transcript form, which has been previously reported (32), and a previously unreported short CDA/lf transcript form that contained the complete sequences of exons 2, 3, and 4 plus up to 30 bp of intron 1 sequence. The presence of intron 1 sequences in the CDA/lf 5′-UTRs implied that this form did not arise from alternate splicing but must be transcribed from its own promoter, presumably in intron 1. Promoter activity was indeed localized to intron 1 by reporter gene assays in transiently transfected HT1080 cells.

What may be the functional significance of the CDA/lf transcript? CDA/lf transcripts encode a homotetrameric protein composed of 16-kDa subunits. Although we were unable to express the CDA/lf protein in a heterologous bacterial system, it is conceivable that in mammalian cells a CDA/lf polypeptide is translated and may function as a regulator of CDA function by forming heterotetramers with the CDA/lf subunits. This possibility is currently being investigated. The low level activity for the CDA/lf promoter is clearly disproportionate to the high levels of CDA/lf transcripts in HT1080 cells. Thus, an enhancer element either up- or downstream of the basal CDA promoter most likely exists. Because the CDA/lf promoter exhibited high levels of promoter activity, it is conceivable that cis-elements in the CDA/lf promoter in intron 1 may also function as enhancers for the weak upstream CDA/lf promoter.

GATA1 encodes a zinc-finger transcription factor that is essential for normal erythroid and megakaryocytic differentiation (19–21). After the first study by Wechsler et al. (13) that acquired somatic mutations in exon 2 of the GATA1 gene were detected exclusively in DS AML cases, subsequent studies have confirmed that GATA1 mutations are detected in essentially all DS AML and transient myeloproliferative disorder cases, whereas GATA1 mutations have not been detected in control populations of non-DS leukemia and non-AML DS cases (14–18). The majority of the GATA1 mutations introduced premature stop codons, which would result in the synthesis of a shorter GATA1 protein with reduced transactivation activity (13–18). Because several GATA1 binding sites can be identified in the putative CDA/lf promoter in intron 1, the presence of mutated copies of GATA1 in DS TMD/AML cases may result in decreased CDA enhancer activity of the CDA/lf promoter and decreased overall CDA expression, accounting for lower CDA transcripts in DS myeloblasts compared with non-DS myeloblasts. In our study, all DS myeloblast samples were the AML phenotype and contained GATA1 exon 2 mutations, whereas no mutations were detected in any of the non-DS AML samples (data not shown).

To better identify the role of GATA1 and transcription of the CDA gene, a reporter gene construct, pBS-1451/-442+L-40/+400, was generated consisting of the upstream sequence of the CDA/lf promoter ligated to the upstream sequence of the 5′-UTR of the CDA/lf promoter. Cotransfection of the pBS-1451/-442+L-40/+400 reporter into D. Mel-2 cells with (wild type) GATA1/lf-stimulated promoter activity in a dose-dependent fashion, whereas transfection with GATA1sf (analogous to the mutated GATA1 gene in DS AML cases) resulted in significantly reduced promoter stimulation. This supports the hypothesis that GATA1 regulates the transcription of the CDA gene, and the basis for the lower CDA transcripts in DS compared with non-DS myeloblasts is likely because of the presence of mutated GATA1 genes, leading to reduced activation of the CDA/lf promoter by the CDA/lf promoter. The relationship between the GATA1 and CDA genes potentially resulting in reduced CDA expression and increased ara-C sensitivity may also account for the observation of a high complete remission rate of adult AML patients whose blast cells lacked GATA1 expression (33). The results of this study also highlight that the patterns of altered gene expression in DS AML are not exclusively related to chromosome 21 because the CDA gene is localized to chromosome 1 and the GATA1 gene is localized to chromosome X.

In conclusion, our study demonstrates that CDA transcripts were significantly higher in non-DS compared with DS myeloblasts and that the ratios of dCk/CDA transcripts significantly correlate with ara-C drug sensitivities and in vitro ara-C-CTP generation. Future studies will examine the basis for the differential expression of the CDA gene between DS and non-DS AML cases and the possible role of both wild-type and mutant GATA1 proteins in regulating CDA transcription. Determining the molecular/biochemical bases for our findings may lead to improvements in the treatment of AML.

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The Role of Cytidine Deaminase and GATA1 Mutations in the Increased Cytosine Arabinoside Sensitivity of Down Syndrome Myeloblasts and Leukemia Cell Lines

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