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Gene-Expression Profiling Reveals Down-Regulation of Equilibrative Nucleoside Transporter 1 (ENT1) in Ara-C–Resistant CCRF-CEM–Derived Cells

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We have investigated the mechanism of resistance of leukemia cells to Ara-C using an in-house cDNA microarray designed for the analysis of leukemia cells. We produced Ara-C–resistant cells from the CCRF-CEM (acute lymphoblastic leukemia) cell line and compared their gene-expression profile with that of wild-type cells. The adenosine deaminase (ADA) gene was highly up-regulated in Ara-C–resistant cells, while equilibrative nucleoside transporter 1 (ENT1) and several cell-cycle–related genes were down-regulated. Of all these genes, ENT1 seemed the most likely to be relevant to Ara-C resistance. To investigate the role of ENT1 in Ara-C–resistant cells, we transfected the cells with the gene. ENT1-transfected Ara-C–resistant cells resembled wild-type CCRF-CEM cells more closely than untransfected Ara-C–resistant cells in terms of growth rate, Ara-C-uptake characteristics, and ADA expression levels. The down-regulation of the ENT1 gene is expected to result in nucleotide deficiency in addition to blockage of Ara-C influx. Accordingly, Ara-C–resistant cells showed low growth rates, which were restored by transfection with ENT1. These low growth rates were also correlated with the phosphorylation level of cell-cycle checkpoint kinase 2. In this study we identified down-regulation of ENT1 as the factor responsible for Ara-C resistance, and this knowledge may be used to devise a clinical regimen that will overcome the resistance.

Key words: adenosine deaminase, Ara-C resistance, CCRF-CEM cells, equilibrative nucleoside transporter 1 (ENT1), gene-expression profiling.

Abbreviations: ADA, adenosine deaminase; ENT1, equilibrative nucleoside transporter 1; Ara-C, 1-β-D-arabinofuranosylcytosine (cytarabine); Ara-CTP, cytosine arabinoside triphosphate; ATM, ataxia-telangiectasia–mutated protein.

The sugar-modified pyrimidine analogue cytosine arabinoside (1-β-D-arabinofuranosylcytosine; cytarabine; Ara-C) is one of the most widely used drugs in the treatment of acute leukemia. Ara-C enters the cell mainly through equilibrative nucleoside transporter 1 (ENT1) (*1*). Inside the cell, Ara-C is phosphorylated to its active form, cytosine arabinoside triphosphate (Ara-CTP), which competes with dCTP for incorporation into DNA, blocks DNA synthesis and causes the cell to undergo apoptosis (*2*). The formation of Ara-CTP can be suppressed by various mechanisms, reducing the effectiveness of Ara-C as a therapeutic agent. For example, pyrimidine nucleotidase I inhibits Ara-CTP formation by opposing the action of deoxycytidine kinase (*3*). In addition, cytidine deaminase and deoxycytidylate deaminase convert Ara-C to Ara-U and Ara-CMP to Ara-UMP, respectively, thereby tending to decrease the concentration of Ara-CTP (*4*, *5*). Several studies suggest that reduced intracellular levels of Ara-CTP are associated with clinical resistance to Ara-C (*2*, *6*). Thus the Ara-C metabolic pathway is closely related to the phenomenon of Ara-C resistance. Furthermore, in freshly isolated blast samples from adult patients with acute leukemia, a low cellular content of nucleoside transporters is correlated with Ara-C resistance (*7*).

Despite the favorable impact of Ara-C on the treatment of acute leukemia over the past three decades, the failure to achieve durable remission remains a significant therapeutic problem (*8*). In this study, we isolated Ara-C–resistant CCRF-CEM cells and compared their gene-expression profile with that of wild-type cells using an in-house cDNA microarray designed for the analysis of leukemia cells (*9*). We used the resulting information to investigate the mechanism of resistance of our Ara-C–resistant cells, as well as their physiological status.

The ENT1 gene appeared to be a cause of the Ara-C resistance. To directly investigate the role of ENT1, we transfected resistant cells with the gene and found that the transfected cells reverted to wild-type character.

MATERIALS AND METHODS

Materials—Ara-C was used as marketed by Nippon Shinyaku Co. under the trade-name Cylocide. [5-^{3}H]Cytosine-β-D-arabinofuranoside ([^{3}H]Ara-C, 15–30 Ci/mmol) was from Moravek Biochemicals (Brea, CA). All other materials were of the highest available commercial grade.

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Cell Culture—CCRF-CEM cells were purchased from the Health Science Research Resources Bank (Japan Health Science Foundation, Tokyo) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C under a humidified atmosphere containing 5% CO_2 in air. To assess cell proliferation, we used an XTT assay kit (Roche Diagnostics, Basel, Switzerland), which is based on the reduction of a tetrazolium salt by metabolically active cells.

cDNA Microarray—An in-house cDNA microarray designed for the analysis of leukemia cells was prepared as described by Takagaki *et al.* (*9*). Briefly, cDNA libraries from five leukemia cell lines (CCRF-CEM, K562, HL-60, RPMI-8226, and MOLT-4) were combined and normalized, and then the cDNA microarray was assembled as described by Katsuma *et al*. (*10*). Partial sequencing of the clones revealed 2,519 different genes on the array. The sequences of these clones have been deposited in the DDBJ (DNA Databank of Japan) database under accession numbers BP428450 through BP432443.

Preparation of Target DNA—Total and poly $(A)^+$ RNA were prepared from cultured leukemia cells as described elsewhere (*10*). Poly $(A)^+$ RNA isolated from four samples of untreated leukemia cells was pooled for each time point and used as a control. Labeled probes were prepared as described by Katsuma *et al.* (*11*) and applied to the microarray.

Hybridization and Scanning—Hybridization and scanning were performed as described by Katsuma *et al.* (*11*) except that hybridized images were scanned with a fluorescence laser scanning device (DNA Microarray Scanner; Agilent, Palo Alto, CA), the signal intensities were quantified with ImaGene software version 4.1 (BioDiscovery, Marina del Rey, CA), and at least three hybridizations were performed for each time point.

Data Analysis—Data analysis was performed as described by Katsuma *et al.* (*10*). In brief, quantified signal intensities were transformed by taking logarithms to base two after eliminating clones with low expression levels. Using transformed data derived from each pair of competitive hybridization images, we prepared scatter diagrams to compare sample signal intensities with those derived from controls. We then carried out regression analysis, and the residuals obtained correspond to the logarithmic gene-expression ratios. From the genes showing statistically significant alterations in expression we selected the thirty most up-regulated and the thirty most down-regulated for further analysis.

Caspase-3 Activity—Caspase-3–like protease activity was determined as the increase in fluorescence observed on cleavage of a fluorogenic substrate at the caspase-3 recognition site using an EnzChek Caspase-3 assay kit (Molecular Probes, Eugene, OR).

Real-Time PCR—From each sample, 5 μg of total RNA was reverse-transcribed for single-stranded cDNA using 1 μM oligo(dT) primer with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), and then used for real-time PCR analysis with a LightCycler™ Real-Time PCR System (Roche Diagnostics, Basel, Switzerland) (*12*). Each single-stranded cDNA was diluted for subsequent PCR amplification by monitoring GAPDH as a quantitative internal control. The PCR primers for ENT1 were 5′-ATCTGCGCTATTGCCAGTG-3′ (sense) and 5′-TCCAACTTGGTCTCCTGCTC-3′ (antisense), those for adenosine deaminase were 5′-CGCTCATCTTCAAGTC-CACC-3′ (sense) and 5′-CGTCTTCAGAGGTTCTGCC-3′ (antisense), and those for GAPDH were 5′-AAGAAGGT-GGTGAAGCAGGC-3′ (sense) and 5′-TCCACCACCCTG-TTGCTGTA -3′ (antisense).

Northern Blotting—Total RNA was prepared with Sepasol-RNA I reagent (Nacalai Tesque, Kyoto, Japan). Northern analysis of poly $(A)^+$ RNA (3 μg) was performed as described previously (*13*). The probe, a 475-bp fragment corresponding to nucleotides 175 to 649 of the cDNA (accession number U81375), was amplified by PCR for Northern analysis. Hybridization signals were detected with a CDP-Star Detection Module (Amersham, Buckinghamshire, England).

Measurement of [^{3}H]AraC Uptake—Assays of [^{3}H]AraC uptake were conducted in RPMI 1640 medium at 37°C as described by Huang *et al*. (*14*). Briefly, an aliquot of 10^6 cells was washed once with RPMI 1640 medium and then resuspended in 600 μl of the same medium. After preincubation for 15 min, ^{3}H-labeled Ara-C (200 nM) was added and the mixture was incubated for 30 min. Uptake was stopped by the addition of 150 μl of mineral oil, followed by vigorous mixing and centrifugation at 8,000 × *g* for 30 s. The supernatant above the oil layer was removed, the cell pellet was washed twice with RPMI 1640 medium, and most of the oil was removed with a final wash. Cell pellets were lysed in 0.5 ml of 0.5 N NaOH for measurement of radioactivity by liquid scintillation counting in the Hionic-Fluor scintillation cocktail (PerkinElmer, Fremont, CA) with a Tri-Carb 2500TR liquid scintillation analyzer (PerkinElmer).

Generation of Stable Transfectants—The gene encoding human equilibrative nucleoside transporter 1 (ENT1) was cloned from a human kidney cDNA library by PCR and then subcloned into the *Nhe*I/*Xbo*I sites of the eukaryotic expression vector pCI-Neo (Promega, Madison, WI). Plasmids were transfected into Ara-C–resistant CCRF-CEM cells with the DMRIE-C transfection reagent (Invitrogen, Carlsbad, CA). Three days after transfection, cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 800 μg/ml Geneticin disulfate (G418; Invitrogen, Carlsbad, CA). Stably transfected cells were cloned by the limiting-dilution method, expanded, and screened for ENT1 mRNA by RT-PCR.

Immunoblotting—Immunoblotting was performed as described by Katsuma *et al.* (*13*) except that membranes were immunoblotted with antibodies against actin (sc-1615), Chk1 (sc-8408 or sc-7898), or Chk2 (sc-5278) from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies against phospho-Chk1 (Ser345; 2341) or phospho-Chk2 (Thr68; 2661) from Cell Signaling Technology (Beverly, MA), or anti-phospho-ataxia-telangiectasia-mutated protein (ATM) from Rockland Immunochemicals (Ser1931; 13647, Gilbertsville, PA).

RESULTS

Isolation of Ara-C–Resistant CCRF-CEM Cells—In order to isolate Ara-C–resistant leukemia cells, we incubated CCRF-CEM cells in the presence of low doses of Ara-C. The addition of 50 nM Ara-C was found keep the growth of CCRF-CEM cells stationary (Fig. 1A), and after

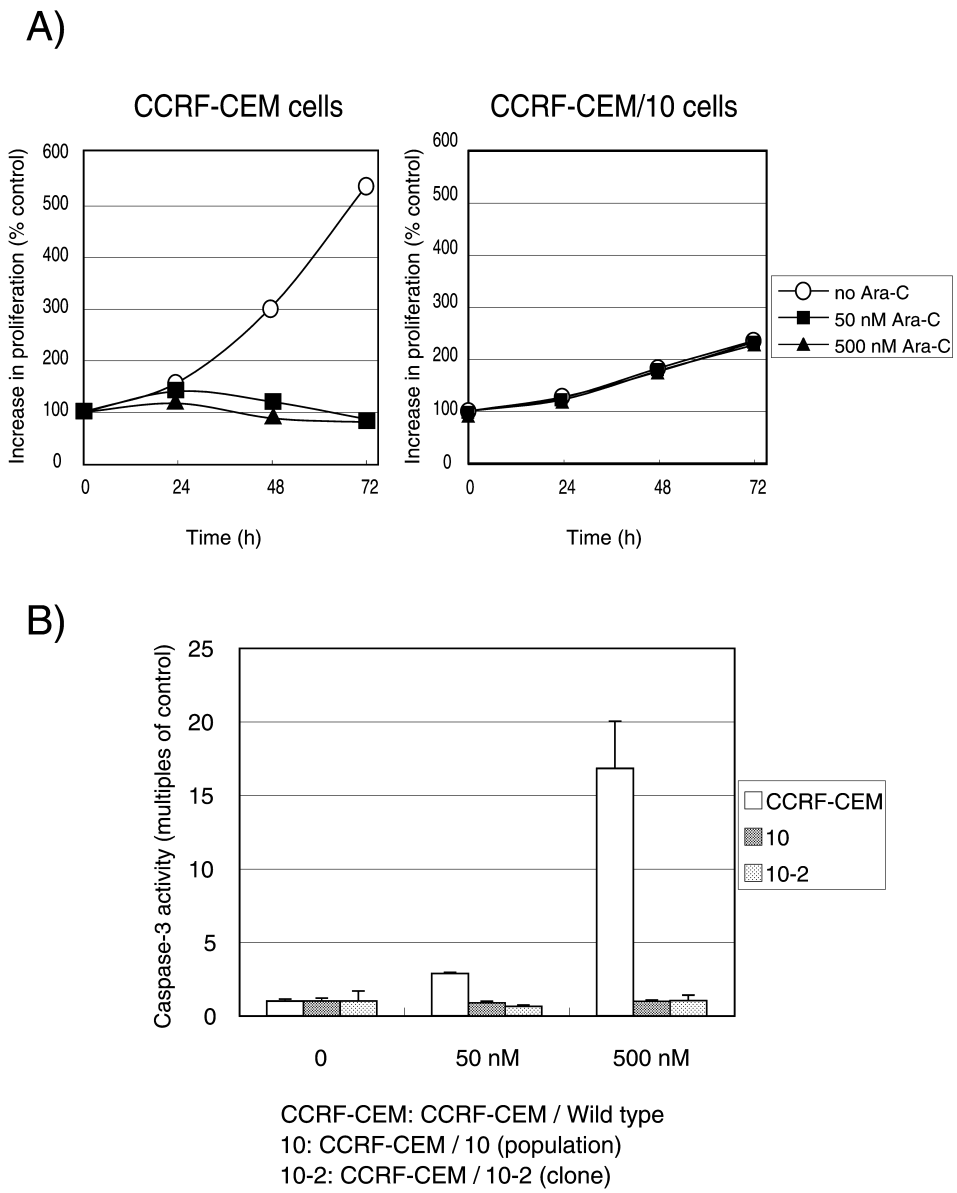

Fig. 1. **Effects of Ara-C on growth and caspase-3 activity of Ara-C–resistant and wild-type CCRF-CEM cells.** (A) Growth. Cells (1.5×10^4) were incubated in 100 μl of culture medium, and after the indicated times cell proliferation was assessed by means of the XTT assay and expressed as the percentage increase compared with the control (no treatment). Each point represents the mean of three measurements. (B) Caspase-3 activity. Activity was measured in cells exposed to 50 or 500 nM Ara-C for 24 h and expressed in multiples of the control (untreated) levels. Each point represents the mean ± SEM of three measurements.

successive stepwise increases of the Ara-C concentration in the culture medium up to 500 nM we obtained a cell population that could grow in the presence of a 10-fold-higher concentration of Ara-C than wild-type cells could. We named this cell population CCRF-CEM/10. We then proceeded to examine whether or not we could isolate highly resistant cell lines from the CCRF-CEM/10 cell population by the limiting-dilution method, and we were able to isolate a cell line that could grow in the presence of a 10-fold-higher concentration of Ara-C than wild-type cells could. We named this cell line CCRF-CEM/10-2, and it showed two noteworthy characteristics. First, the cell line grew only slowly in either the presence or the absence of Ara-C (Fig. 1A). Second, the cells showed no activation of caspase-3 whether or not Ara-C was present (Fig. 1B). When incubated with 50 nM Ara-C, wild-type CCRF-CEM cells showed activation of caspase-3, which is characteristic of apoptosis. However, even when incubated with 500 nM Ara-C, neither CCRF-CEM/10 nor CCRF-CEM/10-2 cells showed any detectable activation of caspase-3 (Fig. 1B), nor did they show apoptosis-specific DNA fragmentation (data not shown).

Gene-Expression Profiles—To monitor alterations in the gene-expression profile of our Ara-C–resistant CCRF-CEM cells on the addition of Ara-C, we used in-house cDNA microarrays designed for the analysis of leukemia cells (*9*). When we hybridized labeled cDNA targets prepared from the mRNA of CCRF-CEM/10-2 cells incubated with and without Ara-C, we found very little difference in the gene-expression profile, consistent with the observed Ara-C resistance of the cells. We then hybridized labeled cDNA targets prepared from the mRNA of wild-type CCRF-CEM cells and Ara-C-resistant CCRF-CEM/10-2 cells isolated after 24 and 48 h of growth. There were some differences between their gene-expression profiles, although the profiles of each cell type were almost the same at 24 and 48 h. The thirty most up-regulated and the thirty most down-regulated genes showing

Table 1. **The thirty most up-regulated and the thirty most down-regulated genes in CCRF-CEM/10-2 cells relative to CCRF-CEM cells after 24 h of incubation.**

Up-regulated genes		fold	
NM_173708	NADH dehydrogenase subunit 1	2.44	mitochondrial
NM_173709	NADH dehydrogenase subunit 2	1.81	mitochondrial
NM_173705	cytochrome c oxidase subunit II	1.76	mitochondrial
GI:4550	16S ribosomal RNA	1.43	ribosomal
NM_001024	ribosomal protein S21 (RPS21), mRNA	2.18	ribosomal
NM_000999	ribosomal protein L38,mRNA, complete cds.	1.52	ribosomal
GI:4549	12S ribosomal RNA	1.51	ribosomal
NM_001028	ribosomal protein S25, mRNA, complete cds.	1.44	ribosomal
NM_012423	ribosomal protein L13a, mRNA, complete cds.	1.43	ribosomal
NM_021109	prothymosin beta 4, mRNA, complete cds.	2.47	cytoskeletal
NM_021109	thymosin, beta 4, X-linked (TMSB4X), mRNA	2.15	cytoskeletal
NM_016337	Enah/Vasp-like (EVL), mRNA	1.65	cytoskeletal
NM_178014	tubulin, beta 5, mRNA, complete cds.	1.83	cytoskeletal
NM_000206	interleukin 2 receptor, gamma (IL2RG), mRNA.	2.45	
NM_002414	CD99 antigen (CD99), mRNA	2.44	
NM_004221	natural killer cell transcript 4 (NK4), mRNA	2.31	
NM_000022	adenosine deaminase (ADA), mRNA.	2.18	ADA
NM_145799	septin 6 (SEPT6), transcript variant I, mRNA	2.08	
NM_000516	GNAS complex locus (GNAS), transcript variant 1, mRNA.	1.83	
NM_002444	moesin (MSN), mRNA.	1.80	
NM_006233	polymerase (RNA) II (DNA directed) polypeptide I, mRNA	1.73	
NM_001636	solute carrier family 25, member 6 (SLC25A6), mRNA.	1.70	
NM_138425	likely ortholog of mouse gene rich cluster, C10 gene (GRCC10), mRNA	1.64	
NM_170697	aldehyde dehydrogenase 1 family, member A2 transcript variant 3, mRNA.	1.61	
NM_138553	B-cell CLL/lymphoma 11A (BCL11A), transcript variant 5, mRNA.	1.56	
NM_153280	ubiquitin-activating enzyme E1 (UBE1), transcript variant 2, mRNA.	1.47	
NM_000732	CD3D antigen, delta polypeptide (CD3D), mRNA	1.45	
NM_002778	prosaposin (PSAP), mRNA.	1.44	
NM_004390	cathepsin H, mRNA, complete cds.	1.41	
NG_001333	T cell receptor beta locus (TRB@) on chromosome 7	1.41	
Down-regulated genes		fold	
NM_000982	ribosomal protein L21 (RPL21), mRNA	0.75	ribosomal
NM_000992	ribosomal protein L29 (RPL29), mRNA	0.73	ribosomal
NM_001002	ribosomal protein, large, P0, mRNA, complete cds.	0.53	ribosomal
NM_000994	Similar to ribosomal protein L32, mRNA, complete cds.	0.69	ribosomal
NM_001010	Similar to ribosomal protein S6, mRNA, complete cds.	0.67	ribosomal
NM_001005	Hums3 mRNA for 40S ribosomal protein s3.	0.66	ribosomal
NM_001514	general transcription factor IIB (GTF2B), mRNA	0.64	
NM_002092	G-rich RNA sequence binding factor 1 (GRSF1), mRNA	0.72	
NM_002568	poly(A) binding protein, cytoplasmic 1 (PABPC1), mRNA	0.62	
NM_002634	prohibitin (PHB), mRNA	0.62	cell cycle or cell growth
NM_003347	ubiquitin-conjugating enzyme E2L 3, transcript variant 1, mRNA	0.74	cell cycle or cell growth
NM_003479	protein tyrosine phosphatase type IVA, member 2 , transcript variant 1, mRNA	0.61	cell cycle or cell growth
NM_006098	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1, mRNA	0.66	cell cycle or cell growth
NM_017838	nucleolar protein family A, member 2 (NOLA2), mRNA	0.75	cell cycle or cell growth
NM_182666	ubiquitin-conjugating enzyme E2E 1 (UBE2E1), transcript variant 2, mRNA	0.75	cell cycle or cell growth
NM_014374	replication initiator 1 (REPIN1), mRNA	0.72	cell cycle or cell growth
NG_000002	immunoglobulin lambda locus (IGL@) on chromosome 22	0.75	
NM_000034	aldolase A, fructose-bisphosphate (ALDOA), transcript variant 1, mRNA	0.58	
NM_000365	triosephosphate isomerase 1 (TPI1), mRNA	0.66	
NM_000616	CD4 antigen (p55) (CD4), mRNA	0.61	
NM_002654	pyruvate kinase, muscle (PKM2), transcript variant 1, mRNA	0.63	
NM_003365	ubiquinol-cytochrome c reductase core protein I (UQCRC1), mRNA	0.68	
NM_004589	SCO cytochrome oxidase deficient homolog 1 (yeast) (SCO1), mRNA	0.74	
NM_005620	S100 calcium binding protein A11 (calgizzarin) (S100A11), mRNA	0.59	
NM_005866	opioid receptor, sigma 1 (OPRS1), transcript variant 1, mRNA	0.75	
NM_006824	EBNA1 binding protein 2 (EBNA1BP2), mRNA	0.62	
NM_012341	GTP binding protein 4 (GTPBP4), mRNA	0.72	
NM_021074	NADH dehydrogenase (ubiquinone) flavoprotein 2, 24 kDa (NDUFV2), mRNA.	0.59	
NM_023033	methyltransferase-like 1 (METTL1), transcript variant 3, mRNA.	0.58	
NM_004955	equilibrative NBMPR-sensitive nucleoside transporter (ENT1), mRNA	0.74	ENT1

The genes are grouped according to their cellular function, along with the RefSeq accession number or NCBI Gene ID and the definition of each gene. The numerical value gives the fold change in mRNA levels for CCRF-CEM/10-2 cells relative to CCRF-CEM cells.

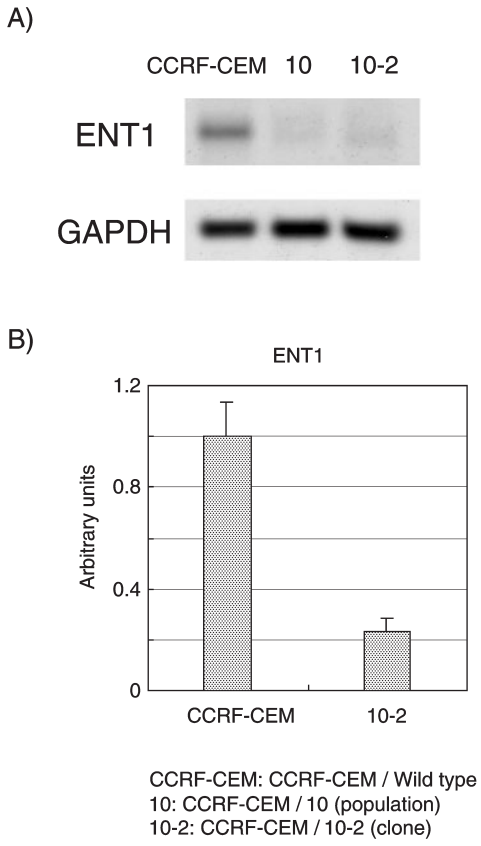

Fig. 2. **Expression of ENT1 in Ara-C–resistant CCRF-CEM cells.** (A) Northern-blot analysis of ENT1 mRNA. (B) Semi-quantitative RT-PCR analysis of ENT1 mRNA.

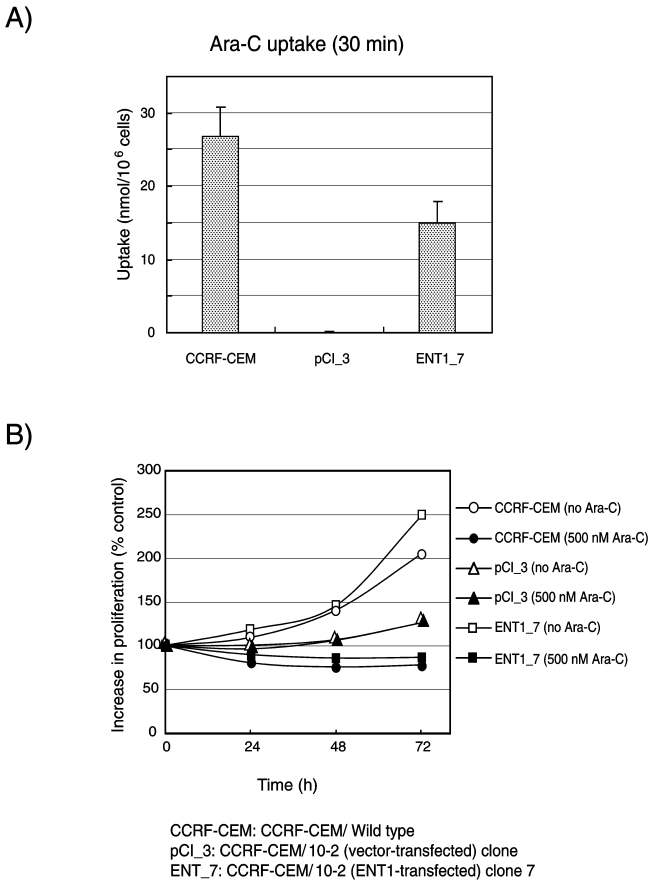

Fig. 3. **Effects of ENT1 transfection on Ara-C uptake and cell growth.** (A) [^{3}H]AraC uptake. Each point represents the mean ± SEM of three measurements. (B) Growth in the presence or absence of Ara-C. Cells (1×10^4) were incubated in 100 μl of culture medium, and after the indicated times cell proliferation was assessed by means of the XTT assay and expressed as the percentage increase compared with the control (no treatment). Each point represents the mean of three measurements.

alterations after 24 h of incubation were selected for further consideration (Table 1). Among the up-regulated genes in Ara-C–resistant cells, the most relevant to Ara-C metabolism appears to be the adenosine deaminase (ADA) gene, while the most relevant down-regulated gene was equilibrative nucleoside transporter 1 (ENT1). Several cell-cycle–related genes were also down-regulated. Because Ara-C mainly depends on ENT1 to enter the cell (*15*, *16*), we focused on the ENT1 gene for further analysis of the Ara-C–resistant CCRF-CEM cells.

Verification of ENT1 Gene Down-Regulation—The changes in ENT1 gene expression levels observed on microarray analysis were subtle, so the changes were further investigated by Northern blotting and real-time PCR analysis (Fig. 2). Down-regulation of the ENT1 gene was clearly observed in Ara-C–resistant CCRF-CEM cells with both methods. When four other genes involved in Ara-C metabolism, namely, those corresponding to deoxycytidine kinase, pyrimidine nucleotidase I, cytidine deaminase, and deoxycytidylate deaminase, were monitored by real-time PCR analysis, their expression levels were found to be unaltered (data not shown).

Establishment of CCRF-CEM/10-2 Cells Transfected with ENT1—To directly investigate the role played by the ENT1 gene in the Ara-C resistance of CCRF-CEM/10-2 cells, we transfected the cells with the ENT1 gene using a CMV-promoter–driven expression vector encoding the gene. We succeeded in isolating cell lines stably expressing the ENT1 gene. When we measured Ara-C uptake for 30 min in wild-type CCRF-CEM cells, vector-transfected CCRF-CEM/10-2 cells, and ENT1-transfected CCRF-CEM/10-2 cells, the vector-transfected CCRF-CEM/10-2 cells showed no uptake of Ara-C, as expected. In contrast, the ENT1-transfected CCRF-CEM/10-2 cells showed about 60% of the Ara-C-uptake levels of wild-type CCRF-CEM cells (Fig. 3A). When the growth of the cells was monitored by means of the XTT assay in the absence of Ara-C, ENT1-transfected CCRF-CEM/10-2 cells (clone 7) were observed to grow at approximately the same relatively rapid rate as wild-type CCRF-CEM cells, in contrast to the slow growth of untransfected CCRF-CEM/10-2 cells. In the presence of Ara-C, neither cell type grew at all (Fig. 3B). The ADA gene, which was up-regulated in Ara-C resistant cells (Fig. 4A), was partially suppressed in ENT1-transfected cells (clone 4; Fig. 4B). In these respects, the ENT1-transfected CCRF-CEM/10-2 cells resembled wild-type CCRF-CEM cells rather than Ara-C–resistant CCRF-CEM/10-2 cells.

Phosphorylation of Cell-Cycle Regulatory Proteins—To briefly investigate the cause of the slow growth of CCRF-

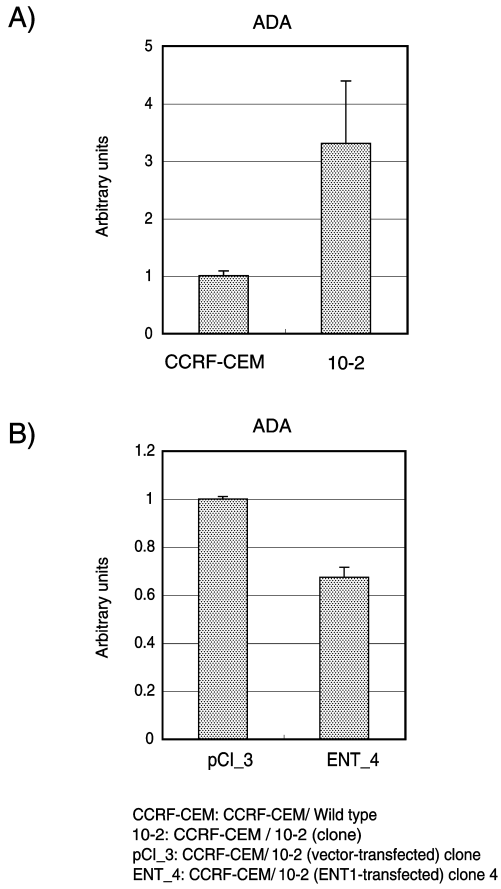

Fig. 4. **Semi-quantitative RT-PCR analysis of ADA mRNA in wild-type CCRF-CEM cells and CCRF-CEM/10-2 cells (A), and ENT1-transfected cells (B).** Each point represents the mean ± SEM of three measurements (A) or the mean ± SD of two measurements (B).

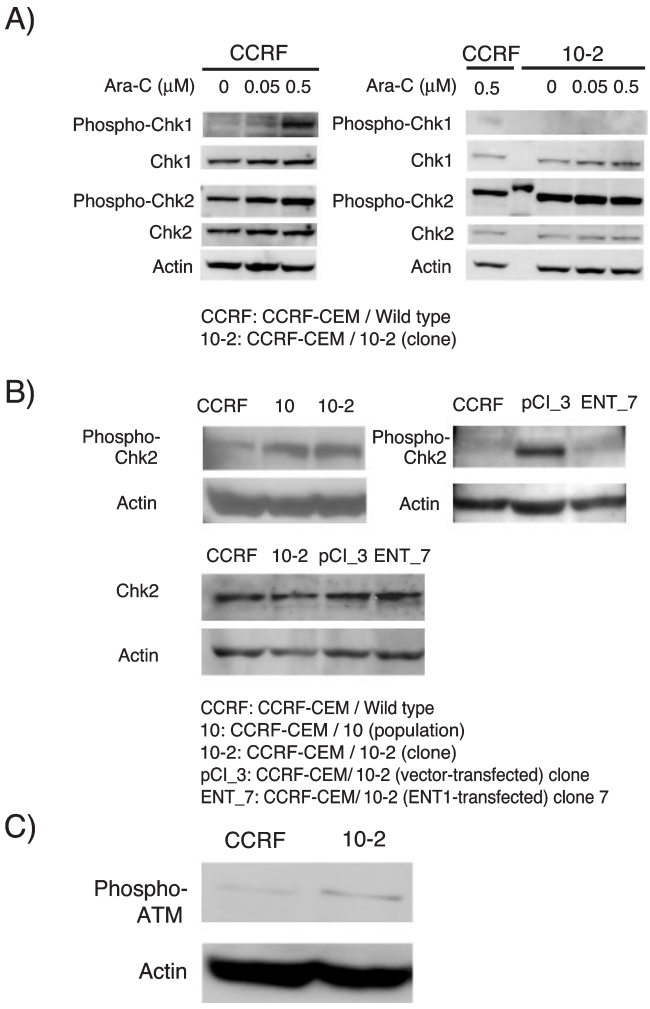

Fig. 5. **Phosphorylation-status of cell-cycle regulatory proteins.** (A) Phosphorylation and total protein levels of Chk1 and Chk2 in wild-type and Ara-C–resistant CCRF-CEM cells. Cells were incubated with various Ara-C concentrations. (B) Phosphorylation and total protein levels of Chk2 in wild-type, Ara-C–resistant, vector-transfected, and ENT1-transfected CCRF-CEM cells. (C) Phosphorylation of ATM in Ara-C–resistant CCRF-CEM cells. Cells were lysed and subjected to immunoblotting with antibodies against Chk1, Chk2, phospho-Chk1, phospho-Chk2, phospho-ATM, or actin.

CEM/10-2 cells, we determined the relative levels of phosphorylation of cell-cycle checkpoint regulatory proteins Chk1 and Chk2 in the three cell types by immunoblotting. The phosphorylation levels of Chk1 and Chk2 increased in wild-type CCRF-CEM cells on incubation with Ara-C (Fig. 5A). However, phosphorylated Chk1 was undetectable in Ara-C–resistant CCRF-CEM/10-2 cells (Fig. 5A), whereas the phosphorylation level of Chk2 was greater in Ara-C–resistant than in wild-type CCRF-CEM cells, even in the absence of Ara-C (Fig. 5, A and B). The total protein levels of Chk1 and Chk2 were about the same in CCRF-CEM cells and CCRF-CEM/10-2 cells (Fig. 5A). In contrast, the phosphorylation of Chk2 was suppressed in ENT1-transfected CCRF-CEM/10-2 cells (clone 7; Fig. 5B), although its total protein levels did not change (Fig. 5B). These results suggest a relationship between the phosphorylation of Chk2 and the slow growth rates of CCRF-CEM/10 and CCRF-CEM/10-2 cells. The phosphorylation level of ataxia-telangiectasia–mutated protein (ATM), a kinase that is upstream of Chk2 in the signaling pathway (*17*), was also found to be slightly greater in Ara-C–resistant CCRF-CEM/10-2 cells than in wild-type cells (Fig. 5C).

DISCUSSION

We have isolated an Ara-C–resistant leukemia cell line, CCRF-CEM/10-2, and compared its gene-expression profile to that of wild-type CCRF-CEM cells. Among the down-regulated genes listed in Table 1, ENT1 seems the most likely to be related to Ara-C resistance, since nucleoside transporters are determinants of Ara-C accumulation in leukemia cells (*18*). We also noted marked up-regulation of the gene for adenosine deaminase (ADA), which converts adenosine to inosine in the purine-salvage pathway. Because CCRF-CEM cells possess no nucleoside transport activity except that mediated by ENT1 (*15*, *16*), down-regulation of the ENT1 gene is expected to induce nucleotide deficiency in addition to blockage of Ara-C influx. Such a scenario would be consistent with the lack of an observed effect of Ara-C on the gene-

expression profile of CCRF-CEM/10-2 cells. A comparison of the gene-expression profiles of wild-type CCRF-CEM cells and Ara-C–resistant CCRF-CEM/10-2 cells also revealed down-regulation of several cell-cycle–related genes, but no alteration in the expression of apoptosis-related genes. The latter finding was expected in view of the lack of activation of caspase-3 in the presence of Ara-C (Fig. 1B).

To directly investigate the role of the ENT1 gene, we transfected the gene into CCRF-CEM/10-2 cells. The ENT1-transfected CCRF-CEM/10-2 cells resembled wild-type CCRF-CEM cells more closely than Ara-C–resistant CCRF-CEM/10-2 ones in terms of growth rate, Ara-C-uptake characteristics, and ADA expression levels. The transfected cells showed about 60% of the Ara-C-uptake levels of wild-type CCRF-CEM cells (Fig. 3A), but they seemed to grow slightly faster than the wild-type cells (Fig. 3B). However, the number of transfected cells evaluated by means of the XTT assay was slightly larger than that of untransfected cells from the beginning of the growth period, so the small observed difference in the growth rate may not be significant. Taken together, these results suggest that the down-regulation of the ENT1 gene accounted for the Ara-C resistance and other observed characteristics of CCRF-CEM/10-2 cells. In agreement with this conclusion, Gati *et al*. (*7*) have shown an increase in the viability of Ara-C–treated CCRF-CEM cells in the presence of graded concentrations of the ENT1-specific inhibitor nitrobenzylthioinosine. The decreased growth rate and the increased expression of ADA seem to be secondary effects of the down-regulation of ENT1. We do not believe that these effects are directly involved in the Ara-C resistance of CCRF-CEM/10-2 cells, but rather that they help the cells survive under low-nucleotide conditions.

As mentioned above, CCRF-CEM/10-2 cells grew slowly, showed no activation of caspase-3 or apoptosis-specific DNA fragmentation, and showed down-regulation of several cell-cycle–related genes. Since cell-cycle regulatory proteins seemed to be involved in the phenomenon of slow growth, we decided to determine the relative levels of phosphorylation of cell-cycle checkpoint regulatory proteins Chk1 and Chk2. We found that, although the phosphorylation-status of Chk1 did not change, the phosphorylation levels of Chk2 increased substantially. When we then checked the phosphorylation levels of ATM, which is upstream from Chk2 in the signaling pathway and which, in the phosphorylated state, phosphorylates Chk2 (*17*), we found that they were also increased, though the observed relative increase in the phosphorylation level of ATM was less than that of Chk2 in CCRF-CEM/10-2 cells. Although Chk2 and ATM are important intermediaries in the DNA damage checkpoint signaling pathway, these proteins also seem to be closely related to the slow growth of our Ara-C–resistant CCRF-CEM/10-2 cells. In addition to ATM, other factors involved in its activation may exist upstream from Chk2. Recently, for example, polo-like kinase 1 was reported to interact with Chk2 (*19*).

The reduced expression of ENT1 and the induced expression of cytoplasmic 5′-nucleotidase detected in leukemic blasts at diagnosis are correlated with a poor prognosis and may play a role in mechanisms of resistance to Ara-C in patients with acute myeloid leukemia (*20*). In these respects, our Ara-C–resistant cell line is a clinically typical resistant cell line. It has been reported that *in vitro* exposure of CCRF-CEM cells to Ara-C results in a substantial Ara-C-concentration–dependent increase in the relative survival of transporter-deficient cells (*21*). Our Ara-C–resistant cells showed similar characteristics. We also obtained information about the physiological status of the resistant cells through gene-profiling analysis. Down-regulation of the ENT1 gene in the resistant cells is expected to cause nucleotide deficiency, and, accordingly, the ADA gene was induced for participation in the purine-salvage pathway. The up-regulation of the ADA gene observed in Ara-C–resistant CCRF-CEM/10-2 cells suggests an approach for overcoming Ara-C resistance: a non-nucleoside ADA inhibitor (*22*) may be effective if it can enter the cell independently of ENT1. Such a compound could serve as an adjunct therapy for nucleotide-transport–deficient Ara-C–resistant leukemias.

The present study has confirmed that gene-profiling analysis can be useful as an exhaustive and unbiased analytical means of investigating the mechanism of drug resistance in leukemia cells. Gene profiling can also be used to gather information on the physiological status of resistant cells, and such information may lead to methods for overcoming clinical resistance to drugs.

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REFERENCES

1. Wiley, J.S., Jones, S.P., Sawyer, W.H., and Paterson, A.R. (1982) Cytosine arabinoside influx and nucleoside transport sites in acute leukemia. *J. Clin. Invest.* **69**, 479–489
2. Kufe, D.W., Major, P.P., Egan, E.M., and Beardsley, G.P. (1980) Correlation of cytotoxicity with incorporation of ara-C into DNA. *J. Biol. Chem.* **255**, 8997–9000
3. Amici, A., Emanuelli, M., Magni, G., Raffaelli, N., and Ruggieri, S. (1997) Pyrimidine nucleotidases from human erythrocyte possess phosphotransferase activities specific for pyrimidine nucleotides. *FEBS Lett.* **419**, 263–267
4. Neff, T. and Blau, C.A. (1996) Forced expression of cytidine deaminase confers resistance to cytosine arabinoside and gemcitabine. *Exp. Hematol.* **24**, 1340–1346
5. Schroder, J.K., Seidelmann, M., Kirch, H.C., Seeber, S., and Schutte, J. (1998) Assessment of resistance induction to cytosine arabinoside following transfer and overexpression of the deoxycytidylate deaminase gene in vitro. *Leuk. Res.* **22**, 619–624
6. Major, P.P., Egan, E.M., Herrick, D.J., and Kufe, D.W. (1982) Effect of ARA-C incorporation on deoxyribonucleic acid synthesis in cells. *Biochem. Pharmacol.* **31**, 2937–2940
7. Gati, W.P., Paterson, A.R., Larratt, L.M., Turner, A.R., and Belch, A.R. (1997) Sensitivity of acute leukemia cells to cytarabine is a correlate of cellular *es* nucleoside transporter site content measured by flow cytometry with SAENTA-fluorescein. *Blood* **90**, 346–353
8. Weick, J.K., Kopecky, K.J., Appelbaum, F.R., Head, D.R., Kingsbury, L.L., Balcerzak, S.P., Bickers, J.N., Hynes, H.E., Welborn, J.L., Simon, S.R., and Grever, M. (1996) A randomized investigation of high-dose versus standard-dose cytosine arabinoside with daunorubicin in patients with previously untreated acute myeloid leukemia: a Southwest Oncology Group study. *Blood* **88**, 2841–2851

9. Takagaki, K., Katsuma, S., Horio, T., Kaminishi, Y., Hada, Y., Tanaka, T., Ohgi, T., and Yano, J. (2003) cDNA microarray analysis of altered gene expression in Ara-C-treated leukemia cells. *Biochem. Biophys. Res. Commun.* **309**, 351–358
10. Katsuma, S., Shiojima, S., Hirasawa, A., Suzuki, Y., Ikawa, H., Takagaki, K., Kaminishi, Y., Murai, M., Ohgi, T., Yano, J., and Tsujimoto, G. (2002) Functional genomic search of G-protein-coupled receptors using microarrays with normalized cDNA library. *Methods Enzymol.* **345**, 585–600
11. Katsuma, S., Nishi, K., Tanigawara, K., Ikawa, H., Shiojima, S., Takagaki, K., Kaminishi, Y., Suzuki, Y., Hirasawa, A., Ohgi, T., Yano, J., Murakami, Y., and Tsujimoto, G. (2001) Molecular monitoring of bleomycin-induced pulmonary fibrosis by cDNA microarray-based gene expression profiling. *Biochem. Biophys. Res. Commun.* **288**, 747–751
12. Wittwer, C.T., Ririe, K.M., Andrew, R.V., David, D.A., Gundry, R.A., and Balis, U.J. (1997) The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* **22**, 176–181
13. Katsuma, S., Hada, Y., Ueda, T., Shiojima, S., Hirasawa, A., Tanoue, A., Takagaki, K., Ohgi, T., Yano, J., and Tsujimoto, G. (2002) Signalling mechanisms in sphingosine 1-phosphate-promoted mesangial cell proliferation. *Genes Cells* **7**, 1217–1230
14. Huang, M., Wang, Y., Collins, M., Gu, J.J., Mitchell, B.S., and Graves, L.M. (2002) Inhibition of nucleoside transport by p38 MAPK inhibitors. *J. Biol. Chem.* **277**, 28364–28367
15. Belt, J.A., Marina, N.M., Phelps, D.A., and Crawford, C.R. (1993) Nucleoside transport in normal and neoplastic cells. *Adv. Enzyme Regul.* **33**, 235–252
16. Crawford, C.R., Ng, C.Y., Ullman, B., and Belt, J.A. (1990) Identification and reconstitution of the nucleoside transporter of CEM human leukemia cells. *Biochim. Biophys. Acta* **1024**, 289–297
17. Zhou, B.B. and Elledge, S.J. (2000) The DNA damage response: putting checkpoints in perspective. *Nature* **408**, 433–439
18. Wiley, J.S., Taupin, J. Jamieson, G.P., Snook, M., Sawyer, W.H., and Finch, L.R. (1985) Cytosine arabinoside transport and metabolism in acute leukemias and T cell lymphoblastic lymphoma. *J. Clin. Invest.* **75**, 632–642
19. Tsvetkov, L., Xu, X., Li, J., and Stern, D.F. (2003) Polo-like kinase 1 and Chk2 interact and co-localize to centrosomes and the midbody. *J. Biol. Chem.* **278**, 8468–8475
20. Galmarini, C.M., Thomas, X., Calvo, F., Rousselot, P., Jafaari, A.E., Cros, E., and Dumontet, C. (2002) Potential mechanisms of resistance to cytarabine in AML patients. *Leuk. Res.* **26**, 621–629
21. Wright, A.M., Paterson, A.R., Sowa, B., Akabutu, J.J., Grundy, P.E., and Gati, W.P. (2002) Cytotoxicity of 2-chlorodeoxyadenosine and arabinosylcytosine in leukaemic lymphoblasts from paediatric patients: significance of cellular nucleoside transporter content. *Br. J. Haematol.* **116**, 528–537
22. Terasaka, T., Nakanishi, I., Nakamura, K., Eikyu, Y., Kinoshita, T., Nishio, N., Sato, A., Kuno, M., Seki, N., and Sakane, K. (2003) Structure-based de novo design of non-nucleoside adenosine deaminase inhibitors. *Bioorg. Med. Chem. Lett.* **13**, 1115–1118