The identification of genes inducing resistance to anticancer chemotherapeutic agents and their introduction into hematopoietic cells represents a promising approach to overcome bone marrow toxicity, the limiting factor for most high-dose chemotherapy regimens. Because resistance to cyclophosphamide has been correlated with increased levels of expression of the aldehyde-dehydrogenase (ALDH1) gene in tumor cell lines in vitro, we tested whether ALDH1 overexpression could directly induce cyclophosphamide resistance. We have cloned a full-length human ALDH1 cDNA and used retroviral vectors to transduce it into human (U937) and murine (L1210) hematopoietic cell lines that were then tested for resistance to maphosphamide, an active analogue of cyclophosphamide. Overexpression of the ALDH1 gene resulted in a significant increases in cyclophosphamide resistance in transduced L1210 and U937 cells (50% inhibition concentration \( IC_{50} \), \( \approx 13 \mu \text{mol/L} \)). The resistant phenotype was specifically caused by ALDH1 overexpression as shown by its reversion by disulfiram, a specific ALDH1 inhibitor. ALDH1 transduction into peripheral blood human hematopoietic progenitor cells also led to significant increases (4- to 10-fold; \( IC_{50} \), \( \approx 3 \) to \( 4 \mu \text{mol/L} \)) in cyclophosphamide resistance in an in vitro colony-forming assay. These findings indicate that ALDH1 overexpression is sufficient to induce cyclophosphamide resistance in vitro and provide a basis for testing the efficacy of ALDH1 gene transduction to protect bone marrow cells from high-dose cyclophosphamide in vivo.

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**MATERIALS AND METHODS**

**Cloning of a full-length human ALDH1 cDNA.** A human liver cDNA library (HL115A; Clontech, Palo Alto, CA) was screened with a partial ALDH1 probe corresponding to 1560 bp of ALDH1 cDNA sequences, lacking 5' coding sequences (a gift from Dr. L.C. Hsu). A partial cDNA clone, pGALDHI-A, was isolated containing 1471-bp coding sequences and 272-bp 3' untranslated sequences, but

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still lacking 46 bp of 5' coding sequences including the translation initiation codon. To obtain the remaining 5' coding sequences, human genomic DNA was amplified by polymerase chain reaction (PCR) using a 5'-end primer spanning 5' flanking sequences and a 3'-end primer internal to the coding sequences in the cDNA. For subcloning purposes, the primers contained a 5' artificial Xba I site and a 3' Bgl II site present in the cDNA. After Xba I and Bgl II digestion, the PCR product was subcloned into pGALDH1-A leading to the 1506-bp full-length ALDH1 cDNA, pGAlldo. The PCR-generated Xba I-Bgl II fragment of pGAlldo was verified by nucleotide sequencing.

Construction of retroviral vectors carrying the ALDH1 gene. An EcoRI-Sal I fragment containing the entire ALDH1-coding domain was isolated from pGAlldo, filled by Klenow, and subcloned into the retroviral vector pLXSN, after digestion with Sfi I and Bgl II and fill-in of the 5' protruding ends (a gift from Dr. D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA) and after removal of the SV40-neoR cassette. The resulting plasmid, pAlldoX, contains the ALDH1-coding domain downstream of the MoMuSV 5' long terminal repeat (LTR).

pN2a-Alldo was constructed by cloning the ALDH1 cDNA into the Xho I site of the pE2a plasmid (a kind gift of Dr. E. Gilboa, Duke University Medical Center, Durham, NC).

To generate amphotropic packaging cell lines, the pAlldoX and pN2a-Alldo plasmids were transfected into PA317 cells (CRL 9078; American Type Culture Collection, Rockville, MD) by the CaPO4 precipitation method. 23,24 pSV2neo, which carries a neomycin-resistance gene (Clontech), was cotransfected in each case to provide a marker for the selection of the transfected cells. After cotransfection, the cells were selected in Iscove's modified Dulbecco's medium (IMDM) containing G418 (active dose, 0.5 mg/mL), 10% calf serum, and a 3' long terminal repeat (LTR).

pN2a-Alldo was constructed by cloning the ALDH1 cDNA into the Xho I site of the pE2a plasmid. 23,24 DNA was isolated from colonies grown in single wells and was subjected to 30 cycles of PCR amplification using probes specific for the transduced ALDH1 sequences. 23,24

Infection of hematopoietic cell lines and identification of ALDH1-transduced clones. L1210 and U937 (American Type Culture Collection) cell lines were transduced by incubation of 106 cells with 1 mL of filtered viral supernatant, in the presence of protamine sulfate (5 µg/mL; Sigma, St Louis, MO), for 24 hours; the incubation was repeated for 3 consecutive days, with replacement of the viral supernatant every 24 hours. Cells were then washed twice with phosphate-buffered saline (PBS) and plated in RPMI 1640 (L1210) or IMDM (U937) medium containing 20% fetal bovine serum, 1% penicillin-streptomycin (GIBCO-BRL, Gaithersburg, MD), and 1% penicillin-streptomycin (GIBCO-BRL, Gaithersburg, MD). High-titer—producing clones were identified by testing the supernatant of multiple neo-resistant clones on NIH3T3 cells and determining the relative number of infected cells by Southern blot analysis using probes specific for the transduced ALDH1 sequences. 23,24

Methsphosphamide cytotoxicity assay in transduced cell lines. Mephosphamide (ASTA Z 7557; a gift of Dr Pohl, Asta Werke, Bielefeld, Germany) was obtained as a pure white powder and kept at −20°C. A 1-mmol/L solution was prepared with PBS and sterilized by filtration (0.25 µm) immediately before use. Mephosphamide resistance was assayed by a modification of the protocol described by Kaizer et al. 25 Briefly, cells transduced with the different vectors were resuspended at 106 cells/mL in regular medium and were incubated with increasing doses of mephosphamide for 30 minutes at 37°C in a water bath with frequent agitation. After this period, cells were cooled on ice, washed twice with cold Hanks' balanced salt solution, and plated in quadruplicate at 5 × 103 (L1210) or 104 (U937) cells/well in a 96-well microtiter plate.

After 3 days of culture, a period during which the growth curve of unselected cells was shown to remain linear (not shown), cell viability was assayed by the tetrazolium salt (MTT; Sigma) colorimetric assay. 26 A total of 20 µL of a 6.5-mg/mL solution of MTT in PBS was added to each well of the 96-well microtiter plate and incubated for 2 hours at 37°C. After this incubation period, 100 µL of solubilization buffer (sodium dodecyl sulfate 20% in 50% N,N-dimethylformamide, pH 4.7) were added, and the incubation continued overnight. The optical densities at 570 nm were then measured using 20 µL of MTT and 100 µL of extraction buffer in complete medium as a blank control.

The calculation of the 50% inhibition concentration (IC50) for each clone was performed with the Probit analysis method. 27 RNA analysis. RNA obtained by the Trizol reagent purification procedure (GIBCO-BRL) was resuspended in diethylpyrocarbonate (Sigma)-treated water and was analyzed by 0.9% agarose, 6% formaldehyde, 1x 3-(N-morpholino)propanesulfonic acid (MOPS), gel electrophoresis. Each lane was loaded with 20 µg of total RNA in 5% formaldehyde and 45% formamide in a 40-µL total volume containing 4 µL of 10× running dye. Gels were run at a constant voltage of 2 V/cm overnight in 1× MOPS running buffer. Overnight transfer to nitrocellulose and subsequent procedures were performed as described. 28 Ethidium-bromide staining was used to determine the integrity of the RNA by inspection of the gels. Filters were hybridized to the ALDH1 cDNA probe as described above. Normalization of the quantity of RNA transferred to the filters was obtained after stripping the filters, as suggested by the manufacturer, and hybridization with a 1.4-kb GAPDH probe as described above.

Disulfiram inhibition assay. Disulfiram (Sigma), a specific inhibitor of aldehyde-dehydrogenase activity, 29 was dissolved in 95% ethanol, used within 1 hour of preparation, and kept at +4°C prior to its use. At the concentrations used, ethanol was not cytotoxic to the cells (not shown). Cells were incubated with disulfiram for 30 minutes at 37°C. Mephosphamide was then added and the incubation was continued for an additional 30 minutes at 37°C.

Assay of ALDH1 activity. Briefly, as previously described, 30 protein lysates from infected clones were incubated in a reaction mixture containing 32 mmol/L sodium pyrophosphate buffer (pH 8.2), 5 mmol/L N-acetyl-L-cysteine (Sigma), 0.1 mmol/L 4-methylpyrazole, 4 mol/L NaAD+, 1 mmol/L disodium salt EDTA, and 4 mmol/L acetaldehyde. A blank reaction, identical to the above but without acetaldehyde, was run for each sample. The reaction was run at 37°C monitoring the change of optical density at a wavelength of 340 nm. A standard curve was calculated with different amounts of yeast-purified ALDH enzyme (Sigma).

Western blotting. Cell extracts from L1210 (106) or U937 (2.6 × 105) cells were analyzed by Western immunoblotting as previously described 23,26 with a polyclonal antihuman ALDH1 rabbit antiserum, 29 (a kind gift of Dr A. Yoshida, Beckman Research Institute of the City of Hope, Duarte, CA) followed by incubation with horseradish peroxidase-conjugated Protein-A (Boehringer Mannheim, Indianapolis, IN) for 60 minutes. Bands were visualized by enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL). Probing with anti-β-actin monoclonal antibody (Sigma) was performed after stripping the nitrocellulose as suggested by the manufacturer.

Isolation and transduction of human HPCs. HPCs were harvested by leukapheresis from the peripheral blood of cancer patients treated with high-dose cyclophosphamide (7 g/m2) and hematopoietic growth factors (recombinant human GM-CSF, 5 µg/kg/d; rh interleukin-3, 5 µg/kg/d) as we have previously described. 21 Leukapheresis aliquots were fractionated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), and the light-density cells were collected and exposed to filtered retroviral supernatants at a multiplicity of infection of 15:1 (4 hours of incubation repeated 3 times) in the presence of protamine sulfate (5 µg/mL; Sigma). The percentage of transduced cells was assessed by PCR as described above.
Maphosphamide-resistance assay in transduced HPCs. To test for cyclophosphamide resistance, transduced HPCs were resuspended at 10^6 cells/mL in regular medium and incubated with increasing doses of maphosphamide for 30 minutes at 37°C in a water bath with frequent agitation. After this period, cells were cooled on ice, washed twice with cold Hank's balanced salt solution, and plated. In some experiments, before maphosphamide exposure, CD34^+ cells were purified from bulk-transduced HPCs by separation on columns containing anti-CD34 antibody (Miltenyi Biotech GmbH, Germany)^26; the percentage of CD34^+ cells was assessed by fluorescence-activated cell sorting analysis. After exposure to maphosphamide, bulk HPCs or CD34^+ HPCs were then tested for their ability to form colonies in semisolid medium, as we have previously described.35 Briefly, cells were plated at 1 to 2 x 10^3 (purified CD34^+ cells) or 1 to 2 x 10^4 (unpurified CD34^+ cells)/35-mm well in IMDM containing 0.3% agar, 20% fetal bovine serum, and 10% conditioned medium from the 5637 cell line as a source of growth factors. After 14 days of incubation at 37°C in 5% CO2, the number of colonies was scored under an inverted microscope.

RESULTS

Construction of retroviral vectors expressing ALDH1. The full-length ALDH1 cDNA (see Materials and Methods) was subcloned into two different retroviral plasmids (Fig 1), which share the backbone of the N2 retroviral vector but differ in their 5` LTR; ie, the pLNSN vector carries the 5` LTR and part of the gag region from Moloney murine sarcoma virus,16 whereas pE2a contains LTR sequences from the Moloney murine leukemia virus.17 After transfection into PA317, two clones were selected for their highest titer and used for subsequent experiments: c1.21 producing a pLNSN derivative (Aldo-X c1.21; titer, 2.5 x 10^7 pfu/mL) and c1.26 producing a pE2a derivative (N2a-Aldo c1.26; titer, 10^6 plaque-forming units [pfu/mL]).

ALDH1 gene transduction and expression into hematopoietic cell lines. L1210, a murine leukemia cell line, and U937, a human monoblastic leukemia cell line, were chosen as targets for ALDH1 transduction because they are representative of hematopoietic precursors, show low levels of ALDH1 RNA, and are sensitive to cyclophosphamide. These two cell lines were transduced with the supernatants of retrovirus-producing PA317 clones. Northern blot analysis of ALDH1 RNA expression in various clones of transduced cells (see Figs 2A and B) showed that endogenous RNA was not detectable in the N2-transduced clones, whereas a 3.4-kb (AldoX) and 5.5-kb (N2a-Aldo) RNA species corresponding in size to the viral transcripts were detectable in all the ALDH1-transduced clones.

To determine whether the ALDH1 protein was expressed in transduced cells, cell extracts from different transduced clones were analyzed by immunoblotting using a rabbit polyclonal antiserum against human ALDH1 (Figs 2C and D). No endogenous protein was detected in N2-transduced L1210 cells, whereas a 55-kD band, corresponding to the human ALDH1 protein, was present in all ALDH1-transduced L1210 cells. N2a-Aldo–transduced clones consistently showed higher levels of exogenous ALDH1 than AldoX-transduced clones (compare lanes 6-8 and 9-11 in Fig 2A with lanes 4-6 and 7-9 in Fig 2C). In U937 cells, we detected a 55-kD band corresponding to endogenously expressed ALDH1 in N2-transduced U937 cells, but the same band was present at higher abundance in the N2a-Aldo–transduced clones showing the expression of the transduced gene.

Assay of ALDH1 activity. A spectrophotometric assay was used to directly quantify the amount of ALDH1 activity present in the soluble fraction of N2-, AldoX-, and N2a-Aldo–transduced cells (Fig 3). Quantitation could be performed in L1210 cells but not in U937 cells because of their high endogenous background, which interfered with the spectrophotometric assay. As shown in Fig 3, ALDH1 activity was absent or minimal in the soluble fraction of N2-transduced L1210 cells, whereas it was increased up to threefold over basal levels on transduction with AldoX or N2a-Aldo vectors.

Maphosphamide cytotoxicity assay. To determine whether increased ALDH1 expression led to increased maphosphamide resistance, N2-, AldoX-, and N2a-Aldo–transduced cells were exposed to increasing doses of maphosphamide, and their IC50 was determined by the MTT or by the colony forming assay. As shown in Figs 4A and B, the IC50 was increased in all ALDH1-transduced, but in none of the control-infected, clones. In both L1210 and U937 ALDH1-transduced cells, the IC50 was approximately 13 μmol/L, representing a fourfold (L1210) and a 1.5-fold (U937) increase over basal values, respectively.

Dose-response curves for representative clones of L1210 and U937 cells (Figs 4C and D) indicate that ALDH1-transduced clones show some level of resistance at up to 20 μmol/L maphosphamide. Taken together, ALDH1 activity and maphosphamide toxicity results indicate that an increase in ALDH1 expression is sufficient to make cells resistant to maphosphamide.

Disulfiram inhibition assay. To assess the specificity of the drug resistance in transduced cells, we tested whether cyclophosphamide resistance could be reversed by pretreatment with disulfiram, a specific inhibitor of cytosolic aldehyde-dehydrogenase. Figure 5 shows that pretreatment with disulfiram caused little or no effect in control, N2-transduced, L1210 or U937 cells, whereas it completely reversed the resistance in ALDH1-transduced L1210 and U937 cells. These results indicate that the cyclophosphamide resistance observed in ALDH1-transduced cells is specifically caused by ALDH1 activity.

ALDH1 gene transduction into human hematopoietic progenitor cells. We next verified whether ALDH1 transduc-
tion could induce cyclophosphamide resistance in primary hematopoietic cells. For this purpose, we used aliquots of primary human HPCs obtained by leukapheresis from the peripheral blood of cancer patients treated with high-dose chemotherapy as part of autologous HPC reinfusion protocols. These cells were infected in vitro with the N2a-Aldo virus or with the N2 virus as a control, were exposed to various doses of maphosphamide, and then were examined for their ability to form colonies in semisolid medium. The results obtained with HPCs from three different patients (see Figs 6A, dose-response experiment performed on HPCs in a representative patient; and B, summary of the IC50 obtained with each patient) indicate that ALDH1 transduction caused a significant increase in the IC50 of all three patients. Similar levels of resistance (IC50, ~3 to 4 μmol/L) were obtained in all three cases, including the case for which HPCs were enriched in CD34' cells before testing for maphosphamide resistance (patient no. 3; see % of CD34 cells shown in Fig 6B insert). In one case (patient no. 2), a sufficient number of HPCs was available to test the efficiency of transduction (measured as % of cells positive for proviral DNA by PCR; see Fig 6B insert) on cells plated in limiting dilution. Because only a fraction (62%) of the cells were positive by this assay, the levels of maphosphamide resistance must be considered as the average between those of transduced and nontransduced cells. Thus, for HPCs from patient no. 2 and most likely for the other two cases, the levels of maphosphamide resistance detected in this assay represent underestimates of the actual levels present in transduced cells. Taken together, these results indicate that ALDH1 transduction can confer cyclophosphamide resistance to primary human hematopoietic cells in vitro.

**DISCUSSION**

In these experiments, we have shown that overexpression of a transduced ALDH1 cDNA in human and murine hematopoietic cell lines and in human HPCs is sufficient to induce increased resistance to the cytotoxic action of maphosphamide, an active cyclophosphamide analog. These results are consistent with previous observations reporting a correlation
between ALDH1 expression and cyclophosphamide resistance and provide a causative link between the two phenomena. Thus, the ALDH1 gene can be considered a member of the drug-resistance gene family that includes the DHFR and MDR1 genes. These findings have implications for the mechanism involved in cyclophosphamide resistance and for the use of ALDH1 in gene therapy strategies in vivo.

The cyclophosphamide-resistance phenotype was obtained in all ALDH1 transduced clones, whereas no spontaneous resistance was detectable in controls. This, together with the specificity of the resistance phenotype shown by the enzymatic inhibition assay, formally shows the direct involvement of ALDH1 in the cyclophosphamide-resistance phenotype. However, it can be noted (see Figs 4A and B) that the maximum levels of IC50 obtained in cell lines appeared to plateau at 12 to 13 μmol/L, despite the fact that some clones had significantly higher levels of ALDH1 RNA and protein. This observation is consistent with previous results showing that levels of aldehyde-dehydrogenase 1,000-fold over the basal activity did not result in a proportional induction of resistance. Thus, it is conceivable that the cyclophosphamide-resistance phenotype may be complex depending on intrinsic properties of the enzyme or its intracellular regulation and turnover and may involve the activation of multiple detoxification biochemical mechanisms. Enzymes involved in the glutathione pathway are good candidates to synergize with ALDH1 in inducing cyclophosphamide resistance.

This hypothesis can be tested by constructing bicistronic retroviral vectors, expressing both the ALDH1 gene and a gene implicated in glutathione metabolism.

Regardless of the possibility of further improving the phenotype, the level of resistance to maphosphamide obtained in ALDH1-transduced cells in vitro may already be adequate to confer drug resistance in vivo. Administration of 1 g/m2 cyclophosphamide, i.e., a dose in the conventional range, is associated with peak serum levels of the active metabolite 4-hydroxycyclophosphamide in the 0.4-μmol/L range. Our results show that transduced cell lines and HPCs are resistant to up to 20 μmol/L and 4 μmol/L maphosphamide, respectively, suggesting that ALDH1 transduction can confer resistance to cyclophosphamide concentration well above those reached in vivo.

This conclusion is also supported by the results obtained by ALDH1 transduction in primary human HPCs. Using heterogeneous HPC populations from three different individuals, ALDH1 transduction resulted in significant increases in maphosphamide resistance (IC50 ≈ 3 to 4 μmol/L) up to 4- to 10-fold over control-transduced HPCs. Several observations suggest that these results underestimate the actual levels of resistance in transduced HPCs as well as the effects that these changes may have in vivo. First, in contrast to experiments performed in cell lines in which clonal populations can be examined, the levels of maphosphamide resistance in HPCs represent averages from cell populations composed of both transduced and nontransduced cells and, thus, underestimate the levels of resistance. Second, it is important to note that the endogenous ALDH1 gene is expressed in early progenitor cells (HPCs), whereas it is turned off during differentiation. Thus, the colony-forming assay used in this study, in which only HPCs are exposed to the drug, fails to document the effect of ALDH1 transduction and enforced expression in mature cells, where it should have the greatest effect because of their lack of endogenous ALDH1 activity. Taken together, these observations suggest that, although the results obtained in HPCs provide evidence that primary hematopoietic...
etic cells can be made more resistant to cyclophosphamide, only in vivo studies in experimental murine systems can provide a reliable measure of the levels and biological effects of this resistance.

The ability to render cells resistant to cyclophosphamide may be clinically useful. Cyclophosphamide, when administered in association with hematopoietic growth factors, causes a rapid decrease in peripheral blood cell counts, followed by a similarly rapid recovery to normal levels. This effect is derived from the ability of cyclophosphamide to kill mature HPCs (e.g., CFU-GM) that lack ALDH1 expression while sparing early progenitor/stem cells expressing high levels of the enzyme. Based on these observations, our strategy will involve the use of autologous transplantation to reconstitute a cyclophosphamide-resistant bone marrow in which the constitutive expression of the transduced ALDH1 gene should protect all mature hematopoietic cells from drug toxicity. This strategy should be useful in cancer therapy by allowing the use of protracted courses of cyclophosphamide administration. In addition, the possibility of selecting transduced hematopoietic progenitors in vivo may have more general applications by allowing the introduction of genetically engineered bone marrow cells expressing other genes of therapeutic relevance.

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Induction of cyclophosphamide-resistance by aldehyde-dehydrogenase gene transfer

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