Anti-Cancer Drugs 1996, **7**, pp. 266–274

Transfection of murine fibroblast cells with human cytidine deaminase cDNA confers resistance to cytosine arabinoside

Richard L Momparler, Josée Laliberté, Nicoletta Eliopoulos, Christian Beauséjour and Denis Cournoyer[1]

Département de pharmacologie, Université de Montréal, Centre de recherche pédiatrique, Hôpital Ste-Justine, 3175 Cote Ste- Catherine, Montreal, Quebec H3T 1C5, Canada. [1] Departments of Medicine and Oncology, Montreal General Hospital, Montreal, Quebec H3G 1A4, Canada. Tel: (+1) 514 345-4840; Fax: (+1) 514 345-4801

One of the major limitations in the use of cytosine arabinoside (Ara-C) in cancer chemotherapy is the hematopoietic toxicity produced by this nucleoside analog. One approach to overcome this problem would be to insert a gene for drug resistance to Ara-C in normal hematopoietic cells to protect them from drug toxicity. An interesting candidate gene for this aim is cytidine deaminase which catalyzes the deamination of Arac-C, resulting in a significant loss of its antineoplastic activity. We have ligated the human cDNA for cytidine deaminase into the plasmid vector pMFG. Transfection of NIH 3T3-derived GP + E86 murine fibroblasts cells with this vector resulted in a marked increase (>50-fold) in the expression of cytidine deaminase. In addition, the transfected cells showed resistance to the cytotoxic action and to the inhibition of DNA synthesis produced by Ara-C. Northern and Western blot analysis of the transfected cells showed increased expression of mRNA for cytidine deaminase and increased immunologically detectable enzyme. The ability to confer drug resistance to Ara-C through gene transfer of cytidine deaminase may have the potential as a selectable marker and for the protection of the bone marrow from the toxicity produced by this analog so as to increase its effectiveness in cancer chemotherapy.

Key words: Cytidine deaminase, cytosine arabinoside, drug resistance, transfection.

Introduction

The development of drug resistance by tumor cells is one of the major factors that limit the effectiveness of cancer chemotherapy. Another important factor is the toxicity of antineoplastic agents to normal cells which limits the dose and frequency of therapy that can be used in patients with neoplastic diseases. Developments in gene therapy have led to interesting approaches to overcome the problem of drug toxicity. One approach is to insert a gene into normal hematopoietic cells to protect them from the toxic action of anticancer agents.[1] The rationale for protection of the bone marrow is that the dose-limiting toxicity of many antineoplastic agents is myelosuppression.

One example of this approach is the insertion of the dihydrofolate reductase gene into murine hematopoietic cells to make them resistant to the toxic effects of methotrexate.[2,3] Another example is the insertion of the human multidrug resistance (MDR) gene into murine bone marrow cells to make them resistant to MDR-responsive drugs.[4–6] Another gene that has the potential to protect the bone marrow from toxicity to alkylating agents is the gene for the Yc isoform of glutathione S-transferase.[7]

An interesting candidate gene for hematopoietic chemoprotection is the cytidine (CR)(1) deaminase gene. This enzyme, which catalyzes the deamination of CR to uridine, plays an important role in cancer chemotherapy since it also deaminates the cytosine nucleoside analog, cytosine arabinoside (Ara-C),[8] resulting in a significant loss of its antineoplastic activity.[9] In addition, the high levels of CR deaminase in human liver produces a rapid deamination of Ara-C and is a major factor responsible for the short plasma half-life of this analog in man.[10] In some patients with acute leukemia that had become refractory to Ara-C therapy, at the time of relapse the leukemic blasts showed high levels of CR deaminase, suggesting that the resulting drug resistance was responsible for the treatment failure.[11,12]

The major dose-limiting toxicity of Ara-C chemotherapy is bone marrow suppression.[13] In order to overcome this problem, it would be of interest to

This work was supported by a grant from The Cancer Research Society.

Correspondence to RL Momparler

make the normal bone marrow cells resistant to the toxic effects of this drug. Our laboratory has recently cloned and expressed the cDNA for human CR deaminase.[14] The objective of this study was to determine if transfection of the CR deaminase cDNA into mammalian cells would make them resistant to Ara-C. Our results show that transfected cells had a increased expression of CR deaminase and showed signs of drug resistance to Ara-C.

Materials and methods

Vector design

The expression vector containing the human CR deaminase cDNA was constructed as follows. The plasmid pBluescript KSII containing the complete cDNA sequence for human CR deaminase[14] was used as the template for PCR amplifying a 465 bp DNA fragment of the CR deaminase protein coding sequence. This PCR was performed in a MJ Research thermocycler using the following oligonucleotide primers:

NcoI

5′-TAC CAC CAT GGC CCA GAA GCG T-3′ (P1)

BamHI

5′-TGG GCA GGA TCC GGC TGT CAC T-3′(P2)

The oligonucleotide P1 contained a *Nco*I recognition site at the 5′ end and the first five codons of the human CR deaminase gene in the sense orientation. Oligonucleotide P2 contained a *Bam*HI recognition site at the 5′ end and the last five codons of the human CR deaminase gene in the antisense orientation. This PCR was performed with 2.5 units ID-Proof (Taq) DNA polymerase (ID Labs Biotechnology, London, Ontario, Canada) using the buffer supplied by the manufacture plus 1.5 mM $MgCl_2$ and 0.1 mM of each of the deoxynucleotide triphosphates in a reaction volume of 50 μl. The template (25 ng plasmid DNA) was denatured for 2 min at 94°C followed by 60°C for 1 min and 72°C for 5 min for one cycle, and amplified for 15 cycles, each cycle consisting of denaturation for 30 s at 94°C, annealing for 30 s at 60°C and extension for 2.5 min at 72°C with a terminal 5 min extension at 72°C. The 465 bp DNA fragment was precipitated with ethanol.

The plasmid expression vectors pMFG-tPA and pMFG-LacZ used in this study were obtained from R Mulligan (Whitehead Institute, Cambridge, MA). In these vectors, expression of the inserted sequence is promoted by the Moloney murine leukemia virus long terminal repeat (LTR). The presence of natural splice donor and acceptor sites, normally used to generate the subgenomic *env* transcript of the virus, appears to improve the expression of cloned inserts.[15,16] To maintain the proper positioning of the cloned insert, the insert was cloned between the *Nco*I and *Bam*HI sites of the vector. This was accomplished by introducing an *Nco*I site at the start codon of the protein coding sequences and a *Bam*HI site shortly after the stop codon.

Both the pMFG-tPA plasmid and the 465 bp PCR amplified CR deaminase fragment were digested with *Nco*I and *Bam*HI, the appropriate fragments were separated by 2% agarose gel electrophoresis, purified with QIAquick spin column (QIAGEN, Chatsworth, CA) and ligated with T4 DNA ligase. Competent *Escherichia coli* were transformed with the construct and individual colonies of transformants were screened for insertion by PCR and restriction enzyme digests. Large-scale preparations of plasmids were produced by standard methods and the plasmids purified with QIAprep plasmid kit (QIAGEN). The resulting plasmid with the CR deaminase gene was named pMFG-CD. The terminal 3′ region of MFG and initial 5′ coding region of the gene were sequenced by the dideoxynucleotide chain termination method using a Pharmacia Automatic DNA Sequencer with fluoro-dATP to verify the desired sequence at the initial ATG codon region of the CR deaminase.

Cell culture techniques

Cells were grown in DMEM medium (Canadian Life Technologies, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated fetal calf serum (Wisent Technologies, St Bruno, Quebec, Canada) and 5 μg/ml gentamycin (Canadian Life Technologies) and incubated at 37°C and 7% CO_2. GP + E86 murine ecotropic packaging cells, which are derived from NIH 3T3 mouse fibroblasts, were obtained from Dr A Bank (Columbia University).[17] The GP + E86 cells were co-transfected with the purified plasmid DNAs pMFG-CD (or pMFG-LacZ) and pSV2-neo in a 10 : 1 molar ratio, using the standard calcium phosphate precipitation method and a calcium phosphate transfection kit (Pharmacia, Baie d'Urfé, Quebec, Canada).[18] At 72 h after transfection, G418 (Geneticin, Promega, Madison, WI) at 400 μg/ml was added to the medium and the cells were selected in this medium for

14 days. Clones of cells resistant to G418 were isolated by ring cloning or by dilution. Thereafter the cells were maintained in medium without G418.

Clonogenic assays were performed as follows. Aliquots of 1 ml of 100 cells/ml were plated in wells of a 12-well Costar dish; 18–20 h later Ara-C was added at the indicated concentrations and the incubation continued for an additional 12–14 days. The well were then stained with 0.5% methylene blue in 50% methanol and colonies of greater than 10^3 cells were counted.

For the histochemical stain for β-galactosidase the monolayer cells were washed with phosphate buffered saline (PBS) and fixed at 4°C in 2% paraformaldehyde/0.2% glutaraldehyde in phosphate buffer. The fixed cells were stained at 37°C with 1 mg/ml X-Gal (Life Technologies) solution containing $KFe(CN)_6$ in phosphate buffer until a blue color develops.

DNA synthesis assays were performed as follows. Cells were diluted to 10^5/ml and 1 ml aliquots were placed in wells of a 12-well Costar dish. After incubation overnight, Ara-C was added at the indicated concentrations with 0.2 μCi [^{3}H]thymidine (20 Ci/mmol) and the incubation continued for an additional 6 h. The amount of radioactivity incorporated into DNA was determined after trypsinization as described previously.[19]

Enzyme assay

In vitro assay for CR deaminase was performed using a modification of a previously described method.[14] Monolayer cells (5×10^7) were trypsinized, centrifuged and washed once in PBS, recentrifuged again and resuspended in 200 μl of 5 mM Tris–Cl, pH 7.4, and 5 mM dithiothreitol. The cell suspension was then subjected to three cycles of rapid freezing and thawing. The mixture was centrifuged at maximum speed in a microfuge at 5°C for 15 min. The molarity of the supernatant (cytosol) was then increased to 50 mM Tris–Cl, pH 7.4. Different dilutions of the cytosol were used in 30 min incubation at 37°C to measure the conversion of [^{3}H]cytidine to [^{3}H]uridine as described previously.[14]

Northern and RNA dot blot analysis

Total RNA was isolated from cells by a modified method of Chomczynski and Sacchi[20] using the Ultraspec-II RNA Isolation kit (ID Labs Biotechnology). Briefly, the cells were lysed with a guanidine solution, the RNA precipitated with isopropanol and purified with a resin. The RNA was eluted from the resin with TE buffer (10 mM Tris, pH 8; 1 mM EDTA) and stored at −70°C.

For dot blot analysis, RNA was diluted in a mixture of 20 × SSC (1 × SSC is 0.15 M NaCl and 15 mM Na citrate at pH 7.0) and 37% formaldehyde. The samples were heated at 65°C for 5 min and spotted on Hybond-N nylon membrane (Amersham, Oakville, Ontario, Canada) in a Hoeffer slot blot apparatus. For Northern blot analysis RNA samples in loading buffer were heated to 65°C for 5 min and loaded onto 1.5% agarose–1.1% formaldehyde gel. Electrophoresis was performed in a 0.035 M sodium borate buffer–1.1% formaldehyde. After UV photography of the gel, the RNA was blotted onto Nytran-N nylon membrane with 20 × SSC using a TurboBlotter device (Schleicher & Schuell, Keene, NH). Before hybridization, the nylon membrane were washed with 20 × SSC, baked at 80°C and cross-linked using a Bioslink UV linker (0.3 J/cm^2).

For synthesis of the probe, the complete cDNA for human CR deaminase was labeled by the random prime method of Feinberg and Vogelstein[21] using the kit from Boehringer Mannheim (Dorval, Quebec, Canada) and [α-^{32}P]dCTP from ICN (Mississauga, Ontario, Canada). The membranes were prehybridized in 6 × SSC, 2 × Denhardt's solution and 1% SDS at 65°C for 2.5 h, and hybridized overnight at 65°C in 10 ml of prehybridization solution containing 2×10^7 c.p.m. of the DNA probe. The membrane was then washed with 2 × SSC and 0.1% SDS at room temperature, and then with 0.2 × SSC; 0.1% SDS at 60°C for 15 min. The blot was exposed to Kodak X-Omat film with intensifying screens at −70°C for 24–48 h.

PCR analysis and Southern blotting

A PCR assay was used to verify the presence of the MFG-CD construct in transfected cells. The oligonucleotides:

5′-GGT GGA CCA TCC TCT AGA CTG-3′ (P3)

5′-AGC AGC TCC TGG ACC GTC ATG-3′ (P4)

were used as primers with genomic DNA in the PCR to amplify a specific 421 bp fragment as predicted by the DNA sequence of the pMFG-CD construct. The sense oligonucleotide P3 was about 270 bp downstream from the splice acceptor region of MFG and 2 bp upstream from the start of the *env* coding region. The antisense oligonucleotide P4 was from

positions 378–398 of the CR deaminase coding region. Genomic DNA was isolated from the GP + E86 cells with In ViSorb DNA Kit (ID Labs Biotechnology) by cell lysis with guanidine thiocyanate, DNA adsorption on silica gel and elution with TE buffer. This PCR was performed as described above with about 1 ng genomic DNA denatured at 95°C for 2 min and amplified for 25 cycles, each cycle consisting of denaturation for 1 min at 94°C, annealing for 1 min at 56°C and extension for 1 min at 72°C with a terminal 5 min extension at 72°C. The reaction mixture was separated on 2% agarose electrophoresis.

For Southern blot analysis, genomic DNA was isolated by cell lysis and digestion with proteinase K followed by extraction with phenol–chloroform–isoamyl alcohol and precipitation with ethanol. The DNA was digested with *Nco*I and *Bam*HI, separated by electrophoresis on a 1% agarose and transferred to a nylon membrane. The *Nco*I–*Bam*HI fragments of MFG-CD containing the open reading frame of CR deaminase were radiolabeled by random prime reaction and hybridized with the membrane as described above. In order to determine copy number of the MFG-CD in genomic DNA, graded quantities of this plasmid were added to 10 μg of genomic DNA from GP + E86 cells followed by digestion with *Nco*I and *Bam*HI.

Western blot analysis

Electrophoreses of 5 μg protein of cytosols from different cells lines were run on 15% polyacrylamide–SDS gels with molecular weight markers and the proteins transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell). After treatment of the membrane with blocking solution, rabbit CR deaminase antiserum (1 : 2000 dilution) was incubated with the membrane. The ECL kit from Amersham was used for antigen detection as described by the manufacturer. Briefly, after washing, the membrane was incubated with goat anti-rabbit IgG–horseradish peroxidase complex (1 : 2000 dilution). The membrane was then washed, incubated with substrate and exposed to film for 10 min for luminescent detection of the antigen. For preparation of antibody to CR deaminase, the 14 amino acid sequence at the C-terminus was selected for immunogenicity as described by Jameson and Wolf.[22] This peptide was linked to keyhole limpet hemocyanin (KLH) and injected s.c. with Freund's adjuvant into rabbits. The antisera was collected at different intervals after immunization.

Results

CR deaminase expression

GP + E86 cells were co-transfected with pMFG-CD and pSV2-neo or with pMFG-LacZ and pSV2-neo. Several clones that showed resistance to G418 were isolated and tested for CR deaminase or β-galactosidase expression. The pMFG-CD transfected clones that showed the highest CR deaminase expression (GP + E86-CD3 and GP + E86-CD4) were selected for further investigation. A histochemical stain showed efficient expression of β-galactosidase in a G418 resistant clone (GP + E86-LacZ) isolated from pMFG-LacZ transfected cells (data not shown).

Enzymatic assays for CR deaminase activity were performed on the non-transfected GP + E86 cells, GP + E86-LacZ, GP + E86-CD3 and GP + E86-CD4 cells (Table 1). CR deaminase activity for the GP + E86 and GP + E86-LacZ cells was very low, of the order of 1–4 units/mg. The same enzyme activity was 1086 units/mg for the GP + E86-CD3 cells and 227 units/mg for the GP + E86-CD4 cells, representing a 271- and 57-fold increase, respectively, in comparison with the control cells.

Table 1. CR deaminase activity in different cell lines

Cell line	CR deaminase activity (units/mg)[a]
GP + E86	4.0 ± 1.0[b]
GP + E86-LacZ	1.3 ± 0.6
GP + E86-CD3	1.086 ± 138
GP + E86-CD4	227 ± 12

CR deaminase activity was measured in cell extracts of different cell lines as described under "Methods".
[a]Units of activity is defined as nmoles CR deaminated per min.
[b]Means ± SD, $n = 6$.

The mRNA expression of CR deaminase of the different clones was also evaluated using the CR cDNA as the probe. In a RNA dot blot analysis, it was not possible to detect CR deaminase expression in 5 μg of total RNA in either the GP + E86 or GP + E86-LacZ cells (Figure 1). In contrast, it was possible to detect CR deaminase expression mRNA in as little as 0.5 μg of total RNA in both the GP + E86-CD3 and GP + E86-CD4 cells. The expression of mRNA was greater in the GP + E86-CD3 cells as compared to the GP + E86-CD4 cells. Similar results were obtained in a Northern blot of these different clones (Figure 2). It was not possible to detect CR deaminase mRNA in cells that had not been transfected with the pMFG-CD construct,

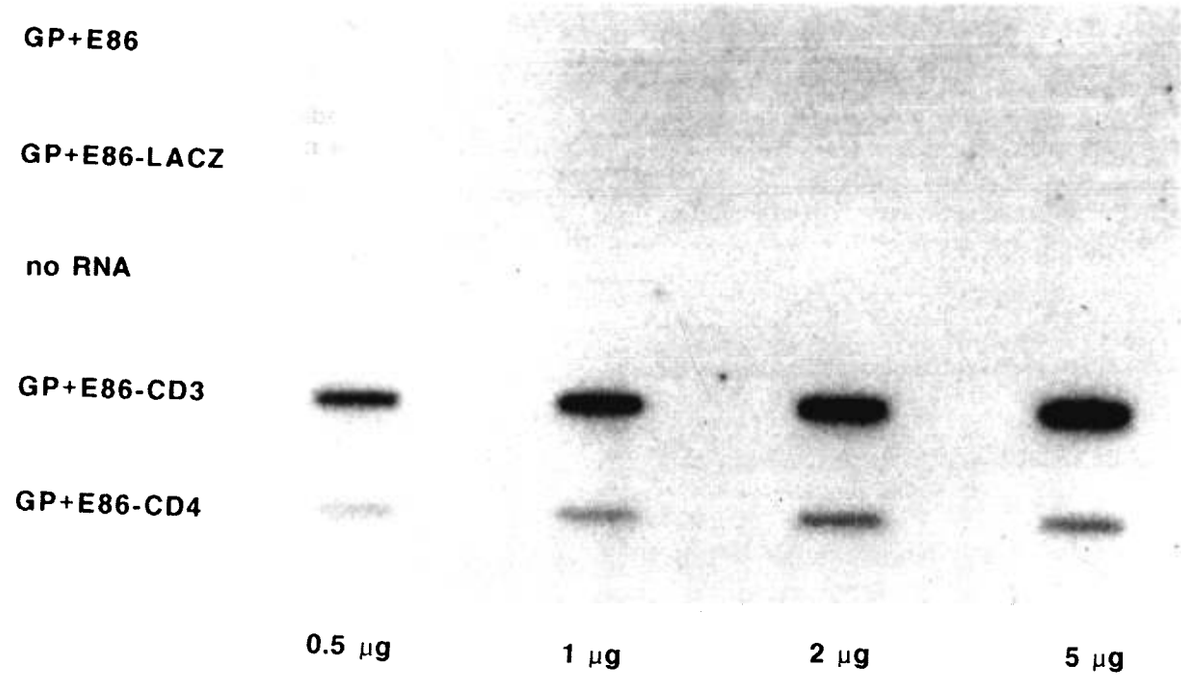

Figure 1. Dot blot analysis of RNA. Different amounts of total RNA from the indicated cell lines were blotted onto a neutral nylon membrane and hybridized with ^{32}P-labeled CR deaminase cDNA probe as described under Methods.

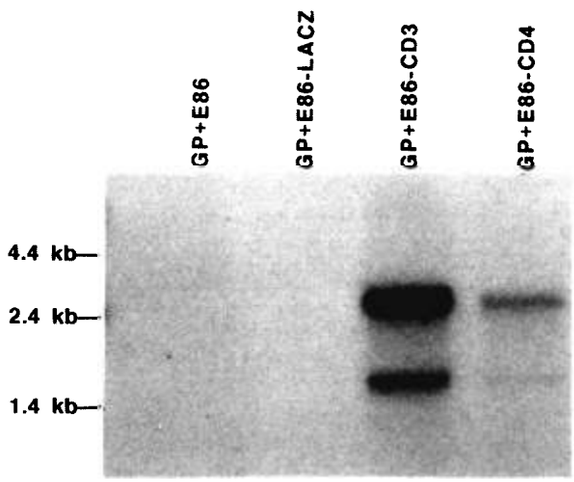

Figure 2. Northern blot hybridization of 5 μg total RNA from each of the indicated cell lines. The membrane was hybridized with ^{32}P-labeled CR deaminase cDNA probe as described under Methods.

whereas the mRNA expression for this gene was greater in the GP + E86-CD3 cells as compared to the GP + E86-CD4 cells. Of note, two transcripts of approximately 2800 and 1900 bp were detected in the GP + E86-CD3 cells. Using the MFG vector, Dwarki *et al.*[16] also detected two transcripts for human factor VIII in 3T3 cells which differ in size by about 0.9 kb.

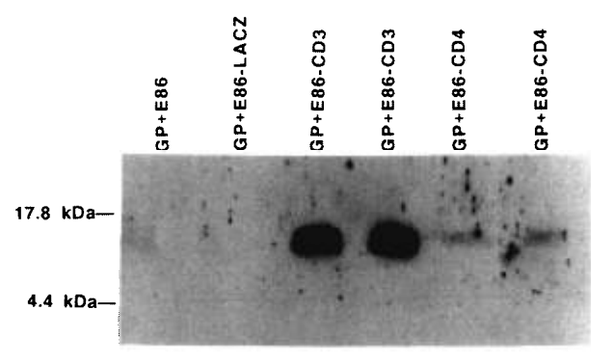

Figure 3. Western blot analysis to detect the expression of human CR deaminase. Initially, 5 μg of protein from the indicated cell lines was separated by polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After incubation with rabbit CR deaminase antiserum, the presence of CR deaminase was detected by chemiluminescence as described under Methods.

The expression of the CR deaminase protein in the different clones was also evaluated by Western blotting. Antibodies to the human CR deaminase were generated in rabbits by injection of a C-terminal peptide fragment coupled to KLH protein. In Figure 3 the Western blot showed that it was possible to detect the presence of the CR deaminase protein in both the GP + E86-CD3 and GP + E86-CD4 cells, but not in the GP + E86 and GP + E86-LacZ cells. The estimated molecular weight of CR

deaminase was about 16 kDa which is the same as the purified protein from human placenta and from the CR deaminase pGEX bacterial expression vector.[14] Again, the expression of CR deaminase was greater in the GP + E86-CD3 cells than the GP + E86-CD4 cells.

PCR and Southern blot analysis

In order to verify the presence of the transfected MFG-CD construct in cells, a PCR was performed using purified genomic DNA from the cells as the template, a sense primer downstream from the MFG splice acceptor region and an antisense primer from the coding region of CR deaminase. A DNA band of the predicted size of 421 bp was amplified from both the GP + E86-CD3 and GP + E86-CD4 cells, but not in the GP + E86-LacZ cells (Figure 4). In a second method to confirm the presence of MFG-CD in the transfected cells, purified genomic DNA was digested with *Nco*I and *Bam*HI and analyzed by Southern hybridization with CR deaminase cDNA probe (Figure 5, upper panel). This analysis revealed a DNA band of approximately 0.45 kb which is similar to the predicted size of the coding region of CR deaminase cDNA. The intensity of the DNA band was greater in the GP + E86-CD3 cells as compared to the GP + E86-CD4 cells, suggesting that the former cells contained more copies of the gene. An estimate of the copy number was performed by loading variable amounts of CR deaminase cDNA corresponding to a specific number of vector copies per cell to a constant amount of genomic DNA from non-transfected cells before Southern blot analysis (Figure 5, lower panel). Densitometric analysis suggests that GP + E86-CD4 cells contain one copy whereas the GP + E86-CD3 cells contain three or four copies of the transfected CR deaminase gene.

Effect of Ara-C on DNA synthesis and colony formation

The effects of Ara-C on DNA synthesis as determined by the incorporation of radioactive thymidine into DNA in both the CR deaminase transfected and non-transfected cells are shown in Table 2. Ara-C at concentrations of 10^{-7}, 10^{-6} and 10^{-5} M produced 14.8, 66.2 and 91.7% inhibition of DNA synthesis, respectively, for the GP + E86 cells. A similar level of inhibition of DNA synthesis with these concentrations of Ara-C was observed with the GP + E86-LacZ cells. For the GP + E86-CD3 cells the same concentrations of Ara-C produced less than 10% inhibition. Ara-C at 10^{-5} M produced 20.3% inhibition of DNA synthesis for the GP + E86-CD4 cells indicating that this clone was more sensitive to this nucleoside analog than the GP + E86-CD3 cells.

Table 2. Inhibition of DNA synthesis by Ara-C in different cell lines

Cell line	Inhibition (%) of DNA synthesis at Ara-C concentration		
	10^{-7} M	10^{-6} M	10^{-5} M
GP + E86	19.1 ± 10[a]	72.3 ± 0.5	93.6 ± 1.0
GP + E86-LacZ	9.0 ± 6.0	68.7 ± 2.6	93.3 ± 2.0
GP + E86-CD3	3.4 ± 5.4	4.7 ± 4.0	7.4 ± 4.6
GP + E86-CD4	2.9 ± 5.0	3.8 ± 4.7	20.3 ± 2.0

Cells were exposed to the indicated concentrations of Ara-C and [^{3}H]-thymidine for 6 hr and DNA synthesis determined as described under Methods
[a]Mean ± SD, $n = 3$.

A clonogenic assay was used to evaluate the cytotoxic action of Ara-C on the non-transfected and CR deaminase-transfected cells (Table 3). Ara-C at concentrations of 10^{-6} or 10^{-5} M completely suppressed colony formation for both the GP + E86 and GP + E86-LacZ cells. At 10^{-6} M Ara-C, colony formation for the GP + E86-CD3 cells and the GP + E86-CD4 cells was greater than 90% of the control value without drug. Colony formation at 10^{-5} M Ara-C was reduced to about 73 and 24% of the control value for the GP + E86-CD3 cells and the GP + E86-CD4 cells, respectively.

Table 3. Effect of Ara-C on colony formation of cells transfected with CR deaminase cDNA

Cell line	Experiment no.	No. of colonies at Ara-C concentration				
		0	10^{-8} M	10^{-7} M	10^{-6} M	10^{-5} M
GP + E86	1	28	33	33	0	0
	2	33	29	30	0	0
	3	36	29	26	0	0
GP + E86-LacZ	1	31	24	16	0	0
	2	32	28	21	0	0
GP + E86-CD3	1	18	18	25	19	19
	2	16	20	15	11	9
	3	21	17	26	20	12
GP + E86-CD4	1	25	28	23	24	3
	2	39	39	34	39	10
	3	27	18	19	29	9

Cells were exposed to the indicated concentrations of Ara-C for 12–14 days and the number of colonies formed determined as described under Methods.

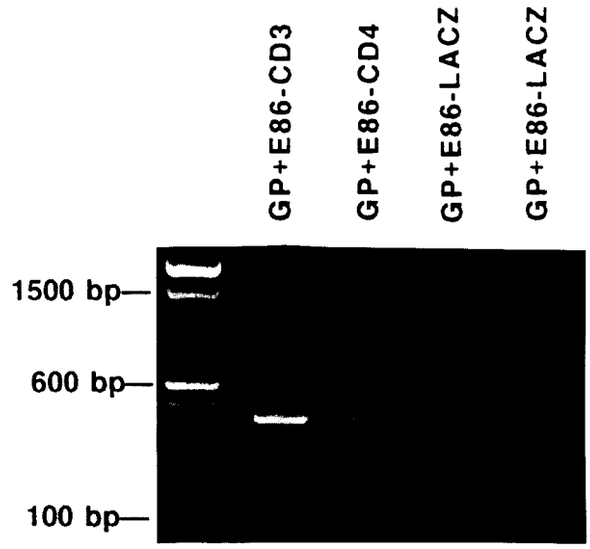

Figure 4. Analysis for the presence of transfected CR deaminase gene in cells by PCR. A sense primer downstream from the MFG splice acceptor region and an antisense primer from the CR deaminase coding region with purified genomic DNA from the indicated cell lines were used in the PCR as described under Methods. Amplification of a specific 421 bp band indicates the presence of MFG-CD DNA.

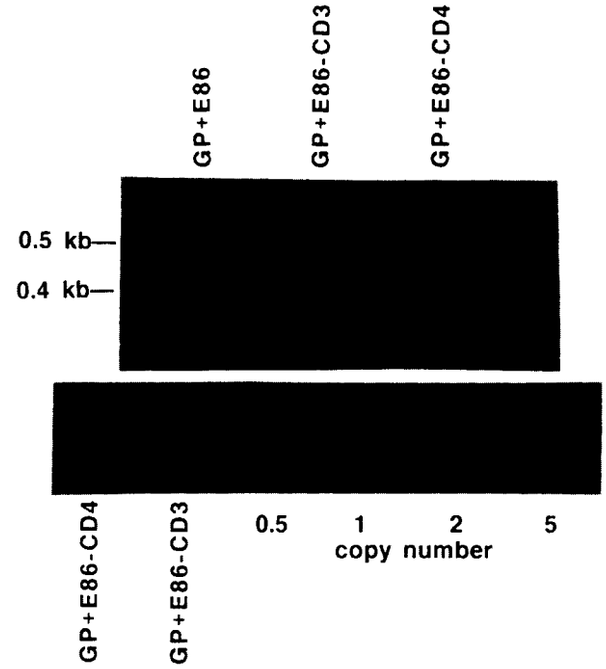

Figure 5. For Southern blot analysis, 10 μg of purified genomic DNA from the indicated cell lines were digested with *Nco*I and *Bam*HI and fractionated on 1% agarose. Following transfer to a nylon membrane, hybridization was performed with ^{32}P-labeled CR deaminase cDNA probe as described under Methods. Southern blot with molecular weight markers (upper panel). Estimation of copy number of MFG-CD by Southern blotting (lower panel) was performed as described above by adding graded amounts of MFG-CD vector to 10 μg of DNA from non-transfected GP + E86 cells prior to digestion with *Nco*I and *Bam*HI.

Discussion

Several lines of evidence indicate that the human CR deaminase plays an important role with respect to the pharmacodynamics of Ara-C in the therapy of leukemia. The *in vitro* incubation of human myeloid leukemic cells with the CR deaminase inhibitor, tetrahydrouridine, resulted in an increased uptake of Ara-C and its conversion to the active inhibitor, Ara-CTP.[23] The use of tetrahydrouridine in patients during the infusion of Ara-C produced a marked increase in the plasma level of analog.[24] Enzyme assays of leukemic cells at the time of relapse showed increased levels of CR deaminase in some patients with refractory myeloid leukemia, suggesting that this enzyme could be involved in drug resistance.[11,12]

Our results demonstrate the development of drug resistance to Ara-C following gene transfer of the human CR deaminase cDNA into NIH 3T3-derived GP + E86 mouse fibroblasts using the pMFG retroviral expression vector. In addition, the level of Ara-C resistance correlated with the level of expression of the transfected CR deaminase plasmid.

The GP + E86 cells were co-transfected with either pMFG-CD or pMFG-LacZ and the pSV2-neo, and G418-resistant clones were isolated. Enzyme assays on two of these clones (GP + E86-CD3 and GP + E86-CD4) showed a 271- and 57-fold increase, respectively, in CR deaminase activity as compared with the parental cells (Table 1). This indicated that the MFG-CD construct was an excellent expression vector for the CR deaminase cDNA. Other investigators have reported good expression of a variety of genes using the MFG vector.[15,16]

The expression of CR deaminase activity in the transfected cells correlated perfectly with the expression of the vector-encoded mRNA. The GP + E86-CD3 cells, which had greater CR deaminase activity than the GP + E86-CD4 cells, also showed more abundant steady state levels of CR deaminase mRNA than the latter cells as demonstrated by the RNA slot blot and Northern blot analysis (Figures 1 and 2). Western blot analysis also showed greater expression of the CR deaminase protein for the GP + E86-CD3 cells as compared with the GP + E86-CD4 cells (Figure 3). These results corre-

late with the enzyme activity and RNA expression as discussed above. Of note, the two different vector transcripts detected by Northern blot analysis in the clone GP + E86-CD3 most certainly represent the spliced and unspliced transcripts resulting from the inclusion of the Moloney virus splice donor and acceptor signals in the pMFG construct. The approximately 0.9 kb difference in size between these two transcripts is also in accord with this interpretation, as this was also the difference in size of the two mRNA species observed when the MFG vector was used to express the human factor VIII.[16]

In order to detect the presence of the MFG-CD construct in the transfected cells, we purified the DNA from these cells to use as the template in a PCR reaction using a sense primer downstream from the MFG splice acceptor region and an antisense primer from the coding region of CR deaminase cDNA. The predicted 421 bp DNA was amplified from the GP + E86-CD3 and GP + E86-CD4 cells, but not from the GP + E86-LacZ cells (Figure 4).

Southern blot analysis was also performed on these cells with purified genomic DNA digested with *Nco*I and *Bam*HI and probed with CR deaminase cDNA. A DNA band of approximately 0.45 kb was detected in both the GP + E86-CD3 and GP + E86-CD4 cells, but not in the GP + E86 cells (Figure 5, upper panel). The intensity of this band, which is similar to the size of the coding region of CR deaminase cDNA, was greater for the GP + E86-CD3 cells than the GP + E86-CD4 cells, indicating that there were more copies of the MFG-CD construct integrated into the genomic DNA of the GP + E86-CD3 cells. A more precise estimate of the copy number for CR deaminase cDNA in these cells is in agreement with our analysis (Figure 5, lower panel). There appears to be only one copy of CR deaminase cDNA in the GP + E86-CD4 cells, whereas the GP + E86-CD3 cells appear to contain three to four copies of the transfected gene.

We have also determined that the increased expression of CR deaminase in the transfected cells conferred drug resistance to Ara-C as evaluated by inhibition of DNA synthesis and a colony formation. Ara-C is a very potent inhibitor of DNA synthesis due to its incorporation into DNA, where it produces a chain termination-like effect.[25] Ara-C at 10^{-5} M produced 91.5% inhibition of DNA synthesis for the parental GP + E86 cells, as compared with only 7.6 and 20.3% inhibition for the GP + E86-CD3 and GP + E86-CD4 cells, respectively (Table 2). In a clonogenic assay, we detected no colonies for the GP + E86 and GP + E86- LacZ cells in the presence of 10^{-6} M Ara-C, whereas the number of colonies for the GP + E86-CD3 and GP + E86-CD4 cells was similar to that of the untreated controls (Table 3). Even at a concentration of 10^{-5} M Ara-C a greater number of colonies appeared for the GP + E86- CD3 cells as compared with the GP + E86-CD4 cells. This correlates with the greater expression of CR deaminase in the former cell line. It is interesting to note that 10^{-6} M Ara-C is in the range of the plasma levels obtained with conventional dose Ara-C whereas 10^{-5} M Ara-C is in the range obtained with high dose Ara-C.[26,27]

The GP + E86-CD clones showed increased expression of CR deaminase and drug resistance to Ara-C for more than 4 months in culture without the presence of a selecting agent indicating that a stable expression of the drug resistance phenotype. The GP + E86 cell line is an ecotropic packaging cell. Our data suggest that the pMFG-CD expression vector was integrated into the genomic DNA of the GP + E86 cells and efficiently expressed. Some investigators have reported the reduction or loss of expression of integrated gene with time.[28,29] We did not observe the silencing of expression of the integrated CR deaminase cDNA in the clones of GP + E86-CD cells. Our preliminary data indicate that the GP + E86-CD clones can by retroviral-mediated transfer confer Ara-C resistance to murine hematopoietic cells, suggesting that the human CR deaminase gene may have the potential for gene therapy.[1] Another important application of this gene may be as a positive selectable marker to enrich the expression of a second gene of interest from a common vector.

Acknowledgments

We thank Mona Greenbaum for technical advice and Louise F Momparler for technical assistance.

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(Received 4 January 1996; accepted 1 February 1996)