Development of a Retroviral Construct Containing a Human Mutated Dihydrofolate Reductase cDNA for Hematopoietic Stem Cell Transduction

By Ming-Xia Li, Debabrata Banerjee, Shi-Cheng Zhao, Barry I. Schweitzer, Shin Mineishi, Eli Gilboa, and Joseph R. Bertino

A double-copy Moloney leukemia virus-based retroviral construct containing both the Neo<sup>+</sup> gene and a mutant human dihydrofolate reductase (DHFR) cDNA (Ser31 mutant) was used to transduce NIH 3T3 and mouse bone marrow (BM) progenitor cells. This resulted in increased resistance of these cells to methotrexate (MTX). The transduced BM progenitor cells were returned to lethally irradiated mice. The recipients were replanted with marrow cells infected with the recombinant virus showing protection from lethal MTX toxicity as compared with mock-infected animals. Evidence for integration of the proviral DNA was obtained by amplification of proviral DNA by polymerase chain reaction (PCR) and Southern analysis. Sequencing a portion of the PCR-amplified human DHFR cDNA showed the presence of the mutation. These studies with the human Ser31 mutant DHFR cDNA gave results comparable with those obtained with the mutant murine DHFR cDNA (Leu to Arg22) in developing MTX-resistant BM. The Ser31 mutant human DHFR cDNA is currently being tested for infection of human CD34<sup>+</sup> human BM and peripheral blood stem cells in vitro.

A n important application for gene therapy is the transduction of hematopoietic progenitor cells with a gene that confers resistance to a drug. If successful, treatment with conventional doses of the drug would be less marrow suppressive, and if marrow were the organ-limiting dose escalation, even higher doses of drug would be tolerated. If the tumor exhibits an increased response as a function of drug dose, then the increased marrow tolerance could result in improved therapeutic benefit. In addition, certain nonmalignant diseases (eg, psoriasis, rheumatoid arthritis, sickle cell anemia) are sometimes treated with anticancer drugs; if host tolerance could be improved, these agents might enjoy even more widespread use.1,6

To date, this approach has focused on the use of an altered dihydrofolate reductase (DHFR) gene to confer methotrexate (MTX) resistance<sup>5,14</sup> and more recently on the use of the multidrug resistance cDNA to confer resistance to drugs that are susceptible to p-glycoprotein mediated efflux.<sup>12,13</sup> Williams et al<sup>10</sup> reported that lethally irradiated mice were protected from MTX toxicity by receiving marrow transduced with a murine 3T6 DHFR gene with a leu → arg mutation in codon 22. We have also reported protection of irradiated mice using this same cDNA contained in a different retroviral construct, containing a SV40 promoter.14

In an attempt to improve the efficiency and usefulness of this approach for human marrow gene transfer, we have developed a retroviral vector containing a human cDNA with a mutation in codon 31 (phe → ser). The use of this cDNA was based on previous studies that showed this mutation did not compromise catalytic efficiency (V<sub>max</sub>/km) of this enzyme to the same degree as did the leu → arg mutation.13,16

In transfection studies using Chinese hamster ovary (CHO) cells lacking dihydrofolate reductase (DHFR), this mutation gave rise to a greater number of resistant colonies than the arg 22 mutation when selected with low levels of MTX.17

The present studies show that viral constructs containing the Phe → Ser 31 mutation (hereinafter referred to as Ser 31) protect mouse marrow progenitor cells from MTX cytotoxicity both in vitro and in vivo. Evidence for integration and expression of the mutated gene product into hematopoietic cells is shown by Southern analysis, polymerase chain reaction (PCR) amplification, and cDNA sequencing.

MATERIALS AND METHODS

Construction of retroviral vectors carrying human mutant DHFR

Double copy (DC) vector (derived from Moloney Murine Leukemia virus<sup>10</sup>) carrying 655 bp of human DHFR cDNA containing a mutation at residue 31 (Phe → Ser) under control of the SV40 promoter were constructed. The DC vector was obtained from plasmid pSV4 HDR Ser31 as shown in Fig 1.

The DC vector contained 564 bp of the coding region and 91 bp 3’ of noncoding region of the DHFR Ser31 cDNA. The expected length of the hDHFR transcript is 1.0 kb.

The construct was packaged to the corresponding virus by a previously described procedure.19 Production of live virus, viral titration, and target cell infection were performed as previously described.19

Immunoprecipitation of the DHFR protein. Lysates from <sup>35</sup>S-methionine labeled cells were used for immunoprecipitation by protein-A sepharose linked rabbit-antihuman DHFR polyclonal antibody. The immunoprecipitates were subjected to electrophoresis on a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel overlayed with a 4.3% stacking gel. After electrophoresis, the gel was fixed in methanol-acetic acid, enhanced with ENHANCE (New England Nuclear, Wilmington, DE), dried and exposed to x-ray film for autoradiography. Two major bands were seen on the autoradiogram, a 22-kD DHFR protein and a nonspecific 40-kD protein. By using the latter 40-kD band as an internal control, the relative ratio of the intensity of the 22-kD DHFR protein band to that of the 40-kD control band was calculated, giving a semiquantitative measurement of DHFR expressed in the virally transduced cells. CHO DG44 cells (lacking DHFR) were used as negative controls and CHO DG44 cells transfected with pSV5 HDR<sup>17</sup> (a mammalian expression vector carrying wild-type human DHFR cDNA) were used as positive controls for immunoprecipitation.

Southern analysis. Genomic DNA from organs and from granulocyte-macrophage colony-forming unit (CFU-GM) colonies were

From the Program of Molecular Pharmacology and Therapeutics, Sloan-Kettering Institute for Cancer Research; and the School of Graduate Studies, Cornell University School of Medicine, New York, NY.

Submitted August 24, 1993; accepted January 31, 1994.

Supported by National Institutes of Health Grants No. CA08010 and CA59350 and a Cancer Center Support Grant (NIH-P30-CA08748).

Address reprint requests to Joseph R. Bertino, MD, Molecular Pharmacology and Therapeutics, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 78, New York, NY 10021.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1994 by The American Society of Hematology.

0006-4971/94/8311-0037$3.00/0
The 3T3 cells infected with retrovectors carrying mutant hDHFR cDNA (Ser31) were selected in G418 selection medium, and the resistant colonies were pooled. The inhibitory effect of MTX on the colony formation of these G418-resistant cells was measured as described in Materials and Methods. The IC50 values are the average of three experiments, and the standard deviations are shown. The parental 3T3 cell line and the cell line transduced by wild-type hDHFR in retroviral vector (DC/SV-hDHFR) were used as controls.

> Table 1. MTX Resistance Level in 3T3 Cells Infected With Mutant hDHFR cDNA in Retroviral Vector

<table>
<thead>
<tr>
<th>Vector</th>
<th>MTX IC50 ± SD (nmol/L)</th>
<th>Resistance Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>3T3</td>
<td>16.0 ± 3.0</td>
<td>1</td>
</tr>
<tr>
<td>DC/SV-hDHFR</td>
<td>19.0±</td>
<td>1.2</td>
</tr>
<tr>
<td>DC/SV-hDHFRSer31</td>
<td>77.01 ± 17.7</td>
<td>4.8</td>
</tr>
</tbody>
</table>

To show that the cDNA was integrated into genomic DNA, DNA was digested with Drai, which cuts once within the hDHFR cDNA, generating a single band of about 4 kb (Fig 2). No gross recombination was observed. DHFR Ser31

To show that the cDNA was integrated into genomic DNA, DNA was digested with Drai, which cuts once within the hDHFR cDNA, generating a single band of about 4 kb (Fig 2). No gross recombination was observed. DHFR Ser31

To show that the cDNA was integrated into genomic DNA, DNA was digested with Drai, which cuts once within the hDHFR cDNA, generating a single band of about 4 kb (Fig 2). No gross recombination was observed. DHFR Ser31
mRNA of expected length was also detected in the transduced cells by Northern analysis (data not shown).

Attempts to construct DC vectors carrying larger cDNAs of the Ser31 mutant human DHFR (800 bp) containing 564 bp of the coding region and 236 bp of 3' untranslated region were unsuccessful. These vectors did not produce the MTX resistance phenotype in transduced cells, although Southern blot analysis of Dra I-digested DNA from transduced cells showed bands of expected sizes, suggesting that failure to produce resistance was likely caused by lack of correct translation or transcription rather than at the DNA level. Attempts to clone both the 655-bp and 800-bp length cDNA's of the Ser31 DHFR in DC vectors in the reverse orientation also failed to impart resistance in transduced cells. Northern analysis showed DHFR mRNA of shorter than expected lengths from constructs containing the 800-bp DHFR Ser31 cDNA. Similar truncated DHFR Ser31 mRNA was also seen from constructs that contained either the 655-bp or 800-bp DHFR Ser31 cDNA in the reverse orientation. Further work with these vectors was not pursued.

Expression of hDHFR Ser 31 in marine bone marrow cells in CFU-GM colonies. MTX resistance of BM cells transduced through coculture with the DC/SV-hDHFRSer31 was determined by CFU-GM assay and compared with the murine cDNA construct, DC/SV-R-mDHFR Arg22. The hDHFR construct produced similar levels of resistance to MTX as the murine construct (DV/SV-R-mDHFR22) (Table 2).

MTX resistance in mice after the transplantation of bone marrow cells transduced by mutant hDHFR. DC/SV-hDHFRSer31 was next used for in vivo BM transplantation studies. Mice transplanted with marrow transduced by DC/SV-hDHFRSer31 survived MTX selection with both low-dose (Fig 3A) or delayed high-dose schedules (Fig 3B and C), whereas control mice transplanted with untransduced marrow died. However, immediate high-dose selection of mice transplanted with transduced marrow resulted in 100% lethal toxicity within 3 weeks (Fig 3C).

Hematocrits and WBCs; at day 28 the mice that survived the MTX treatment were all from the group transplanted with marrow transduced with the retroviral vector construct, DC/SV-hDHFRSer31. The hematocrits and WBCs had returned to normal. In the experiment shown in Fig 3B, after a period of low-dose selection, the animals were challenged with a high-dose regimen of MTX (200 mg/kg twice weekly × 8 doses); this dose schedule, which causes essentially 100% lethal toxicity in normal animals, was well tolerated by the animals with marrow transduced with the DC/SV-hDHFRSer31 vector. A third experiment was also performed in which mice receiving the transduced marrow were allowed to recover from the effects of whole body irradiation for 4 weeks; during this time, no MTX was administered. At the end of this time, they were treated with the same high-dose schedule used in the experiment shown in Fig 3B; of interest was that 6 of 9 mice survived.

Evidence for integration and expression of DC/SV-hDHFR31. As the vector contained an internal Neo gene, Southern analysis using a Neo<sup>k</sup> cDNA probe was performed in spleen colonies obtained at day 12 from irradiated mice who had received transduced BM. Using EcoRI and Dra I restriction enzymes, evidence for integration of the Neo<sup>k</sup> gene into DNA from 18 of 44 colonies was obtained (Fig 4, A and B). The expected sizes of the bands hybridizing to a Neo<sup>k</sup> gene probe after EcoRI digestion and Dra I digestion are ~1.5 and 4 kb, respectively. EcoRI enzyme cuts just outside the 5' and 3' regions of the Neo<sup>k</sup> gene generating a 1.5-kb fragment. Dra I on the other hand cuts once within the DHFR gene. However, because of the double copy nature of the vector, Dra I digestion of integrated virus actually cuts out an approximately 4-kb fragment, which contains the Neo<sup>k</sup> gene also.

The presence of the hDHFR cDNA in spleen and peripheral nucleated cells was confirmed by PCR amplification and by sequencing the portion of the gene containing the mutation (data not shown). Evidence for the presence of the transduced hDHFR cDNA was also found in liver; in subsequent experiments when the liver was perfused with saline before DNA was isolated, no PCR product was detected, indicating that peripheral blood mononuclear cells present in unperfused liver cells were the likely source of the PCR signal.

The integration of the proviral DNA in MTX-resistant CFU-GM colonies of recipient mice 5 weeks after the BM transplant and low-dose MTX selection was also seen by PCR and Southern blotting (data not shown).

DISCUSSION

Constructs with longer DHFR cDNA (800 bp) containing ~236 bp 3' untranslated region, as well as the Ser31 mutant human DHFR (655 bp) cloned in the reverse orientation, did not produce any significant resistance in transduced 3T3 cells. This failure could be attributed to the lack of formation of DHFR mRNA in these transduced cells of the expected size. No rearrangement at the DNA level was observed. A DC vector carrying less 3' untranslated region, and with only 655 bp of the Ser31 mutant human DHFR cDNA expressed the mutant DHFR effectively. The message of expected length was transcribed and the human Ser31 DHFR protein was translated.
The expression of the altered hDHFR (Ser31) cDNA construct was compared with the altered mDHFR (Arg22) in the CFU-GM assay after the coculture infection of murine BM cells with the amphotropic producer lines. The percentage of the resistant CFU-GM colonies of the hDHFR construct tested was similar to the construct DC/SV/R-mDHFR, which had been previously shown to confer MTX resistance in vitro and in vivo.14

The MTX-resistant phenotype induced by transfection with the mutant hDHFR in vivo. The animal survival data after BM transplant with low-dose MTX selection showed that animals receiving the BM transduced by altered mDHFR were more tolerant of MTX treatment. The hematocrit and WBC count of most of these animals returned to normal 4 weeks after BM transplant and MTX treatment, whereas all control animals died of anemia, GI bleeding, and marked weight loss within the same time.

The transduced BM not only protected the animal from the marrow toxicity of MTX, but GI toxicity as well. Similar observations were reported by Williams et al.10 and Corey et al. The prevention of prolonged and marked leukopenia by the transduced gene probably contributes to the protection of the GI tract.
Integration of the retroviral vector in the hematopoietic cells of the recipient mice is necessary for the stable and long-term expression of the mutant DHFR. PCR and Southern analysis confirmed the presence of the Neo<sup>R</sup> gene in the spleen and peripheral nucleated cells of the recipient mice, spleen CFU (CFU-S) colonies, and in the MTX-resistant CFU-GM colonies from the recipient mice marrow. DNA sequence analysis of the PCR amplification product of the vector DHFR cDNA confirmed the existence of the mutant human DHFR in the recipient mice.

The high-dose MTX protocol tested whether the MTX resistance conferred to BM by gene transfer protects animals from toxicity of a lethal dose of MTX. These data show that protection was afforded even after challenge with high doses of MTX. Current studies are directed toward using this construct to infect human CD34<sup>+</sup> selected...
hematopoietic precursor cells, with the eventual goal of clinical trials.

REFERENCES

Development of a retroviral construct containing a human mutated dihydrofolate reductase cDNA for hematopoietic stem cell transduction

MX Li, D Banerjee, SC Zhao, BI Schweitzer, S Mineishi, E Gilboa and JR Bertino