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Insertion of New Genes into Bone Marrow Cells of Mice

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A. Summary

Drug resistance genes such as those coding for a methotrexate-resistant dihydrofolate reductase (DHFR) or the thymidine kinase from herpes simplex virus can be used to confer a proliferative advantage on bone marrow cells of mice. As a result of this proliferative advantage, transformed cells become the predominant population in the bone marrow. Efficient gene expression was obtained for both the thymidine kinase and DHFR genes inserted into mouse bone marrow. Such gene insertion techniques may ultimately lead to the cure of life-threatening globinopathies such as sickle cell disease or the beta thalassemias. They may also be useful in reducing the hematopoietic toxicity of anticancer drugs.

B. Introduction

We have developed techniques for the insertion of new genes into bone marrow stem cells of living mice. By selective procedures we can favor the proliferation of transformed stem cells carrying the inserted genes (Cline et al. 1980; Mercola et al. 1980). We have shown that such cells transformed with the genes coding for DHFR or herpes simplex virus thymidine kinase (HSV-tk) produce high levels of the active enzymes specified by these genes (Salser et al., to be published).

In these experiments we focused on procedures which might eventually have clinical relevance for the treatment of genetic diseases of the hematopoietic system such as sickle cell disease. This constrained out approach to procedures which could potentially be used to insert functioning globin genes into the great majority of bone marrow stem cells.

We utilized the technique of cell transformation by calcium precipitates of DNA as developed by Bachetti and Graham (1977) and modified by Wigler et al. (1978). This procedure allows the insertion of quite large DNA sequences; the mouse DHFR gene which we have inserted is at least 42 kilobases (kb) in size (Nunberg et al. 1980).

C. Use of Selection Techniques

An essential component of our strategy has been to develop selective procedures favoring the proliferation of transformed cells in living animals. In this way even a small number of cells with the desired gene insertions could gain a proliferative advantage so that they would ultimately predominate in the bone marrow population. We selected a strategy based on selective pressure by the chemotherapeutic agent, methotrexate (MTX). MTX binds to the enzyme DHFR and inhibits the synthesis of thymidine monophosphate (TMP) and DNA synthesis. Our first strategy involved the insertion of altered DHFR genes coding for an enzyme resistant to MTX. Our second strategy involved the insertion of HSV-tk genes which code for the enzyme that catalyzes the conversion of thymidine to TMP and enables the cells to grow in the presence of MTX by utilizing the salvage pathway of DNA synthesis more efficiently. The HSV-tk enzyme has a greater affinity for its substrate than does the normal mammalian enzyme. We reasoned that this might confer an additional advantage to cells incorporating and expressing the viral gene. The K_m for the virus enzyme is estimated to be

0.4 μM thymidine (Klemperer and Haynes 1967) whereas that for the normal mouse enzyme is estimated as $9\mu M$ thymidine (Chang and Prusoff 1974).

D. 3T6-Rl Cells Have a MTX-resistant DHFR

We isolated DNA from mouse 3T6 cells containing reiterated DHFR structural genes (Kellums et al. 1979). These cells, designated 3T6-Rl, contain about 30 copies of the DHFR structural gene and produce correspondingly elevated levels of the enzyme (Kellums et al. 1979). We found that the 3T6-Rl DHFR activity is strikingly resistant to MTX (Salser et al., to be published).

The 3T6-Rl cells appear to contain two DHFR activities; one, comprising about 25% of the total activity, is sensitive to MTX; the other, which represents about 75% of the total activity, is highly resistant to MTX.

E. Transformation of Mouse Bone Marrow Cells with DHFR Genes

We established an escalating schedule of MTX treatment which would select for drug-resistant hematopoietic cells without killing animals (Cline et al. 1980). We used a syngeneic pair of mouse strains (CBA/Ca and CBA/T6T6), one of which carries a distinctive T6T6 chromosomal marker. This enabled us to follow the two cell types independently and determine whether the transformation procedure was successful. In a successful transformation the cell type receiving the methotrexate-resistant (MTX^R) genes should show a marked growth advantage and come to predominate in recipient animals treated with MTX. This cell type could be identified by its distinctive karyotype.

The experiment shown in Table 1 illustrates the type of results obtained. The T6T6 marked cells transformed with MTX^R DHFR genes showed 75%–96% predominance in the bone marrow of recipient animals after treatment with MTX for 33 to 65 days. Control experiments show that these results are not due to any inherent advantage of the T6T6 cells over Ca cells.

Treatment of the recipient animals with MTX is essential if the cell type transformed with MTX^R DNA is to become dominant. When MTX treatment is omitted the MTX^R DNA-transformed cell karyotypes are typically found at levels of only 33%–40% (Cline et al. 1980).

Success of these experiments was judged by appropriate karyotype predominance. It was not feasible to establish insertion of a new DHFR gene by Southern hybridization analysis because of the origin of the mutant DHFR gene from murine sources. In independent experiments it was possible to demonstrate insertion of the human beta globin gene by cotransformation with MTX^R DHFR (Salser et al., to be published).

Table 1. Karyotype analysis of marrow cells of CBA/Ca mice receiving a 1:1 mixture of control Ca and T6 marrow cells transformed with MTX-resistant DNA (Ref. 1)

Recipient	Period with Mtx (days)	Period without MTX (days)	Karyotype (%T6)
Primary 1	0–33	–	79
Primary 2	0–40	–	75
Primary 3	0–47	–	75
Primary 3	0–47	48–68	83
Secondary 3	47–61	–	88, 88, 100[a]
Primary 4	0–54	–	75
Secondary 4	54–72	–	83
Primary 5	0–65	–	96
Primary 5	0–65	66–113	63

[a] Three secondary recipients

Spleens were removed from primary and secondary recipients of transformed marrow from four independent experiments and assayed for DHFR. MTX treatment was terminated 5–7 days before collection of tissue from these mice for the DHFR assays. Spleens were collected from 12 control animals chosen to match those of the experimental group. A radiometric assay for DHFR was performed on sonicated cell-free extracts of spleen (Hillcoat et al. 1967; Hayman et al. 1968). DHFR-specific activity was two- to fourfold greater in the spleen extracts of animals receiving transformed bone marrow than in controls (Cline et al. 1980).

F. Transformation of Mouse Bone Marrow with HSV-tk Genes

The strategy of the experiments was similar to that employed for selection of expression of the DHFR gene. Mouse bone marrow cells with the T6T6 chromosomal marker were treated in vitro with a calcium microprecipitate of the HSV-tk gene. The treated marrow was mixed in a ratio of 1:1.5 with "mock" transformed CBA/Ca marrow cells lacking the chromosomal marker. Karyotype analysis was used to determine whether there was a predominance of T6T6 marked cells indicating successful gene therapy. As shown in Table 2, high levels of the T6T6 karyotype were frequently observed in the treated animals and occasionally in untreated animals, but never in controls which received mixtures of Ca and T6T6 marked cells in which *both* cell types had been mocked transformed. In selected cases where T6T6 predominance was observed, DNA was extracted and then digested with appropriate restriction enzymes and subjected to Southern blot analysis in order to test for the presence of HSV-tk gene sequences. HSV-tk gene sequences were shown to be present, confirming the success of the transformation procedure (Mercola et al. 1980).

Table 2. Karyotype analysis of bone marrow cells of irradiated CBA/Ca mice receiving A 1:1.5 mixture of CBA/T6 marrow transformed with HSVtk gene and "mock" transformed CBA/Ca (Ref. 2)

Recipient	Period with Mtx (days)	Karyotype (% T6)
Primary 1	0–32	74
Primary 2	0–47	84
Primary 3	None	35
Controls (20)	0–90	38 ± 10

Pyrimidine kinase assays showed that the spleen extracts from transformed animals contain the expected unique enzyme activity which can convert ^{125}I dC to ^{125}I dCMP, and activity not seen in extracts similarly prepared from normal mice (Salser et al., to be published).

We concluded that the HSV-tk gene confers a proliferative advantage on mouse bone marrow cells in the presence and sometimes in the absence of selective drug therapy with MTX. This may indicate that the salvage pathway for DNA synthesis is somewhat limiting to the growth of normal bone marrow cells and that cells with an enhanced salvage pathway capacity have an advantage during repopulation of the irradiated bone marrow.

G. Discussion

We have shown the insertion of genes into the bone marrow of living mice is feasible. Because cells which have been altered in a desired way may have a proliferative advantage, they may eventually constitute the majority of the cells in the bone marrow. We have also shown that these procedures can be used to introduce a gene such as that coding for human beta globin, which by itself would confer no selective advantage on the recipient cells (Salser et al., to be published).

It is our hope that such gene insertion techniques may eventually be used to treat a variety of human diseases more effectively. The hemoglobinopathies and some hematopoietic malignancies are natural targets for such an approach.

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