IN VITRO STUDY ON THE CLONING AND TRANSDUCTION OF HUMAN O^6-METHYLGUANINE-DNA-METHYLTRANSFERASE cDNA INTO HUMAN UMBILICAL CORD BLOOD $CD34^+$ CELLS

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ABSTRACT

Objective: To explore whether human umbilical cord blood hematopoietic progenitor cells transduced with human O^6-methylguanine-DNA-methyltransferase (MGMT) gene could increase resistance to 1,3-Bis(2-Chloroethyl)-1-Nitrosourea (BCNU). Methods: The cDNA encoding the MGMT was isolated by using RT-PCR method from total RNA of fresh human liver, the fragment was cloned into pGEM-T vector and further subcloned into G1Na retrovirus vector. Then the G1Na-MGMT was transduced into the packaging cell lines GP+E86 and PA317 by LipofectAMINE. By using the medium containing BCNU for cloning selection and ping-ponging supernatant infection between ecotropic producer clone and amphotropic producer clone, high titer amphotropic PA317 producer clone with the highest titer up to 5.8×10^5 CFU/ml was obtained. Cord blood $CD34^+$ cells were transfected repeatedly with supernatant of retrovirus containing human MGMT-cDNA under stimulation of hemopoietic growth factors. Results: The retrovirus vector construction was verified by restriction endonuclease analysis and DNA sequencing. PCR, RT-PCR, Southern Blot, Western Blot and MTT analyses showed that MGMT drug resistance gene has been integrated into the genomic DNA of cord blood $CD34^+$ cells and expressed efficiently. The transgene cord blood $CD34^+$ cells conferred 4-folds stronger resistance to BCNU than untransduced cells. Conclusion: The retrovirus vector-mediated transfer of MGMT drug resistance gene into human cord blood $CD34^+$ cells and its expression provided an experimental foundation for gene therapy in clinical trial.

Key words: MGMT gene, Gene clone, Retrovirus vector, Gene therapy, Hematopoietic stem cell, Cord blood

Myelosuppression is often a dose-limiting side effect of chemotheropeatic drugs in the treatment of cancer. Transducing resistance genes into hematopoietic precursors that confer protection against the hematotoxicity of anticancer agents has been proved to reduce myelosuppression. In this regard, efforts have been made to potentiate the resistance conferred by this method. Chemotheraopeatic drugs such as alkylating agents (temozolomide, BCNU, mitozolomide, chlorozotocin and rocarbazine) are cytotoxic as a consequence of forming O^6-alkylguanine DNA adducts. O^6-methyguanine-DNA-methyltransferase (MGMT) mediates resistance to alkylating agents by an irreversible covalent transfer of an alkyl group from the O^6 position of guanine to a cysteine residue within its active site. Resistance to these agents is principally due to the action of MGMT and expression of this protein protects otherwise sensitive cells against the toxic, mutagenic, clastogenic and carcinogenic effects of O^6-alklguanine (O^6-alkG), which is the primary DNA lesion of biological consequence generated by the O^6-alhylating agents.[1,2] Additionally, the clinical use of O^6-alhylating agents is associated with a risk of therapy-related malignancy.[3] Human hematopoietic progenitors have low level expression in the DNA repair enzymes, retroviral-mediated transduction has been successful in increasing the expression of human MGMT in murine hematopoietic progenitors and providing enhanced resistance to BCNU treatment.[4]

To examine the feasibility of this strategy, we have isolated human O^6-methylguanine-DNA-methyltransferase (MGMT) gene by using RT-PCR

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method from total RNA of fresh human liver, and constructed G1Na-MGMT retrovirus vector. Transduced human cord blood 34^+ cells by using the lipofectAMINE-mediated method. To explore whether transgene cells could increase resistance to BCNU.

MATERIALS AND METHODS

Construction of Vectors

The human MGMTcDNA was synthesized by reverse transcriptase chain reaction (RT-PCR) from a patient with cholelithiasis liver tissue cells. The primers for MGMT are 5'AAA, ATG GAC AAG GAT TGT GAA A3' and 5' CAT CCG ATG CAG TGT TAC ACG 3'. These primers gave a PCR product with a size of 684bp. The MGMT-cDNA was cloned into pGEMT vector (Promage Company) and confirmed by DNA sequencing (ABL PRISM TM 377DNA Sequencer). The MGMT-cDNA was subcloned into the G1Na retroviral expression vector (Kindly provided by Dr. Lu Da-ru, Fudan University) at the Not I cloning site in the sense orientation, vector plasmid made in this study was G1Na-MGMT, and was verified by restriction endonuclease analysis. The schematic structures of the retroviral expression vector is presented in Figure 1.

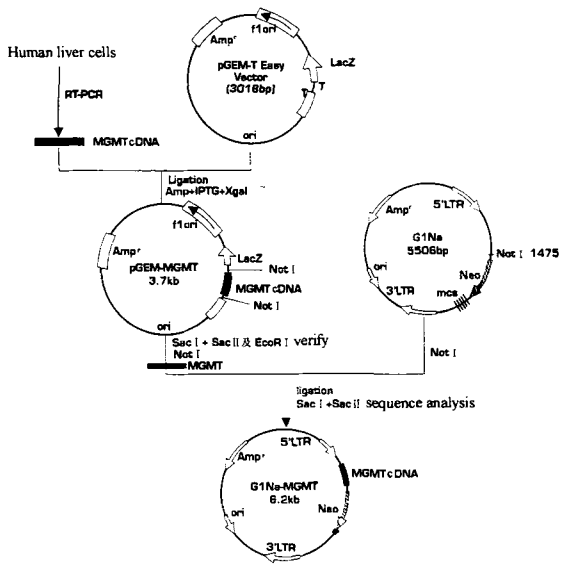

Fig. 1. The construction of retroviral vector G1Na-MGMT

Cell Lines and Cell Culture

The amphotropic retrovirus packaging cells PA317, the ecotropic packaging cells GP+E86, and the mouse fibroblast NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco/BRL) with 10% newborn calf serum. All cells were cultured at 37°C with 5%CO_2 in humidified atmosphere.

Establishment of Amphotropic Viral Producer Cells

To generate MGMT retrovirus, the PA317 and GP+E86 packaging cell lines were transferred with G1Na-MGMT using LipofectAMINE(GIBCO/BRL) followed to select with G418 (8mg/ml). To increase viral titer, a modified supernatant "ping-pong" method[5] was used and the packaging cells GP+E86 or PA317 were selected by vincristine (VCR) (10~200ng/ml) and/or BCNU (10-50μm/L. After transfected with this successfully constructed vector that carried MGMT drug resistance gene, the titer was calculated by transducing NIH 3T3 cells and the replication-competent retrovirus (RCR) was detected by nested PCR analysis of env gene.

Transfection of G1Na-MGMT Retroviral Vector into $CD34^+$ Cells

Cord blood $CD34^+$ cells were enriched with a high-gradient magnetic cell sorting system (MACS) and then transfected with supernatant of retrovirus containing human MGMT cDNA. $CD34^+$ cells were prestimulated for 48hr in presence of FL (100ng/ml), SCF(100ng/ml), IL-3(50units/ml), IL-6 (100units/ml). Retrovirus-containing supernatant was changed double a 24-hr interval, five days after the transgene cells were harvested.

Analysis of Trangene $CD34^+$ Cells by PCR Assay

The integration of MGMT gene in transduced $CD34^+$ cells genomic DNA was analyzed by PCR as described,[6] the MGMT gene specific primers as described above and PCR were performed on an DNA thermalcycer (Gene Amp9600, Perkin Elmer). The products were run on a 1.5% agarose gel and the specific fragments were detected after ethidium blomide staining under an UV light.

Southern Blot Analysis Genomic DNA

The PCR products were separated on 1.5% agarose gel after denaturing and neutralization steps, the DNA was transferred by capillary blotting onto a Hybond-N nylon membrane and covalently crosslinked to the membrane by ultraviolet light and backed at 120°C for 20minutes. The blots were hybridized overnight at 65°C with constant agitation

using ^{32}P-labeled MGMT-cDNA and washed three times in room temperature before being autoradiographed. Exposure to X-ray film was performed using two intensifying screen at -70°C.

Reverse Transcription (RT)- PCR Analysis

To investigate the expression of MGMT-mRNA in transducegene CD34$^+$ cells, total RNA was extracted by using the Trizol reagent purification (GIBCO-BRL), the cDNA synthesis was carried out with 200 U Moloney mouse leukemia virus-reverse transcriptase (Life Technologies) at 37°C for 1hr. After cDNA synthesis, the full-length MGMT transcript was amplified with MGMT gene specific primers as described above.

Western Blot Analysis Transducegene CD34$^+$ Cells

Western blotting was performed with cell extracts resolved by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) (12%polyacrylamide) as described previously.[7] Proteins were transferred onto nitrocellulose membranes, the bolted membranes were blocked with 5% dry milk in TBS buffer and then probed for 1 hr with the monoclonal antibody MT3.1 (1:1000) (NEOMarker) which is specific for human cellular MGMT (for detecting Human alkyltansferase). After three 10 minutes washed with TBS-Tween20 (0.05%), the blots were incubated with secondary antibody (antimouse horseradish peroxidase [HRP]-labeled anti-IgG) for 1 hr. Antibody binding was visualized by enhanced chemiluminescence (ECL) (Amersham Life Science), According to the manufacturer's instructions.

Chemosensitivity Assay

The drug resistance of the cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-dipheny-kterazoliumbromide (MTT assay) (Sigma). Three thousand cells, in a volume of 100 μl were plated in 96-well plates and the tested drugs were added at varying concentrations. After 3–4days, the number of viable cells was determined by colorimetric assay, and the IC_{50} and index of resistance were calculated.

RESULTS

Retroviral Vector Construction and Generation of Amphotropic Producer Cell Lines

The retrovirus vector pGEM-T-MGMT/G1Na-MGMT was constructed successfully and was verified by DNA sequencing and restriction endonuclease anlysis in Figure 2. The purified expression vector was transfected into ecotropic GP+E86 cells mediated by LipofectAMINE. The resistant colonies were yielded by selecting with BCNU (10 μm/L), and the supernatants were harvested with titer of 1.6×10^5 CFU/ml for transduction. To generate high titer amphotropic MGMT retrovirus, PA-317 cells were infected with six rounds of exposure to supernatants from GP+E86 cells. After continuous selecting with 20 μm/L BCNU, we have obtained PA-317/MGMT producer cells with a titer of 5.8×10^5 CFU/ml assayed on NIH 3T3 cells. By using nested PCR analysis, wildtype retrovirus env gene transfer was not found in G1Na-MGMT-containing retrovirus transduced cells, indicating no helper virus presence in this system.

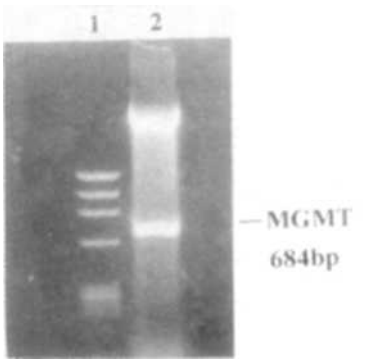

Fig. 2. Restriction endonuclease analysis of re-combinatant pGEM-T-MGMT digestion wih NotI 1Φ× 174 DNA/HaeIII Marker; 2 pGEM-T-MGMT/NotI

Integration and Overexpression of MGMT Transgene in Human Cord Blood CD34$^+$ Cells

Integration of MGMT provirus was verified by PCR with specific primers in CD34$^+$/G1Na-MGMT cells, which produced 684bp amplification bands. In addition, Southern Blot using ^{32}p-labeled MGMT-cDNA showed evidence of proviral integration. MGMT-mRNA expression in CD34$^+$ cells transduced with the vector was detected by RT-PCR (Figure 3).

Sensitivity to Cytotoxic Drugs

To examine the effectiveness of G1Na-MGMT vector, we determined the extent of drug resistance in MGMT gene transfected packaging cells and the human cord blood CD34$^+$ cells transduced with the vector. The research results shown that the mean BCNU IC_{50} of transgene CD34$^+$ cells was 16 μm/L, and was 4-flods higher resistance than that untransduced CD34$^+$ cells.

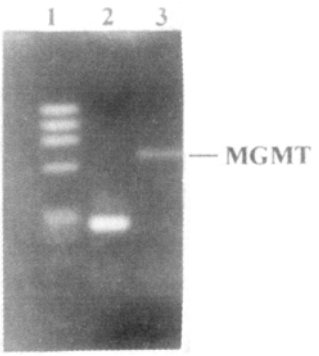

Fig. 3. Expression of MGMT mRNA detected by RT-PCR in umbilical cord blood $CD34^+$ cells transduced wihG1Na-MGMT retroviral vector.
1. Φ× 174DNA/HaeIII Marker; 2. β-actin; 3. $CD34^+$ cells/G1Na-MGMT

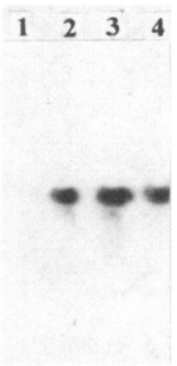

Fig 4. Southern Blot analysis MGMT gene integration in transducegene $CD34^+$ cells
1. $CD34^+$ cells untransduced; 2, 3, 4. $CD34^+$ cells transduced with G1Na-MGMT

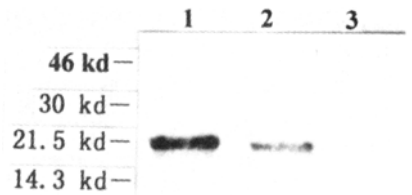

Fig. 5. Western Blot analysis of AGT protein expression from PA317 and $CD34^+$ cells
1. PA317 cells transduced wihG1Na-MGMT
2. $CD34^+$ cells transduced wih G1Na-MGMT
3. $CD34^-$ cells untransduced

DISCUSSION

The occurrence of drug resistance phenotype is the major reason for the failure of chemotherapy in cancer patients. Bone marrow suppression is the main dose-limiting toxicity of many clinically useful chemotherapeutic drugs. Standard treatments for this complication involve the use of transfusion support, hematopoietic cytokines, pharmacological rescue and bone marrow transplantation. These measures do not eliminate chemotherapy-induced hematopietic suppression nor do they confer long-term multilieage protection from multiple cycles of chemotherapy. A mumber of drug resistance genes have been identified that may be useful in gene therapy approaches to ameliorate chemotherapy toxicity. Hematopoietic tissue is the most suitable target for drug resistance gene therapy. The introduction of chemoresistance genes into hematopoietic cells may offer a way to overcome these limitations. Clinical trials of the MDR1 gene transferring into normal hematopoietic precursors have already started.[8,9]

BCNU is one of the most commonly used alkylating agents in chemotherapeutic regimens because it has a broad anti-tumor spectrum, a low risk of inducing chemoresistance, and a limited hematopoietic toxcity. Its use in high-dose regimens, in association with hematopoietic growth factors, has led to improve therapeutic responses. Our study was aimed at directly testing whether the overexpression of the MGMT gene could induce BCNU resistance as a basis for the eventual use of MGMT in gene therapy strategies *in vivo*. For this reason, we have cloned a full-length MGMTcDNA and used retroviral vectors to transduce it into human cord blood $CD34^+$ cell which were then tested for resistance to BCNU. The preliminary results of PCR, Southern Blot, PT-PCR, Western Blot and MTT analysis indicated a successful integration of MGMT gene in the genomic DNA of these transduced $CD34^+$ cells and efficiently expressed. The expression level of MGMT in transduced $CD34^+$ cells were high enough and showed 4-folds drug resistance to BCNU in comparison with untrasduced cells. In addition, the experiment of gene transfer safety was performed and replication-competent virus could not be found in the supernatant of the transferred NIH3T3 and K562 cells by nested PCR and rescue assay. The results suggest that retroviral vector system is effective and safe.

Transfer of drug resistance genes for the chemoprotection of normal hematopoietic cells is a promising strategy for gene therapy of cancer. Characteristics required for candidate drug resistance genes are as follows: the drug is effective against the patient's tumor, high-dose administration of the drug

does not cause severe toxicity to other tissues, efficient introduction of the drug resistance gene results in reasonably high degrees of drug resistance in hematopoietic cells, and expression of the gene in hematopoietic cells does not disturb normal function. In this work, we developed retroviral vector system satisfies all of the above criteria. These data suggest the possibility of using retroviral-mediated drug resistant gene transfer for protection against the myelosuppression caused by chemotherapy.

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