Acta Medica et Biologica
Vol. 43, No. 4, 217-222, 1995

Augmentation of Gene Transfer Efficiency into Human Hematopoietic Stem Cells by the Spin Transduction Method

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Received May 31 1995; accepted August 29 1995

Summary. **In order to improve the efficiency of gene transduction into human hematopoietic stem cells by retrovirus vectors, we conducted an *in vitro* study to determine the optimal conditions. Cells employed as the target were K562, a human myeloblastoid cell line. Retrovirus vectors used were LNL6 and GlNa40, both carrying the Neo^R (Neomycin-resistant) gene as a genetic marker. LNL6 was provided by Genetic Therapy Inc. (Gaithersburg, MD, U.S.A.) as culture supernatants of the producing cells; GINa40 was of the supernatants alike and their concentrates from the same Inc. Transduction efficiency varied from 1.0% to 59.1% depending upon the factors and procedures in the experiments, which comprised MOI (multiplicity of infection) values, the duration of exposure of cells to the vectors, and conduct/non-conduct of centrifuge of cells during the exposure. An optimal transduction was achieved by daily supplementation up to three days of the vectors to cells, together with a centrifuge of the cells at 2,500 rpm for 90 min during their exposure to the vectors. Along with the Neo^R gene transduction, a new assay system was introduced as a related matter of importance.**

Key words—retrovirus vectors, spin transduction, Neo^R gene, hematopoietic stem cells, PCR.

INTRODUCTION

The efficiency of foreign gene transduction into human hematopoietic stem cells is a factor directly limiting the outcome of clinical gene marking and gene therapy. Retrovirus vectors are now known for their gene transduction capability. However, when they are applied to human hematopoietic stem cells enriched with CD34$^+$ population, the efficiency of transduction remains far removed from the desired extent.[1-3] Also, the retrovirus vectors, as employed for the gene transfer into prulipotent stem cells (CFU-S: colony-forming unit in spleen), are markedly hindered in the rate of transduction, probably due to the Go phase refractoriness in most of the recipient cell population.[4] Thus, even with the retrovirus vectors, further improvement of the gene transduction efficiency is needed when the hematopoietic stem cell lineages are targeted.

In this study, we investigated experimental conditions for improving the gene transduction efficiency into hematopoietic stem cells *in vitro*. Experiments were conducted by employing a human myeloblastoid cell line, K562 as target, coupling this with retrovirus vectors, LNL6 and GlNa40, both carrying Neo^R (Neomycin-resistant) gene as a genetic marker. Results, together with a new assay system for the Neo^R gene transduction, are described and discussed.

MATERIALS AND METHODS

Cells

Cells employed as target for gene transduction were K562, a human myeloblastoid cell line which was derived from a patient of CML (chronic myelogenous leukemia)-blastic crisis. Cells were grown as a suspension culture in plastic plates using RPMI-1640 medium supplemented with 10% heat-inactivated FBS (fetal bovine serum), 1% penicillin, and 1% streptomycin (growth medium, below) at 37℃ in 5% CO2 atmosphere.

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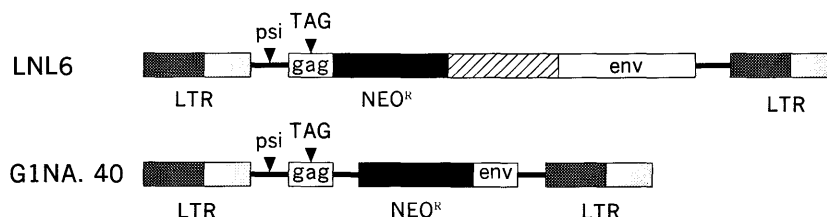

Fig. 1. Schematic diagrams of the retrovirus vectors used. LTR: a fused structure of Moloney murine leukemia virus and Moloney murine sarcoma virus LTRs; psi: packaging signal; gag: truncated gag gene; TAG: mutated staring codon in the gag gene sequence; Neo^R: bacterial Neomycin-phosphotransferase gene; env: truncated env gene.

Retrovirus vectors

Two types of retrovirus vectors, LNL6 and GlNa.40, both carrying Neo^R gene as a genetic marker (Fig. 1), were prepared by Genetic Therapy Inc. (Gaithersburg, MD, U.S.A.) as culture supernatants (with the growth medium constituents) of the producing cells, each giving potencies of 5×10^5 cfu (colony forming unit)/ml and 1×10^7 cfu/ml. Another preparation of GlNa. 40 vector was a concentrate (5×10^8cfu/ml) of the same vector material by ultracentrifuge. All these vector preparations were confirmed to be free of replication-competent helper virus by the S^+L^- complementation assay,[5] and then provided as clinical-grade materials in the form of frozen vector solutions at -70℃. Solutions were thawed immediately before use.

Retroviral transduction

This followed the method of spin transduction by Kotani et al.[6] For the trunk protocol, K562 cells were suspended in the vector solution at a density of 1×10^5 cells/ml using Falcon 3303 plastic tubes, supplemented with polybrene at 8 μg/ml, and centrifuged at 2,500 rpm for 90 min at room temperature for tight contact of cells and vectors. Resulting cell pellets were resuspended in a fresh growth medium at the same density for 72 hours' cultivation at 37℃ in 5% CO_2 atmosphere. At the end of cultivation, the cells were washed twice with RPMI-1640 medium by light centrifuge, then subjected to the assays for gene transduction. In the experiment, this protocol was modified for the centrifuge of cell-vector mixture by substituting daily repeations of it with fresh vector solutions for four days, so as to determine optimal conditions for the transduction.

Assay for Neo^R gene transduction

Cells to be assayed were plated in 35 mm plastic petridishes with 0.3% agar medium containing G418 at 0.5 mg/ml, then cultured for six to seven days at 37℃ in 5% CO_2 atmosphere. At the end of cultivation, cell colonies grown were counted as those that originated from the transduced cells with the Neo^R gene.

PCR (polymerase chain reaction)

To confirm the Neo^R gene transduction by PCR, individual cell colonies in the soft agar plates were randomly collected into separate microcentrifuge tubes using a capillary pipette, washed twice with the growth medium by light centrifuge, transferred into 20 μl of distilled water for swelling, then lysed by the addition of 10 μg Proteinase K, followed by serial heating at 95℃ for 10 min, 55℃ for one h, and 95℃ for 15 min. Resulting cell lysates underwent PCR amplification for the Neo^R gene according to 30 thermal cycles at 94℃ for one min, 64℃ for two min, and 72℃ for three min, with a pause of five sec between the cycles. Primers employed were Neo-1 (3′-CAAGATGGATTGCAGCAGG) and Neo-5 (5′-CCCGCTCAGAAGAACTCGTC).[7] Products obtained were confirmed for their complementarity to the Neo^R gene by electrophoretical analysis in 1.5% agarose.

RESULTS

Cumulative susceptibility of cells to retroviral transduction

Prior to the application of spin transduction, K562 cells were examined for the cumulative susceptibility to the retrovirus vectors during the course of cell culture. Cells pelleted by light centrifuge were suspended in a given vector solution at 1×10^5 cells/ml

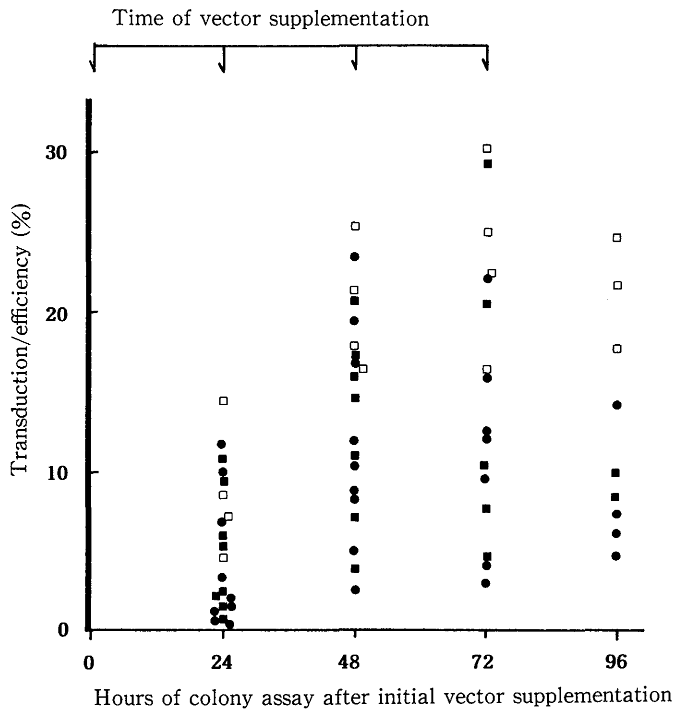

Fig. 2. Effects of daily supplementation of the vectors on the transduction efficiency. Applications of LNL6 (●) and GlNa.40 (■) followed the text; that of GlNa.40 with spin (□) followed the spin transduction in the MATERIALS AND METHODS. Transduction efficiency (%) was figured out according to the formula of: (colony number in the plate with G418/colony number in the plate without G418). ×70

for 24 hours' cultivation, followed by repeation of the same procedure every 24 h with fresh vector solutions for four days. Daily samples after the initial culture were assayed for the Neo^R gene transduction of cells by the G418 resistancy (Fig. 2).

The proportions of cells transduced increased up to three days of culturing and then declined at four days, giving respective maximal mean values of 12.1% and 14.8% by the LNL6 and GlNa.40 vectors.

Effects of MOI (multiplicity of infection) values upon transduction

A higher MOI value of the vector may also augment the efficiency of transduction. A range of MOI values of the vectors was examined for their effects upon the transduction, in combination with the daily supplementation of vectors to confirm the cumulative susceptibility of cells observed. The experiment followed the above method; Fig. 3 shows the results.

For both the vectors, LNL6 and GlNa.40, the efficiencies of transduction increased proportionally to the MOI values, and these MOI-responding kinetics were further amplified by the daily refreshing of the vectors for three days. The maximal values for the transduction reached were 29.6% by the LNL6 vector at a MOI of 5 and 21.5% by the GlNa.40 vector at a MOI of 50.

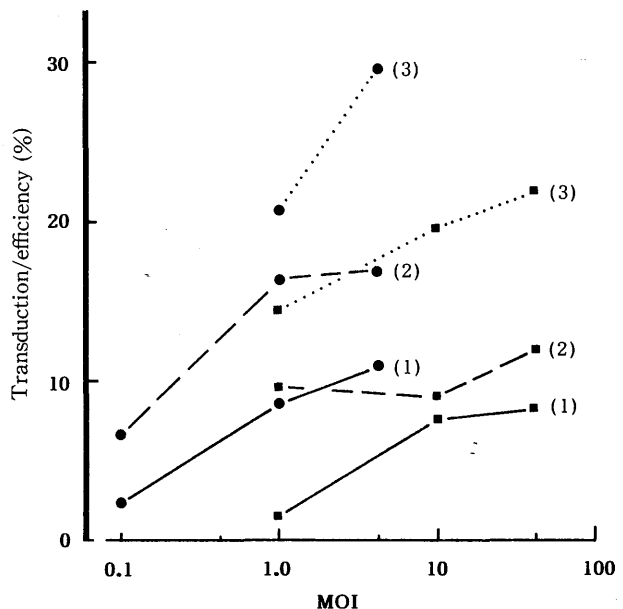

Fig. 3. Effects of MOI values on the transduction efficiency. ●: LNL6; ■: GlNa.40. Numerals in the parenthesis indicate cumulative days for vector supplementation. Percent values for the transduction efficiency were plotted as the respective means for three samples.

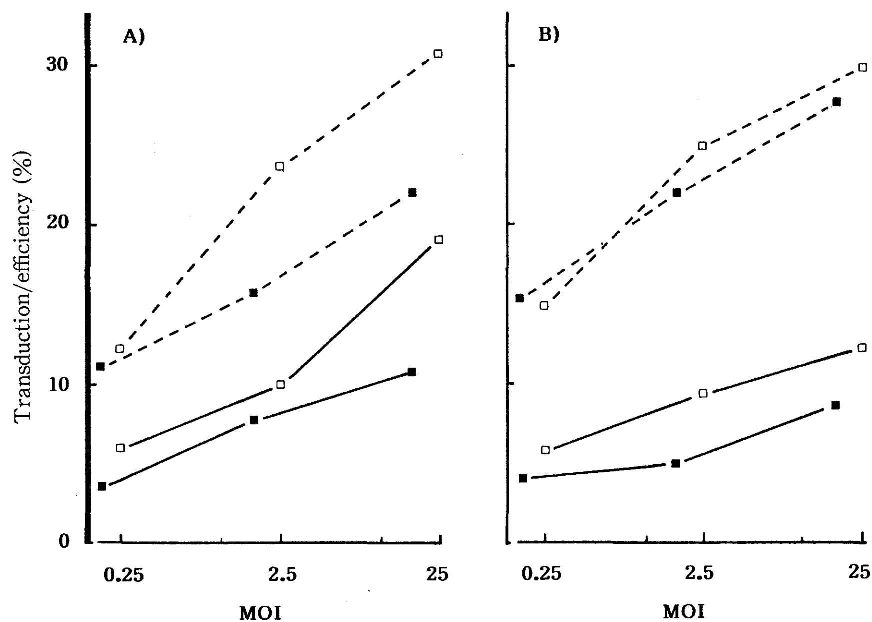

Fig. 4. Augmentation of the transduction efficiency by the spinning of the vector-cell mixture. ■: GlNa. 40 as culture supernatant; □: GlNa.40 as partially purified aliquot by ultracentrifuge. Applications of the vector materials without the spinning (——) followed the text; that with the spinning of vector-cell mixture (------) following the spin transduction in the MATERIALS AND METHODS. Cells were cultured for 48 h **A** or 72 h **B** after initial supplementation of the vectors. For other details, see Fig. 3.

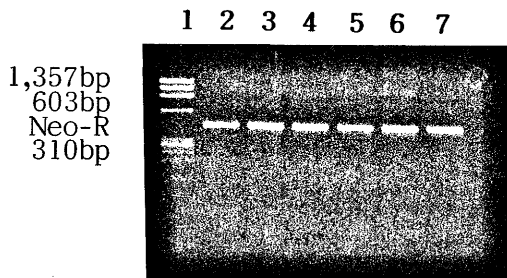

Fig. 5. Confirmation by PCR of the Neo^R gene transduction of cell clones resistant to G418. Lane 1: molecular marker; Lanes 2-7: PCR products from separate cell clones, those that originated randomly from the experiments of Fig. 2 to 4 and then grown in the G418-containing soft-agar plates.

Spin transduction

Under the experimental conditions, it was evident that the duration of exposure of cells to the vectors and the MOI values directly defined the extents of transduction. On the other hand, Kotani et al.[6] reported that the spinning of cell-vector mixture upon infection markedly enhanced the gene transfer. These factors and the procedure were both subjected to an integral analysis to determine their optimal conditions. The method followed the retroviral transduction in the MATERIALS AND METHODS, with two forms of GlNa.40 vector material, culture supernatant and partially purified vector aliquot of this as obtained ultracentrifuge being applied at varying MOI values to K562 cells, which thereafter were cultured for 48 and 72 h.

In the experiment for 48 hours' culture (Fig. 4A), the efficiencies of transduction were again proportional to the MOI values of the vectors. Of the two forms of vectors, the partially purified aliquot duly surpassed the culture supernatant in the rate of transduction at each MIOs, mostly at a MOI of 25.

In this experiment, however, the most marked finding was the promotion of transduction by the spinning of the vector-cell mixture. This promotion was observed at whole MOI values, giving the maximal transduction of 31.0% at a MOI of 25, as compared to 18.2% without spinning at the same MOI. A similar dose response to the MOI values, exceeding

Table. 1. Quantitation of the transduction efficiency of K562 cells with the Neo^R gene by colony and well assays

Vector	(MOI)	Transduction efficiency (%)			
		Colony assay		Well assay	
		Non-spin	Spin	Non-spin	Spin
GlNa.40*	(20.0)	6.0	21.0	31.3	59.1
	(2.0)	5.8	16.4	—	—
GlNa.40**	(25.0)	18.7	30.5	33.6	50.0
	(2.5)	9.4	23.0	—	—
	(0.25)	6.1	—	—	—

*: Prepared as culture supernatant; **: prepared as partially purified aliquot by ultracentrifuge. Applications of the vectors by "Non-spin" followed the text for the 72 hours' culture; that by "Spin" followed the spin transduction in the MATERIALS AND METHODS for the same duration of culturing. Colony assay followed the MATERIALS AND METHODS; the Well assay followed the DISCUSSION using micro-well culture plates containing G418 at 1.0 mg/ml in the fluid medium. For calculation of the percent values, see Fig. 2. Each value is tabulated as the means for three samples.

the partially purified vector aliquot, and promotion by the spinning of the vector-cell mixture, were both also observed for the transduction in the 72 hours' culture (Fig. 4B). The magnitude of promotion by the spinning in this experiment was larger by a factor of 2-5, again yielding the maximal transduction of 29.8% at a MOI of 25, in contrast to 12.6% without the spinning. This promotion of transduction by the spinning was confirmed as well in the experiment shown in Fig. 2. The spinning of the vector-cell mixture at the time of the supplementation of vectors considerably heightened the cumulative susceptibilities of cells throughout the range of assays (GlNa.40 with spin in Fig. 2).

Confirmation of Neo^R gene transduction by PCR

In the PCR amplification to confirm the Neo^R gene transduction, all of the six cell colonies that originated from random collections through the experiments and were grown in the G418-containing soft-agar plates, yielded identical products. The products in electrophoresis (Fig. 5, Lanes 2 to 7) were 460 bp in the molecular size and homogenous in the signal intensity, which matched well with the Neo^R gene complement estimated.

DISCUSSION

In this study, we attempted to find the *in vitro* conditions optimal for gene transduction into hematopoietic stem cells by retrovirus vectors. Cells employed as recipient were K562, a human myeloblastoid cell line. The retrovirus used were LNL6 and GlNa.40, both of which carried Neo^R gene as a genetic marker and were prepared as clinical-grade materials.[5] Conditions approved are thus directly applicable to clinical trials.

In vitro conditions that are influential upon the retrovirus vector-mediated transduction of hematocyte species are diverse. Cassel et al. reported on the Go phase refractoriness in the growth cycle of bone marrow stem cells.[3] In our observation, the efficiency of Neo^R gene transduction increased steadily up to three days by the continual culturing of cells together with daily supplementations of the vector solutions (Fig. 2). During the period of this culture, a part of cells could turn to the growing phase, shifting to become susceptible to the retrovirus vectors.

The efficiency of transduction increased proportionally to the MOI values of the vectors. The maximal rate of transduction attained in this study was 31.0% at a MOI of 25 with the spinning (Fig. 4A), and the maximal MOI of 50 yielded 22.3% of transduction without the spinning (Fig. 3). However, these maximal values were not those at the plateau levels of transduction. Theoretically, it is possible to further improve the rate of transduction by increasing the MOI values of vectors for infection.

Kotani et al.[6] were the first to report on the spinning of vector-cell mixture to promote gene transduction (spin transduction). In their report, cells employed were of mouse NIH3T3. We applied the procedure to a CD34-enriched population of human hematopoietic stem cells in a separate study and confirmed the promotion.[8] In this study, the procedure was adopted to a human myeloblastoid cell line, K562, giving a marked promotion of Neo^R gene transfer through a series of experiments. It should be worthwhile to apply the procedure to wider cell species of myelogenous and lymphatic origins to determine its total usefulness.

Taking the above results together, the integral protocol, which seems workable for clinical application, is to daily supplement vectors to cells up to three days, together with a light centrifuging of the vector-cell mixture at 2,500 rpm for 90 min upon the supplementation. For further refining of this trunk protocol, improvement of the vector materials and procedures may be desirable.

Cell colony formation in the soft-agar plates in the presence of G418 (Neomycin) is a usual assay for the Neo^R gene transduction of hematopoietic cell species. However, the drug, when combined with the agar constituents, may impede cell growth due to the unspecified cytotoxicity, eventually reducing the rate of transduction. This issue was examined by modifying the conventional protocol toward seeding cells first in the soft-agar plates, but voiding the drug, then transfering the cell colonies grown into micro-well culture plates containing the drug in the fluid medium for selective cell growth by transduction. Results obtained are given in Table 1 as an additional finding, where the maximal rate of transduction was markedly elevated by the modified protocol (well assay in the Table 1) to 59.1% from 21.0% by the conventional assay (colony assay in the Table 1) for the matched counterpart. Although this observation is separate from that on the vector-cell interaction, the investigation[9] is thought important for knowing the actual features of transduction in the quantitative aspect.

Acknowledgements. We sincerely thank Dr. H. Kotani and Dr. P. Tolstosher for their help with this study. This work was supported in part by a grant in aid for Cancer Research from the Gakushi-kai of the Niigata University School of Medicine in Niigata.

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