

Successful transfer of ADA gene in vitro into human peripheral blood CD34⁺ cells by transfecting EBV-based episomal vectors

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Abstract We report a novel non-viral system for transfecting human immature hematopoietic cells in vitro. Epstein-Barr virus (EBV)-based episomal vectors carrying human adenosine deaminase (ADA) gene cDNA were transfected by electroporation into human peripheral blood (PB) CD34⁺ cells. The transgene-specific mRNA were detected from 37 to 100% of CFU-c (colony forming unit in culture) colonies derived from the transfected cells. A two-fold increase in enzyme activity was also found. These results indicate the successful transfer and expression of genes in human immature hematopoietic cells using the EBV-based episomal vector system.

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Key words: Epstein-Barr virus vector; Hematopoietic progenitor cell; Gene therapy

1. Introduction

An efficient gene transfer system for human hematopoietic progenitor cells is required for gene therapy of hematopoietic diseases. Many of the systems currently available utilize the replication-incompetent retroviral vectors, while other recombinant viral vectors such as lentiviral vectors are also being developed [1,2]. We focused on plasmid vectors; if hematopoietic progenitor cells can be transfected with plasmid vectors by a simple method like electroporation, the system may be of practical value, so that gene therapy will become routine clinical work in the future. We engaged the Epstein-Barr virus (EBV)-based episomal vector, the plasmid vector carrying the EBV nuclear antigen (EBNA)-1 gene and the origin of plasmid replication (oriP), because this vector is very effective in transfecting human cells [3–5].

We have previously shown that the gene transfer/expression efficiency with the EBV-based episomal vectors was extremely high, when the plasmids were transfected by electroporation into lymphoid cell lines of human origin [6,7]. Various human primary cells were also successfully transfected with the EBV-based episomal vectors, by combining the plasmids with cationic liposome or hemagglutinating virus of Japan (HVJ) liposome [8]. Here, we transferred adenosine deaminase (ADA) gene into human peripheral blood (PB) CD34⁺ cells in vitro and estimated the transfection efficiency.

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Abbreviations: ADA, adenosine deaminase; CFU-c, colony forming unit in culture; EBNA, EBV nuclear antigen; EBV, Epstein-Barr virus; PB, peripheral blood

2. Materials and methods

2.1. Plasmid vectors

The ADA gene-expression vectors (Fig. 1A) are composed of (i) the human ADA gene cDNA located between the SR α promoter [9] and the SV40 poly(A) additional signal; (ii) EBV oriP [3]; (iii) the EBV EBNA1 gene [3] under the control of SR α promoter (pSES.ADA), cytomegalovirus (CMV) promoter (pCES.ADA), chicken β -actin promoter (p β ES.ADA), CAG promoter [10] (pCAGES.ADA) or SV40 promoter (pSVES.ADA); and (iv) the ampicillin resistant gene and the replication origin for *E. coli*. Another plasmid, pSES.CD8 α [8], possesses murine CD8 α cDNA located downstream of the SR α promoter (Fig. 1B).

2.2. PB CD34⁺ cells

Prior to the experiments, informed consent was obtained from 6 patients with malignancies (3 with non-Hodgkin's lymphoma, 1 with ALL (L2), 1 with small-cell lung cancer and 1 with Ewing's sarcoma) in complete remission. The cancer cells from all the patients were CD34⁻ (data not shown). The PB CD34⁺ cells were obtained from these patients as described [11–13]. Briefly, patients were administered subcutaneously with 5 μ g/kg of recombinant human G-CSF (granulocyte-colony stimulating factor) after high dose chemotherapy. Using a CS-3000 blood cell separator (Fenwal, Deerfield, IL, USA), peripheral mononuclear cells were collected on 2–3 consecutive days during the recovery phase from myelosuppression. The CD34⁺ cells were enriched using a magnetic cell sorting system (Miltenyi Biotech, Gladbach, Germany). More than 95% of the recovered cells were positive for CD34.

2.3. Transfection, cell culture and RT-PCR analysis

Electroporation was performed as described previously [7] using a Gene Pulser apparatus (Bio-Rad). After the transfection, the cells were cultured in Iscove's modified Dulbecco's medium (IMDM) (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% FCS, 10 μ g/ml of IL-3 (Gibco-BRL), 10 U/ml of IL-6 (Gibco-BRL) and 10 U/ml of stem cell factor (SCF) (Pepro Tech, Rocky Hill, NJ, USA). Three, 6 or 13 days later, RNA was extracted and treated with DNase I (Boehringer Mannheim, Germany), followed by reverse transcription (RT) by Superscript II (Gibco-BRL). PCR was then performed using human β -actin gene-specific sense (5'-GTGCT-ATCCCTGTACGCCTC-3') and antisense (5'-AGTCCGCCTAGAGCATTGTG-3') primers, murine CD8 α gene-specific sense (5'-TCGCTGAACCTGCTGCTGCT-3') and antisense (5'-GTTGTAGC-TTCCTGGCGGTG-3') primers, or a combination of human ADA gene-specific sense (5'-TTACTGATGCTCTGACCCC-3') and SV40 poly(A)-specific antisense (5'-GCAGTAGCCTCATCACT-3') primers. The reactions were performed for 30 cycles (92°C for 1 min, 56°C for 1 min and 72°C for 2 min) and the PCR products were electrophoresed on a 1.5% agarose gel and stained by ethidium bromide.

2.4. PCR analysis from colony forming unit in culture (CFU-c) colonies

Cells were seeded into 0.9% methylcellulose/IMDM supplemented with 3 U/ml of erythropoietin, 10 ng/ml of IL-3, 10 ng/ml of GM-CSF and 50 ng/ml of SCF. Sixteen days later, single CFU-c colonies were picked up under a phase-contrast microscope, heated for 1 min at 90°C and then incubated for 1 h at 37°C with 10 U of DNase I

(Boehringer) and 0.3 U of RNase inhibitor (Toyobo, Osaka, Japan). After boiling, cDNA synthesis and a first PCR were performed with the Definitive RT-PCR KIT (Biotech International), using murine CD8 α gene-specific sense (5'-TCGCTGAACCTGCTGCTGCT-3') and antisense (5'-GTTGTAGCTTCTGGCGGTG-3') primers, or a combination of ADA gene-specific sense (5'-TTACTGATGCTCC-TGACCCC-3') and SV40 poly(A)-specific antisense (5'-GCAGTAG-CCTCATCACTACT-3') primers. The reactions were performed with a thermal cycler (TaKaRa, Tokyo, Japan) using the following cycle profiles: 60°C for 30 min, 94°C for 2 min and 60°C for 1 min, followed by 60 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The PCR products were subjected to a second PCR with Ex Taq DNA polymerase (TaKaRa) using murine CD8 α gene-specific sense (5'-CACAGGCACCCGAACTCCGA-3') and antisense (5'-TC-TGACTAGCGGCCTGGGAC-3') primers, or a combination of ADA gene-specific sense (5'-GTCCATTCTGCACACACGT-3') and SV40 poly(A)-specific antisense (5'-TGCTCCATTCATCA-GTTCC-3') primers.

2.5. Enzyme assay

The ADA enzyme assay was performed as described [14,15] with slight modifications. Briefly, aliquots of cell lysate were added to a reaction mixture (100 mM Tris-HCl, pH 7.5, 500 μ M adenosine, 33 μ M [8-¹⁴C]adenosine (2.2 GBq/mmol)) in a total volume of 20 μ l. After incubation at 37°C for 30 min, the reactions were subjected to descending paper chromatography. Radioactive counts in adenosine, inosine and hypoxanthine were measured by a BAS 2000 imaging analyzer (Fuji Photo Film, Tokyo, Japan) and the amount of inosine and hypoxanthine generated from adenosine (pmol) was calculated by the formula: $10^4 \text{ (pmol)} \times (I+H)/(I+H+A)$, where 10^4 pmol is the amount of adenosine initially added to the reaction and I, H, and A are the radioactivities for inosine, hypoxanthine and adenosine, respectively.

3. Results

3.1. Expression of the marker gene mRNA in CD34⁺ cells transfected with EBV-episomal vector

We first examined whether CD34⁺ cells transfected with the EBV-based vectors expressed the transgene mRNA. RNA was extracted from CD34⁺ cells which had been transfected with pSES.CD8 α and cultured for 3 days with IL-3, IL-6 and SCF. RT-PCR was performed using mouse CD8 α gene-specific primers. As shown in Fig. 2A, the transfected cells expressed the marker gene mRNA on day 3 post-transfection. The intensities of the bands correlated with the voltage at which the cells were pulsed. In contrast, no expression was observed in cells which had been given the plasmid DNA but pulsed. To investigate if the expression would continue for longer culture

Table 1
RT-PCR analyses of single colonies derived from transfected PB CD34⁺ cells

Experiment	Plasmid	Primers (sense/antisense)	Colonies			
			CFU-GM	CFU-mix	BFU-e	CFU-Mk
1	pSES.ADA	ADA/SV40 ^a	6/8 ^b	3/8	3/8	ND ^c
		CD8 α /CD8 α ^d	0/8	0/8	0/8	ND
2	pSES.ADA	ADA/SV40	6/8	4/8	4/8	ND
		CD8 α /CD8 α	0/8	0/8	0/8	ND
3	pSES.ADA	ADA/SV40	4/8	5/8	4/8	ND
		CD8 α /CD8 α	0/8	0/8	0/8	ND
4	pSES.CD8 α	ADA/SV40	0/8	0/8	0/8	ND
		CD8 α /CD8 α	8/8	4/8	3/8	ND
5	pSES.CD8 α	ADA/SV40	0/8	0/8	0/8	0/4
		CD8 α /CD8 α	8/8	6/8	6/8	3/4

PB CD34⁺ cells were transfected with indicated plasmids, and CFU-c colonies derived from the cells were individually picked up and analyzed by RT-PCR.

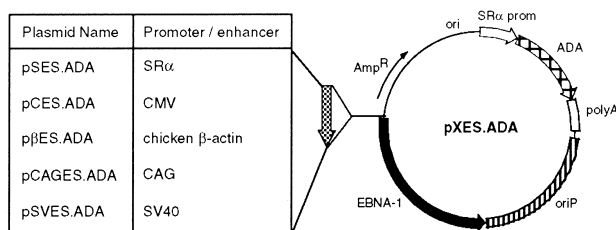
^aThe combination of human ADA gene-specific sense primer and SV40 poly(A) specific antisense primer was used.

^bNumber of positive colonies/number of colonies tested.

^cND: Not determined.

^dThe mouse CD8 α gene specific sense and antisense primers were used.

A



B

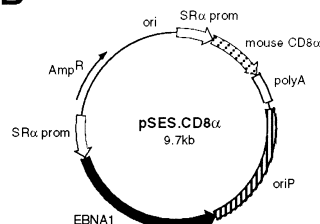


Fig. 1. Plasmids used in this study. Maps of pXES.ADA (A) and pSES.CD8 α (B) are shown. Prom: promoter; polyA: SV40 poly(A) additional signal.

periods, CD34⁺ cells were transfected with pSES.ADA, pCAGES.ADA or pSES.CD8 α and cultured for 6 or 13 days. Cells transfected with pSES.ADA or pCAGES.ADA expressed the transgene mRNA on both day 6 and 13, while non-transfected or pSES.CD8 α -transfected cells showed no evidence of mRNA expression (Fig. 2B). These results indicated the successful transfection, expression and maintenance of the marker genes in the CD34⁺ cells.

3.2. Transfection efficiency into CFU-c by the EBV-based episomal vectors

Next we investigated whether immature cells with colony forming activity could be transfected using this system. PB CD34⁺ cells transfected with pSES.ADA or pSES.CD8 α were allowed to form CFU-c colonies and single CFU-c colonies were picked up and individually analyzed to estimate the transfection efficiency into each type of CFU-c. Fig. 2C shows the results of a representative experiment. In this case (when

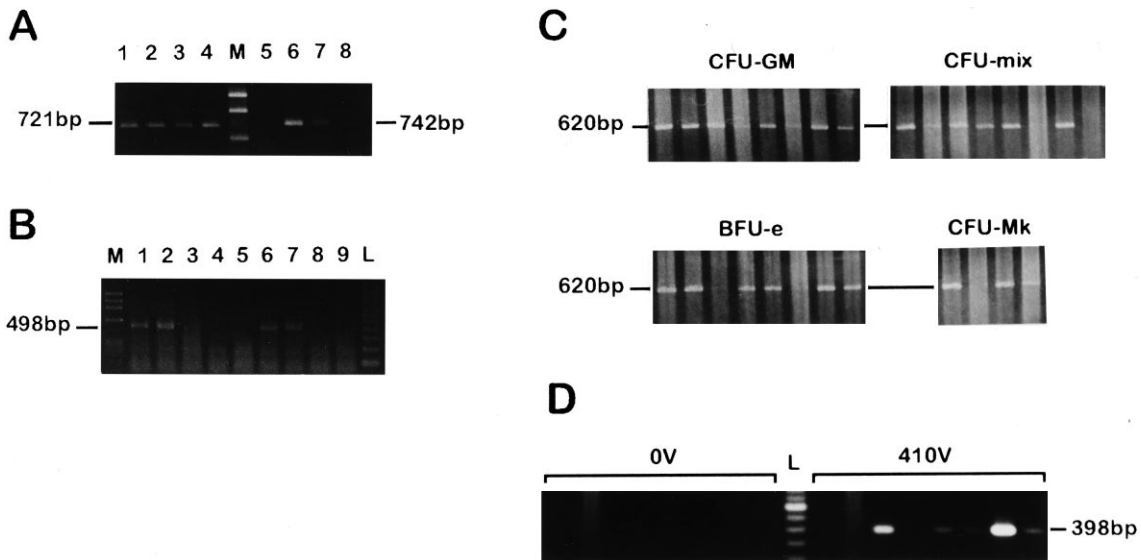


Fig. 2. RT-PCR analyses of transfected PB CD34⁺ cells and single CFU-c colonies derived from them. A: PB CD34⁺ cells were mixed with pSES.CD8 α and pulsed at 410 V (lanes 2 and 6), 370 V (lanes 3 and 7), 330 V (lanes 4 and 8) or, as control, 0 V (lanes 1 and 5). After 3 days of culture, RNA was extracted and subjected to DNase I treatment. RT-PCR was performed with primers specific for human β -actin gene (lanes 1–4) or murine CD8 α gene (lanes 5–8). B: Cells were mixed with pSES.ADA (lanes 1, 4 and 6), pCAGES.ADA (lanes 2 and 7) or pSES.CD8 α (lanes 3, 5, 8 and 9), and pulsed at 410 V (lanes 1–3, and 6–8) or 0 V (lanes 4, 5 and 9). After 6 (lanes 1–5) or 13 (lanes 6–9) days of culture, RNA extraction, DNase I treatment and RT-PCR were performed. ADA gene-specific sense primer and SV40 poly(A)-specific antisense primer were used. C: PB CD34⁺ were transfected with pSES.CD8 α and allowed to form CFU-c colonies. Sixteen days later, single CFU-c colonies were picked up and after extraction and DNase I treatment RT-PCR was performed using murine CD8 α gene-specific primers. Each lane represents single colonies of the indicated type. D: PB CD34⁺ were mixed with pSES.ADA and pulsed at 410 V (right 8 lanes) or 0 V (left 8 lanes). Sixteen days later, single CFU-mix colonies were picked up and after extraction and DNase I treatment RT-PCR was performed with ADA gene-specific sense primer and SV40 poly(A)-specific antisense primer. Each lane represents single colonies. ϕ : ϕ X174 DNA, *Hae*III digested; L: 123-bp DNA ladder.

pSES.CD8 α was transfected), the murine CD8 α mRNA was detected in 8 out of 8 GM-CSF colonies, 6 out of 8 CFU-mix colonies, 6 out of 8 BFU-e colonies and 3 out of 4 CFU-Mk colonies. Similar results were obtained from 4 independent experiments, as summarized in Table 1. No band was seen when colonies derived from the pSES.ADA-transfected PBSC were analyzed with murine CD8 α -specific primers, and vice versa. To confirm the specificity of the PCR, we also analyzed the CFU-c colonies derived from PB CD34⁺ cells which had been mixed with pSES.ADA but pulsed. We did not detect any band in all samples tested (the representative data are shown in Fig. 2D).

3.3. Elevation in ADA activities in PB CD34⁺ cells transfected with the EBV-based episomal vectors carrying the ADA gene expression units

To analyze the transgene product at the protein level, we measured the ADA enzyme activity of PB CD34⁺ cells transfected with pXES.ADA. As shown in Fig. 3A and B, the ADA activity of the pSES.ADA-transfected cells was 1.4–2 times higher than that of the pSES.CD8 α -transfected cells (endogenous ADA activity). The degree of the elevation depended on the vector constructs, suggesting the importance of a promoter/enhancer for EBNA1 gene in transfection/expression efficiency (Fig. 3B). The elevation in enzyme activity was also observed in pooled CFU-c colonies derived from PB CD34⁺ cells transfected with pSES.ADA (Fig. 3C).

4. Discussion

In the present study, we found that human immature hem-

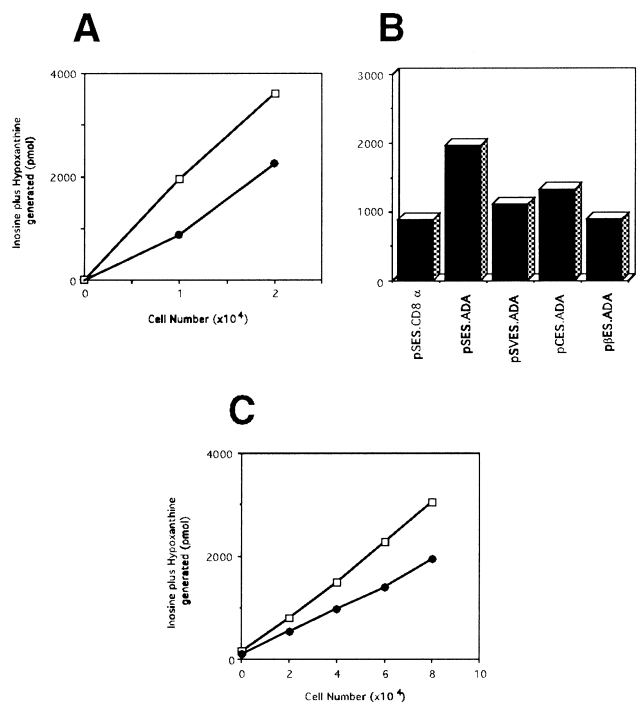


Fig. 3. ADA activity of the transfected cells. PB CD34⁺ cells were transfected with pSES.ADA (open squares in A and C), pSES.CD8 α (closed circles in A and C), or indicated plasmids (in B). Cells cultured for 3 days (A and B) or pooled CFU-c colonies derived from the transfected cells (C) were lysed and ADA enzyme activities were assayed as described in Section 2.

atopoietic cells could be successfully transfected in vitro with the EBV-based vector system. The transfection efficiency to CFU-c was estimated to be more than 37%. The expression of the transgene product was confirmed by the elevated ADA activity.

We transfected PB CD34⁺ cells by electroporation because it is a safe, easy, inexpensive and highly reproducible technique and thus well suited to ex vivo gene transfer. The cell viability is 50–70% after the electroporation and the number of CFU-c decreased in parallel (data not shown). Practically, the number of PB progenitor cells harvested from a donor is usually much higher than that transplanted to a recipient [16,17]. Recently, we have combined the EBV vector with other gene delivery systems, i.e. cationic liposome, HVJ liposome, and succeeded in transfecting human bone marrow cells [8]. This may indicate that the EBV episomal vector in combination with various delivery vehicles could be a useful tool for gene transfer into human hematopoietic cells.

Compared to bone marrow (BM) transplantation, PB stem cell transplantation has some advantages: ease of collection, rapid engraftment and less possibility of tumor cell contamination in the graft [16,18,19]. Thus, PB progenitor cells may be an ideal target for gene therapy for hematopoietic as well as malignant diseases.

It is known that the PB CD34⁺ cells contain higher proportion of lineage committed cells than BM CD34⁺ cells do. More than 98% of PB CD34⁺ cells prepared by our hands expressed CD38, as revealed by 2-color flow cytometric analysis (data not shown). We are planning to perform LTC-IC assay to examine if cells more immature than CFU-c can also be transfected by our system.

Recently, it has been revealed that the hematopoietic stem cells with long term repopulating activity belong to the CD34⁻ population [20–23]. Thus, this population should be regarded as the target for gene transfer. We are planning to transfect CD34 positive and negative cells and transplant them into NOD (non-obese diabetes)/SCID (severe combined immunodeficiency) mice, to examine the in vivo expression and long term maintenance of the transferred gene [23].

The EBV-based vectors are plasmid vectors, producing no infectious virus particles. The EBNA1 is the only viral protein encoded by the plasmid. Although the EBV is an oncogenic virus, it is reported that EBNA2, LMP (latent membrane protein)1 and other latent gene products play essential roles in B-cell transformation, while EBNA1 has no transformation activity [24–28]. On the other hand, it is well known that EBNA1 escapes recognition by host immune systems [29–32]. Thus, the EBV-based episomal vectors may be neither oncogenic nor immunogenic, although their safety must be confirmed by in vivo experiments.

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