Hematopoiesis & Growth Factors

original paper

haematologica 2001; 86:13-16
http://www.haematologica.it/2001_01/0013.htm

Efficient lentiviral transduction of primary human acute myelogenous and lymphoblastic leukemia cells

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Background and Objectives. Gene manipulation and cell vaccines represent innovative strategies to enhance the immunogenicity of cancer cells. We adopted a defective lentivirus derived from the human immunodeficiency virus (HIV)-1 backbone and carrying the enhanced green fluorescent protein (EGFP) gene to transduce primary human acute myelogenous leukemia (AML) and B-precursor acute lymphoblastic leukemia (ALL) cells.

Design and Methods. AML blasts were maintained with or without cytokines (stem cell factor, FLT3 ligand and interleukin 3) for 48 hours, and successively infected with two spin infection cycles. ALL blasts were cultured on a murine S17 stromal cell line.

Results. As regards AML cells, the efficiency of infection at 7 days varied from 8.4 to 37%. As confirmed by cell cycle analysis, cells were, in most of the cases, blocked in different phases of the cycle and did not proliferate during culture: the infection was therefore obtained in the absence of cell proliferation. In contrast, the maintenance of optimal cell viability was of fundamental importance for obtaining good infection levels. As regards ALL blasts, the percentages of infection after 3 days varied from 4.4 to 21%.

Interpretation and Conclusions. These preliminary data suggest that gene delivery into primary human AML and B-precursor ALL cells by an HIV-1 derived lentiviral vector could represent a strategy for engineering leukemic cells for use as cancer vaccines.

Key words: retroviruses, gene therapy, cancer vaccines, human leukemia

Actual therapeutic results obtained with standard protocols in acute myelogenous (AML) and lymphoblastic (ALL) leukemia strongly demand introduction of innovative strategies, including cell vaccines and gene manipulation. The use of irradiated autologous cancer cells transduced with genes encoding cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4, IL-12 or tumor necrosis factor-α (TNF-α) or co-stimulatory molecules (such as B7-1 and B7-2) could represent a valid approach to create efficient cell vaccines able to elicit anti-leukemia immunity.[1-5] Gene transduction has been traditionally achieved with Moloney-derived retroviral vectors, which have the advantage of stable expression but can only infect dividing cells. In most cases, however, the fresh leukemia samples from acute leukemia cannot be easily propagated in mass culture *in vitro*, even in the presence of growth factors.

Since human-immnodeficiency-virus (HIV)-based lentiviral vectors are believed to transduce non-cycling cells,[6,7] we have explored the feasibility of a gene transfer protocol in primary human AML (of different FAB origin) and B-precursor ALL cells, using a defective lentivirus derived from the HIV-1 backbone and carrying the enhanced green fluorescent protein (EGFP) gene under the phosphoglycerokinase promoter (pRRLsinhPGK-GFP-pre transfer vector).[6,8-10]

Design and Methods

Cells

AML and ALL cells were all derived from frozen samples of bone marrow aspirates obtained at diagnosis from pediatric patients admitted to our Pediatric Hematology Department: in all cases, the phenotypic analysis with standard appropriate monoclonal antibodies showed over 95% of leukemic cells in the tested populations (data not shown).

Infections

Human AML blasts were plated at 1×10^6/mL in a 12-well plate in Iscove's modified Dulbecco's medium

(IMDM) supplemented with 20% fetal calf serum (FCS), glutamine and gentamycin. The cells were maintained in the presence or absence of cytokines (CK) or growth factors (stem cell factor at 100 ng/mL, FLT3-ligand at 50 ng/mL and IL3 at 20 ng/mL)[11] for 48 hours at 37°C with 5% CO_2, and successively infected with two spin-infection cycles at 6-hours' interval. The HIV-1 derived pRRLsinhPGK-GFPpre transfer vector (10 μg/plate) was transiently transfected, by means of the calcium phosphate precipitation method, into the 293T epithelial cell line together with the packaging vector pCMVΔ8.74 (6.5 μg/plate) and the vescicular stomatitis virus (VSV)-env-encoding plasmid pMD.G for VSV pseudotyping (3.5 μg/plate): the 293T cell line was seeded (the day before the transfection) at 5×10^6 per 100 mm Petri dish in 10 mL of IMDM supplemented with 10% FCS, glutamine and gentamycin. All plasmids and cells were kindly provided by Dr. L. Naldini, Turin, Italy.[6,8] The spin-infection procedure consisted in centrifuging the cells at 1,800 rpm for 45 minutes at room temperature, placing the cells in contact with 1 mL of viral supernatant and then replacing the supernatant with 1 mL of fresh complete RPMI 1640 supplemented with 20% FCS, glutamine and gentamycin, as previously detailed.[6,8-10] In all the experiments the quality and the titer of viral preparations were always tested by concomitant infection of the T-lymphoblastoid human line CEM, as previously reported.[9,10] In the entire set of experiments the viral titer was around 1×10^7 infective particles/mL, which is equivalent to a multiplicity of infection (MOI) of 10.[4,5] MOI was calculated as the number of infectious units divided by the number of leukemic cells exposed to the vector.

The viability of leukemic cells was monitored in all cases by the trypan blue exclusion test before infection and after 3 and 7 days of subsequent culture. Cell-cycle status was also analyzed before infection with propidium iodide staining and FACS analysis according to standard procedures.[12] At the end of the infection, myelogenous blasts were returned to identical culture conditions adding fresh CK twice a week. EGFP expression was evaluated by flow cytometry 7 and, in some cases, 3 and 14 days after infection, adopting standard procedures of FACS analysis and commercial phycoerythrin (PE)-conjugated antibodies (Becton Dickinson).

Human ALL blasts were cultured at 1×10^6/mL in a 12-well plate on murine S17 stromal cell line to preserve leukemia cell viability using the above mentioned culture-medium:[13] the stromal cells were plated at 2×10^4/well and adopted for ALL culture at a confluence grade of 80%. The cells were analyzed by double immunofluorescence for the expression of CD19 and EGFP to exclude any stromal cells from the analysis: in this setting the flow-cytometry analysis was performed only after 3 days of infection due to the extreme fragility of these cells upon *in vitro* culture and the difficulty in maintaining a high cell viability; indeed the observation of over 50% dead cells after 5 days precluded the analysis at 7 days.

Results

In this study, primary human leukemic cells from a total of 8 AML patients and 4 B-precursor ALL pediatric patients were analyzed. It was not possible to propagate primary acute leukemic cells easily *in vitro* even in the presence of cytokines, growth factors or a feeder cell layer. As illustrated in Table 1, the FACS analysis of S/G2/M cycling cells performed 48 hours after thawing either in the presence or in the absence of CK showed no significant difference in the 5 AML cases. Moreover the cell number did not change significantly during culture, varying from $6\pm2.3\times10^5$/mL on the day of infection to $5\pm2.6\times10^5$/mL at day 3, to $5.3\pm1.6\times10^5$/mL at day 7 in the absence of CK, or to $5.8\pm2.9\times10^5$/mL at day 3 and to $7\pm2\times10^5$/mL at day 7 in the presence of CK.

In all the cases reported the cell viability exceeded 80% on the day of infection (data not shown). Three additional cases of AML (FAB M2, M3 and M4) and one case of ALL showed a very poor viability immediately after thawing and which decreased to less than 20% on the day of infection: the EGFP gene expression at day 3 was extremely low (below 1%, data not shown). These four cases were therefore excluded from the analysis.

In spite of such difficult culture conditions and in the absence of detectable cell proliferation, we were able to transduce primary leukemic cells using the HIV-1 derived lentiviral vector with a good efficiency and to a variable degree for different patients. As shown in Table 1, transduction of the EGFP gene was best appreciated 7 rather than 3 days after infection, in agreement with previous reports showing a rather slow expression of

Table 1. Transduction efficiency of primary human acute myeloblastic and B-precursor lymphoblastic leukemia cells.

			% EGFP expressing cells (MFI)			
	% SG2M cells*		at 3 days		at 7 days	
Patient # (disease)	Without CK	With CK	Without CK	With CK	Without CK	With CK
1 (AML M1)	5	13	8	15	8.4 (72)	17 (88)
2 (AML M4)	38	38	3.6	4.7	29 (186)	32 (198)
3 (AML M5)	9	9	11	8	37 (547)	21 (421)
4 (AML M2)	4	4			16 (110)	15 (101)
5 (AML M4)	14	17			9 (104)	12.6 (110)
6 (B-ALL)°		10.2 (145)				
7 (B-ALL)°		4.4 (119)				
8 (B-ALL)°		21 (173)				

**The cell cycle analysis was performed after 48 hours of culture, just before the infection procedure for both populations cultured either in the presence or in the absence of CK; °the B-ALL populations were grown on a feeder cell layer (S17 murine cell line). CK = cytokines; MFI = mean fluorescence intensity (numbers in parentheses).*

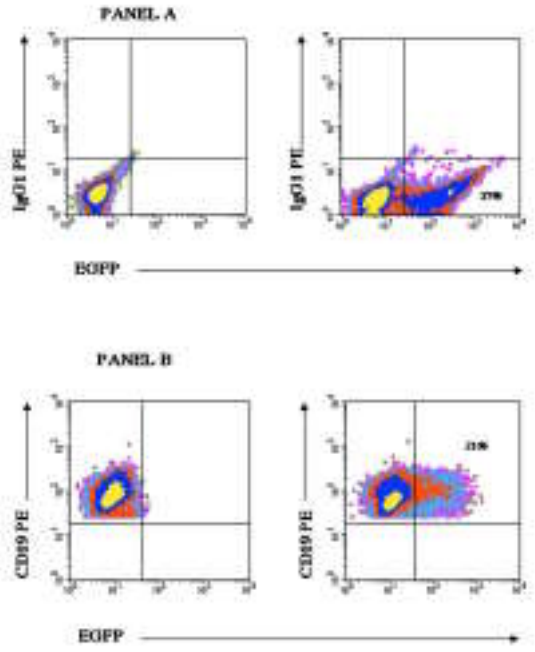

Figure 1. Transduction of EGFP gene into primary AML (panel A) and ALL (panel B) blast cells. X-axis: EGFP fluorescence. Y-axis: isotype monoclonal antibody (panel A) or anti-CD19-PE (panel B). Left panels: negative controls. Right panels: cells transduced with HIV-1-derived lentivirus vector.

transduced genes after infection with lentivirus vectors.[14] A representative experiment of EGFP gene expression in transduced AML blasts (patient #3) is shown in Figure 1, panel A. Transduction efficiency at day 7 ranged from 8.4 to 37% in the absence of CK and from 12.6 to 32% in the presence of CK (Table 1). Thus the presence of CK did not significantly improve infection levels. In two cases (patients #1 and #3) it was possible to infect a second aliquot from the same patient: after 7 days, patient #1 showed 37.6% EGFP positive cells in the presence and 20% in the absence of CK and patient #3 showed 21.7 % in the presence and 20% in the absence of CK. These data, if compared with those listed in Table 1, demonstrate that the cells from these patients were reproducibly infected to a significant degree although some variability was observed. In the entire set of experiments, samples were infected with viral supernatants of comparable titer as documented by parallel infections of the CEM T-lymphoblastoid human cell line that we use as our internal standard, as previously reported.[9,10]

As regards AML samples, the intensity of the signal (mean fluorescence intensity-MFI) at 7 days varied significantly in different patients, ranging from 72 to 547 (Table 1). The limited number of samples analyzed precludes further speculation, even if the adopted internal promoter (pgk) may work optimally in different FAB subtype groups.

In the three evaluable ALL cases cultured on a murine fibroblast feeder layer, only the day 3 time point could be analyzed due to poor viability at later time points. The efficiency of infection ranged in this case from 4.4 to 21% and was comparable to that observed for AML cells at the equivalent time point (Table 1). A representative experiment of EGFP gene expression in transduced ALL blasts (patient #8) is shown in Figure 1, panel B. This capacity of infection of the ALL samples is particularly significant in view of the fact that only 60 to 70 % of the leukemic cells plated were actually recovered on day 3 and less then 20% after 7 days of culture (data not shown). Thus also in this case infection took place to a significant extent in the absence of *in vitro* proliferation.

Discussion

Altogether, these data suggest that, with a standardized and reproducible protocol, it is possible to transduce a significant percentage of primary human AML and ALL blasts such that the preparation of engineered leukemic cells for use as vaccines can be envisaged. In our experience, and that of other groups,[4,5] it is clear that primary leukemic cells (especially ALL blasts) are very fragile and can be sustained in a viable state for only a few days *in vitro*; in addition we observed a great variability in cell survival between different patients. The lentivirus (derived from the HIV-1 backbone)[6,7] proved to be efficient for the transduction of primary leukemic cells. These encouraging results, which would not have been possible with the Moloney virus-based vectors, are probably explained by the active metabolic state of the leukemic cells, a state which needs to be induced in G_0 resting cells in order to obtain lentiviral infection.[15] Future developments will probably include the further optimization of such vectors in order to obtain more stable and regulated expression of the transduced gene.[16]

Contributions and Acknowledgments.

EB, MI and AB designed the study. FB and CC standardized the method of spin–infection adopting the HIV1-derived lentivirus. EB and FB carried out all the set of experiments. GG analyzed all the samples by FACS and contributed to the study design. EB and MI prepared the manuscript. AB, MI and JG were involved in critically revising the content of the manuscript. AB and MI gave the final approval for its submission. The order in which the Authors appear depends on the importance of their contributions. MI was the most important contributor to the study design and critical revision and final approval of the manuscript: he, therefore, appears last.

We thank E. Erba (Istituto M. Negri) for his technical support with the cell cycle analyses.

Funding

This work was supported by the "Associazione Italiana Ricerca sul Cancro", the "Associazione Paolo Belli", the "Fondazione Tettamanti" and MURST. F.B. and C.C. are Fellows of the Fondazione Tettamanti.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Prof. Pier Giuseppe Pelicci, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Pelicci and the Editors. Manuscript received September 12, 2000; accepted November 15, 2000.

Potential implications for clinical practice

The defective lentivirus (derived from the HIV-1 backbone) proved to be efficient for transduction of primary human AML and ALL leukemic cells: gene manipulation represents an innovative strategy to enhance the immunogenicity of cancer cells, such that the preparation of engineered leukemic cells for use as vaccines can be envisaged.

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