Bone Marrow Transplantation (2003) 32, S33–S35

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Transduction of hematopoietic stem cells with a retroviral vector expressing the neomycin phosphotransferase gene

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Summary:

Transduction of stem cells with a marking gene holds promise to determine if tissue repair or regeneration is derived from the adult hematopoietic stem cell and if relapse of an autoimmune disease should occur whether relapse arises from the stem cell compartment or from lymphocytes surviving the conditioning regimen. New safety concerns about gene-modified stem cell would entail new safety testing such as documentation of the insertional site prior to release.
Bone Marrow Transplantation (2003) **32,** S33–S35. doi:10.1038/sj.bmt.1704178
Keywords: stem cells, gene transduction; autoimmune disease

Remarkable results have been achieved in patients with a variety of autoimmune disorders treated with hematopoietic stem cell transplantation (HSCT).[1,2] However, some patients do not respond or their responses are not durable.[3] The mechanism of relapse or resistance to the therapy is not well understood. The conditioning regimens may not be effective in eliminating lymphocytes and immune effector cells that are recognizing self-antigens. Immune cells derived from stem cells may become re-educated against the self-antigens and induce disease relapse or persistence. Recently, interesting data suggesting that epithelial cells may arise from the hematopoietic stem cell (HSC) compartment have been reported by Korbling *et al.*

In an attempt to further understand the nature of autoimmune diseases and tissue regenerative processes, a portion of autologous stem cells provided to these patients may be transduced with a neomycin phosphotransferase resistance (Neo^R) gene. The patients could then be screened for the presence of specific immune cells and/or epithelial cells derived from Neo^R marked stem cells. To this end, a Neo^R gene construct based on a murine embryonic stem cell virus (MESV) retroviral backbone was developed (Figure 1).[4–6]

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Methods

We constructed a chimeric retroviral helper virus plasmid, AM3–internal ribosome entry site–zeocin resistance gene (pAM3-IRES-Zeo) and established packaging cell lines by transfecting 293 human embryonic kidney cells with the helper virus construct. Stable integrants were selected with zeocin. The presence of zeocin gives a selective advantage only to cells expressing helper virus since both pAM3 and the zeocin resistance (zeo^R) gene are transcribed from the 5′ long terminal repeat (LTR) promoter, and therefore, eliminates cells that have methylated the transgene. Single cell-derived 293.AMIZ (abbreviation from AM3–IRES–Zeo) packaging cell lines with the highest level of expression of helper virus were identified.[5] Retroviral vector producer cells (VPC) were established by transfection of the long terminal repeat–neomycin resistance gene–long terminal repeat (LNL) vector construct into 293.AMIZ packaging cells followed by selection of stable integrants in the presence of both G418 and zeocin. The LNL vector is based on a modified murine embryonal stem cell virus (MESV) pR338 backbone. Rapid molecular screening of single cell-derived clones allowed for identification of several 293.AMIZ-based VPC clones with titers of $1–3 \times 10^7$ colony forming units (CFU)/ml. Retroviral particles produced by these clones are resistant to inactivation by human serum.

Mobilized human hematopoietic progenitor cells (HPC) were isolated from peripheral blood (PB) apheresis product using the high-performance cell sorter with a purity of resulting cell population not less than 98% of $CD34^+$ cells. HPCs were separated by fluorescence activated cell sorter (FACS), plated in Iscov's modified Dulbecco medium (IMDM) or X-VIVO-15 tissue culture media, and supplemented with 5% fetal bovine serum. The following combination of human recombinant cytokines was used to activate/expand mobilized human $CD34^+$ cells: FLT3 ligand (FLT3-L), stem cell factor (SCF), interleukin-6 (IL-6), interleukin-11 (IL-11), leukemia inhibiting factor (LIF). Under these conditions, an average four-fold increase of the cell number was detected by 72 hours of cell culture. The phenotypes of the cells growing in the cultures were analyzed every 24 hours. The clonogenic potential of activated/expanded cells was estimated in colony assay, performed in semisolid Methocult growth media H4434 (Stem Cell Technologies, Vancouver, Canada).

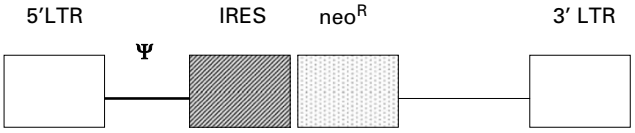

Figure 1 Structure of LNL construct used for HPCs transduction. 5′LTR = 5′ long terminal repeat from murine embryonal stem cell virus; Ψ = packaging signal sequence; IRES = internal ribosome entry site; Neo^R = neomycin resistance gene, 3′ long terminal repeat derived from myeloproliferative sarcoma virus.

The samples of HPCs for colony assay were collected just before the beginning of cell activation (0 hour), after 72 hours of activation/expansion and immediately after the transduction procedure (106 hours from initiating activation).

Transduction of CD34$^+$ cells was performed after activation for 72 hours as described above. In brief, the cells were collected, pelleted, resuspended in vector containing media supplemented with cytokines and placed in the wells of 24 × flat-bottomed multiwell plates coated with RetroNectin for 12 hours. Those procedures were repeated three times in each experiment (three cycles of transduction).

Results

To facilitate clinical applications of retroviral-mediated gene transfer into human hematopoietic stem cells, retroviral vectors and their cellular producers should meet several important requirements. The most important is that extinction of integrated provirus must not occur during CD34$^+$ cell differentiation *in vivo*. Efficient *ex vivo* transduction of CD34$^+$ cells requires high titers of retroviral vectors. Resistance of these vectors to complement-mediated inactivation by human serum would allow for presence of autologous serum in the culture medium used for transduction, thereby creating a more permissive enviroment for *ex vivo* CD34$^+$ cell manipulation. We have developed the LNL monocistronic vector based on a murine embryonal cell virus (MESV) backbone[6] by expressing the *neo*^{R} gene after the internal ribosomal entry site (IRES) (Figure 1). A MESV 5′LTR was utilized because of higher transgene expression in stem cells compared to other retroviral 5′LTRs. An IRES was built in because it enables ribosomes to bind in cap-independent fashion and start translation at the next UAG codon downstream.

The LNL construct possesses the following features: 5′LTR derived from MESV, packaging signal sequence, IRES, Neo^R gene, 3′LTR from myeloproliferative sarcoma virus, and elimination of a negative regulatory element (NRE) coincident with the proline transfer ribonucleic acid (tRNA) primer-binding site (PBS) of Moloney murine leukemia virus (MoMLV) (NRE is a potent repressor of LTR-mediated transcription in embryonic stem cells). Furthermore, the vector contains high-affinity binding site for the specific protein 1 (SP1) transcription factor.

Our results indicate that under activation conditions described in the Methods section, the immature phenotype of the cells was preserved for not more than 3 days.

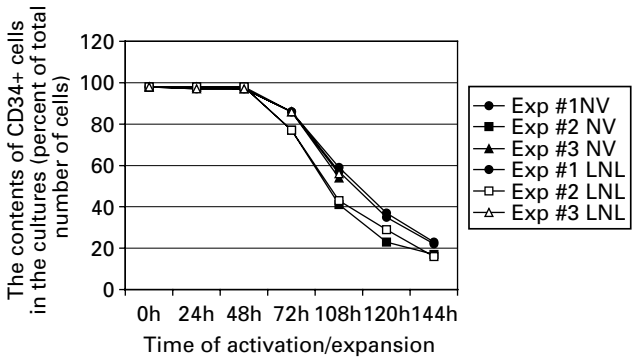

Figure 2 Kinetic of CD34 surface antigen expression by HPCs during their activation/expansion *ex vivo* in the presence of SCF, IL-6, FLT-3L, LIF and IL-6 estimated in three independent experiments. Abbreviations: NV = non-transduced cells; LNL = CD34$^+$ cells transduced with Neo^R gene.

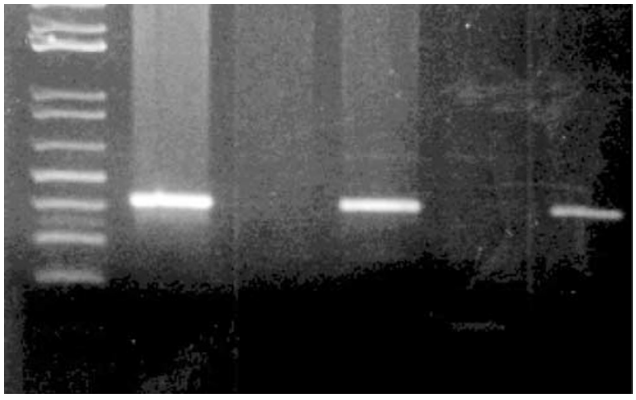

Figure 3 The results of PCR analysis of Neo^R gene integration into genomic DNA of CD34$^+$ PB cells. The DNA isolated from LNL transduced or nontransduced (negative control) HPCs have been assayed by PCR followed by gel-electrophoresis analysis in 1.5% agarose gel. Track 1: DNA size markers; track 2: positive control (PCR analysis of genomic DNA derived from HL-60 cells transduced with Neo^R gene followed by selection in the presence of G418); track 3: negative control for the first transduction experiment (CD34$^+$ cells activated by cytokines); track 4: activated CD34$^+$ cells cultured in the presence of retroviral vector containing media; track 5: second transduction experiment – negative control (CD34$^+$ cells, activated by cytokines); track 6: second transduction experiment (cells cultured in the presence of retroviral vector containing media).

After 72 hours (from 72 to 120 hours) of culture, the number of CD34-positive cells dropped dramatically from 100 to not more than 30% (Figure 2). In contrast, the number of cells expressing such differentiation markers as CD13, CD33, CD36, CD41a, CD45^{high} increased progressively. We observed that no more than 8–10% of cells growing in the tissue cultures expressed CD34$^+$ surface marker at day 6 after initiation of activation.

Furthermore, the number of erythroid burst forming units (BFU-e) per 2×10^3 HPCs derived from expanding cell cultures has not changed for up to 72 hours after cytokine activation compared with the original nonactivated CD34$^+$ cell population. After 106 hours of activation/expansion the number of BFU-e decreased dramatically indicating loss of pleuropotentiality. In contrast, the number of granulocytes colony forming unit (CFU-G) and granulocytes macrophages colony forming

unit (CFU-GM) in expanded $CD34^+$ cell population increased significantly after 106 hours of activation by the cytokine combination described above. The successive integration of Neo^R gene into genomic DNA derived from HPCs and collected 24 hours after the end of last transduction cycle was confirmed by PCR analysis (Figure 3).

Conclusions

The Neo^R gene could be successfully integrated into human $CD34^+$ cells growing in the presence of SCF, FLT3-L, IL-6, IL-11 and LIF using MESV retroviral vector. The immature phenotype of blood-derived mobilized HPCs expanded in the media containing the combination of cytokines listed above could be preserved for up to 72 h of activation/expansion. The optimal period for the HPCs transduction, therefore, falls in a narrow time window from 0 to 72 h of their activation/expansion.

The contribution of Neo^R marked stem cells to the mechanism of autoimmune disease recurrence and organ-specific tissue regeneration in patients undergoing immune ablation followed by stem cell rescue could be evaluated by infusion of gene marked HPCs. This study may permit insight into the mechanism of disease progression and tissue regeneration after myeloablative therapy and such insights might allow for the design of new and/or improved treatments.

New safety concerns with stem cell gene therapy marking experiments have moved to the forefront. Recently, two children treated for severe combined immune deficiency with gene therapy developed leukemia. Both children received retrovirally transduced stem cells expressing the therapeutic transgene. In both instances, a transforming event occurred by insertional mutagenesis of the transgene into a known oncogene. These findings prompted the US FDA in January 2003 to suspend gene therapy and marking trials of $CD34^+$ stem cells. In studies involving gene marking without therapy, the threshold for safety has increased. Additional measures and safety studies will likely be required before product release in human clinical studies in order to justify this genuine risk to patients. In the immediate future, gene marking of stem cells may be useful in animal models to evaluate ''transdifferentiation'' of adult hematopoietic stem cells into epithelial cells.

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