Lentiviral-mediated gene transfer into haematopoietic stem cells

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Objectives. Lentiviral vectors can transduce nondividing cells. As most haematopoietic stem cells (HSCs) are nondividing in vivo, lentiviral vectors are promising viral vectors to transfer genes into HSCs.

Design and Setting. We have used HIV-1 based lentiviral vectors containing the green fluorescent protein (GFP) gene to transduce umbilical cord blood CD34+ and CD34+/CD38– cells prior to transplantation into NOD/SCID mice.

Results. High level engraftment of human cells was obtained and transgene expression was seen in both myeloid and lymphoid lineages. Bone marrow from the primary transplant recipients mice was transplanted into secondary recipients. GFP expression was seen in both lymphoid and myeloid cells in the secondary recipients 6 weeks posttransplantation. Human haematopoietic progenitor colonies were grown from both primary and secondary recipients. Over 50% of the haematopoietic colonies in these recipients were positive for the GFP transgene by PCR. Following inverse PCR, amplified fragments were sequenced and integration of the vector into human genomic DNA was demonstrated. Several vectors containing different internal promoters were tested in NOD/SCID mice that had been transplanted with transduced CD34+ and CD34+/CD38– cells. The elongation factor-1α (EF-1α) promoter gave the highest level of expression, both in the myeloid and lymphoid progeny of the engrafting cells.

Conclusions. These data collectively indicate that candidate human HSCs can be efficiently transduced with lentiviral vectors and that the transgene is highly expressed in their progeny cells.

Keywords: gene therapy, haematopoietic stem cells, lentiviral vectors.

Introduction

The development of gene therapy strategies to correct haematopoietic and genetic disorders has been hampered by the low level of gene transfer into human haematopoietic stem cells (HSCs) using vectors derived from oncoretroviruses such as the Moloney murine leukaemia virus (MoMLV) virus [1, 2]. Oncoretroviruses require cell division for integration and as repopulating HSCs are largely quiescent, oncoretroviral vectors are largely inefficient in these targets [3]. Thus, much interest has recently been focused on vectors derived from lentiviruses such as HIV-1, which have been shown
to transduce a variety of nondividing cells [4, 5]. Furthermore, lentiviral vector viral particles pseudotyped with VSV-G can enter a large variety of targets as a result of the ubiquity of the VSV-G phospholipidic receptor, and can be easily concentrated by ultracentrifugation. Lentiviral vectors are generated using a transient transfection packaging system where the transfer vector plasmid is transfected into 293-T cells with plasmids encoding the accessory genes of HIV, quiescent CD34+, CD38−, Thy1+ human hematopoietic progenitors could be transduced. Stable expression of the transgene was seen in progeny cells from these purified progenitors, sorted initially as single cells, after growth on stromal cells for 5–6 weeks [10]. In similar studies, Case et al. demonstrated stable transduction for 15 weeks in progeny cells from nondividing CD34+, CD38− cells. The gene transfer efficiency into extended long-term culture initiating cells was approximately 10–30% [11]. One recent study has reported efficient gene transfer of SCID repopulating cells (SRCs) [12]. CD34+ cells were transduced with an GFP gene containing HIV vector and transplanted into NOD/SCID mice. Transgene expression was seen in peripheral blood of the mice up to 22 weeks after transplantation, and 30–40% of the human hematopoietic colonies from the bone marrow cells contained the transgene. Despite these encouraging results, a recent study compared the transduction efficiency of human hematopoietic progenitor cells at various stages of the cell cycle using HIV vectors. The results show that although CD34+ cells in G0 can be transduced, CD34+ cells in G1 or S/G2/M are much more effectively transduced than when they are in G0 [13].

Transplantation of human hematopoietic cells into sublethally irradiated NOD/SCID mice provides the best assay system for candidate human HSCs currently available. There are indications suggesting that the engrafting cells, defined as SCID-repopulating cells (SRCs), are biologically distinct from and more primitive than the hematopoietic cells that can be assayed in vitro [14, 15]. In this paper, we demonstrate transduction of primary and secondary SRCs with high efficiency. Expression of the transgene was seen in both myeloid and lymphoid cells of mice that were transplanted with a very low dose of transduced CD34+ cells. An ‘inverted’ PCR technique was used to confirm the integration of the vector provirus in the chromosomal DNA of hematopoietic colonies derived from the NOD/SCID mice.

Engraftment of transduced CD34+ cells in primary NOD/SCID recipients

The transduction conditions were designed to maintain the in vivo repopulating ability and viability of the HSC whilst minimally stimulating the cells into the S/G2/M phase of the cell cycle. Freshly isolated CD34+ BM or CB cells were transduced overnight in serum-free conditions in the presence of mast cell growth and differentiation factor (MGDF). Cell cycle analysis demonstrated that the transduction conditions used promoted some activation of the cells into G1 but they did not enter the S/G2/M phase of the cell cycle during transduction for 18 h with MGDF. To assess the transduction efficiency of candidate human stem cells in the CD34+ population, CD34+ cells transduced overnight in the presence of MGDF were transplanted into sublethally irradiated NOD/SCID mice. After 5–6 weeks, the mice were killed and the bone marrow analysed by fluorescence-
activated cell sorter (FACS) for engraftment of human cells by staining with a monoclonal antibody directed against human CD45. The level of engraftment reflected the number of cells transplanted. Mice receiving a transplantation dose of 100 000 cells, the number of CD45+ cells ranged from 3 to 13%. Multilineage haematopoiesis was obtained in all the animals. GFP+ cells were found in all lineages tested and at comparable frequencies. GFP+ CD34+ cells were detected in all animals suggesting that transduced progenitor cells were maintained in the BM of NOD/SCID mice. The percentages of GFP+ cells in clonogenic myeloid progenitors was assessed in methylcellulose cultures that only support outgrowth of human progenitors. When scoring by fluorescence microscopy, 23–48% of the colonies were GFP+. By PCR analysis of randomly picked individual colonies the GFP gene was detected in 46 ± 23% (range: 16–80%) of the CFU-GM colonies.

Engraftment of secondary NOD/SCID mice with cells from primary recipients

Serial transplantation was performed to assess the transduction efficiency into cells that can sustain long-term lymphomyelopoiesis. Bone marrow mononuclear cells from seven primary recipients were transplanted into seven secondary sublethally irradiated animals. No growth factors were administered to any of the primary or secondary recipients. The secondary transplant recipient mice engrafted with human cells in the range of 1–6%. FACS analysis of GFP expression in the CD45+ population showed that 26 ± 10% of the human cells in the secondary recipients were GFP+. Multilineage engraftment was obtained in all secondary transplant recipients and the transgene expressed in all the following tested lineages CD19, CD33, CD14, CD38 and CD34. Clonogenic progenitor cells from the secondary transplant recipients were assessed in methylcellulose cultures and scored for presence of the GFP gene by PCR. A very high number of colonies was positive for the vector (64 ± 13%, range: 56–78%; n = 6).

Cell-dose limiting dilution assay in NOD/SCID recipients

In order to address the issue of clonality in the reconstitution of transplanted NOD/SCID mice a cell-dose limiting dilution experiment was performed. Here mice were transplanted with reducing numbers (5000–400 000) of lentivirally transduced CD34+ cells. Only the groups with 100 000 and 400 000 cells transplanted had all four mice positive for engraftment defined as the presence of five lymphoid (CD19) and five myeloid (CD33/15) cells present per 20 000 mouse mononuclear cells and the generation of CFU-GM colonies in methylcellulose. Groups with lower cells doses had reduced numbers of positive engrafting mice with the group with only 5000 cells transplanted having no positive engrafting mice. A linear relationship exists between the cell transplantation dose and engraftment level obtained by percent CD45+ cells. This suggests that either one or a low number of SRC reconstituted the positive engrafting mice in these groups. Interestingly, one mouse had 91% GFP expressing cells and all BM derived CFU-GM colonies visually green. Lineage analysis of this unique mouse shows lymphoid and myeloid cells both expressing GFP at 91 and 93%, respectively. This mouse may have been repopulated by a single transduced SRC whose myeloid and lymphoid progeny cells are expressing the transgene. Collectively, these data indicate that very primitive candidate human stem cells have been transduced and that these cells can repopulate NOD/SCID mice and generate myeloid and lymphoid progeny.

Lentiviral transgene integration in CFU-GM colonies

To show that proviral integration in primitive haematopoietic cells had taken place, we developed an ‘inverted’ PCR assay to detect integrated vector copies in individual colonies derived from NOD/SCID mice. The results demonstrate vector integration in visually green CFU-GM colonies derived from the three primary recipient NOD/SCID mice. Vector genomic junctional fragments from a few colonies were sequenced and demonstrated the sequence of the HIV-1 LTR in tandem with sequences from human genomic DNA.

Lentiviral vector design for optimal expression in haematopoietic cells

In experiments with lentiviral vectors in murine and human haematopoietic cells, we noted that the expression level of the transgene was lower per proviral copy number than is usually seen with
oncoretroviral vectors that express the transgene from the viral long terminal repeat (LTR). Therefore, we designed several lentiviral vectors and tested their expression in NOD/SCID mice in cells that are progeny of SCID repopulating cells. We compared vectors with different internal promoters. The phosphoglycerate kinase (PGK) promoter, the cytomegalovirus (CMV) promoter and the elongation factor-1α (EF-1α) promoter were compared driving expression of the GFP gene following transplantation of the transduced cells into NOD/SCID mice. The EF-1α promoter turned out to be the most effective promoter, both in myeloid and lymphoid cells 6 weeks after transplantation. When vectors with the EF-1α promoter with or without a self-inactivating (SIN) deletion in the LTR were tested, the EF-1α SIN vector turned out to give higher expression. The EF-1α SIN vector gives 7–8 times higher expression that the PGK vector in NOD/SCID mice.

Conclusions and future directions

Lentiviral vectors have been used to transduce very primitive human haematopoietic progenitors or candidate stem cells that are capable of repopulating NOD/SCID mice after serial transplantation (secondary transplant SRC). It has also been demonstrated that the lentiviral vector is integrated into the chromosomes of progenitor cell colonies derived from the NOD/SCID mice. The immunophenotype of the engrafted cells was analysed by FACS. The results showed that all animals analysed in this way had both lymphoid and myeloid human cells. Cells of the B cell lineage (CD19 positive) were the most abundant and a surprisingly high proportion of CD34+ cells was detected. Many cells with a myeloid cell surface phenotype were also detected. GFP positive cells were seen in all these fractions detected with antibodies towards antigens characterizing different lineages or stages of maturation. CD34+, CD19+, CD38+, CD33+ and CD14 cells were all found to be positive and the proportion of positive cells was rather similar in all these fractions, even in the secondary NOD/SCID mice after serial transplantation. These results suggest that we are transducing secondary SRC that can serially engraft in NOD/SCID mice and develop into both lymphoid and myeloid lineages, we performed limiting dilution experiments where reducing numbers of transduced CD34+ cells were transplanted into primary recipients. The results clearly show that there is a direct relationship between the number of recipients that engraft human cells and the dose of cells transplanted. Furthermore, there is also a relationship between the number of cells transplanted and the number of animals expressing the GFP transgene. In one recipient which received only 35 000 cells, the engraftment was relatively low (3.8%) as expected but 91% of the human cells were expressing the transgene. All the CFU-GM colonies from this animal were visually green and almost all myeloid and lymphoid cells expressed the GFP as determined by FACS. The most likely interpretation of these results is that one transduced SRC has generated the myeloid and lymphoid progeny that is expressing the transgene. Collectively these data indicate that very primitive repopulating cells that are able to differentiate into both myeloid and lymphoid progeny cells have been successfully transduced [16].

Several different lentiviral vector constructs were tested to determine what regulatory elements within the context of an HIV 1-based lentiviral vector would lead to optimal expression in transduced human haematopoietic cells that engraft NOD/SCID mice. Different promoters were tested and the vectors were with or without a self-inactivating deletion (SIN) in the LTR or the Woodchuck hepatitis virus posttranscriptional regulatory element [17, 18]. After comparing several internal promoters, the EF-1α promoter was found to be the best of the promoters that were tested. The best expression vector was found to be the one that contained the EF-1α promoter with a self-inactivating deletion in the LTR. This vector expresses 7–8 times better than the standard PGK promoter vector. The EF-1α SIN vector expresses the transgene as well in haematopoietic cells as the best oncoretroviral vectors. Therefore, we have shown that lentiviral vectors can be used to transfer genes efficiently into candidate human stem cells and a vector design has been identified to allow high level expression in the transduced cells.

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References


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