The Stimulatory Effect of MIP-1α on the Gene Transduction Efficacy of Cord Blood CD34+ Cells by a Pseudotype Retroviral Vector

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Abstract

Introduction: Hematopoietic stem cells are always in a quiescence state. Since they need retroviral transduction to infect dividing cells, they are resistant to retrovirus transduction. They need to be pre-stimulated by a cytokine cocktail. For proliferation without maturation, we suggest MIP-1α as a novel factor.

Material and methods: Retroviral vector produced by PG13/LN C8 cells titter on Hela cells. Then, the $CD34^+$ cells of cord blood can be pre-stimulated in a serum- free media supplemented with SCF, Flt3,TPO,IL6 in the presence and absence of 50 ng/ml MIP-1α. Transduction efficiency was assessed by a semi-quantitative PCR for the neomycin gene.

Results: A PCR analysis of the neomycin gene in $CD34^+$ cells revealed an improved transduction of cord blood cells in the presence of MIP-1α 65%, in comparison to its absence: 40.7%.

Conclusion: the addition of MIP-1α to the cytokine cocktail improves the transduction efficiency of cord blood hematopoietic progenitor cells. Further studies are required to clarify its effect on the functional properties of $CD34^+$ cells.

Key Word: Gene Transduction, MIP-1α, Retroviral Vector

Received: 17, Feb, 2009
Accepted: 20, Mar, 2009

Introduction

Hematopoietic stem cells (HSCs) have certain properties including high proliferative capacity, a differentiation of myeloid and lymphoid lineages and a self renewal feature to reconstitute the hematopoietic system.(1,2) the gene transfer approach to HSCs such as CD34+ cells is promising for the treatment of inherited and acquired disease.(3) Using viral vectors, especially retroviral vectors, has enabled some progress to be made.(4) Retroviruses carry genetic information in RNA form; upon their entrance into the host cell, genetic information is transcribed to the DNA and integrates into the DNA of the transuded cell.(5) the Moloney murine leukemia virus (Mo-MuLV) has been studied extensively in experimental gene transfer studies and clinical gene therapy trials.(6) It has been shown that retroviral vectors pseudo-typed with the gibbon ape leukemia virus (GALV) envelope cause a significant increase in the transduction efficiency in primate and human cells due to the existence of their receptors on a wide variety of cells.(7)

Most HSCs transduction protocols have used MLV-based vectors; however, the major drawback of this method is the requirement of active cell division during the vector entrance to gene integration. The quiescence state of the HSCs makes them refractory to transduction and causes a low efficiency of the transduction.(8,5)

An activation of cell division by a cytokine cocktail improves gene transduction.(9) Optimizing growth factor cocktails to help expansion without differentiation is the aim of researchers.(3,9,10) The beneficial effects of stem cell factor(SCF), interleukins 3 and 6 (IL-3 and IL-6) on stem cell survival in retroviral transduction efficiency were enumerated.(11) the addition of Flt-3L (FL) and thrombopoietin (TPO) to this cocktail improved the induction of cycling stem cells, while preserving pluripotency, self-renewal and the engraftment capacity of stem cell properties.(12)

Macrophage inflammatory protein-1α (MIP-1α) a cysteine cysteine (CC) chemokine,(13) have various inhibitory and stimulatory effects on primitive and more mature stem cells, respectively.(14,15) It has

been shown that the addition of MIP-1a to primitive progenitor cells of cord blood cells(16) inhibits cell entry into the G1cycle.(17) Given this, MIP-1α may enhance the transduction efficiency and long-term expression of transuded cells. Hematopoietic inhibitors such as MIP-1α, the transforming growth factor ß (TGF-ß) and TNF-α have differential effects on the proliferation of cord blood CD34+ cells.(18) As known, the combination of MIP-1α with TGF-β and TNF-α have the capacity to modulate the cytokine-induced proliferation of cord blood cells.(19)

Moreover, previous reports show that the combination of SCF, IL-3, IL-6, FL, TPO and MIP-1α is optimal for the short-term serum-free expansion protocol of CB CD34+ cells.(18,20) Additionally, MIP-1α can prolong the survival of LTC-IC in suspension cultures.(13) Furthermore, the increased CD34+ CD38-cells transduction observed following the antisense to p27, a protein that promotes cellular quiescence in HSC cells in combination with antibodies to neutralize TGF-ß.(21)

According to this evidence, we have attempted to improve the retrovirus-mediated gene transfer efficacy into HSCs via stimulatory cytokine cocktail and inhibitory role of MIP-1α on differentiation.(20) We claim MIP-1α as a candidate for increasing transduction efficiency without the induction of maturation.

Materials and methods

Isolation of HSCs from cord blood cells: $CD34^+$ cells were isolated from umbilical cord blood after cesarian delivery. Mononuclear cells were obtained by density gradient centrifugation. Progenitor cell enrichment was performed using the magnetic affinity cell sorting (Dayna beads,GIBCO-BRLGrand Island, NY, USA). Briefly, 100 µl Dyna beads was added to the cell suspension and vortex for 2-3 seconds. The mixture was incubated at 2-8°C for 30 min. with occasional rotation. The tube was filled with cold buffer to the height of the magnet and the cell-bead complexes was resuspended. The tube was placed for 2 minutes in a magnet and then the supernatant was discarded. The bead-bound cells were resuspended in 2 ml buffer by vortexing or pipetting. Following three cycles of magnetic attraction, the beads were detached from the cells with an excess of biotin (Life Technologies, Grand Island, NY) and separated from the cells magnetically. The purity of the $CD34^+$ population was evaluated by flowcytometry using phycoerythrin (PE)-conjugated anti-CD34+ monoclonal antibodies (Dako, Denmark).

Retroviral vector production and viral titration: The oncoretroviruses were produced from PG13/LN C8 cells (ATCC-CRL-10686) containing the neomycin gene, which is resistance to G418 (GIBCO-BRLGrand Island, NY, USA). Cells were cultured in a HAT/HT medium with 10^{-7} M methotrexate for 5, 2 and 5 days, respectively. Viruses containing supernatant were titrated using G418 resistance colony assay on Hela cells. Hela cells were seeded at 1×10^5 cells per 6 cm dish on day 1 in a Dulbecco-modified Eagle medium (DMEM) (GIBCO-BRLGrand Island, NY, USA) with high glucose, supplemented with a 10% fetal bovine serum. On day 2, the test LN virus supernatant was added, and on day 3, the cells were trypsinized and seeded at 1:10 to 1:500 dilutions into 6 cm dishes in a medium containing G418 at a concentration (active) of 1000 µg/ml. Colonies of G418-resistant cells transduced with the LN vector were stained and counted on day 14. A virus titer in a colony forming units per ml (CFU/ml) was calculated by dividing the number of colonies by the volume (in ml) of virus used for infection and multiplying by the dilution factor used after trypsinization of the infected cells prior to selection.

Preparation of retronectin-coated dishes: Tissue culture plates were coated with a CH296 fragment of Retronectin (RetroNectin; Takara Shuzo, Otsu, Japan) according to this protocol. Briefly, the wells were coated for 2 hours at room temperature with CH296 (1-20µg/cm^2). An excess protein solution was aspirated, and the remaining free sites were blocked with a 2% bovine serum albumin (BSA) in hanks solution for 30 minutes at room temperature. The excess BSA solution was aspirated and the wells were washed with Hanks' balanced salt solution.

Gene transduction of CD34+ cells: CD34+ cells were added to a retonectin coated plate in a StemSpan® Serum-Free Expansion Medium (SFEM) (Stem Cell Technologies Inc, Canada) supplemented with 50 ng/ml SCF, Fl,TPO, IL6 in the presence and absence of 50 ng/ml MIP-1α) (all purchased from R&D Systems, Minneapolis, MN, USA) and 8µg/ml protamin sulfate (Sigma, St. Louis, MO, USA) for 24 hrs. A viral supernatant was added 24 and 72 hours post-incubation. Then, cells were harvested from the plate and seeded onto new dishes in the absence of a viral supernatant. Mock-infection control was conducted during each experiment. After the transduction, cells were cultivated for one week in a liquid culture with 50

ng/ml of the above mentioned cytokine cocktail with or without 50 ng/ml MIP-1α in the absence of the viral supernatant for one week.

Semiquantitative PCR analysis: Genomic DNA was prepared using proteinase K, DNA was extracted following standard procedures from equal numbers of infected and mock-infected cells.(22) the DNA was amplified in a 25 µl reaction with the primers specific for the short tandem repeat (STR) as internal control(23) and neomycin genes as a selectable marker of the LN vector in eppendorf master cycler gradient. The sequences of the primers were the following: 5'-GATCCCAAGCTCTTCCTCTT-3' (STR F), 5'-ACGTTTGTGTGTGCATCTGT-3' (STR R) (http://www.cstl.nist.gov/strbase/str_fact.htm) and 5'-CTGAAGCGGGAAGGGACT-3' (neo F), 5'-GGCCACAGTCGATGAATC-3' (neo R).(24) The amplification profiles were 1 min 94^0C, 1 min 55^0C,1 min 72^0C for 40 cycles. The expected sizes of the amplification products were 141-173 bp (STR) and 359 bp (neo). The Amplified products were analyzed in a 2% agarose gel run in a TBE buffer and stained with ethidium bromide. A semiquantitative PCR analysis was performed on a scanned agarose/ polyacril amide gel using multi-analyst software version1.1 (Bio-Rad laboratories Inc, Life Science Group 2000).(25)

Results

The purity of cord blood CD34+ cells in isolation: The purity of CD34+ cells population isolated by dynal bead was enumerated using phycoerythrin (PE)-conjugated anti-CD34+ monoclonal antibody (Dako). Flowcytometry results indicated a purity of more than 90 percent in the isolated cells (Fig 1.).

Transduction efficiency: A supernatant of PG13/LN C8 cells containing retroviral vector (LN) was collected and stored at-80. For the detection of viral titer in G418 containing media, Hela cells were used. After 14 days, a resistance colony was counted and the multiplicity of infection (MOI) was: $1.7\times10^5\pm3.6$.

Semiquantitative PCR analysis: To elucidate the efficacy of the Neo gene transfer from the LN vector, extracted DNA from hematopoietic cells were amplified via PCR. The specificity of the amplified products was confirmed by STR amplification. The expression of Neo in comparison to STR as an internal control in amplified products shows a 13.1% and 31.34% gene expression in the absence and presence of MIP-1α in an agarose gel electrophoresis of PCR products.

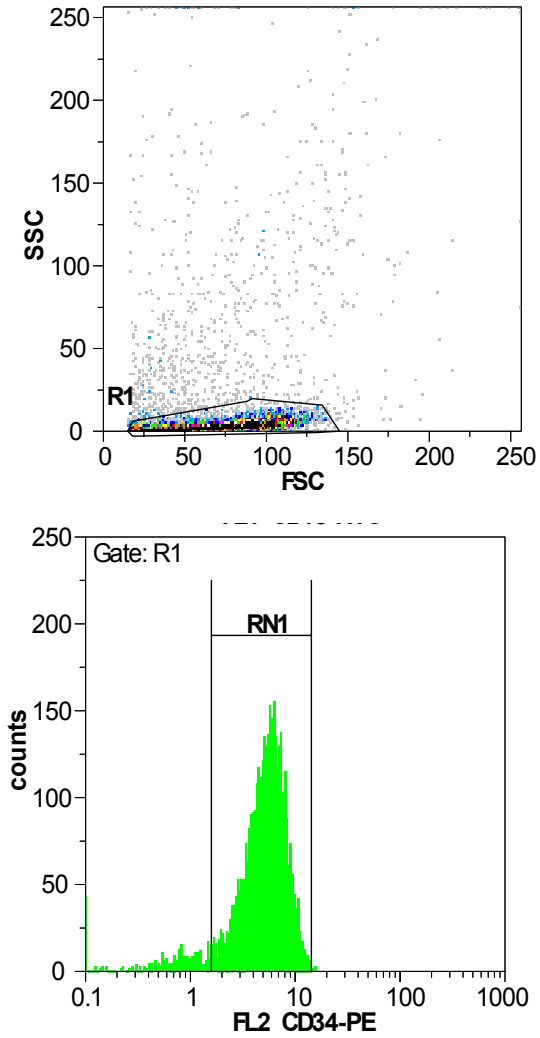

Figure1. Flowcytometry analysis of CD34+ cells after sorting with dynal bead.

The polyacrimamid gel analysis of the PCR products shows that the range of transduction was 35.5±5.0% and rose to 65.1±0.05% after an exposure to MIP. Both figure 2 and 3 demonstrates that MIP-1α exposure resulted in a more efficient transduction of HSCs.

Discussion

Human hematopoietic stem cells gene transfer attracted scientific attention as a novel strategy for the treatment of hematologic disorders.

A low efficiency of stem cell gene transfers is the major impediment to this approach.(3) Changes to the cultivation condition of stem cells including growth factor, stromal cells and physical status, have been done in many studies.(9,26) Nevertheless, owing to the quiescence state of HSCs, ex vivo strategies to induce the cycling required for MLV transduction cause loss of HSC self-renewal and engraftment capabilities.(3)

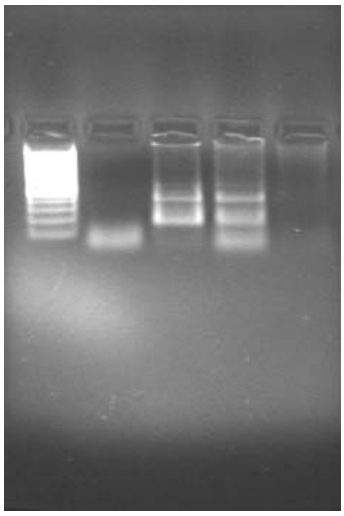

Figure 2. PCR analysis of neomycine gene in cord blood $CD34^+$ cells in the absence and presence of MIP-1α. ($CD34^+$=13.11%, $CD34^+$ MIP-1α =31.34%) from left to right (ladder=359bp, con pos, without MIP, with MIP, con neg)

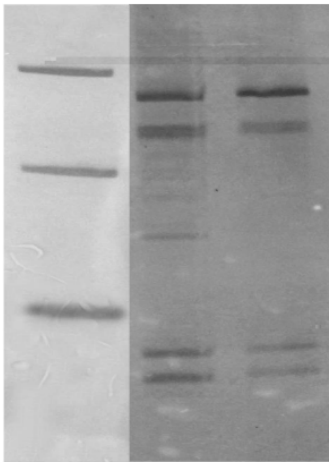

Figure 3. PCR detection of neomycine gene after transduction to cord blood $CD34^+$ cells in the presence and absence of MIP-1α in polyacryleamd gel with STR size marker141-173 bp ($CD34^+$=40.7% [Internal control: 28%,31.1%] $CD34^+$MIP=65% [Internal control:19.5,15.3]).

Cytokine combinations that induce HSC division without severe loss of engraftment potential improved HSC transduction efficiency.(9)

Importantly, stimulatory cytokines in retroviral transduction of stem cells lead to extended cell division and also their differentiation, which later is easy for stem cell gene transfer. Since, the in vivo condition of the hematopetic system is the balance of inhibitory and stimulatory regulatory factors, mimicking this microenvironment in ex vivo cultures could circumvent difficulties in gene transduction. Therefore, employing inhibitory factors of primitive progenitor cell differentiation like MIP-1α,(27) could be a modulating agent to maintain the balance of proliferation and differentiation.

This acts principally by reducing the cycling(28) and preventing hematopoetic cells from entering the G1,(17) as the best studied chemokine inhibitor of hematopoiesis.(29)

To ascertain the effect of the combination of stimulatory and inhibitory growth factors on stem cell gene transduction, we had chosen a cytokine cocktail of SCF, Flt3, TPO and IL6 plus MIP-1α, respectively.

In order to assess the possible role of MIP-1α on the efficacy of transduction, cord blood CD34+ enriched cells were transduced with GaLV. Co-cultivation was carried out on retronection coated plates and in the presence of protamin sulfate as stimulatory agents for transduction.(5,30)

The results of viral vector titration on Hela cells show reasonable infective titer for CD34+ transduction compared with results obtained by the previous report, 5×10^5,(9) and 7×10^5.(31) Higher titers obtained in other studies are the results of different methods of vector preparation, mostly using ultracentrifugation for concentrating supernatant containing viral vector. However, because of wanting to preserve the maximal biological activity of the viral vector, we did not concentrate it.

The data of the semi-quantitative PCR analysis revealed a positive regulation of neomycin gene expression in the cord blood CD34+ cells treated additionally by exposure to MIP-1α. We found that the MIP-1α could enhance transduction with efficiencies up to 31.3%. Our results are in alignment with previous reports indicating positive effects of this cytokine cocktail plus the MIP-1α on the CD34+ expansion,(32) as well as other cytokine cocktails,(33) such as IL3 and MIP-1α which are able to maintain a myeloid and lymphoid differentiation capacity.(34) This implicates the inhibitory effect of MIP-1α on the differentiation which is primarily factor in stem cell gene transduction.

The efficacy of the gene transfer to stem cells varies in different studies, due to diverse approaches they had used. In one study, Kalle et al, showed that in the presence of IL-l, IL-3, IL-6, SCF, and PG13/LN vectors, bone marrow CD34 enriched cells were effectively transduced (19.35%) greater than PA317/LN (11.5%),(10) which highlights the advantage of the PG13 producer cell line. Also, enhanced retroviral gene transfers in vivo (20%) into baboon marrow in the presence of IL-6, SCF, FL, and megakaryocyte growth and development factors (MGDF) was demonstrated.(9)

Although there is no direct report in accordance our study about the modulatory effect of MIP-1α on the CD34+ cells gene transduction, but indirect evidences and our results support this hypothesis. We have developed conditions that mimic the bone marrow microenvironment by employing positive and negative factors including cytokines and chemokine MIP-1α, respectively. Choosing a LN retroviral vector produced by PG13/LN c8 cells was another advantage in our study, because of the supportive evidence of its positive role compared to other vectors for HSCs transduction.(10,31) Nevertheless, further studies are required to optimize this condition; more confirming analysis on the functional behavior of transduced cells is required.

In conclusion, we have defined the novel role of MIP-1α on gene transduction efficacy, as a result of the modulation of the proliferation caused by stimulatory cytokine and remaining in its quiescence because of MIP-1α. The enhanced transduction efficacy in transduced cells encouraged us to further study the mechanisms in detail in future experiments.

Acknowledgment

This work was performed under a grant by the Molecular Medicine Network of Iran and Hematology- Oncology and Stem Cell Research Center of Tehran University of Medical Sciences.

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