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RESEARCH ARTICLE

Isolation and cultivation of murine hematopoietic stem cells and expression of hFIX mediated by recombinant lentiviral vectors *in vitro*

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Abstract Hematopoietic stem cells (HSCs) are an attractive target for gene therapy, especially for inherited blood diseases. Moreover, recombinant lentiviral vectors are considered to be prospective in HSCs gene therapy for the high efficiency of infection. In this study, murine mononuclear cells (MNCs) were isolated from bone marrow and cultured in suspension, and then $Lin^{-}CD117^{+}$ HSCs were isolated by immunomagnetic beads. During culturing, cells and colonies increased in HSCs supplied with cytokines while no change was observed in the control group without cytokines. FUXW recombinant lentiviral vectors were produced by calcium phosphate-mediated transient cotransfection infected MNCs from ICR and C57 mice. The hFIX expressions were 41.7 ± 4.2 ng / mL and 34.5 ± 6.6 ng/mL in supernatant on 7d. The hFIX expressions of HSCs infected by FUXW recombinant lentiviral vectors were 46.6 ± 5.7 ng/mL (with cytokines) and 33.3 ± 4.8 ng/mL (without cytokines) in supernatant on 7d. Results indicate that recombinant lentiviral vectors can infect murine MNCs and $Lin^{-}CD117^{+}$ HSCs efficiently, and expression of the transgene can be improved when supplied with cytokines.

Keywords mononuclear cells, $Lin^{-}CD117^{+}$ HSCs, recombinant lentiviral vectors, hFIX

1 Introduction

Hematopoietic stem cells (HSCs) are ideal target cells for gene therapy due to their ability to self-renovate and differentiate into various mature cells in the blood and the immune system (kurre, 2000; Bank, 2003). In addition, HSCs are heterogeneous cells lacking direct morphological characteristics, and are found in small amounts in marrows, umbilical cords and peripheral blood. Recently, various specific antigens against HSCs and other blood cells have been isolated, that have facilitated the identification and purification of these cells. CD117 (c-kit or stem cell factor receptor, SCF-R) is the main surface marker on murine HSCs (Pei, 2003). Lentiviral vectors based on HIV-1 have become the hotspot for gene therapy via HSCs instead of traditional retroviruses because of their ability to infect non-dividing cells. Lentivirus-mediated gene therapy via HSCs has achieved some headway in blood diseases such as Sickle Cell Disease (Pawliuk et al., 2001) and β-thalassaemia (May et al., 2000). In this work, we studied the gene therapy of hemophilia B by lentivirus via HSCs. First, murine mononuclear cells (MNCs) were isolated from bone marrow and cultured in suspension, and then highly pure $Lin^{-}CD117^{+}$ HSCs were isolated by immunomagnetic beads. Finally, MNCs and HSCs were infected with the recombinant lentiviral vector FUXW, and, in addition, the effect of cytokines on the expression level of hFIX was studied. This report will directly benefit animal trials of hemophilia B gene therapy.

2 Materials and methods

2.1 Materials

(1) Plasmids: Lentiviral vector C-FUW, packaging plasmid pCMV△R8.93 and envelope plasmid VSV-G were kindly provided by Dr Carlos Lois of the California Institute of Technology, USA. Vector plasmid FUXW was constructed by Chen Xiaoguang by inserting hFIX cDNA fragment into the MCS of C-FUW; (2) Cell line resources: 293T cells were stored in our lab; (3) Lymphocyte separating medium system (density = 1.077g/mL) was purchased from Shanghai Huajing Biological Technology Company; (4)

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MACS were purchased from Miltenyi Company, Germany; (5) Recombinant cytokines (murine IL-3, human IL-6 and murine SCF) were purchased from PeproTech Company; (6) C57 and ICR mice were purchased from the Animal Center of the Second Military Meclical University, Shanghai, China.

2.2 Murine marrow collection and mononuclear cells (MNCs) suspension preparation

The mice were sacrificed and their femurs and tibias were taken out. The bones were cut off at both ends and were flushed repeatedly with media. The cell suspension was then slowly added to a lymphocyte separating media system and centrifuged at 2000 rpm for 20 min. After that, the white cell layer in the middle was collected, which was mononuclear cells (MNCs). HSCs were further purified from MNCs by the MACS. Lin^-CD117^+ cells were harvested as described in the protocol.

2.3 The isolation and purification of $Lin^-c\text{-}kit^+$ HSCs

(1) Removing Lin^+ cells (negative selection): Mature cells, such as T, B lymphocytes, granulocytes, macrophages and their precursor cells, were marked with a biotin-labeled antibody cocktail and were attached to anti-biotin microbeads; when placed in a magnetic field, those cells which were not absorbed by the column included HSCs, marrow intermediary stem cells, etc. (2) Collecting $CD117^+$ cells (positive selection): $CD117^+$ cells were attached to the microbeads that bind to CD117 antibodies. Those absorbed by the column in a magnetic field were $CD117^+$ cells. (as described in the MACS protocol from Miltenyi Biotec Company)

2.4 The cultivation of MNCs and HSCs *in vitro*

(1) MNCs cultivation *in vitro*: MNCs were cultured in high-sugar DMEM medium containing 10% calf serum, and plated on 24-well-plates with a density of 5×10^4 / mL and 1 mL per well. (2) HSCs cultivation *in vitro*: Lin^-CD117^+ HSCs were cultured in high-sugar DMEM without any serum and plated on 24-well-plates with a density of 1×10^4 /mL. One group was cultured without cytokines, while the other was supplied with cytokines as follows: 20 ng/mL IL-3, 20 ng/mL IL-6 and 50 ng/mL SCF. Both kinds of cells were cultured at 37ºC in a 5% CO_2 incubator.

2.5 The preparation, titer and in-vitro infection of lentiviral vectors

Vector plasmid FUXW, packaging plasmid CMVΔR8.93 and envelope plasmid VSV-G were identified by restriction nuclease analysis, and were then co-trans-infected into 293T cells by calcium phosphate to prepare lentiviral vectors. Northern dot blot was used to titer the FUXW lentivirus (Gong et al., 2004). MNCs and HSCs were cultured *in vitro* for 12h and then lentiviral vectors (MOI ＝100) ($n = 4$) were added to the medium.

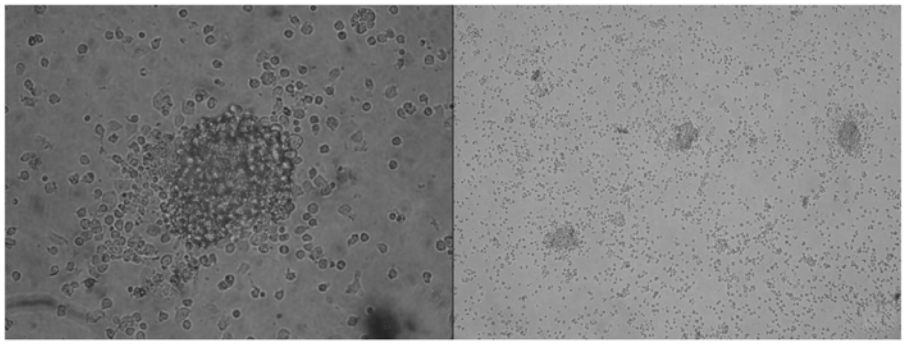
2.6 Detection of hFIX expression

hFIX expression in seven day old cell supernatant was detected by ELISA as described in the literature (Wang et al., 1997).

3 Results

3.1 The isolation and purification of MNCs and their cultivation *in vitro*

The mononuclear cells (MNCs) were isolated from C57 and ICR mice using lymphocyte separating medium. It was found that, on the average, $1\sim2\times10^7$ MNCs could be isolated from the femurs and tibias of individual mice. The MNCs separated from C57 murine marrow were cultured in suspension. CFU-GM (colonies formed by over 40 cells, yellow in color) were observed to form after 2~3 days in the groups with or without cytokines. MNC cultures could be maintained with activity for at least 3~4 weeks (Fig. 1), then the number of colonies would gradually start decreasing.



(a): 100 × (b): 40 ×

Fig. 1 CFU-GM in murine bone marrow MNCs culturing on day 14

3.2 The isolation and purification of HSCs and their cultivation *in vitro*

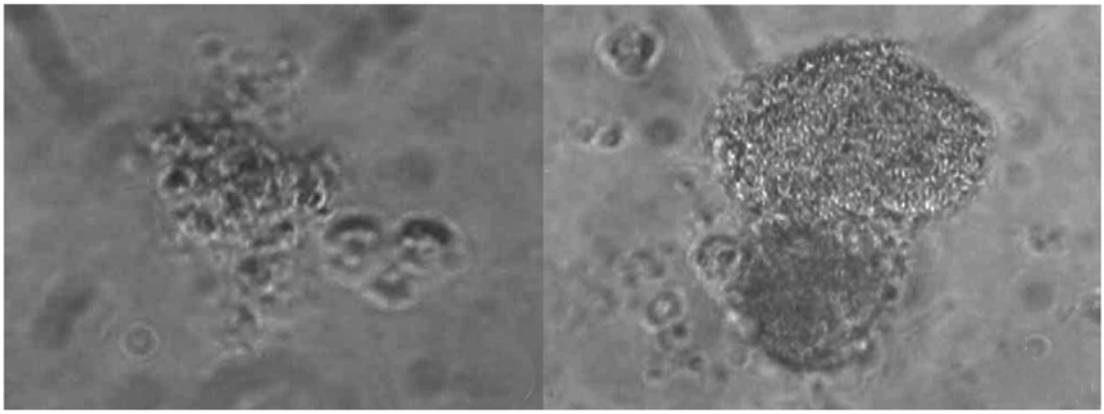
$Lin^{-}CD117^{+}$ HSCs were isolated from C57 murine marrow by MACS and cultured in suspension. In the group supplied with cytokines, the number of HSCs and colonies increased gradually (Fig. 2). By contrast, the number of HSCs did not significantly increase in the control group without cytokines, and the number of colonies was much less than in the cytokine-treated group. Almost all of the cells in the control group died during the third week.

3.3 hFⅨ gene expression of MNCs and HSCs mediated by lentivirus

MNCs isolated from ICR and C57 mice were infected respectively by FUXW lentiviral vectors prepared by calcium phosphate-mediated transient transfection. hFIX expression after day 7 was measured at 41.7 ± 4.2 ng/mL and 34.5 ± 6.6 ng/mL in MNCs taken from ICR and C57 mice respectively. For C57 murine HSCs infected by FUXW lentivirus, hFIX expression in the cytokine-treated group was 46.6 ± 5.7 ng/mL, while in the control group this was 33.3 ± 4.8 ng/mL (Fig. 3). Our results demonstrated that recombinant FUXW lentiviral vectors could efficiently infect murine MNCs and HSCs, and that cytokines could increase the expression of the transgene.

4 Discussion

The number of HSCs is low in marrow cells, which is an obstacle for the isolation and purification of HSCs. The percentage of HSCs is 0.5 % in marrow cells and even lower in peripheral blood. Magnetic cell sorting (MACS) is a simple and feasible technology which is widely used in clinical gene therapy to obtain highly purified HSCs without affecting their activity. In order to prove originality and pluripotentiality, the detection of hematopoietic ability *in vivo* using NOD/SCID mice transplantation is required (Dick et al., 1997). We are currently in the process of doing this work.



(a): without cytokines (b): with cytokines

Fig. 2 Colonies in murine bone marrow HSCs culturing on day 14 (400×)

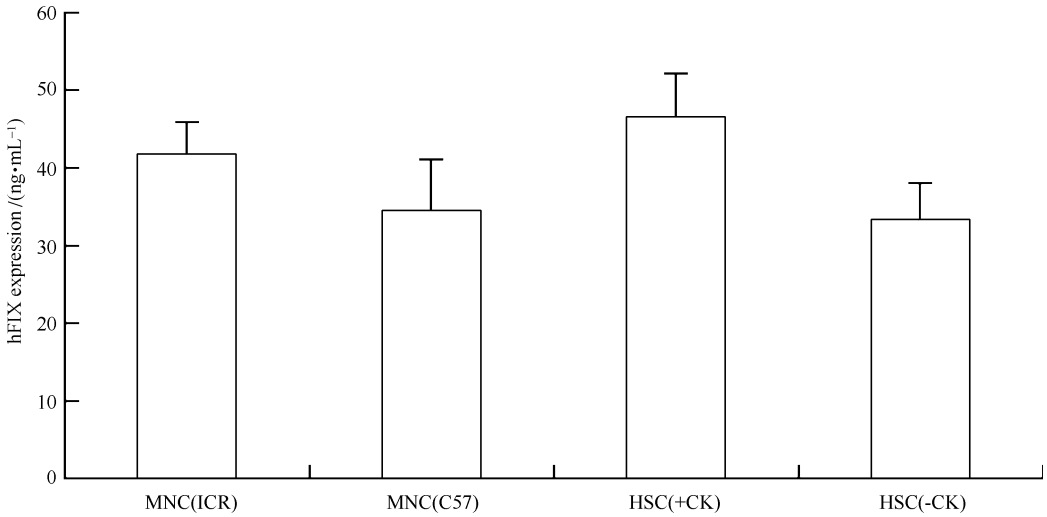


Fig. 3 Expression of hFⅨ in MNCs and HSCs infected by FUXW lentiviral vectors on day 7

The promoters and cis-elements of lentiviral vectors are the main factors for the expression of the transgene in HSCs. The Ubiquitin promoter used in this report can regulate the expression of foreign genes in various tissues (Lois et al., 2002). We used Ubiquitin promoter to regulate hFIX expression in MNCs and HSCs for the first time. The interior promoters used in HSCs include CMV promotor, MSCV promotor, PKG promotor and EF1-α promotor. Among them, CMV promotor has the lowest efficiency, while EF1-α promoter has the higest (Mikkola et al., 2000). It will increase hFIX expression to select a more powerful promoter.

In addition, the cis-element discovered in the pol region of HIV-1 genome called the central polypurine tract (cPPT) has been proven to be an important factor for lentivial vectors' infection into non-dividing cells including HSCs. Kay et al. infected C57Bl/6 mice with cPPT-included lentiviral vectors and achieved hFIX expression of 65 ng/mL (Park et al., 2001). At present, we are constructing lentiviral vectors with cPPT to increase infection efficiency.

Finally, the optimization of infection conditions is also a way to increase the expression of the transgene. Until now, it was unclear whether the treatment of cytokines is required for lentiviral infection of HSCs. This study demonstrated that VSV-G phenotyped lentiviral vectors could infect murine HSCs without cytokines but the efficiency was increased when cytokines were added. The reason may be that cytokines stimulated the proliferation of HSCs and therefore increased the infection efficiency of the lentivirus. Similar results were reported by Sutton et al. that only a part of HSCs could be infected by lentivirus when no suitable cytokines induced cell proliferation (Sutton et al., 1999). However, stimulation by cytokines would lead to the differentiation of HSCs and their failure of homing ability. Moreover, it will reduce the long-term reconstitution ability of the hematopoietic system.

We successfully isolated murine MNCs and HSCs and studied the lentivirus-mediated gene transfer *in vitro*. Relatively high expression of hFIX was detected and the effect of cytokines on gene expression was also studied. This work has achieved *in vitro* expression of hFIX in HSCs mediated by lentivirus and may be helpful in animal testing of hemophilia B gene therapy mediated by lentiviral vectors via HSCs.

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