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THE PROLIFERATION OF HEMATOPOIETIC STEM CELLS FROM UMBILICAL CORD BLOOD WAS ENHANCED BY USING PEPTIDE SL-13R

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ABSTRACT

Umbilical cord hematopoietic stem cells (UC-HSCs) are capable of self-regeneration and differentiation into many different types of blood cells, helping to treat blood diseases such as leukemia, Hodgkin's disease and non-Hodgkin's lymphoma, as well as for transplantation. However, the number of UC-HSCs is still very small due to limitations in isolation and proliferation. In this study, UC-HSCs were cultured with the bioactive SL-13R peptide to examine whether SL-13R enhances UC-HSC proliferation. Here, we showed that $CD34^+$UC-HSCs can be enriched by up to 47.08% by culturing total mononuclear cells (MNCs) from umbilical cord blood (UCB) in serum-free StemMACS medium supplemented with a cytokine cocktail. We demonstrated that SL-13R enhanced the proliferation of $CD34^+$UC-HSCs by 1.21-1.44 times compared to that of the controls. These results suggested that StemMACS medium supplemented with cytokines and SL-13R can be used to expand $CD34^+$UC-HSCs *ex vivo* for medical treatment and transplantation.

Keywords: Umbilical cord blood, mononuclear cells, hematopoietic stem cells, SL-13R peptide, cell proliferation.

INTRODUCTION

HSCs are considered the progenitors of all types of blood cells in the body. HSCs represent the origin of a complex hierarchy that creates functional blood cells. These HSCs are produced from the bone marrow and mostly maintain a silent stage (nonproliferative) until receiving signals from growth factors or cytokines to generate all blood cell lineages (Gvaramia *et al*., 2017). Therefore, HSCs have been applied

for many purposes in regenerative medicine, such as transplantation to treat cancer, injured cells and hematological disorders (Juric *et al*., 2016). Among the various sources of HSCs, those from the UCB are of greatest concern because of the safety and convenience of the collection process, the permissiveness of HLA mismatch and the lower risk of graft-versus-host disease compared to other sources of HSCs (Cutler *et al*., 2001). However, unlike cultured mesenchymal stem cells, HSCs do not attach to the plastic layer of culture dishes but rather to suspensions. This makes it difficult to culture and expand HSCs. On the other hand, the number of HSCs cultured from UCB is limited, leading to decreased efficacy in clinical treatment. To overcome these disadvantages, many studies have been performed to determine the optimal conditions for expanding UC-HSCs (Miller *et al*., 2013). There are studies on the expansion of HSCs by using cytokines such as IL-3, GM-CSF, IL-4, and ILF-γ (Ogawa *et al*., 1993, de Bruin *et al*., 2013). Recently, small molecules such as bioactive peptides have been shown to be effective at promoting the proliferation of stem cells (Dayem *et al*., 2023). SL-13R is an artificial bioactive peptide that was developed by extraction from a sequence of the extracellular domain of the DLK1 protein (Nii *et al*., 2021). Bioactive peptides are short fragments of peptides that are often hidden in the structures of the protein of origin and have many applications in metabolic, immune system, antimicrobial, proliferation and differentiation mechanisms in stem cells (Akbarian *et al*., 2022). It plays crucial roles in regulating stem cell pools and tissue differentiation as well as contributing to maintaining the stemness of many stem cells. It also acts as a paracrine factor secreted by the stem cell niche to maintain aggressive stem cell characteristics in tumor cells (Grassi *et al*., 2021). In this study, we tested the ability of a specific culture medium supplemented with a cytokine cocktail to increase the number of total MNCs and thereby enrich CD34+UC-HSCs. In addition, we investigated the effects of the bioactive peptide SL-13R on the proliferation of CD34+UC-HSCs at different culture time points and peptide doses.

MATERIALS AND METHODS

Isolation of $CD34^+$HSCs from UCB

Human umbilical cord samples were collected from women who presented at the Hanoi Obstetrics and Gynecology Hospital. The study was performed in accordance with protocols approved by the ethics committee of the Hanoi Hospital of Obstetrics and Gynecology (Ethical issue: No. 2206 CN/PS). Umbilical cord blood samples were drawn directly into a 50 ml tube containing anticoagulants and immediately transferred to the laboratory at 4 degrees Celsius, and HSC collection experiments were conducted within 4 hours of sample collection. A buffer solution including PBS (pH 7.2), $Ca2^+$, Mg^{2+}-free, 0.5% (w/w) BSA and 2 mM EDTA (with the buffer kept at 2-8°C) was prepared, and then the blood sample was diluted with this buffer at a ratio of 1:1. Then, 15 ml of Ficoll-Paque solution (p = 1.077 g/ml; Sigma–Aldrich) was added to a 50 ml Ficoll tube, 35 ml of diluted blood was carefully added on top, and the mixture was centrifuged at 400 × g for 35 minutes at 20°C using a swinging bucket rotor without braking. The sample inside the tube was separated into 4 layers. The serum in the first layer was gently removed, and the serum in the second layer containing the mononuclear

cells was collected in another 50 ml centrifuge tube. The cells were washed with buffer, mixed and centrifuged at 300 × g for 10 minutes at 20°C. The pellet was resuspended in media to culture mononuclear cells (MNCs). UC-HSCs were isolated with a CD34 Microbead kit (Miltenyi Biotec, Germany) and a Macs multistand cell isolation kit (Miltenyi Biotec, Germany) according to the manufacturer's protocol.

Flow cytometry

After 3 weeks of culture, $CD34^+$UC-HSCs were collected from culture wells, and the cell suspension was centrifuged at 300 × g for 10 minutes to completely remove the supernatant. Then, the cells were resuspended in 100 µl of buffer, and 10 µl of CD34 antibody or 10 µl of buffer was added to the control samples. The solution was mixed well and incubated for 10 minutes in the dark at a cold temperature (2–8°C). One milliliter of buffer was added, the cells were centrifuged at 300 × g for 10 minutes to wash the cells, and the excess antibody was added to the cells. Then, 0.5 ml of PBS buffer containing 4% paraformaldehyde was added, and the cells were mixed well and incubated at 4°C for 15 minutes. The cells were centrifuged at 1.500 rpm for 5 minutes, after which all the supernatants were removed. Then, the cells were resuspended in 0.5 ml of PBS buffer and placed on a Macs QuantVYB flow cytometer (Miltenyi Biotec, Germany).

Examination of $CD34^+$UC-HSCs proliferation with the SL-13R peptide

UC-HSCs obtained after magnetic separation were grown at a concentration of 5000 cells/ml in 24-well plates using serum-free StemMACS HSC Expansion media XF (Miltenyi Biotec, Germany) supplemented with 20 ng/ml Flt3-L cytokine, 20 ng/ml IL-6, 50 ng/ml SCF, and 10 ng/ml TPO. The culturing method used for UC-HSCs was previously described (Nii *et al.*, 2021). The medium was changed every 2-3 days. After culture, UC-HSCs were analyzed for the expression of the specific marker CD34 using flow cytometry. CD34+ UC-HSCs were cultured at 5000 cells/well either with or without SL-13R and harvested them at 5 different time points (1, 3, 5, 7 and 9 days). The cell plates were cultured at 37°C with CO_2 maintained at 5% and 90% humidity. The medium was changed every 2-3 days. CD34+ UC-HSCs were also cultured at 5000 cells/well with different concentrations of SL-13R (1, 5, 10, 20 and 50 µg/mL). After the specified time points, the number of cells was counted by using a red blood cell counting chamber, and the cell number was calculated.

Statistical analysis

The statistically significant differences between groups were assessed by Student's t-tests. The data are shown as the means ± standard deviations (SD). P values < 0.05 were considered to indicate statistical significance.

RESULTS AND DISCUSSION

Isolation and culturing of MNCs from UCB for enriching $CD34^+$UC-HSCs

Because the percentage of $CD34^+$UC-HSCs from UCB was very low, at only 0.1-0.5%, the total MNCs isolated from UCB were cultured in StemMACS HSC Expansion media XF medium supplemented with a cytokine cocktail to enrich UC-HSCs. After

3 weeks of culture, optical microscopy revealed that the number of MNCs increased (Figure 1A). Flow cytometry analysis revealed that 47.08% of the total MNCs were $CD34^+$ (Figure 1B). These results demonstrated that the number of UC-HSCs in the MNC population increased many times. There is still controversy regarding the preselection of cells for HSC expansion. Some reports have shown that enrichment of HSCs by culturing total MNCs may be a good method for obtaining high amounts of HSCs (Briddell *et al.* 1997, Chivu *et al.*, 2004, Koller *et al.*, 1998, Madkaikar *et al.*, 2007). In addition, different cytokine combinations/cocktails in culture media can affect HSC proliferation (Möbest *et al.*, 1999, Petzer *et al.*, 1996). Cytokines, including IL-3, IL-6, SCF, TPO, FLT3L, and G-CSF, are preferred for use in HSCs. In this study, we used a cytokine combination described by Nii *et al.* (2021). However, the effects of the concentration and ratio of cytokines as well as the doses of growth factors on the proliferation and fate of HSCs should be further investigated.

A **B**

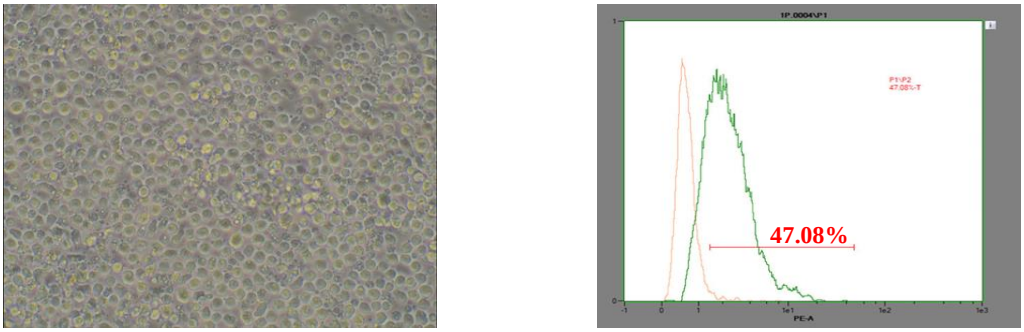

Figure 1. (A) MNCs observed under a 20x objective optical microscope; (B) $CD34^+$UC-HSCs analyzed by flow cytometry. The results are representative of three independent experiments.

Effects of the SL-13R peptide on the proliferation of $CD34^+$UC-HSCs at different time points

UC-HSCs stimulated with SL-13R were cultured for different durations to examine changes in cell number during culture. Table 1 shows that the number of SL-13R-stimulated UC-HSCs was significantly greater than that of SL-13R-nonstimulated UC-HSCs by 1.21-, 1.40-, 1.34-, 1.39- and 1.44-fold after 24, 72, 120, 168, and 216 hours of culture, respectively. These results suggested that the SL-13R peptide enhanced the proliferation of UC-HSCs.

Table 1. Number of $CD34^+$UC-HSCs according to time points at which proliferation was stimulated with or without the SL13R peptide.

Culture time (hrs)	**Cells cultured without SL-13R (10 µg/ml)**		**Cells cultured with SL-13R (10 µg/ml)**		**Cells cultured with SL-13R/without SL-13R (fold)**
	Cell numbers	Doubling time (hrs)	Cell numbers	Doubling time (hrs)	
0	5000		5000		1.0

24	5780 ± 20	114.8 ± 2.8	7033 ± 57*	48.7±1.2	1.21
72	9833 ± 76	73.8 ± 0.9	13790 ± 128*	49.2 ± 0.5	1.40
120	20600 ± 625	58.8 ± 1.3	27633 ± 1115*	48.7±1.1	1.34
168	40467 ± 1976	55.7 ± 1.3	56300 ± 3915*	48.1±1.4	1.39
216	79367 ± 1124	54.1 ± 0.3	114600 ± 10671*	47.9±1.4	1.44

*$p < 0.05$.

Effects of different concentrations of the SL-13R peptide on the proliferation of CD34$^+$UC-HSCs

Table 2 shows that after 5 days of culture, the number of cells stimulated with 1, 5, 10, 20, or 50 µg/ml SL-13R was significantly greater than that of nonstimulated SL-13R cells. The cell density of the UC-HSCs reached a stationary phase starting at 10 µg/ml.

StemMACS expansion medium was demonstrated to effectively proliferate HSCs (van Til *et al.*, 2014). In the present study, the results of an examination using the additional bioactive peptide SL-13R indicated that a greater number of total HSCs were present. In addition, it is necessary to evaluate the ability of CD34$^+$UC-HSCs to differentiate into many other types of blood cells for specific applications (Viswanathan *et al.*, 2017). In the future, the combined effects of the SL-13R peptide need to be further investigated for supporting therapies involving HSCs (Esmaeili *et al.*, 2019).

Table 2. Number of CD34$^+$UC-HSCs stimulated with different concentrations of the SL-13R peptide after 5 days of culture.

Concentration of SL-13R (µg/mL)	Cell numbers	Doubling time (hours)
0	19933 ± 400	60.2 ± 0.9
1	22433 ± 513*	55.4 ± 0.8
5	26100 ± 794*	50.4 ± 0.9
10	28000 ± 700*	48.3 ± 0.7
20	28500 ± 500*	47.8 ± 0.5
50	28200 ± 1014*	48.1 ± 1.0

*$p < 0.05$.

CONCLUSION

UC-HSCs were enriched from UCB, with 47.08% of the total MNCs. The proliferation rate of CD34$^+$UC-HSCs cultured with SL-13R was approximately 1.21-1.44 times greater than that of CD34$^+$UC-HSCs cultured without SL-13R. These results suggested that long-term culture of MNCs and the bioactive peptide SL-13R can be used as tools to stimulate the proliferation of CD34$^+$UC-HSCs to obtain a large number of cells for medical applications.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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