Tissue and Cell 48 (2016) 305–311

Contents lists available at ScienceDirect

Tissue and Cell

journal homepage: www.elsevier.com/locate/tice

Evaluation of umbilical cord blood CD34+ hematopoietic stem cells expansion with inhibition of TGF-β receptorII in co-culture with bone marrow mesenchymal stromal cells

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ARTICLE INFO

Article history:
Received 23 September 2015
Received in revised form 5 June 2016
Accepted 7 June 2016
Available online 8 June 2016

Keywords:
CD34+ hematopoietic stem cells
Mesenchymal stromal cells
Cord blood

ABSTRACT

Background: Umbilical cord blood (UCB) is an important source of hematopoietic stem cells (HSCs). However, low number of HSCs in UCB has been an obstacle for adult hematopoietic stem cell transplantation. The expansion of HSCs in culture is one approach to overcome this problem. In this study, we investigated the expansion of UCB-HSCs by using human bone marrow mesenchymal stromal cells (MSCs) as feeder layer as well as inhibiting the TGF-β signaling pathway through reduction of TGF-βRII expression.
Materials and methods: $CD34^+$ cells were isolated from UCB and transfected by SiRNA targeting TGF-βRII mRNA. $CD34^+$ cells were expanded in four culture media with different conditions, including 1) expansion of $CD34^+$ cells in serum free medium containing growth factors, 2) expansion of cells transfected with SiRNA targeting TGF-βRII in medium containing growth factors, 3) expansion of cells in presence of growth factors and MSCs, 4) expansion of cells transfected with SiRNA targeting TGF-βRII on MSCs feeder layer in medium containing growth factors. These culture conditions were evaluated for the number of total nucleated cells (TNCs), CD34 surface marker as well as using CFU assay on 8th day after culture.
Results: The fold increase in $CD34^+$ cells, TNCs, and colony numbers (71.8 ± 6.9, 93.2 ± 10.2 and 128 ± 10, respectively) was observed to be highest in fourth culture medium compared to other culture conditions. The difference between number of cells in four culture media in 8th day compared to unexpanded cells (0 day) before expansion was statistically significant ($P < 0.05$).
Conclusion: The results showed that transfection of $CD34^+$ cells with SiRNA targeting TGF-βRII and their co-culture with MSCs could considerably increase the number of progenitors. Therefore, this method could be useful for UCB-HSCs expansion.

1. Introduction

As an important source of stem cells, umbilical cord blood has attracted much attention mostly due to its application in HSCs transplantation (Hofmeister et al., 2007; Broxmeyer et al., 1989; Gordon, 2008; Laughlin et al., 2001). There are a number of reasons that have made UCB a good option for transplantation purposes, including no ethic problems, no need for perfect HLA compatibility, no risks for donor, low risk of infection transmission, freezing capacity, availability and immediate use (Laughlin et al., 2001; Gluckman, 2000; Gluckman et al., 2001; Wagner et al., 2002). However, the limited number of hematopoietic progenitors in UCB is regarded as a limitation for their application in adult transplantation and since the number of these cells is the most critical parameter for a satisfactory transplantation, solutions should be adopted to deal with these issues (Migliaccio et al., 2000; Gilmore et al., 2000; Kelly et al., 2009). One such solution is the ex vivo expansion of HSCs by providing favorable conditions in culture

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http://dx.doi.org/10.1016/j.tice.2016.06.003
0040-8166/

medium so as to improve the outcome of HSCs transplantation (Tung et al., 2010). HSCs have a unique niche in bone marrow consisting of growth factors, cytokines and distinct types of cells that provide desired conditions for their growth, expansion and self-renewal (Jing et al., 2010). MSCs play a prominent role in survival and expansion of HSCs. They can enhance the survival and self-renewal of HSCs through secretion of cytokines, growth factors and cell to cell contact via adhesion molecules. Utilization of MSCs as a feeder layer interacting with HSCs and progenitor cells can support the growth and proliferation of these cells (Jing et al., 2010; Robinson et al., 2006). Cytokines can have a crucial role in cell fate as their in vitro release or inhibition can be remarkably effective upon cell function. Transforming growth factor (TGF)-β1 is an important cytokine and a negative regulator of primitive HSCs expansion (Karlsson et al., 2007; Larsson et al., 2003). TGF-β1 can keep HSCs in static mode by cell cycle arrest and is also able to inhibit the in vitro expansion and self-renewal of these cells (Larsson et al., 2003; Larsson and Karlsson, 2005). After TGF-β1 binding to TGF-βRII, type I and type II receptors make a heterodimeric complex. TGF-βRI leads to R-Smads phosphorylation, which is hetero-oligomerized with Smad4 and eventually controls the gene expression (Oshima et al., 1996). Using antisense oligonucleotide and TGF-β neutralizing antibodies in HSCs causes an increase in the number and size of colonies derived from primitive hematopoietic cells in response to different cytokine combinations (Ruscetti et al., 2008; Hatzfeld et al., 1991). Treatment of HSCs with TGF-β1 neutralizing antibodies to inhibit autocrine TGF-β1 greatly increases the transplantation rate of these cells in a specific period of time (Bartelmez and Iversen, 2001; Ruscetti et al., 2005). Given the aforementioned literature and due to the importance of the number of HSCs in transplantation, in this study, we aimed at investigating the effects of TGF-β signaling inhibition together with MSCs and growth factors on the proliferation of HSCs derived from UCB.

2. Materials and methods

2.1. Isolation of CD34⁺ cells from umbilical cord blood

Three UCB specimens were collected from the newborns immediately after delivery by obtaining informed consent from parents. Hydroxyethyl starch (Stem Cell Technologies, Copenhagen, Denmark) was used to precipitate the red blood cells. After isolation of RBCs, mononuclear cells were isolated from cell suspension using Ficoll (Sigma, St. Louis, MO, USA). In order to isolate $CD34^+$ cells, anti-human CD34 antibody conjugated with Microbeads (Miltenyi Biotec, Auburn, CA, USA) and Magnetic-activated cell sorting (MACS) method (Miltenyi Biotec, Auburn, CA, USA) were used (Yang et al., 2009). The isolated cells were counted by hemocytometer and the viability of cells was assessed using trypan blue (Stem Cell Technologies, Copenhagen, Denmark) staining. Finally, anti-human CD34/FITC monoclonal antibody (Dako, Copenhagen, Denmark) and flow cytometric analysis (BD Bioscience, San Diego, CA, USA) were used to evaluate the purity of $CD34^+$ cells (Tung et al., 2010).

2.2. Purity and viability of isolated CD34⁺ cells from cord blood

The purity and viability of $CD34^+$ cells were evaluated using flow cytometric analysis and trypan blue staining, respectively, on an average of three samples. Both assays were performed after isolating the mononuclear cells from three UCB samples. Purity and viability of these cells were $89.6 \pm 2.5\%$ and $91.0 \pm 3.0\%$, respectively.

2.3. Isolation of bone marrow MSCs

Bone marrow specimens (with heparin) were collected from three healthy donors following written consent. Mononuclear cells were isolated using Ficoll and were then cultured in 25 cm^2 culture flasks containing 5cc Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO, USA) \low Glu\10% fetal bovine serum (FBS) and incubated at 37 °C and 5% CO_2. To remove the non-adherent cells, the culture medium was withdrawn after 72 h and refreshed every 3 days for 14 days. The cells were passaged when the confluency of MSCs in the bottom of flask reached 80%. We used flow cytometric analysis and osteoblast-directed differentiation to confirm whether isolated cells from bone marrow are MSCs or not. To evaluate MSCs, we used CD44, CD166, CD90, CD105, CD45 and CD34 antibody (Dako, Copenhagen, Denmark) and to differentiate the cells into osteoblast, DMEM sterile medium, 1 μL dexamethasone 10 mM (Chemicon, Billerica, MA, USA), 1cc FBS and 100 μL glycerol phosphate 1 M (Chemicon, Billerica, MA, USA) were used. Also, Alkaline Phosphatase and Red Alizarine specific stainings were used for confirmation of osteoblasts (Jing et al., 2010; Oswald et al., 2004).

2.4. Transfection of CD34⁺ cells with SiRNA against TGF-βRII

Stealth™ select RNAi targeting TGF-βRII was used to transfect $CD34^+$ cells, and Negative Control Stealth™ RNAi was the negative control. We also used Block-iT™ Alexa Fluor Red (conjugated RNAs with Fluor Red) to evaluate the efficiency of cell transfection. Finally, Lipofectamine™ RNAi MAX was utilized to transfect SiRNA into cells (all these materials were obtained from Invitrogen Corporation, CA, USA). We selected three independent UCB samples that were experimented in duplicate. 500 μL of Stemspan (serum-free) medium (Life Technologies, Burlington, Ontario, Canada) containing $60–80 \times 10^3$ $CD34^+$ cells was added to each 24-well plate. The wells were divided into three groups including SiRNA targeting TGF-βRII, Negative Control, and Block-iT™ Alexa Fluor Red. To transfect cells in each well, 50 μL of Opti-MEM solution (Gibco, Karlsruhe, Germany) and 8 pmol of double-stranded SiRNA were added in a microtube and 50 μL of Opti-MEM were mixed with 1 μL of lipofectamine in another microtube. These two microtubes were mixed together and incubated (25 min) to form SiRNA-lipofectamine complexes (according to the kit protocol). The formed complexes were added to the three aforementioned groups and after 8 h, the culture medium was replaced with fresh Stemspan medium.

2.5. Evaluation of the TGF-βRII suppression using quantitative real-time PCR

Cellular RNA was extracted using RNA extraction kit (Qiagen, Hilden, Germany) according to the kit protocol at 48, 72 and 96-h intervals after transfection of cells with SiRNA. Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) enzyme (Fermentase, PA, USA) was used for cDNA synthesis. 25 μL of Master Mix (Roche, Basel, Switzerland), 2 μL (10 pmol) Primer and 2 μL (100 ng) cDNA were mixed, and the volume reached to 50 μL with distilled water.

Oligo 6 software was used to design specific primers. The sequence of the primers designed for TGF-βRII gene was as follows:

TGF-βRII: Forward

5′-TTTTCCACCTGTGACAACCA-3′

TGF-βRII: Reverse

5′-GCTGATGCCTGTCACTTGAA-3′0

Real-time PCR reaction was performed according to the following conditions: initial denaturation at 95 °C for 5 min, secondary