Communication of substance P, calcitonin-gene-related neuropeptides and chemokine receptor 4 (CXCR4) in cord blood hematopoietic stem cells

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Abstract
Background: Modulation of the expression of CXCR4 as a critical adhesion molecule on cord blood (CB) CD34+ cells could overcome delay following cord blood transplantation. Identification of beneficial effects of growth factors including cytokines and neuropeptides on CXCR4 expression would enable our understanding of this complicated network. Therefore, we aimed to assess the role of substance P (SP) and Calcitonin gene related peptide (CGRP) on CXCR4 levels.

Material and methods: CD34+ cells purified from CB were cultured in a serum-free liquid culture system. Different concentrations of SP and CGRP were used in combination with cytokine cocktail. Expression of CXCR4 at protein and genomic levels was assessed by flow cytometry and real time RT-PCR.

Results: Our results indicate increased CXCR4+ CD34+ cells after 7 days cultivation with SP and/or CGRP. Increased gene expression of the CXCR4 molecule was observed at 10^{-9} M either SP or CGRP individually, by day 11 as compared to control group.

Conclusions: Our study indicates that SP and CGRP induce CXCR4 protein expression in short term culture, and stimulate its expression. Consequently, the increased expression of CXCR4 could improve engraftment of CB CD34+ cells.

1. Introduction

The central nervous system can regulate hematopoiesis through cytokine, chemokine and neurotransmitter release. Neuropeptides exist in the immune system as well as the central and peripheral nervous system. Innervations of the hematopoietic microenvironment and its local production by resident cells suggests its hematopoietic contribution (Nowicki et al., 2007). Interestingly, SP stimulates hematopoiesis through secretion of stimulatory cytokines and by initializing proliferative signal (Murthy et al., 2007). Noticeably, collocation of SP and calcitonin-gene-related peptides (CGRP) in the nerve ends of sensory fibers suggests their possible contribution in physiological as well as the hematopoietic process (Wu et al., 2007). Therefore, these agents can be considered as non hematopoietic growth factors in regulation of hematopoietic stem cell’s function.

Cord blood (CB) stem cells are an alternative source of hematopoietic stem cells (HSCs) for transplantation. However CB contains limited amounts of stem cells and when used in transplantation demonstrates delayed engraftment, attributed to the due to immaturity of HSCs with low expression of adhesion molecules (Zheng et al., 2003; Zheng et al., 2004). Among adhesion molecules, CXCR4 is known to play a critical role in homing and repopulating of stem cells (Burger et al., 2003). Activation of CXCR4 receptors results in activation of G proteins and downstream signals leads to cell activation and upregulation of NF-κB activity and increased intracellular calcium. Also, CXCR4 can activate other adhesion molecules involved in homing process such as integrins, particularly, very late antigen 4 (VLA-4) and VLA-5 integrin on human CD34+ cells (Balkwill 2004). Cytokines such as stem cell factor and interleukin (IL)-6,
IL-4, and IL-10 can induce transcription of CXCR4 mRNA (Zhang et al., 2007).

Interestingly, there is positive correlation between Tac-1 as SP producer gene and CXCR4 expression, dose dependently, in response to low concentration of SDF1 as specific ligand for CXCR4 in BM microenvironment, while higher concentration showed reverse effects (Corcoran et al., 2007). Antagonists to SDF and SP such as dipeptidylpeptidase IV/CD26 and MMP-2/9 enhance CXCR4 expression and HSCs/HPCs mobilization (Watt, 2008). Negative regulatory role of CD26 may also occur via degradation of SP and consequently, SDF cleavage. Additionally, there is evidence about the inhibitory effect of SP on MMP-2/9 (Cohen et al., 2007). Cleavage of CXCL12 N-terminal residues by MMP-2/9 also enhance HSCs/HPCs mobilization (Watt, 2008).

Noticeably, SP could be considered in mobilizing HSCs via enhancing GM-CSF to stimulate HSCs mobilization and hematopoietic stimulation (Corcoran et al., 2007). Additionally, SP inhibits TGF-β1, VEGF and BFGF production that can stimulate CXCR4 up regulation while increasing IFN-α and γ which show negative effect on CXCR4 expression (Balkwill 2004).

These controversial effects and many unknown aspects of effects of SP on CXCR4 demand more research. In spite of the existence of little information about the effect of SP in SDF1 and CXCR4 regulation, there is no evidence about CGRP as its collocated neuropeptide.

Therefore, because of the importance of interaction of growth factors with CXCR4 in hematopoietic microenvironment, we aimed to explore effects of SP and CGRP either individually or combined on genomic and protein expression of CXCR4 on CB CD34+ cells.

2. Materials and methods

2.1. CD34+ cell purification

Following RBC depletion of cord blood samples by Hydroxy Ethyl Starch 6% (HAS), leukocyte enriched supernatant was collected and diluted in Phosphate-Buffered Saline (PBS) containing 1% Bovine Serum Albumin (BSA) (Sigma) and EDTA (2 mM). The mononuclear cells were isolated by Histopaque-1077 (BAG, Germany) density gradient centrifugation.

Magnetic cell sorting (MACS) system (Miltenyi Biotech; Bergisch Gladbach, Germany) was used for CD34+ cells isolation, according to the manufacturer’s instructions with some minor modifications. Briefly, up to 1006 MNCs in 300 μl were incubated with 100 μl of FcR Blocking Reagent and 100 μl of CD34 MicroBeads for 30 min at 4°C. For removing free magnetic particles, the cells were washed twice with cold PBS-EDTA (2 mM)-BSA 0.5% at 400 g for 7 min in 4°C. Sample was loaded on prepared LS columns, where CD34+ cells passed through the column while the positive fraction was trapped. Then, column was removed from Midi MACS and CD34+ cells isolated by pushing plunger. The viability and count of cells was determined by trypan blue dye exclusion method.

2.2. CD34+ cell cultivation

Purified CD34+ cells/ml were cultured in 24-well plate in StemSpan® Serum-Free Expansion Medium (SFEM) (Stem Cell Technologies Inc., Canada) containing SCF at 50 ng/ml, FL at 25 ng/ml, TPO at 25 ng/ml, IL3 and IL6 each at 10 ng/ml. In addition to cytokine cocktail, SP and CGRP were used in 10−7, 10−9 and 10−11 M concentrations either separately or combined (SP plus CGRP; SG). Control groups were treated only with cytokine cocktail. The cultures were maintained at 37°C and 5% CO2 in a humidified atmosphere for either 7 or 11 days. After the incubation period, cells were harvested and evaluated for CXCR4 expression by flowcytometric analysis.
2.3. Analysis of CXCR4 expression by flow cytometry

For analysis of CXCR4 expression, CD34+ cells, either before or after culture were analyzed. Cells were stained with monoclonal antibodies (mAbs) conjugated to phycoerythrin (PE) and fluorescein isothiocyanate (FITC). The mAbs included PE mouse anti-human CXCR4 (CD184) (Clone 12G5) and isotype mouse IgG2a (BD Pharmingen San Diego, CA, USA). FITC conjugated Ab CD34 (Clone AC136) and isotype mouse IgG2a, were purchased from Miltenyi Biotech. Approximately 10^5–10^6 cells were double stained by primary Abs and incubated at 4°C in the dark for 15 min with occasional shaking. For excluding nonspecific binding, staining was performed by isotype control for both PE and FITC fluorescence. Cells were washed twice with buffer PBS-1% BSA-0.01% NAN3, then fixed in 1% paraformaldehyde and analyzed by flow cytometry.

2.4. Real time RT-PCR analysis for the expression of CXCR4 gene

To analyze the expression of CXCR4 gene, 1 µg RNA of non-expanded cells was extracted and reverse transcribed. The resulting cDNA was quantitatively analyzed by real time PCR using Light Cycler instrument (Roche Molecular Biochemicals, Mannheim, Germany) with the DNA-binding dye SYBR Green I (Roche, Germany). The amounts of CXCR4 were normalized by GAPDH as housekeeping. The sequences of the primers were as follows: (GAPDH-F) 5’–GCA GGG GGC ACC CAA AAG GGT–3’, (GAPDH-R) 5’–TGG GTG GCA GTG AAT GCA TGG–3’, (CXCR4-F) 5’–GAACCT GTTTGTCTGAAGA–3’ and (CXCR4-R) 5’–CTTGCTGCTCCTGTTCT–CA–3’. The 20 µl PCR reaction mixture consisted of 1 pmol/L of each primer, 4 µl Master Mix, 12 µl H2O and 2 µl of templates. Samples were amplified with 40 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 2 s, and extension at 72°C for 10 s. Data was analyzed by 2–ΔΔCT method.

3. Results

3.1. Flow cytometry of CXCR4 molecule

To enumerate the frequency of expression and antigen density of the CXCR4 molecule, flow cytometry was performed. Purity of isolated CD34+ cells was more than 90%. Results indicated an increase in the percentage of CD34+ CXCR4+ cells (about 3-fold) at all concentration of SP treatment cells compared with the control group by day 7. Also, a significant increase was observed by day 7 in the 10^-7 and 10^-9 M CGRP and SG treated cells. Moreover, the frequency of this population was statistically (P < 0.05) significant increased in 10^-9 M CGRP and 10^-11 M SP by day 7 compared with day 0 (Figs. 1 and 3). Additionally, a significant rise relative to

![Fig. 3](image-url) Flowcytometric analysis of frequency of CD34+ CXCR4+ cells following SP and/or CGRP treatments. Flowcytometric patterns of CD34+ CXCR4+ cells on days 7 (A), and 11 (B) in cells treated with various concentrations of SP and/or CGRP in comparison to the control group. Data present randomly one independent sample of each group.
day 0 was observed after 11 days cultivation of $10^{-7}$ and $10^{-11}$ M CGRP, and $10^{-11}$ M SP. Antigen density (MFI) of CXCR4 molecules in $10^{-9}$ M SP and SG treated cells also increased significantly by day 7 compared with day 0 (Figs. 2 and 3).

3.2. Real time RT-PCR analysis for the expression of CXCR4 gene

To determine expression of CXCR4 mRNA, Real time RT-PCR analysis was performed. Results indicate that by 11 days cultivation, $10^{-9}$ M SP and CGRP treated cells expressed CXCR4 statistically significant higher than their control group and freshly purified cells by day 0 (Fig. 4).

4. Discussion

Compared with BM and mobilized peripheral blood (mPB), delayed hematopoietic recovery was observed following transplantation using CB. It has been suggested that increased adhesion molecule expression may be beneficial to accelerate engraftment (Zheng et al., 2004). Low expression of CXCR4 has been identified on CB-CD34 (bright) and they suggested lower expression of adhesion molecule is a major cause of delayed hematopoietic reconstitution after CB transplant (Zheng et al., 2004). There is also a correlation between neutrophil and platelet synthesis with CXCR4 as major cell deficiency after CB transplantation (Liu et al., 2003). The critical role of CXCR4 has been clarified in adhesion and engraftment of stem cells to extracellular cell adhesion matrix (Voermans et al., 1999; Zheng et al., 2003). Therefore clarifying the role of different factors in regulating CXCR4 expression will...
be helpful. Increased expression of CXCR4 following SCF + FL + TPO + IL-6 cultivation has been reported after 7 day expansion (Mao et al., 2006). SCF has also been shown to induce upregulation of CXCR4 expression (Cancelas et al., 2006).

According to our previous studies about expanding capabilities of these neuropeptides and stimulating adhesion molecules expression on CB stem cells (Shahrokhi et al., 2010) as well as involvement of SP in complicated network of SDF-CD26-CXCR4 (Corcoran et al., 2007), we aimed to evaluate the effect of SP and CGRP – its physiological collocate neuropeptide – on genomic and protein level of CXCR4 molecule on CB stem cells.

We used mAb 12G5 for flowcytometry enumeration, which binds epitopes of CXCR4, that serves for SDF-1 binding and signaling (Balkwill 2004). Our flowcytometric results indicated that neuropeptide–cytokine treatment in short time cultivation enhances the frequency of CXCR4+ CD34+ cells. Therefore, this expanded population in ex vivo condition could help improved engraftment due to increased CXCR4 expression. These findings are in accordance with expansion of SP and CGRP treated cells in short time cultivation (7 days) (Shahrokhi et al., 2010). Short term cultivation of stem cells with cytokines up regulates CXCR4 expression. Following CXCR4 over-expression, migration and repopulating capacity of CXCR4+ CD34+ cells increases at low concentration of SDF. These cells migrate toward BM, proliferate extensively (Kahn et al., 2004) and induce Tac1 expression. Consequently, stimulation of hematopoiesis occurs through SP receptor, NK1 (Surbek et al., 2000; Lee et al., 2004; Murthy et al., 2007). Also, SDF1 could be induced by SP as well (Greco et al., 2004). Here, we observed this cooperative effect between SP, CGRP and CXCR4. Future studies are required to examine the response of neuropeptide treated cells to SDF1. If proven, these ex vivo neuropeptide treated cells could be used in clinical transplantation procedures.

The correlation of CXCR4 over-expression and expansion of CD34+ CD38− cells in neuropeptide treated cells (Shahrokhi et al., 2010) is in agreement with similar reports on cytokine cocktail treatment (Jo et al., 2000).

With regards to CXCR4 genomic expression, enhanced gene expression was observed in 10−9 M of SP and CGRP following 11 days culture. These changes were not observed in protein level. The discrepancy between intensity of CXCR4 molecule and its genomic expression might be due to the short half life of CXCR4 mRNA or impaired translation from mRNA to protein.

Communication between inhibitory and stimulatory role of cytokines and neuropeptides and other molecules can regulate expression of cell surface molecules such as CXCR4. One possible mechanism of CXCR4 over-expression is its G protein coupled receptor structure, consequently, favoring responsiveness and migration of these cells to its ligand, SDF1. Since G protein-coupled receptors, such as neuropeptide and chemokine receptors are involved in a wide range of regulatory processes their action time is limited and rapidly down regulated (Balkwill 2004).

We could show positive regulatory role of SP and/or CGRP on protein expression of CXCR4. However, further studies are required to understand the basic mechanisms underlying these processes and the consequent in vivo responses.

Conflict of interest

The authors declare no potential conflicts of interest.

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