Original Article

Effect of Staurosporine on Neural Differentiation of CD133+ Umbilical Cord Blood Cells

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Abstract

Objective: CD133+ umbilical cord blood cells were identified as a hematopoietic stem cell which has the capacity for extensive self-renewal and differentiation. The aim of this study was to identify the effect of staurosporine (STS), a well-known protein kinase inhibitor on differentiation of CD133+ cells into neural cells.

Materials and Methods: CD133+ cells were enriched by immunomagnetic beads from human mononuclear cells of umbilical cord blood and the purity of higher than 94% was achieved by flowcytometry. Induction of differentiation was performed by addition of STS (12.5, 25, and 50 nM). The differentiated cells were evaluated by immunofluorescence and RT-PCR for neuron-specific proteins and transcripts.

Results: STS-treated CD133+ cells expressed mRNA transcripts for neuron-specific neurofilament protein (NFM), and several basic helix-loop-helix (bHLH) transcription factors important for early neurogenesis, including Otx2, Wnt1, and Hash1. The structural proteins characteristics of neurons including β-tubulinIII and Microtubule-Associated Protein-2 (MAP-2), were shown by immunocytochemistry. STS-treated CD133+ cells also expressed the astrocyte-specific marker, glial fibrillary acidic protein (GFAP) by immunofluorescence.

Conclusion: The human cord blood-derived CD133+ hematopoietic stem cells could differentiate into neural cell types of neuron-like cells and astrocytes by STS treatment.

Keywords: Staurosporine, CD133+ Cells, Umbilical Cord Blood, Neural Differentiation

Introduction

Umbilical cord blood (UCB), used to be discarded as postpartum medically waste product in delivery rooms, was experimentally proved to be a rich source of hematopoietic stem/ progenitor cells by Broxmeyer et al. (1) in 1989. In the same year, Gluckman et al. (2) reconstituted the hematopoietic system of a child with Fanconi anemia by means of UCB from an HLA- identical sibling. Since then, human UCB substituted successfully for bone marrow cells (BMCs) in blood transplantation therapies and accordingly, many related and unrelated cord blood transplantations have been performed worldwide (3).

This source of hematopoietic stem/ progenitor cells has a number of advantages over the other blood sources (e.g. bone marrow) such as relative ease of procurement, high contents of primitive pluripotent cells, low probability of transferring of infectious agents such as cytomegalovirus and Epstein-Barr virus (2), better toleration across the HLA barrier and milder incidence of graft- versus- host disease (GVHD) due to the naiveté and immaturity of these cells (3).

Previous studies have shown that human UCB-derived mononuclear cells (MNCs) can differentiate into different types of cells (1),

33

and they also have the capability to express different neural markers such as β-Tubulin III and glial fibrillary acidic protein (GFAP) by retinoic acid (RA) (4), β-mercaptoethanol (5), and epithelial growth factor (EGF) as well as basic fibroblast growth factor (bFGF) (6). As a whole, these findings suggest that the neonatal blood system has the capacity to differentiate into neural cells under specific conditions.

CD133/AC133 positive cells are self-renewing noncommitted stem/ progenitor cells that have the potentials to differentiate into myelo-monocytic lineage (7), endothelial cells (8), cardyomyocytes (9) as well as neural cells in the presence of different inducers. Jang et al in 2004 demonstrated that the induction of CD133+ cells by RA, as a neurogenic stimulator causes the expression of neural markers and trans-differentiation of these cells into neuron-like and glial cells (10). On the other hand, Belicchi et al. showed that the human skin-derived CD133+ cells have the capacity to differentiate into neural cells in the presence of bFGF (8).

Moreover, many recent studies approved the effect of Staurosporine (STS), a well-known protein kinase inhibitor (11,12) and apoptosis inducer (13,14) on induction of neurite outgrowth in several types of cells. Induction of differentiation by this bacterial alkaloid on neuroblastoma cell lineages (13), embryonic stem cells (12) and dorsal root ganglion of the chick embryo (14) as well as human prostatic cancer cells (11), C6 glioblastoma (15), and PC12 pheochromocytoma (16) caused neurite outgrowth and expression of different neural markers. Although the detailed mechanism of STS action as a neurogenic morphogen remains unclear, it seems that it associates with the activation of some protein kinases which may contribute to neurite outgrowth (4).

Due to the capacity of neural differentiation of UCB-hematopoeitic stem cells and neurogenetic influence of STS, the purpose of this study was to determine the effect of STS on differentiation of CD133+/AC133+ human UCB stem/ progenitor cells into neural cells.

Materials and Methods

Isolation of CD133+/AC133+ cells from human umbilical cord blood

After full- term delivery, human Umbilical Cord Blood (UCB) was collected from the umbilical vein of the neonatal. After achieving maternal informed consent, the use of cells for research was approved by Royan Institute Ethics Committee. Blood samples were collected by anticoagulant- treated sterile syringe. The collected samples were transferred into sterile tubes (Falcon, England), kept in the flask containing ice bag and processed within 4 hr after collection. In the lab, the volume reduction of the blood was performed by using 10% hydroxyethylstarch (Fresenius, Germany), then the rest of the cells were diluted in washing buffer containing phosphate buffered saline (PBS), supplemented with 0.2% EDTA, 100 U/ml penicillin, 0.1 mg/ml streptomycin (all materials from Sigma, USA) and 15% fetal calf serum (FCS) (Gibco, England). Then mononuclear cells (MNCs) were obtained after centrifugation over Ficoll-Hypaque (Inno- train, Germany) gradients at 1500 rpm for 15 min.

CD133+ cells were isolated from other MNCs by Magnetic Activated Cell Sorting System (MACS) (Miltenyi Biotec, Germany). By this method, MNCs (1× 10^8 cell/ml) were incubated for 30 min at 4 °C with FcR- Blocking Reagent, followed by 30 min incubation with a solution containing magnetic microbead-conjugated antibody raised against CD133. Then the MNCs were passed through a magnetic separation column that sits in a magnetic block and CD133+ cells were obtained by flushing the column with CliniMACS washing buffer (Miltenyi Biotec, Germany).

Isolated CD133+ cells (10^6 cell/ml) were grown in Iscove´s Modified Eagle Medium (IMDM) (Gibco, England) supplemented with 50 μg/ml stem cell factor (SCF) (R&D, USA), 1% L-glutamine (Gibco, England), 2 mM β-mercaptoethanol (Sigma, USA), 25 μg/ml Thrombopoitin (TPO, R&D, USA), 1 μl/ml Non Essential Amino Acids (NEAA 100X) (Gibco, England), 20% FCS (Gibco, England), 100 U/ml Penicillin and 0.1 mg/ml Streptomycin (both from Sigma, USA) and

kept in humidified chamber at 37 °C and 5% CO_2.

Flowcytometry

Mono- color flowcytometry was carried out within 24 hr post isolation. Cells which were isolated by MACS were preincubated with 10% mouse serum for 1 hr at 4 °C and then incubated with mouse anti- human CD133 monoclonal antibody conjugated with Phycoerythrin (10:100; MiltenyiBiotec, Germany). Then the cells were fixed with 2% paraformaldehyde. Isotype-matched mouse IgG1/RPE (MiltenyiBiotec, Germany) was used as control. The fluorescence intensity was quantified by flowcytometer (Becton- Dickinson, USA) and data analysis was performed by WINMDI software. This process repeated for 4 different samples.

Treatment by STS

After one week, the proliferated CD133+ cells were transferred to DMEM (Gibco, England) supplemented with 20% FCS and Methylcellulose (R&D, USA). Then the plates were kept in humidified chamber at 37 °C and 5% CO_2. After one week, the cells attached well at the bottom of the plate, then the medium was exchanged by fresh supplemented DMEM, and the cells were exposed to different doses of STS (12.5, 25, and 50 nM) (Sigma, USA). Afterwards the plates were transferred to humidified chamber at 37 °C and 5% CO_2. Immunocytochemistry and RT-PCR was performed 48 hr post induction by STS.

Immunofluorescence staining

CD133+ enriched cells (10^6 cell/ml) were plated in a 4-well plate containing supplemented DMEM. By 48 hr post induction by STS, the cells processed to determine the neural markers. To immunofluorescence staining, the cells were fixed in 4% paraformaldehyde for 10 min at 4 °C, washed in PBS, then permeabilized with 0.2% Triton X-100 in PBS for 1 min at room temperature, washed in PBS again, then incubated with 10% goat serum (Gibco, England) and placed in a humidified chamber for 60 min at room temperature. Afterwards, the samples were incubated for 45 min at 37°C with monoclonal

primary antibodies, including goat anti-mouse β-tubulinIII (1:250) (Sigma, USA) and goat anti-mouse microtubule associated protein II (MAPII; 1:200) (Sigma, USA), as well as goat anti-mouse Glial Fibrillary Acidic Protein (GFAP; 1:50) (Chemicon, USA). Then the cells were washed in PBS and incubated with FITC-conjugated secondary antibody (1:200) (Sigma, USA) for 45min at 37 °C and stained with Propidium Iodide (Sigma, USA) to identify cellular nuclei. Immunofluorescent cells were visualized by Olympus DP70 fluorescent microscope.

RT- PCR

Total RNA was extracted from cell cultures using RNX- Plus™ kit (Cinnagen, Iran), then 1μg of total RNA was reverse transcribed into first- strand cDNA by RevertAid™ first strand cDNA synthesis kit (Fermentase, Canada). Reverstranscription was carried out with MLV reverstranscriptase (Fermentase, Canada) for 60 min at 42 °C. The cDNA was amplified by 34 cycles of PCR using SmarTaq kit (Cinnagen, Iran). The primers were designed from end to end exons of corresponding human genes: Oct-4 (Embryonic stem cell transcription factor), CXCR4, AC133/CD133 (Both hematopoietic stem/progenitor markers), Otx-2, Wnt1, Hash1 (Neural progenitor transcription factors), Nestin (Neural progenitor cell marker), GFAP, and MAPII, as well as NFM (Neural cell markers) (Primers sequences have been shown in tableI). PCR products were visualized on 1.8% agarose gel, under ultraviolet gel document device (Uvidoc, England).

Statistical analysis

Results are expressed as the mean± standard error of mean (SEM). All data were analyzed using Excel Software, Microsoft, USA.

Results

Isolation and Culture of Hematopoietic Stem Cells

Human umbilical cord blood-derived MNCs were passed twice through a magnetic separation column that sits in the magnetic
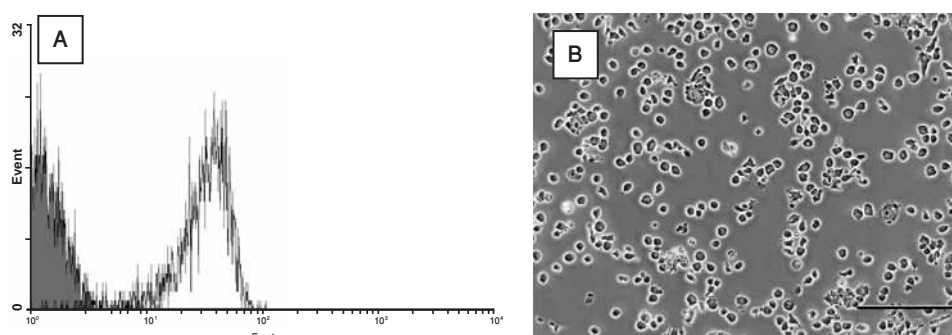
35

Fig 1: Flow cytometry and Cell culture of MACS-sorted CD133⁺ cells. (A) Representative flowcytometry of CD133 expression on UCB-derived cells. The cells expressing CD133 compared with isotype control (red peaks) were termed CD133⁺ population. (B) Isolated CD133⁺ cells were grown for 7 days in methylcellulose medium containing growth factors.

block. 94.43± 0.28% of isolated cells were CD133⁺ (Fig 1A).

After 7 days of continued growth in culture, the cells attached readily to the surface of culture dishes. Most attached cells were either round (Fig 2A). CD133⁺ cells were treated with different concentrations (12.5, 25, and 50 nM) of STS for 2 days (Fig 2). After STS treatment, CD133⁺ cells became bipolar and extended long processes (Fig 2B, D), whereas fewer cells were bipolar in the absence of STS (Fig 2A). After STS treatment for 2 days, the expression of β-tubulinIII (Fig 2F, H), MAP2 (Fig 2J, L),

and GFAP (Fig 2N, P), three most popular neural markers were detected in the treated samples and a slight expression of them was observed in the control groups (Fig 2E, I, M).

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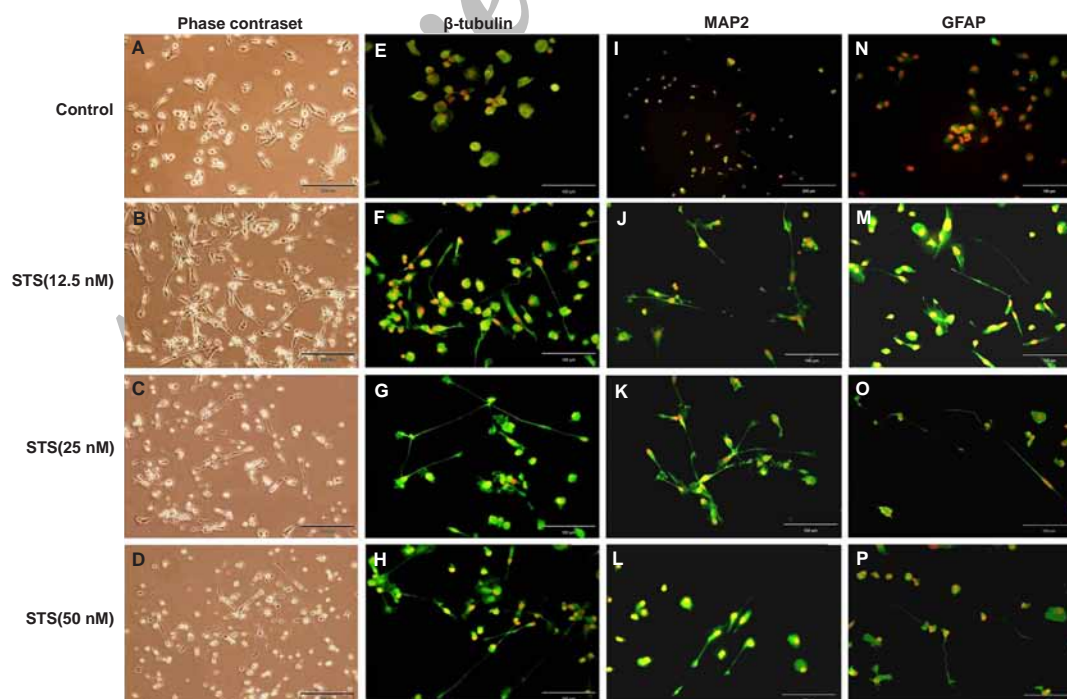

Fig 2: Cellular morphology and immunostaining of CD133⁺ cells after STS treatment. (A) CD133⁺ cells without addition of STS to the culture medium and only cultured for 7+2 days. They are some bipolar or round-shaped. (B-D) CD133⁺ cells were exposed to different concentrations (12.5, 25, and 50 nM) of STS for 2 days. Extensive outgrowth of cellular processes is noted under phase contrast microscope. The expression of β-tubulinIII (F-H), MAP2 (J- L) and GFAP (N-P) were detected by STS treatment. Few expression of the markers was observed in control group (E-I- M).

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Expression of stem cell and neural markers

The slight expression of Oct4 mRNA, as an embryonic stem cell marker was detected in fresh isolated CD133+ cells but no trace of this marker was observed in the treated groups, which had been induced by 12.5 nM, 25 nM and 50 nM of STS as well as the control group (Fig 3). CXCR4 antigen as a hematopoietic stem/ progenitor marker was expressed in all the treated and non- treated groups (Fig 3). Although the expression of AC133 (CD133) marker was detected both in the fresh isolated CD133+ cells and the control group, its elimination in the treated CD133+ cells was remarkable (Fig 3).

Fig 3: RT- PCR analysis of cell type- specific markers. The expression of these markers were investigated on Fresh isolated CD133+ cells and treated CD133+ cells with 12.5 nM, 25 nM and 50 nM STS. Control is consisted of CD133+ cells which underwent spontaneous differentiation in the absence of STS.

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Although NFM expression in CD133+ cells was not detected by RT-PCR before STS treatment, the expression was induced by this alkaloid after treatment (Fig 3). In addition, expression of β-tubulinIII, MAP2, and GFAP as specific neural markers, were detected in CD133+ cells after STS treatment (Fig 3). Such STS-induced expression of β-tubulin-III

and MAP2 and GFAP were also reproduced with immunostaining results (Fig 2E, P). It is noteworthy to say that the expression of these neural markers was also observed in control group in RT-PCR (Fig 3). Although immunostainig results showed that almost all the treated and non-treated CD133+ cells were immunoreactive for neural markers, the morphology of neuron- like cell beside the expression of neural markers (β-tubulinIII, MAP2 and GFAP) were indicated only in the treated groups (Fig 2E, H, I, L, M, P).

We investigated expression of several basic helix-loop-helix **(**bHLH) transcription factors central to neural differentiation of embryonic or neural stem cell, including Otx2, Wnt1, and Hash1 (Fig 3). Otx2, a bHLH transcription factor important in regulation of the early phase of neural plate formation (17), was not expressed in non-stimulated CD133+ cells (control) or in the treated groups. Expression of Wnt1, which controls the initial phase of neural plate formation (18), was not detected in the control fresh CD133+ cells but the expression was induced by STS treatment or long term culture of CD133+ cells. It is interesting to note that Hash1 (human achaete-scute complex homolog 1), which is known to play an important role in neuronal differentiation (19,20) was expressed in non- stimulated CD133+ cells (control) and the primarily STS- treated sample (12.5 nM), but not in the fresh isolated CD133+ cells.

Discussion

We investigated that cell populations of CD133+, has the capability for trans-differentiation into neural cell types. Using magnetic bead-based cell sorting, human hematopoietic stem cells were isolated from umbilical cord blood based on presentation of CD133, a hematopoietic stem cell marker, on the surface of hematopoietic stem cells. Purified human hematopoietic stem cells isolated via CD133 were exposed to STS, a universal protein kinase inhibitor (11,12). Cells with neuronal and glial phenotypes were induced, and several cell type-specific markers for neurons and glial cells were identified in these cells by RT-PCR, and immunocytochemistry.

37

A previous study with human UCB-derived CD133⁺ hematopoietic stem cells showed that these cells could transdifferentiate into neural cell types of neuron-like cells, astrocytes, and oligodendrocytes by retinoic acid treatment (10). Moreover, the expression of NFM, MAP2, and GFAP was detected in fresh isolated UCB mononuclear cells without any treatment (5). Terskikh et al. showed a genetic program overlap between human hematopoietic and neural stem cells by DNA microarray (21). Moreover, it was shown the down-regulation of genes related to blood cell lineage in accompany with the expression of neural stem cell markers such as β- tubulin III and GFAP, during neurogenesis (22).

Constitutive expression of Hash1, a member of proneural genes in nonstimulated CD133⁺ cells implies that this stem cell has the potential to differentiate into neural cells. Wnt1 signaling pathways are expressed in developing and mature CNS. It is interesting that Wnt1 appears to be a common transcription factor between mesodermal and neural cell lineages, because it seems to be active in inducing the dorsal cells of somites to become muscle and is involved in the specification of the midbrain cells too (23); so the expression of this marker in our cell culture could be another evidence of the existence of genetic program overlap between mesodermal lineage and neural cells, and co- expression of proneural and adult neural markers (e.g. Wnt1& MAPII) by the same cell is not unusual in progenitor cells during early development, because it may reflect a transitional state in the cellular differentiation process.

In our study, STS-treated CD133⁺ cells expressed mRNA transcripts for Nestin, a cell type-specific marker for neural stem cells (24) and neuron specific markers NFM, and MAP2. Structural proteins characteristic of neurons including β-tubulinIII and MAP-2 were also revealed by immunocytochemistry. STS -treated CD133⁺ cells also expressed the astrocyte-specific marker, GFAP, as demonstrated by RT-PCR and immunocytochemistry. Notably, expression of CXCR4 and Oct-4 was observed in CD133⁺ cells before treatment.

Although STS is known as a universal protein kinase inhibitor (11,12) and apoptosis inducer (13,14), studies indicate that it can arrest cell cycle progression (11) and induce neuronal and glial differentiation of murine embryonic stem cells (12), C6 glioblastoma (15) and PC12 pheochromocytoma (16) in vitro. Detailed mechanism of STS function as a neurogenic morphogen remains unclear. Although this alkaloid has been known as a protein kinase inhibitor, it has been associated with the activation of some protein kinases as like as c- Jun N- terminal kinase (25) and MAPK (26). STS specifically activation of c-Jun N- terminal kinase may contribute to the neurite outgrowth from PC12 cells (25). MAP kinase is also one of the most important signal transduction pathways which can activate several regulatory molecules in the cytoplasm and in the nucleus to initiate cellular processes such as proliferation, differentiation, and apoptotic pathway (27) as well as development (28,29). This protein kinase activates ERK (Extracellular Regulated Kinase) pathway, which plays important roles in the induction of neuron-specific proteins in Mesenchymal Stem Cells (MSCs). Sustained activation of this kinase is required for neuronal differentiation. The blockade of Erk/ MAPK actvation impaired both the morphological changes and the expression of neuronal proteins (30). So, all together, it seems that STS as a chemical substance which can impress protein kinase activation, is also capable to induce neural differentiation via MAPK pathway (10) or by changing in microfilaments rearrangements. It was suggested the treatment of the stem cells with toxic compounds disrupt the actin cytoskeleton and may cause cell shrinkage and neuron-like morphology of treated cells (31, 32).

Conclusion

Although our study supports this idea that human umbilical cord blood may be a possible source of neurogenenic progenitor cells, we believe that more precise quantitative methods are required to investigate the expression of neural markers in treated samples in correspondance with true neural cells, because beyond the expression of

neuron-like morphology of the treated CD133+ stem/ progenitor cell with STS, there is constitutive expression of neural markers in this type of cell. On the other hand, assessment of the electrophysiological capabilities of this neuron- like cell is also suggested.

Acknowledgement

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