

Article

Are globoseries glycosphingolipids SSEA-3 and -4 markers for stem cells derived from human umbilical cord blood?

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Umbilical cord blood (UCB) is an efficient and valuable source of hematopoietic stem cells (HSCs) for transplantation. In addition to HSCs it harbours low amounts of mesenchymal stem cells (MSCs). No single marker to identify cord blood-derived stem cells, or to indicate their multipotent phenotype, has been characterized so far. SSEA-3 and -4 are cell surface globoseries glycosphingolipid epitopes that are commonly used as markers for human embryonic stem cells, where SSEA-3 rapidly disappears when the cells start to differentiate. Lately SSEA-3 and -4 have also been observed in MSCs. As there is an ongoing discussion and variation of stem-cell markers between laboratories, we have now comprehensively characterized the expression of these epitopes in both the multipotent stem-cell types derived from UCB. We have performed complementary analysis using gene expression analysis, mass spectrometry and immunochemical methods, including both flow cytometry and immunofluorescence microscopy. SSEA-4, but not SSEA-3, was expressed on MSCs but absent from HSCs. Our findings indicate that SSEA-3 and/or -4 may not be optimal markers for multipotency in the case of stem cells derived from cord blood, as their expression may be altered by cell-culture conditions.

Keywords: umbilical cord blood, hematopoietic stem cells, mesenchymal stem cells, SSEA-3, SSEA-4

Introduction

Stem cells are potential therapeutic agents that offer great promise in regenerative medicine. They have a unique ability to both self-renew and differentiate into diverse cell types. During the past decade, research on stem cells has rapidly expanded. Currently, multipotent stem cells isolated from umbilical cord blood (UCB) and bone marrow (BM) are routinely used in medical therapies for leukaemia and other bone and blood cancers. By definition, multipotent cells are capable of generating a limited range of differentiated cell lineages as a result of appropriate stimulus. UCB harbours two types of multipotent stem cells: hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) (Flynn et al., 2007; Ruhil et al., 2009). UCB has been shown to be a valuable source of HSCs ever since the first UCB transplantation in 1989 (Gluckman et al., 1989). Until now, UCB transplantation has mainly been used in treating paediatric patients due to the small size of the transplant. As a stem cell source, UCB has several advantages over BM and peripheral blood (PB):

(i) UCB is harvested after delivery of both the infant and the placenta and therefore poses no risks to the donor, (ii) UCB is thoroughly tested and cryopreserved in cord blood banks, which makes it safe and rapidly available, (iii) UCB transplantation permits a higher degree of HLA disparity and most importantly, (iv) UCB causes less serious acute and chronic graft-versus-host disease while maintaining a strong graft-versus-leukaemia effect (Brown and Boussiotis, 2008).

Despite the recent advances in the stem cell field, many issues of stem cell biology and their clinical use remain unresolved. Stem cells are currently defined by a combination of physical, phenotypic and functional properties (Flynn et al., 2007). Novel cell-surface markers are therefore needed for quick characterization and isolation of different stem cell populations. As stem cells can only be identified by differential expression of a combination of multiple cell-surface epitopes, minor changes in, for example, stem-cell differentiation status, are not easily detected. A single cell-surface 'stemness' marker that could be used to directly and efficiently identify and extract different stem-cell populations has not yet been identified.

The outermost layer of cells is a dense glycocalyx that is composed of a complex network of carbohydrates linked to proteins and lipids decorating the cell surface. The glycocalyx is

characteristic to, and different in, every cell type (Cummins, 2009). The glycans on cell-surface serve as receptors and participate in multiple adhesion events. They also mediate signals in and out of the cell as well as diversify and fine tune the functions of cell-surface proteins and lipids. We have previously reported comprehensive glycome analysis of human stem cells from various sources (Heiskanen et al., 2007; Hemmoranta et al., 2007; Satomaa et al., 2009). In conclusion, we have shown that the cell-surface glycans in stem cells have special characteristics. More specifically, stem cells display more high-mannose-type N-glycans and terminal α 2,3-sialylation than their differentiated counterparts. MSCs also harbour asialylated N-acetyllactosamines in great amounts.

Data collected on cell surface glycans can be exploited to isolate, characterize and identify different cell populations (Goetz et al., 2009; Nakatani et al., 2010). A novel platform to detect even subtle changes in cell behaviour, and thereby track changes in stem-cell differentiation, is introduced by studying cell-surface glycosylation. Indeed, many currently used stem-cell markers are glycan epitopes attached to either cell-surface proteins or lipids (Lanctot et al., 2007). For example, the globoseries glycosphingolipids SSEA-3 and -4 (stage-specific embryonic antigen-3 and -4) are widely used markers for embryonic stem cells (Kannagi et al., 1983; Muramatsu and Muramatsu, 2004; Wright and Andrews, 2009).

Glycans that are displayed on cell surfaces are assembled in the ER and Golgi compartments and attached to naïve proteins and lipids. Some distinct glycans can also appear on cells as a consequence of metabolic incorporation. We, among others, have previously reported that cell-culture conditions can change the nature of cell surface glycans. The best example is the non-human sialic acid Neu5Gc, which is incorporated on the surface of human stem cells from animal products used in cell-culture media (Bardor et al., 2005; Martin et al., 2005; Heiskanen et al., 2007; Nystedt et al., 2010).

In order to more efficiently identify, isolate, characterize and culture stem cells from UCB, we have now analysed in detail the globoseries glycosphingolipids SSEA-3 and SSEA-4 expression in human UCB-derived mesenchymal and hematopoietic stem cells. On the basis of these results, the usefulness of SSEA-3 and SSEA-4 epitopes in the characterization of UCB-derived stem cells is questioned, and the effect of cell-culture conditions on the cell-surface expression of these epitopes is discussed.

Results

Glycosyltransferases involved in SSEA-3, SSEA-4 and Globo-H synthesis display a characteristic expression pattern in cord-blood stem cells

Globoseries glycosphingolipid epitopes SSEA-3 and -4 are generated by the sequential action of a specific set of glycosyltransferases (Figure 1). β 1,3-galactosyltransferase-V (β 3GalT-V) is the main enzyme responsible for generating galactosylgloboside (Gb5, SSEA-3) by the transfer of galactose to the terminal N-acetylgalactosamine of globoside (Gb4) (Zhou et al., 2000). The SSEA-4 epitope (monosialylgalactosylgloboside, MSGb5) has a terminal α 2,3-linked sialic acid in

SSEA-3 backbone, generated *in vivo* by ST3Gal-II (α 2,3-sialyltransferase-II) activity (Saito et al., 2003). *In vitro*, ST3Gal-I, ST3Gal-II and ST3Gal-IV can utilize the Gal β 1-3GalNAc β sequence as acceptor (Harduin-Lepers et al., 2001). Alternatively, fucosyltransferase-1 (FUT-1) or -2 (FUT-2) may construct Globo-H (fucosylgalactosylgloboside) from SSEA-3 by adding a terminal α 1,2-linked fucose (Chang et al., 2008).

Multiple previous studies have shown that the expression of distinct glycan epitopes on cell surfaces may be correlated to the expression and activity levels of appropriate glycosyltransferases (Hemmoranta et al., 2007; Nairn et al., 2008). We thus studied the mRNA levels of the glycosyltransferases involved in the synthesis of the SSEA-3, SSEA-4 and Globo-H epitopes (Figure 2). mRNA expression levels were characterized both by using a public domain microarray expression data set (Kilpinen et al., 2008) and by analysing in-house-generated gene-expression microarray data from UCB-HSCs and UCB-MSCs. The data in the public domain are composed of normalized gene expression data of 9783 samples from 175 healthy and pathological cell/tissues. The expression levels of the glycosyltransferases of interest were investigated within the data set. The expression level of ST3Gal-II was observed to be

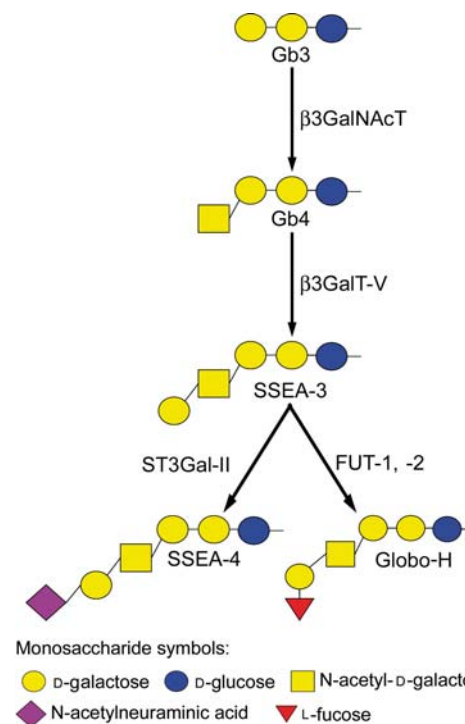


Figure 1 Schematic diagram of the biosynthesis of globoseries glycosphingolipids. Globoseries of glycosphingolipids are synthesized in a sequential manner by specific glycosyltransferases. SSEA-3 (Gb5), the precursor of SSEA-4 (MSGb5) and Globo-H, is synthesized from globoside (Gb4) by β 3GalT-V. Either ST3Gal-II or FUT-1/2 transfers a terminal sialic acid or a fucose, respectively, into SSEA-3 (Gb5) backbone in order to create SSEA-4 (MSGb5) or Globo-H. Glycosidic linkages are indicated by lines connecting the monosaccharides (horizontal lines: α/β 1–4 linkage; slanting lines: α/β 1–3 linkage; vertical lines: α 1–2 linkage).

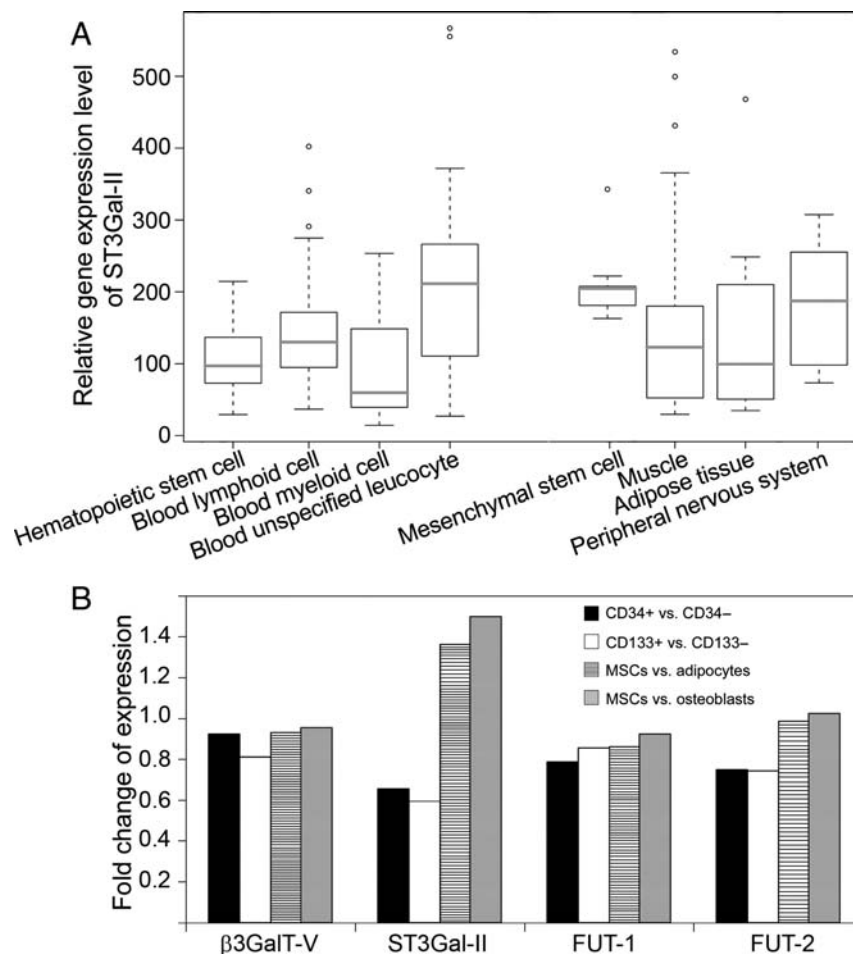


Figure 2 Expression pattern of glycosyltransferases involved in the biosynthesis of SSEA-3 and -4 epitopes in stem cells. **(A)** Boxplot visualization of mRNA expression data are shown for SSEA-4 synthase ST3Gal-II, retrieved from a public domain microarray data. **(B)** Fold change enrichment of mRNA expression levels from in-house microarray analysis for the glycosyltransferase genes involved in the biosynthesis of SSEA-3, -4 and Globo-H epitopes. The expression levels in UCB-derived CD34+ cells are compared with the expression levels in CD34- cells (black bars), the expression levels in CD133+ cells to those in CD133- (white bars), and the expression levels in MSCs to those in adipocytes (striped bars) or osteoblasts (grey bars).

elevated in MSCs in relation to differentiated cell types arising from them, namely adipose tissue and muscle, and to cells from the nervous tissue (Figure 2A). Similar type of elevation was not observed for HSCs in relation to differentiated blood cells (Figure 2A). In case of β 3GalT-V, FUT-1 and -2, no elevated expression was observed in any of these cell types (data not shown). Since the public domain mRNA data for adult stem cells is most likely from cells originating from the BM, we further explored the glycosyltransferase expression levels in our own mRNA expression data bank, which has been generated using umbilical-cord-blood-derived HSCs (Hemmoranta et al., 2006; Jaatinen et al., 2006) and MSCs (unpublished data; Figure 2B). When comparing fold changes of mRNA expression levels between each stem cell class and their differentiated counterparts, we were able to clearly see that the SSEA-4 synthase, ST3Gal-II, is markedly enriched in UCB-MSCs, whereas in HSCs no enrichment can be detected. Instead, slight down-regulation could be observed (Figure 2B). Of these genes, ST3Gal-II displayed the highest expression levels in all studied stem cell populations (data not shown).

SSEA-4, but not SSEA-3 or Globo-H, is expressed in cord blood-derived MSCs

Since the mRNA expression of SSEA-4 synthase is clearly enriched in UCB-MSCs, we studied further the cell surface expression of related glycosphingolipid epitopes in living cells. Flow cytometric analysis of *in vitro* expanded UCB-MSCs was performed using commercial antibodies against SSEA-3, SSEA-4 and Globo-H epitopes. As expected, MSCs were positive for SSEA-4, but quite surprisingly negative for SSEA-3 and Globo-H epitopes (Figure 3). Immunofluorescence staining of unpermeabilized cells confirmed further that the SSEA-4 antibody gives a positive staining (Figure 4), while SSEA-3 and Globo-H antibody stainings were negative (data not shown). SSEA-4 antibody distinctively labels the cell surface.

It is generally known that glycan antibodies are difficult to generate, and may display broad specificity (Manimala et al., 2007). The SSEA-3 binding antibody clone MC-631, used in this study, has been reported to recognize both Gb5 and MSGb5, with higher affinity to Gb5. The specificity of the SSEA-4 binding antibody clone used in this study, MC-813-70, has been reported to

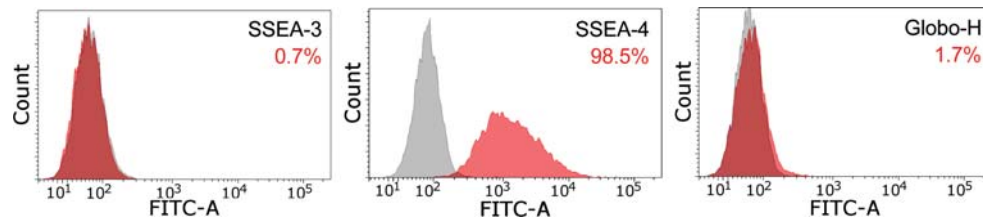


Figure 3 Flow cytometric analysis of SSEA-3, SSEA-4 and Globo-H cell-surface expression in UCB-MSCs. UCB-MSCs were analysed by flow cytometry. Antibodies against SSEA-3 (red, left panel), SSEA-4 (red, middle panel) and Globo-H (red, right panel) were used. Filled grey histograms represent unstained cells in all panels. A total of 10000 cells were collected for analysis in each. The data are representative of three individual experiments and two independent UCB-MSC lines.

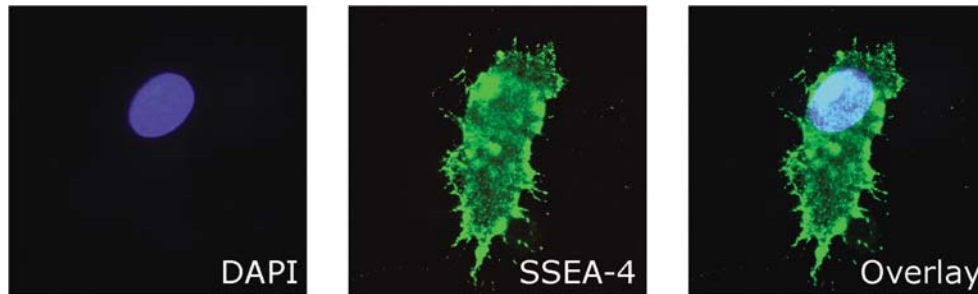


Figure 4 Immunofluorescence staining of SSEA-4 in UCB-MSCs. Unpermeabilized UCB-MSCs were stained with anti-SSEA-4 antibody followed by Alexa 488 coupled secondary antibody (green, middle panel) and mounted with media containing DAPI in order to visualize the cell nucleus (blue, left panel). Overlayed image is shown in the right panel.

include certain ganglioseries glycolipids with terminal NeuNAc α 3Gal β 3GalNAc. SSEA-3 and -4 antibodies do not bind, or bind only weakly, to Globo-H (Kannagi et al., 1983).

To confirm our flow cytometric findings on the SSEA-4 epitope, UCB-MSC glycosphingolipids were extracted and purified. The glycan portions were released using an endoglycoceramidase enzyme and profiled by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. In the negative ion spectra used to analyse acidic glycans, a signal at m/z 1159.5, corresponding to the mass of the SSEA-4 epitope, MSGb5, was observed (Figure 5). In the positive ion spectra, used for analysis of neutral glycans, masses corresponding to Gb4, SSEA-3 (Gb5) and Globo-H epitopes were also detected, but the data cannot be interpreted unambiguously due to interfering signals arising from the endoglycoceramidase enzyme preparation (data not shown). In conclusion, our findings suggest that SSEA-4 is the best marker among the antigens of these three antibodies for UCB-MSCs.

SSEA-3, SSEA-4 and Globo-H are not expressed in cord-blood-derived HSCs

The majority of stem cells in UCB are multipotent HSCs, commonly identified as positive for CD34 and CD133 cell-surface markers. mRNA analysis showed that transferases synthesizing SSEA-3 and -4 epitopes as well as Globo-H were down-regulated in CD34/CD133 positive cells (Figure 2). To verify the lack of epitope expression on cell surface, UCB mononuclear cells were isolated by Ficoll-Paque centrifugation and flow cytometric analysis was performed. HSCs were first identified as positive for both CD34 and CD133 antigens (Figure 6A) and this cell population was further analysed by using antibodies against SSEA-3, SSEA-4 and Globo-H epitopes (Figure 6B). All antibodies gave negative staining within this

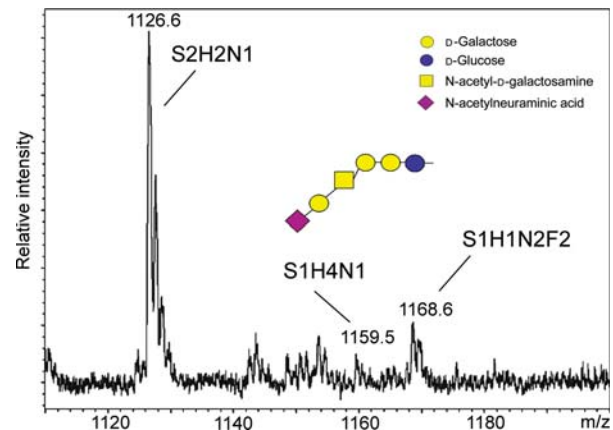


Figure 5 Mass spectrometric analysis of acidic lipid linked glycans from UCB-MSCs. UCB-MSCs derived acidic glycosphingolipid glycans were analysed by MALDI-TOF mass spectrometry. m/z (mass to charge ratio) is indicated on the x-axis and relative intensity on the y-axis. The glycan signal that corresponds to the mass of SSEA-4 (MSGb5) is annotated.

cell population and thereby confirmed that freshly isolated HSCs do not display SSEA-3, SSEA-4 or Globo-H epitopes on their surfaces. However, we cannot exclude the fact that minor populations carrying these epitopes and differential potency may exist.

SSEA-3 and -4 expression may be influenced by cell-culture conditions

UCB-MSCs are typically *in vitro* expanded and have been passaged several times in cell culture conditions prior to therapy. UCB-HSCs used in therapy are, in contrast, freshly isolated and undergo no cell culturing in general. It is well known that

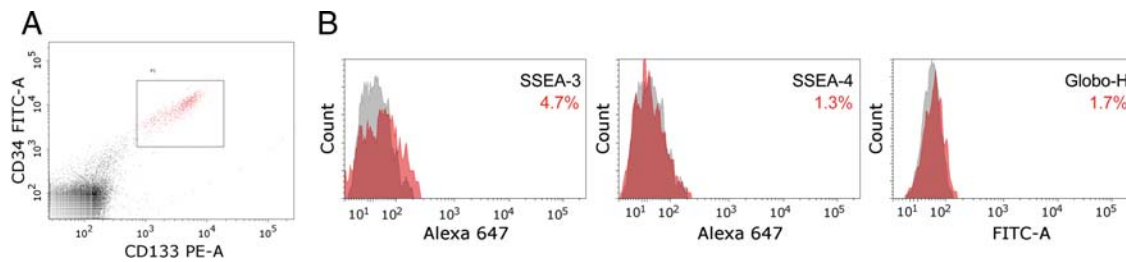


Figure 6 Flow cytometric analysis of SSEA-3, SSEA-4 and Globo-H cell surface expression in freshly isolated UCB-HSCs. **(A)** UCB mononuclear cells were isolated by Ficoll-Paque centrifugation and HSCs identified by positivity for cell-surface markers CD34 and CD133. **(B)** Double positive cells were further analysed for SSEA-3 (red, left panel), SSEA-4 (red, middle panel) and Globo-H (red, right panel) expression. Filled grey histograms represent unstained cells. The data are representative of three individual experiments, at least 3000 double positive cells were analysed in each.

culture conditions affect cell surface glycans, and metabolically incorporated non-human glycans may contaminate cells. Therefore, we decided to study if cell-culture media, and foetal calf serum (FCS), in particular, would affect the differential display of SSEA-3 and SSEA-4 epitopes in different UCB stem cells, namely MSCs and HSCs. First we tested whether FCS contains glycosphingolipid SSEA-3 or SSEA-4. The overall glycosphingolipid portion of FCS was extracted and analysed by thin layer chromatography (TLC) with immunodetection (Figure 7). To our surprise, we discovered detectable bands of globoseries glycolipids; several bands were visualized by anisaldehyde staining and a band corresponding to a size of MSGb5 was recognized by the SSEA-4 antibody.

In order to see if culture conditions affect the presentation of SSEA-3 or -4 epitopes on cell surface, we cultured freshly isolated cord-blood HSCs, originally negative for both epitopes, overnight in the presence of FCS and reanalysed these cells by flow cytometry (Figure 8). We detected a clear elevation in SSEA-3 epitope cell-surface expression. Taken together, our results indicate that exposure to FCS could alter the cell-surface expression of globoseries glycosphingolipids in cord-blood-derived stem cells.

Discussion

Stage-specific embryonic antigens-3 and -4 are carbohydrate epitopes of globoseries glycosphingolipids, which were originally discovered on the surface of embryonal carcinoma cells (Kannagi et al., 1983). Today, they are generally used markers of human embryonic stem cells. It has been suggested that these two glycosphingolipid epitopes play an important role in early embryogenesis, but no specific function for the glycosphingolipids carrying the SSEA-3 and -4 epitopes has yet been identified. It is known that SSEA-3 is extinguished more rapidly from the cell surface than SSEA-4 if glycosphingolipid synthesis is blocked by inhibitors, and a similar phenomenon was also detected during embryonic stem-cell differentiation (Draper et al., 2002; Brimble et al., 2007). Depletion of these two epitopes from human embryonic stem cells seems to have no apparent effect on the pluripotency of these cells (Brimble et al., 2007).

In addition to embryonic stem cells, SSEA-3 and -4 have, in some cases, also been described in MSCs from different origins (Gang et al., 2007; Guillot et al., 2007; Sun et al., 2007; Sobiesiak et al., 2010). Lately, SSEA-4 has also been suggested as a general ‘stemness’ marker for adult MSCs (Gang et al.,

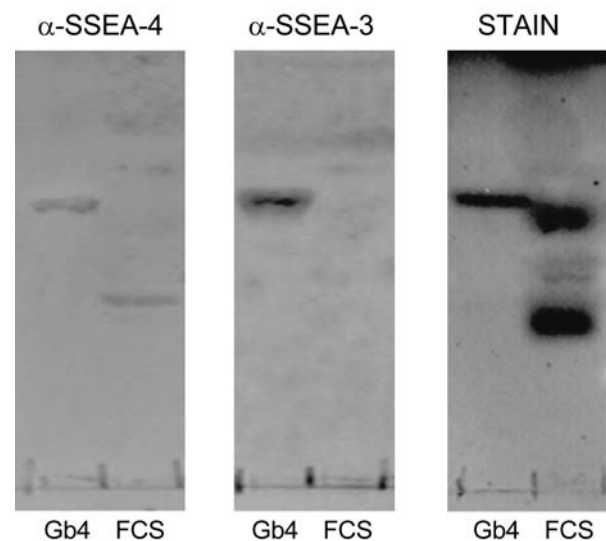


Figure 7 TLC analysis of SSEA-3 and -4 in cell culture medium. Anisaldehyde staining of TLC plate (right panel) indicated the presence of several glycosphingolipids in FCS. Immunodetection using SSEA-3 antibody (middle panel) and SSEA-4 antibody (left panel) was performed.

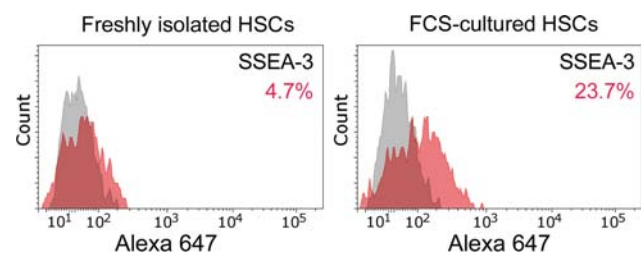


Figure 8 Flow cytometric analysis of SSEA-3 in UCB HSCs cultured with FCS. UCB mononuclear cells were isolated by Ficoll-Paque centrifugation and analysed by flow cytometry. HSCs were identified by double positivity for cell surface markers CD34 and CD133 and further analysed for SSEA-3 antibody staining within this cell population (red histograms, left panel). After overnight incubation in the presence of 10% FCS, cells were similarly reanalysed (right panel). Filled grey histograms represent unstained cells. The data are representative of three individual experiments, at least 3000 double positive cells were analysed in each.

2007). MSCs were first discovered in the BM, but they have also been identified in many other adult and foetal tissues such as UCB. MSCs are multipotent and can differentiate towards, for example, osteogenic, chondrogenic and adipogenic lineages. There is an ongoing debate over the cell surface epitope profile that would uniquely define MSCs. A controversy between different cell preparations and laboratories exists (Kuhn and Tuan, 2010). This discrepancy may be due to different origins of the MSCs, variable cell-isolation protocols, differential cell-culture conditions and/or a potentially different specificity between antibody clones, sources and conjugates.

We have now studied the expression of SSEA-3 and SSEA-4 epitopes in both UCB-derived multipotent stem cells (MSCs and HSCs) in more detail. On mRNA level, it was clearly seen that the SSEA-4 (MSGb5) synthase, ST3Gal-II, is enriched in UCB-MSCs, whereas for glycosyltransferases synthesising SSEA-3 (Gb5) and Globo-H, no differences were detected when compared with differentiated cells. A mass spectrometric glycan signal corresponding to the mass of the SSEA-4 epitope (MSGb5) could be detected in mass spectrometric profiling of glycosphingolipid glycans. Furthermore, we were able to show by flow cytometry that UCB-MSCs are positive for SSEA-4, but negative for the two other terminal epitopes of globoseries glycosphingolipids, SSEA-3 and Globo-H. We demonstrated by immunofluorescence microscopy that the SSEA-4 antibody specifically labels the MSC surface. In contrast to MSCs, SSEA-3 and -4 epitopes were not detected in HSCs. On the mRNA level the appropriate glycosyltransferases were barely expressed and we could not observe cell surface expression of SSEA-3 or -4 or Globo-H by flow cytometry.

All the data, detailed above, indicate that freshly isolated HSCs from UCB express neither SSEA-3, -4 nor Globo-H epitope, while UCB-MSCs cultured in the presence of FCS display SSEA-4 on their surface. The mononuclear cell population from an UCB unit contains both of these cell types, non-adherent HSCs in relatively large quantities and a barely detectable population of adherent MSCs. We wanted to explore the possibility that SSEA-3 or SSEA-4 cell-surface expression in HSCs could be altered by culturing these cells, as we had discovered that FCS contains detectable amounts of globoseries glycosphingolipids and a specific band corresponding to the size of SSEA-4 could be detected in immuno-TLC. In order to see whether cell-surface glycosphingolipid display could be altered by cell-culture conditions, we cultured freshly isolated HSCs overnight in the presence of FCS. When reanalysed, the cells displayed SSEA-3 on their surface. Several different mechanisms may have contributed to this altered cell-surface expression: incorporation of glycosphingolipids from FCS, induction of gene expression involving glycosyltransferases, differential expansion of SSEA-3⁺ cell populations or FCS-induced stimulation of enzymes involved in the biosynthesis of SSEA-3.

UCB is effectively and widely used for HSC transplantation in paediatric patients. For adult patients, the cell dose per one unit of UCB remains low and results in delayed engraftment. This leads to an increased risk of infections, morbidity and mortality (Brown and Boussiotis, 2008). Attempts to expand UCB stem-cell units *ex vivo*, in order to generate increased numbers of stem/progenitor cells to improve hematopoietic recovery after UCB

transplantation, have been recently documented (Delaney et al., 2010). In contrast to HSCs, which still are mainly used in therapy in the context of a naïve and uncultured cell population, MSCs need to be expanded *ex vivo* in order to produce a therapeutically relevant amount of material. *In vitro* expanded MSCs are also widely used for research purposes. In order to evaluate the influence of cell culture conditions on the properties of the MSC surface, a study using primary UCB-MSCs would be of crucial importance. However, as the isolation process currently relies on the adherence properties of the cells and selective cell surface markers have not been described, it is nearly impossible to identify sufficient amounts of primary cells from UCB.

It is a well-known fact that cell-culture conditions can modify primary cells and their cell-surface phenotype. Especially, animal products used in cell-culture medium cause serious alterations of the cell surface glycans. Most widely studied is the incorporation of the non-human sialic acid Neu5Gc into cell surface glycans from animal derived products (Martin et al., 2005; Heiskanen et al., 2007). In addition, certain bovine serum ganglioseries glycolipids containing Neu5Gc have previously been reported to be incorporated into cultivated malignant cell lines, and even cell-type-specific sialylation of the glycolipids by an endogenous cellular sialyltransferase has been described (Furukawa et al., 1989). In addition, human erythrocytes have been shown to intake glycosphingolipids with blood group antigens from plasma (Marcus and Cass, 1969).

The present results indicate that cell surface glycosphingolipid markers such as SSEA-3 and -4 may display altered expression profiles in response to cell-culture conditions. Generally accepted, standardized and safe protocols have not yet been established for the isolation and culturing of human stem cells. In order to create safe protocols for the therapeutic use of stem cells, attention should be paid to even minor changes in the cells: a small change on the cell surface may have a huge impact on the behaviour of the cell and the therapeutic outcome.

Materials and methods

Cells

UCB was obtained after normal vaginal delivery, with full consent of informed and healthy volunteers, at the Helsinki University Central Hospital, Department of Obstetrics and Gynaecology, and Helsinki Maternity Hospital. Ten UCB units were collected for this study, six were used for studies involving HSCs and four were used to create the MSC-lines described below. The study protocol was accepted by the ethical review board of the Helsinki University Central Hospital and the Finnish Red Cross Blood Service. UCB was collected and processed essentially as described (Kekarainen et al., 2006).

UCB mononuclear cells were isolated by Ficoll-Paque density gradient centrifugation (Jaatinen and Laine, 2007). The HSCs used in this study were identified from UCB mononuclear cells by flow cytometric analysis (see below). To evaluate the effect of cell-culture conditions on cell-surface antigen expression, the cells were incubated overnight in IMDM supplemented with 10% FCS and penicillin–streptomycin (Gibco, Invitrogen by Life Technologies, Carlsbad, CA, USA). The MSC-lines used in this study were prepared as previously described (Laitinen and

Laine, 2007; Laitinen et al., 2011) and the mesenchymal phenotype was confirmed by flow cytometric analysis of a panel of cell-surface markers (Dominici et al., 2006). Briefly, the cells gave negative staining for CD14, CD19, CD34, CD45 and HLA-DR, and positive staining for CD73, CD90 and CD105. In addition, the cells were shown to be able to differentiate along osteogenic, adipogenic and chondrogenic lineages, as described below.

To assess the osteogenic and adipogenic potential of the UCB-derived MSCs, the cells were seeded at the density of 3×10^3 cells/cm² and grown to confluency. The osteogenic differentiation was induced by changing the media to α -MEM supplemented with 10% FCS, 20 mM HEPES, 2 mM L-glutamine and penicillin–streptomycin (all from Gibco, Invitrogen by Life Technologies), 0.1 μ M dexamethasone, 10 mM β -glycerophosphate, 0.05 mM L-ascorbic acid-2-phosphate (all three from Sigma-Aldrich, St. Louis, MO, USA). For adipogenic differentiation, the cultures were first placed in adipogenic induction media and after 3 days the media was replaced with terminal differentiation media. The induction media and terminal differentiation media were based on basal media: α -MEM supplemented with 10% FCS, 20 mM HEPES and penicillin–streptomycin, 0.5 μ g/ml insulin (Promocell GmbH, Heidelberg, Germany) and 0.1 mM indomethasin (Sigma-Aldrich). The induction media contained in addition 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 0.4 μ g/ml dexamethasone (both from PromoCell GmbH). The terminal differentiation media was supplemented with 3 μ g/ml Ciglitazone (PromoCell GmbH). The cells were incubated in differentiation media for up to 4 weeks, replacing the medium twice a week. Osteogenic differentiation was demonstrated by staining the mineralized calcium phosphate accumulation with von Kossa staining, and adipogenic differentiation was demonstrated by staining the lipid-rich vacuoles of the adipogenic differentiated cells with Sudan III stain (Sigma-Aldrich).

Antibodies

Antibodies in this study were used according to manufacturers instructions and purchased as follows: APC-conjugated CD34-antibody was from BD Pharmingen (BD Biosciences, San Jose, CA, USA), PE-conjugated CD133-antibody and FITC-conjugated CD34-antibody from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany), unconjugated SSEA3 antibody (clone MC-631) from R&D Systems Inc. (Minneapolis, MN, USA) and Chemicon International (Millipore, Billerica, MA, USA), unconjugated SSEA4 antibody (clone MC-813-70) from Chemicon International (Millipore, Billerica, MA, USA), Alexa Fluor 647 conjugated antibodies against SSEA-3 (clone MC-631) and -4 (clone MC-813-70) were from Biolegend (San Diego, CA, USA), Globo-H antibody (clone MBR1) from Alexis Biochemicals (Enzo Life Sciences, Farmingdale, NY, USA), Alexa 488 goat-anti mouse from Molecular Probes (Invitrogen by Life Technologies), FITC-conjugated anti-rat-antibody from Sigma-Aldrich and HRP-conjugated goat anti-rat and anti-mouse antibodies from AbD Serotec (Düsseldorf, Germany).

RNA microarray analysis

Collection of hematopoietic CD133⁺ and CD34⁺ cells, their control samples and the microarray analysis have been described in detail (Hemmoranta et al., 2006). Total RNA for microarray was isolated

using RNase Mini Kit (Qiagen, Hilden, Germany). Sample labelling and hybridization were carried out at the Finnish DNA Microarray Centre at Turku Centre for Biotechnology, Turku, Finland. In brief, 2 μ l of total RNA from each sample was used and samples prepared using One-Cycle Target Labeling protocol (Specific Protocols for Using the GeneChip Hybridization, Wash and Stain Kit) and hybridized to GeneChip[®] Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). RNA and cDNA concentrations were checked with Nanodrop ND-1000 (Thermo scientific, Wilmington, DE, USA) and quality controlled by Experion Electrophoresis station (Bio-Rad Laboratories, Hercules, CA, USA). GeneChip Fluidics Station 450 was used to wash and stain the arrays and GeneChip Scanner 3000 with AutoLoader was used to scan the arrays. GeneChip Fluidics Stations and Scanner were controlled with GeneChip Command Console (AGCC) software version 1.0. Hybridization data for MSCs and cells from osteogenic and adipogenic differentiation were obtained from four different MSC-lines. For the gene expression analysis, AffyReader (a Microarray Pipeline component) was used to extract gene expression measures from the Affymetrix CEL-files. The Affymetrix probeset expression data of transcripts were newly clustered to represent genes by recombining the probes that represented the same gene using GeneChip library files (Custom CDF. version 10) with esembl gene ID (Dai et al., 2005, 2007). All samples, including expression data from hematopoietic samples (Hemmoranta et al., 2006), were normalized using Robust-Multi-array (RMA) Average background adjustment for intensities, quantile normalization and median-polish summarization (Wu and Irizarry, 2004). The RMA normalization was implemented using Bioconductor R, package *affy*. Values were transformed into log₂.

Flow cytometry and immunofluorescence

Cells were stained for flow cytometric analyses using standard protocols and analysed by FACSria and FACSDiva[™] Version 5.0.2 software (BD Biosciences). Histogram overlays were created after FACSDiva analysis with Adobe Photoshop CS3 version 10.0.1 in order to better visualize the collected data. HSCs were identified from Ficoll-Paque isolated UCB mononuclear cells for flow cytometric analysis by positivity for cell-surface markers CD34 and CD133. In all experiments, 3000–5000 double positive cells were analysed from three separate cord blood units. In case of UCB-MSCs, 10000 cells in passages 4–7, from two separate cord-blood unit derived cell lines, were analysed.

For immunofluorescence stainings, UCB-MSCs were grown on coverslips for 48 h. Unpermeabilized cells were first immunostained on ice using standard protocols and fixed after staining with 4% PFA. For visualization of the nucleus, coverslips were mounted with Vectashield mounting media containing DAPI (Vector Laboratories, Peterborough, UK). Cells were visualized by Zeiss Axioskop 2 plus microscope equipped with Zeiss AxioCam MRC-camera and AxioVision software 3.1/4.0 (Carl Zeiss Vision GmbH, Germany).

Mass spectrometry of lipid linked glycans

Glycosphingolipids were isolated from cell pellets stored in -70°C prior to analysis by organic solvent extraction and phase partition (Folch's phase) followed by solid-phase extraction using a

Sephadex G-25 column (Amersham Pharmacia, GE Healthcare Bio-Sciences AB, Uppsala, Sweden; Miller-Podraza et al., 2005). Lipid-linked glycans were detached by *Macrobodella decora* endoglycosidase (Calbiochem, Merck, Darmstadt, Germany) digestion from isolated glycosphingolipid fractions. The released glycans were purified for analysis mainly by miniaturized solid-phase extraction steps as previously described (Hemmoranta et al., 2007). MALDI-TOF mass spectrometry was performed with a Bruker Ultraflex TOF/TOF instrument (Bruker Daltonik GmbH, Bremen, Germany). Neutral glycans were detected in positive ion reflector mode as $[M + Na]^+$ ions and acidic glycans were detected in negative ion reflector or linear mode as $[M - H]^-$ ions.

Glycosphingolipid analysis by immuno-TLC

Glycolipid composition of FCS was analysed by immuno-TLC essentially as described before (Miller-Podraza et al., 2000). Glycolipids were separated on silica gel 60 HPTLC plates (aluminum sheets) (Merck, Darmstadt, Germany) using chloroform-methanol-0.25% KCl aq (by vol.) as a developing solvent. The plates were dried in air and covered with a plastic layer by dipping in 0.3% polyisobutylmethacrylate (Aldrich Chemical Company, Inc., Milwaukee, WI, USA) in diethyl ether-hexane, 5:1 (by vol.) for 1 min. The fixed plates were dried and incubated in a blocking solution (2% BSA and 0.1% Tween 20 in PBS) at room temperature for 1 h. The plates were then overlaid with a primary antibody (either anti-SSEA-3 or anti-SSEA-4) in PBS for 2 h. After that the plates were gently washed with PBS, overlaid with secondary HRP-labelled antibody in PBS, and incubated at room temperature for additional 2 h. Finally, the plates were washed four times with PBS and visualized by incubating at room temperature (in dark) in 0.02% DAB (3,3'-diaminobenzidine tetrahydrochloride; Pierce, Rockford, IL, USA) in PBS containing 0.03% H_2O_2 for 5–10 min. A parallel control plate was used for chemical visualization of organic bands using anisaldehyde (4-methoxybenzaldehyde; Merck, Darmstadt, Germany).

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