Expansion of mesenchymal stem cells from frozen UCB

Clausen C*. and Madsen CNP.°

* M.Sc, M.D., Group leader at Bioneer A/S, Denmark, °M.Sc.,Laboratory Manager at StemCare A/S, Denmark

Abstract

Umbilical cord blood (UCB) has recently been the focus of clinical applications due to its relative accessibility, its high number of primitive progenitor cells and its relatively low frequency of graft-versus-host-disease. UCB contains two types of stem cells: hematopoietic stem cells (HSC) and mesenchymal stem cells (MSC). HSC have been used for treatment of leukemia since the 1960's. Clinical studies shows that MSC can be used in regenerative medicine hereby treatment of cardiac diseases, diabetes and certain neurological diseases. However, due to the low number of MSC in UCB, ex vivo expansion is required. It has previously been demonstrated that MSCs can be isolated from fresh UCB, however limited data exists with isolation of MSC from frozen UCB samples. The aim of this project is to establish an optimal protocol for isolation of MSCs from frozen UCB, and characterize their MSC specific phenoptype. The MSCs were derived from thawed UCBs by using a known thawing protocol and Ficoll separation of the mononuclear fraction. We used flow cytometry to analyze the expanded cells for MSC surface markers (CD73, CD90 and CD166) and the presence of non-specific MSC markers like CD34 and CD31. The protocol used in this study resulted in adherent cell populations with MSC morphology. $CD90^+$, $CD73^+$, $CD166^+$, $CD34^-$, $CD31^-$ cell populations were identified with flow cytometry analysis. In conclusion, this study demonstrated that the used freezing, thawing and MSC isolation protocol resulted in MSC populations that could be used for regenerative medicine.

Discussion/Conclusion

The study demonstrates that MSCs can be isolated from cord blood samples cryopreserved and thawed in accordance with the Stemcare process. The flow cytometry analysis demonstrated a classical surface marker profile (CD73, CD90 and CD166) comparable with MSC populations isolated from e.g. bone marrow or adipose tissue. Additional studies are needed in order to fully determine the potential of the established hucMSC population, e.g. multipotential differentiation. The seeding of MNCs into the culture flasks can also be optimized with respect to medium composition, surface coatings of plastic substrate etc.
In conclusion this project has demonstrated that MSCs can be established from the UCB samples from Stemcare, however more optimization is needed in order to establish a robust procedure. A possible donor to donor variation in the number of hucMSCs in the collected umbilical cord blood should also be considered. The isolation of MSCs from frozen umbilical cord blood samples pave the way for using cord blood samples as a source for regenerative medicine.

Method

Proccessing

The collected umbilical cord blood (UCB) is processed in the laboratory in Copenhagen, Denmark. The overall aim of the process is to separate the UCB into plasma, red blood cells and the buffy coat in which the stem cells are concentrated. The total nucleated cells (TNC), the viability and the number of CD34 positive cells are counted.

Freezing

Before the freezing of the buffy coat, cryopreservation (DMSO/Dextran) is added. Hereafter the final product is placed in a freezing machine (IceCube, Sylab) and frozen down under controlled conditions to a temperature of minus 130ºC. The frozen UCB is stored in the vapor phase in a nitrogen tank.

Thawing

As advised by Stemcare the thawing/wash-SOP was followed just after each UCB-sample was thawed. Thawing of each UCB sample was done in a 37 degrees water bath under gently agitation. After washing according to the SOP provided by Stemcare each UCB sample was processed according to protocols designed by Bioneer. These protocols are based partly on a literature survey and partly on in-house know-how.

Ficoll

The Ficoll gradient separation method has previously been applied to fresh UCB for MSC isolation. MSC isolation from the interphase of the Ficoll gradient is based on their ability to adhere to plastic surfaces. The mononuclear fraction were isolated be the Ficoll gradient separation method, leading to a well defined layer after centrifugation containing the mononuclear cell fraction from the thawed umbilical cord blood sample.

Cultivation/Expansion

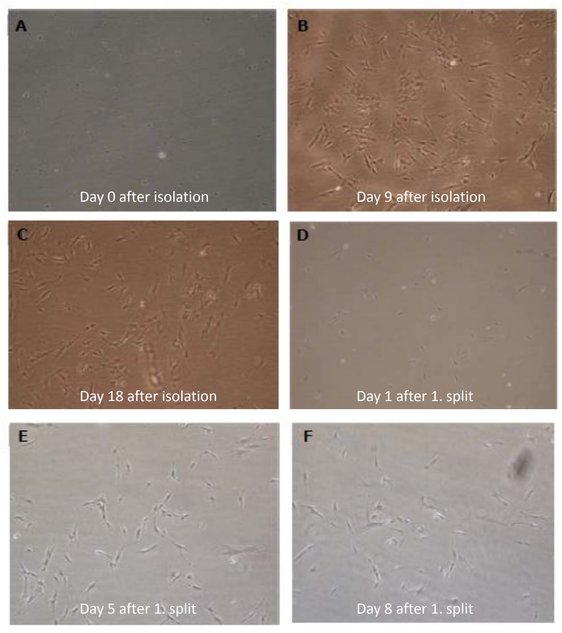
Mononuclear cells were washed and seeded into T25 Nucleon cell culture flasks at $1x10^6$ cells/ml and placed in a standard CO_2 cell incubator. After 24 hours the growth medium (DMEM/LG + 10% FBS) was changed carefully, washing out the non-adherent cells in the culture. The first developing colonies with MSC morphology were observed after 8-12 days. ucMSCs were expanded and able to being passaged by conventional trypsin treatment.

Analysis

The surface marker profile of ucMSC cultures were analysed with flow cytometry (BD FACSarray). Monoclonal antibodies relevant for the MSC phenotype; CD90, CD73 and CD166 as well as endothelial- and hematopoietic cells were tested on the expanded ucMSC populations. In addition relevant isotypic controls were included. Data were analysed with FlowJo and overlay histograms were generated with the isotypic signal as background.

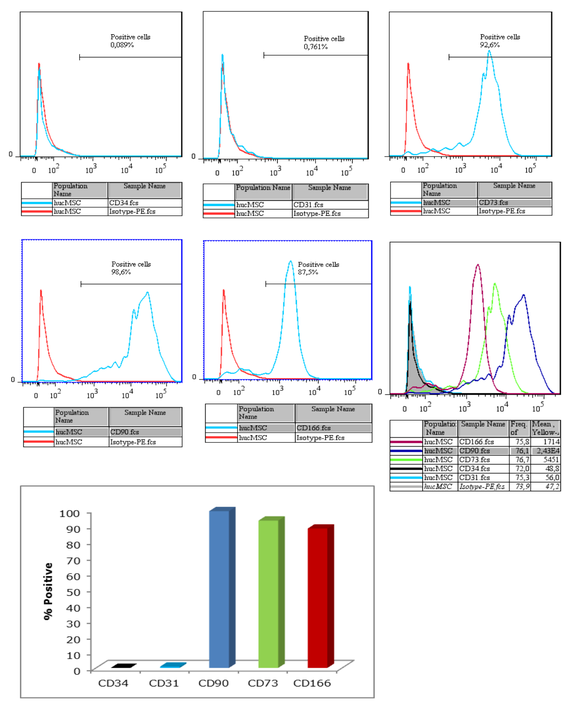
Furthermore all the established UCB cultures were followed by classical light microscopy.

Results



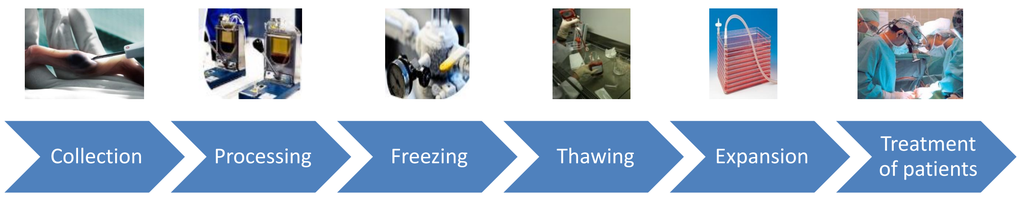
Expansion

We were able to etablish adherent MSC-like colonies that continued to divide and eventually establish a confluent monolayer. At day 8-12 typically MSC colonies were observed (Panel B). Morphologically, the cells resembled the characteristics of human MSCs from bone marrow. The UCB derived MSC culture, hucMSC (passage 0 (P0), was passaged and the cell population continued their dividing capacity after splitting (see figure).



Flow cyto analysis

Flow cytometry analysis (BD FACSArray) was done using a gating strategy, in which a cell population (FSS^{high} SSC^{high}), excluding the FSS^{low} SSC^{low} fraction, was gated and analyzed further in histogram-setting with the FlowJo software. As illustrated in the figure, the gated population was negative for CD34 (hematopoietic marker) and CD31 (endothelial marker) and positive for the classical MSC markers; CD73 (ecto-5'-nucleotidase), CD90 (Thy-1) and CD166 (ALCAM).



References

1. Kim, et al., "Therapeutic Potential of Umbilical Cord Blood-Derived Mesenchymal Stem Cells in Ischemic Myocardium", Korean Circ J 2008;38:446-454.
2. Basford, et al., "The Cord Blood Separation League Table: a Comparison of the Major Clinical Grade Harvesting Techniques for Cord Blood Stem Cells", International Journal of Stem Cells Vol. 3, No. 1, 2010
3. Peters et al., "Efficient Generation of Multipotent Mesenchymal Stem Cells from Umbilical Cord Blood in Stroma-Free Liquid Culture", 2010; PLoS ONE 5(12): e15689
4. Kekarainen et al., "Optimization of immunomagnetic separation for cord blood-derived hematopoietic stem cells", BMC Cell Biology 2006, 7:30
5. Kang et al., "Differentiating characterization of human umbilical cord blood-derived mesenchymal stem cells in vitro", Cell Biology International 30, 2006, 569e575.
6. Lee et al., "Stem and progenitor cells in human umbilical cord blood", Int. J Hematol, 2010, 92:45-51.
7. Robinson et al.,"Superior *ex vivo* cord blood expansión following co-culture with bone marrow-derived mesenchymail stem cells.

About the companies:

StemCare A/S is a private cord blood bank in Denmark. The quality system is approved by the Danish Medical Agency. In addition StemCare is co-founder of Cord Blood Europe which is an association of cord blood banks in EU.
Bioneer A/S is an independent, not-for-profit research based service company (CRO) within biomedicine. Bioneer has more than 25 years of experience in developing platform technology to the pharmaceutical -and biotech industry.