The expansion and differentiation potential of CD271+(LNGFR) marrow stromal cells (MSCs) vs. MSCs isolated by plastic adherence

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Introduction

In bone marrow (BM), stem and progenitor cells with haematopoietic differentiation potential exist in close proximity to another heterogeneous group of stem cells with nonhaematopoietic (NH) differentiation potential termed marrow stromal cells (MSC). A subpopulation of these marrow stromal cells with unknown phenotype, can be isolated by plastic adherence (PA). Many attempts have been made to find the most specific antigen for the isolation of MSCs, such as CD105, CD117, CD271(LNGFR), Stro-1 and D7-Fib, a fibroblast marker. Isolated CD271+ bone marrow cells, as compared to plastic adherent bone marrow cells, were reported to show a one to three log greater expansion of MSCs in culture and a greater capacity to differentiate to adipocytes and osteoblasts. Therefore CD271 has become one of the most promising markers for MSC isolation [1,2].

Methods

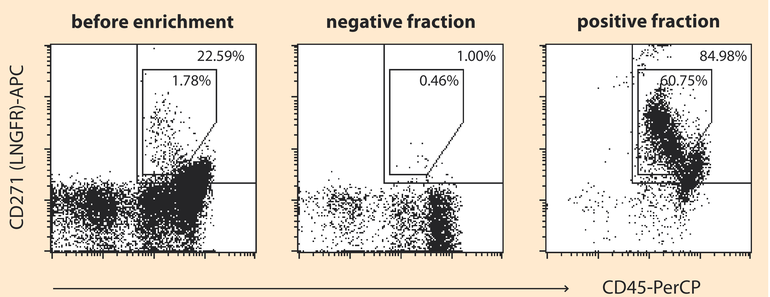
MSC isolation and cultivation were performed using the MSC Research Tool Box – CD271 (LNGFR) (Miltenyi Biotec GmbH) containing CD271 (LNGFR)-APC and Anti-APC MicroBeads for separation and NH Expansion Medium supplemented with CytoMix – CD271 (LNGFR) for cultivation.

2×10^7 bone marrow mononuclear cells (BM MNC) were separated using CD271 (LNGFR)-APC and Anti-APC MicroBeads. The negative and positive fractions were cultivated with NH Expansion Medium with and without CytoMix – CD271 in order to assess their proliferative capacity. These fractions were compared with MSCs isolated by PA and expanded using the same cultivation conditions as described for separated cells. The phenotype and population doubling (PD*) as well as the cumulative population doubling (CPD**) levels were determined over 41 days of cultivation using the equations shown below[3]. To assess clonogenic potential, a colony-forming unit fibroblast (CFU-F) assay was used. The assay was performed with 1×10^6 BM MNC, 1×10^6 cells of the negative fraction or a sample of the positive fraction which were cultivated in NH Expansion Medium for 14 days. Furthermore, multipotent differentiation potential was investigated by culturing 5×10^4 MSCs in NH AdipoDiff and NH OsteoDiff Medium (Miltenyi Biotec GmbH). In order to observe chondrogenic differentiation a micromass[4] culture with 2×10^5 MSCs in NH ChondroDiff Medium (Miltenyi Biotec GmbH) was performed. A negative control was cultured in NH Expansion Medium only.

* PD for each subcultivation: $[\log10(N_e)-\log10(N_i)]/\log10(2)$
N_i = inoculum number of cells; N_e = cell harvest number
** CPD for 41 days of culture: Σ(PD)

Results

1 Positive selection of CD271(LNGFR)+ cells from BM MNC

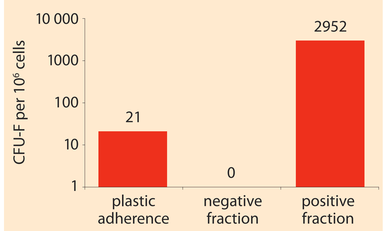


2×10^7 BM MNC were isolated using CD271(LNGFR)-APC and Anti-APC MicroBeads. Samples of all fractions were labelled with CD45-PerCP. PI immunofluorescence and light scatter signals were used for gating live cells. The positive fraction contained CD271(LNGFR) positive cells with a purity of about 85% (83% ± 4,1%, n=4). This population contains about 61% CD271(LNGFR)^{bright}/CD45^{dim} cells and about 22% CD271(LNGFR)^{dim}/CD45^{bright} cells.

2 Colony-forming unit fibroblast (CFU-F) assay

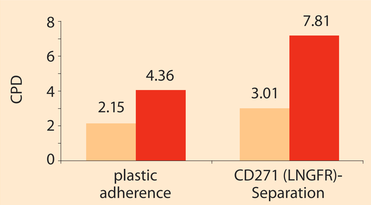
A)

B)



All fractions were giemsa stained (A) and CFU-F numbers were counted after 14 days of incubation (B). The numbers of CFU-F rise by about 2 log when MSCs are isolated using CD271(LNGFR) as compared to PA. No CFU-Fs were detected in the CD271-negative cell fraction.

3 Expansion of MSCs isolated by CD271 separation vs. plastic adherence and assessment of the CPD



CD271(LNGFR) separation, as well as CytoMix, have a positive effect on the MSC expansion potential. CD271(LNGFR) enriched cells show a 50 % increased CPD* in comparison to MSCs isolated by PA. When cultivated with CytoMix, the CPD level of CD271+ cells more than doubles.

* CPD for 41 days of culture: Σ(PD**)
** PD for each subcultivation: $[\log10(N_e)-\log10(N_i)]/\log10(2)$
N_i = inoculum number of cells; N_e = cell harvest number

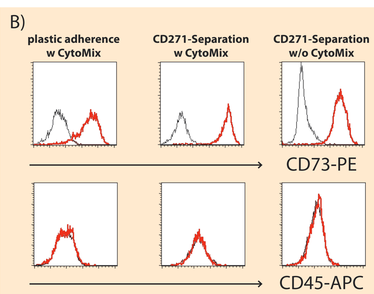
w/o CytoMix w CytoMix

4 Phenotyping of *in vitro* expanded MSCs isolated by CD271 separation and plastic adherence

A)

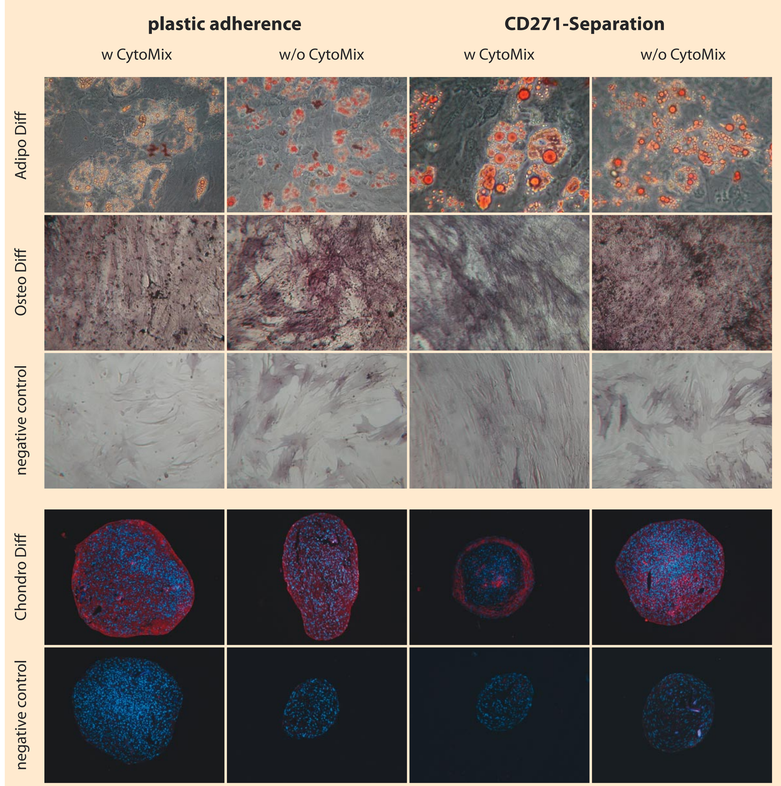
MSC Fraction	CD29	CD44	CD73	CD105	CD166	CD14	CD34	CD45
PA w CytoMix	+	+	+	+	+	–	–	–
CD271+ w CytoMix	+	+	+	+	+	–	–	–
CD271+ w/o CytoMix	+	+	+	+	+	–	–	–

B)



After 41 days of culture using NH Expansion Medium (with and without CytoMix), MSCs were trypsinized and stained with antibody conjugates for different markers (——) and the corresponding isotype controls (——). *In vitro* expanded MSCs are CD29+, CD44+, CD73+, CD105+, CD166+ and CD14–, CD34–, CD45– [5]. Independent on whether MSCs were isolated by PA or CD271 separation, and cultivated with or without CytoMix, cells matched these criteria after expansion (A). The histograms (B) show, as an example, CD73 and CD45 staining for all samples.

5 Differentiation potential of MSCs isolated by CD271 separation vs. plastic adherence



After 18 days of cultivation in NH AdipoDiff Medium, all MSCs showed an increased accumulation of intracellular lipid vacuoles, as revealed by Oil red-O staining.
After 10 days of cultivation in NH OsteoDiff Medium all MSCs showed a high alkaline phosphatase (AP) activity. Detection of AP activity was performed with Fast BCIP/NBT substrate.

After 24 days of cultivation in NH ChondroDiff Medium the nodule was processed immunohistochemically. In order to detect chondrocyte specific aggrecan, an indirect staining with anti aggrecan antibody and Rhodamine-Red labelled secondary antibody was used. Cell nuclei were stained with 4'-6-Diamidino-2-phenylindole (DAPI).
Negative controls showed no differentiation.

Conclusion

Highly purified CD271+ cells show greater expansion in number of fibroblastic cells compared to MSCs isolated by plastic adherence. They possess the ability to give rise to adipocyts and osteoblasts.

A number of methods relying on specific physical properties have been used historically to isolate MSCs from sites at which they reside. The problem with this type of approach is that no physical properties have been uniquely ascribed to MSCs. Therefore many different cell types are co-isolated, resulting in a mixed population of cells.

The purification approach embodied in the CD271(LNGFR) isolation results in a cell population containing approximately 1000-fold greater concentration of MSCs compared to conventional methods of isolation. This enables isolation of a homogeneous, and consequently more effective NH stem cell population.

References:
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2. Quirici *et al.* (2002) Experimental Hematology 30:783–791
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4. Johnstone *et al.* (1998) Exp. Cell res. 238:265–272
5. Pittenger *et al.* (1999) Scienc. 284:143–146