CD29 of human umbilical cord mesenchymal stem cells is required for expansion of CD34+ cells

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Abstract

Objectives: Human umbilical cord mesenchymal stem cells (hUCMSCs) play a critical role in expanding haematopoietic stem cells (HSCs) by providing the essential microenvironment for haematopoiesis. In this study, we sought to investigate whether CD29 of hUCMSCs would play a key role in the ability of hUCMSCs to help expand HSCs in vivo and in vitro.

Material and methods: To investigate whether CD29 of hUCMSCs would play a key role for the ability of hUCMSCs to expand HSCs, soluble anti-CD29 antibody was added to co-cultures of hUCMSCs and cord blood (CB) CD34+ cells. It significantly blocked expansion of CB CD34+ cells induced by hUCMSCs. Using CD29-deficient hUCMSCs models, long-term culture-initiating cell and non-obese diabetic/severe combined immunodeficient disease mouse repopulating cell assay, revealed that CB CD34+ cells co-cultured with CD29-deficient hUCMSCs only retained the capacity of multipotent differentiation for 5 weeks at the most.

Results: Soluble anti-CD29 antibody significantly blocked expansion of CB CD34+ cells induced by hUCMSCs. CB CD34+ cells co-cultured with CD29-deficient hUCMSCs only retained the capacity of multipotent differentiation for 5 weeks at the most. 

Conclusions: CB CD34+ cells co-cultured with CD29-deficient hUCMSCs gave rise to all major haematopoietic lineages, but failed to engraft long term.

Introduction

Haematopoietic stem cells (HSCs) have extensive regenerative potential which makes them attractive targets for cell and genetic therapies; however, their rarity limits their use for therapeutic application. Large ex vivo HSC expansion has been one of the major goals of stem cell research (1–4). Any expansion protocol must attempt to simulate (as close as possible) in vivo haematopoiesis, to maintain stemness properties of the HSCs (4–6).

Human mesenchymal stem cells (MSCs) play a critical role in providing the essential microenvironment for haematopoiesis, which has been successfully used in vitro as a scaffold for stromal support and expansion of HSCs via cell/cell contact (7–11). Biological interest in MSCs, first described by Friedenstein et al. (12), has risen dramatically over the last decade. Although bone marrow (BM) remains a major source of MSCs for most investigations, and primary marrow stromal cells are able to support HSC expansion in vitro, it has been difficult to isolate adequate primitive BM stromal cells for expansion (13,14). Umbilical cord (UC), rich in MSCs, and UC-derived MSCs (UCMSCs) have been shown to be easy to isolate and culture (15,16). Increasing amounts of data have shown that adhesion and direct cell/cell contact between UCMSC and feeder layers supports ex vivo expansion, migratory potential and stemness of HSCs (17,18). Although beneficial effects of human UC mesenchymal stem cells (hUCMSCs) on their supportive role in haematopoiesis is known, molecular regulation of interaction between MSCs and HSCs up to now still needed to be elucidated.

CD29, a binding subunit of the β1 integrin family receptors, binds various types of ligand such as vascular...
adhesion molecule (VCAM)-1 and extracellular matrix proteins, produced by many stromal cells, and mediates niche interactions (11,19). To investigate molecular regulation of the supportive role of hUCMSCs in haematopoiesis, we formed the hypothesis that CD29 would play a key role in the ability of hUCMSCs to support it, as it mediates niche interactions and is highly expressed by hUCMSCs (10,20,21). To test the hypothesis, first we proved that CD29 was important for the ability of hUCMSCs to support haematopoiesis, by the addition of soluble anti-CD29 antibody to co-cultures of hUCMSCs and CB CD34+ cells. Using CD29-deficient hUCMSCs models, long-term culture-initiating cell (LTC-IC) and non-obese diabetic/severe combined immunodeficient disease (NOD/SCID) mouse repopulating cell (SRC) assay revealed that CB CD34+ cells co-cultured with CD29-deficient hUCMSCs only retained the capacity of multipotent differentiation for 5 weeks at the most. CB CD34+ cells co-cultured with CD29-deficient hUCMSCs gave rise to all major haematopoietic lineages, but failed to engrraft long term. CD29-deficient hUCMSCs may interact more loosely with CB CD34+ cells, which would promote efficient transition from long-term to short-term HSCs, then speed up efficient and continuous differentiation of HSCs.

In addition to being important for mediating HSC–niche interactions, our data raise the possibility that CD29 in hUCMSCs may also be necessary for the ability of hUCMSCs to expand CB CD34+ cells.

Materials and methods

In this study, experimental protocols concerning humans were approved by the Ethics Committee of Peking University. Before experiments, subjects were informed of the objectives, requirements and procedures of the experiments. All subjects gave informed written consent to participate in the study.

Experimental protocols concerning animals had been approved by the Institutional Authority for Laboratory Animal Care, of Peking University.

Isolation and culture of hUCMSCs and cord blood (CB) CD34+ cells

After washing in Hanks balanced salt solution to remove contaminating blood, UCs were cut into 1 cm pieces, and vessels were removed to avoid endothelial cell contamination. Tissue pieces were placed in six-well plates for culture expansion in low-glucose Dulbecco’s modified Eagle’s medium (L-DMEM) (Hyclone, Logan, Utah, USA) supplemented with 10% foetal bovine serum (FBS). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO2. Medium was changed every 2–3 days. After approximately 2 weeks, cells were found at the edge of the tissue fragments. When colonies of fibroblast-like cells appeared and cells in wells reached 70% confluence, cultures were detached using 0.25% trypsin-EDTA, and reseeded in 10 cm dishes for optimal proliferation.

Human CB samples were obtained as described previously (4,5). Briefly, CB mononuclear cells (MNCs) were isolated using lymphocyte separation medium (1.077 g/ml) (TBD Biotech, Tianjing, China), and were immunomagnetically enriched for CD34+ cells using MACS CD34+ Cell Isolation Kit (Miltenyi Biotech Inc., Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Purity of CD34+ cells was in the order of 80–90%, determined by flow cytometry (FCM).

CD29 shRNA design, construction and packaging of shRNA vectors

The two CD29-specific small hairpin RNAs (KD1 and KD2) oligomers were designed using online RNAi design software. These shRNA sequences excluded all sequence homology with any other human coding sequences in BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Details of shRNA sequences are provided in Table 1. Sense and antisense oligomers were used to produce double-stranded oligomers, and these were inserted into retroviral vector RNAi-pSIREN-RetroQ, which drives shRNA production from the U6 promoter and also contains puromycin resistance (Clontech, San Francisco, USA). Inserts were confirmed by sequencing (ABI PRISM 310 Genetic Analyzer, Foster, CA, USA). If not otherwise mentioned, RNAi-pSIREN-RetroQ vectors containing scrambled target sequences not complementary to any known miRNA were served as controls (CTRL). Phoenix packaging cell line was co-transfected with RNAi-pSIREN-RetroQ retroviral plasmid and viral packaging plasmid by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s instructions. Viral supernatants were collected at 48 or 72 h after transfection and stored at −80 °C until future use.

Table 1. Primer used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>KD1</td>
<td>CCACAGACATTTACATTAAA</td>
</tr>
<tr>
<td>KD2</td>
<td>CCACAGATGCCGGGGTTCAC</td>
</tr>
<tr>
<td>CTRL</td>
<td>ACACGTCCGAACATTAAA</td>
</tr>
<tr>
<td>CD29</td>
<td>F: 5'-GCATACAATCTCCCCCTTCTCTCA-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-AATAACCTCTACCTTCCCTCGT-3'</td>
</tr>
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RNA extraction and real-time RT-PCR
Total RNA was extracted using trizol reagent (Invitrogen) and real-time RT-PCR analysis was performed using Bio-Rad iCycler with iQSYBER green supermix (Bio-Rad, Hercules, CA, USA) as described previously (4,5). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was the endogenous control. Standard curves for internal control and tested genes were measured each time to determine relative level of the respective transcript. Relative expression was normalized to endogenous control levels.

Co-culture of hUCMSCs with human CB CD34+ cells
Co-culture assay was performed as described previously (4,5). Briefly, indicated hUCMSCs were irradiated at 40 Gy, then seeded in 24-well plates (5.0 × 10^5/well) overnight. CD34+ cells (2.0 × 10^5/well) in Iscove’s modified Dulbecco’s medium (IMDM) (Bio-Whittaker, Walkerville, MD, USA) were supplemented with 10% FBS, 10^-3 M 2-mercaptoethanol, 2 mM L-glutamine, 5 mg/ml insulin, 100 U/ml penicillin and 100 mg/ml streptomycin, as well as a cytokine cocktail consisting of Flt ligand (FL; 10 ng/ml), SCF (10 ng/ml), thrombopoietin (TPO, 10 ng/ml) and interleukin (IL)-6 (10 ng/ml), all of which were purchased from Peprotech Inc, Rocky Hill, NJ, USA. After 14 days culture, non-adherent and adherent haematopoietic cells loosely attached to stromal cells, were harvested by gentle pipetting, and counted.

Long-term culture-initiating cell (LTC-IC) assay
LTC-IC assay was performed as described previously (4,5). Briefly, 1.0 × 10^6 CB CD34+ cells were pipetted into six-well plates containing nearly confluent, irradiated CD29-deficient hUCMSC or control hUCMSC monolayers in LTC medium (Myelo-Cult, StemCell Inc., Vancouver, BC, Canada), along with 10^-6 M hydrocortisone sodium hemisuccinate (Sigma, St. Louis, MO, USA), LTC medium contained horse serum, foetal bovine serum, 2-mercaptoethanol and α-MEM. 50% of the medium was replaced weekly with fresh. Both non-adherent and adherent cells were harvested weekly over 3–7 weeks culture, in complete methylcellulose medium containing 2.8% BSA, 30% FBS (Hyclone), 50 ng/ml SCF, 20 ng/ml IL-3, 20 ng/ml GM-CSF, 20 ng/ml IL-6 and 3 U/ml EPO (Kirin, Tokyo, Japan), at 37 °C with 5% CO2. After 14–16 days, colonies with greater than 50 cells were counted, to assess LTC-IC activity. Colony-forming units (CFU-Cs), colony-forming unit-mix (CFU-Mix), colony-forming unit-erythrocyte (CFU-Es) and burst-forming unit-erythrocyte (BFU-Es) were counted to calculate frequency of LTC-IC, according to the manufacturer’s instructions (StemCell Inc.).

NOD/SCID repopulating cell (SRC) assay
Total of 5.0 × 10^4 CB CD34+ cells were co-cultured with indicated stromal cells for 4 weeks, harvested as described above, and injected intravenously (i.v.) into 8-week old, sublethally irradiated (3.5 Gy) NOD/SCID mice. Peripheral blood was obtained by non-lethal eye-bleeds under anaesthesia with isoflurane, per institutional guidelines. Mice were sacrificed 12 weeks post-transplantation. Mononuclear cells were harvested and analysed by FCM. Human cell repopulation was assessed using anti-human-CD45-fluorescein isothiocyanate (FITC; 555482), anti-human-CD34-phycoerythrin (PE; 550761), anti-human-CD36-PE-cyanin 7 (PC7; 563666) and anti-glycophorin A (GPA)-allophycocyanin (APC; 551336) antibodies. Isotype controls were mouse IgG1 conjugated to PE, APC, PC7 or FITC. All antibodies were from Becton Dickinson, Franklin L. New Jersey, USA.

Statistical analysis
Results are expressed as mean ± SD. Statistical comparisons were performed using two-tailed Student’s t-test.

Results

Ex vivo expansion of CB CD34+ cells with hUCMSCs
To investigate how hUCMSCs affected proliferation and differentiation of HSCs, CB CD34+ cells were cultured with or without hUCMSCs for 14 days in vitro. As expected, total nucleate cells expanded significantly more when in cultures supported by hUCMSCs (39.40 ± 3.84-fold versus 16.83 ± 3.84-fold; P < 0.05; Fig. 1a). Then, we examined CD29 expression of hUCMSCs by FCM. Consistent with prior work, hUCMSCs constitutively highly expressed CD29 (Fig. 1b).

CD29 was required for haematopoiesis-supporting activity of hUCMSCs
As hUCMSCs expressed high levels of CD29, we first investigated whether the support of haematopoiesis by hUCMSCs resulted from CD29-mediated interaction between hUCMSCs and CB CD34+ cells. Interestingly, addition of soluble anti-CD29 antibody to cocultures significantly blocked expansion of CB CD34+ cells, specially CD34+/CD38- populations (Fig. 2a). Unexpectedly, although the mechanism by which
soluble isotype IgG1 promotes CD34+ cell expansion is not yet clear, addition of soluble isotype IgG1 led to increased expansion of CD34+ CD38+ cells. These results suggest that antibody treatment altered the culture milieu, which specifically promoted or inhibited CD34+ cell expansion, depending on effects on interactions between hUCMSCs and CB CD34+ cells. Taken together, the results strongly suggest that CD29 is important for the ability of hUCMSCs to support haematopoiesis.

To further investigate the function of CD29 on haematopoiesis support by hUCMSCs, CD29 knockdown models were created using hUCMSCs, by using retroviral vectors. CD29 levels in hUCMSCs transduced with the indicated virus were determined using real-time RT-PCR. We observed clear down-modulation of CD29 in KD cells compared to CTRL (Fig. 2b). Based on shRNA influence on CD29 expression, KD1 was chosen for further studies.

Effect of CD29 knockdown in hUCMSCs on LTC-IC activity of CB CD34+ cells

To test capability of CD29 of hUCMSCs in supporting self-renewal and maintaining multipotent differentiation of HSC, we performed LTC-IC assay. CB CD34+ cells were co-cultured with KD1 or CTRL cells in LTC-IC medium for 3–7 weeks, then subjected to CFU assay. After 14–16 days culture, colonies with more than 50 cells were counted. As shown in Fig. 3, numbers of total CFCs and CFU-GMs from cells co-cultured with KD1 or CTRL cells were similar to those from cells co-cultured with CTRL cells. However, numbers of total CFCs and CFU-GMs from cells co-cultured with KD1 cells decreased rapidly at week 6 then progressively lost their multipotency of differentiation over time of culture. CFCs and CFU-GMs were close to undetectable at week 7 co-culture. Consistently, although numbers of CFU-Mix and BFU-Es from cells co-cultured
with KD1 cells for 3–4 weeks were similar with those from cells co-cultured with CTRL cells, numbers of CFU-Mix and BFU-Es from cells co-cultured with KD1 cells decreased rapidly at week 5. It was noted that compared to CB CD34+ cells co-cultured with CTRL cells (which retained the capacity of multipotent differentiation for at least 6 weeks), CB CD34+ cells co-cultured with KD1 only retained the capacity of multipotent differentiation for 5 weeks at the most. This was because numbers of CFU-Mix and BFU-Es from these cells were not detectable at week 6. These results suggest that CD29 in hUCMSCs play an important role in the ability of hUCMSCs to support haematopoiesis.

Effect of CD29 knockdown of hUCMSCs on expansion of SRCs

To further support our in vitro expansion and LTC-IC results, we examined engraftment of CB CD34+ cells after co-cultured with KD1 or CTRL cells, in NOD/SCID mice, by temporal monitoring of peripheral blood and BM of recipients, for 12 weeks. Sublethally irradiated NOD/SCID mice were transplanted with 5.0 × 10^4 CB CD34+ cells from the co-cultures, with irradiated KD1 or CTRL cells, for 4 weeks. Although levels of chimaerism gradually increased in the peripheral blood of mice transplanted with CB CD34+ cells co-cultured with CTRL cells, engraftment of CB CD34+ cells co-cultured with KD1 cells peaked between 2 and 4 weeks then declined (Fig. 4a). Total human cell engraftment was composed of CD45+, CD45+CD36- and CD36-GPA+ cells, and CD45+CD34+ populations were assessed in BM mononuclear cells of engrafted mice 12 weeks post-transplant, by FCM. At week 12, total human cells from mice injected with CD34+ cells co-cultured with KD1 was significantly lower than that of mice injected with CD34+ cells co-cultured with CTRL (Fig. 4b; P = 0.026). Statistical analysis was performed for comparison between mice injected with CD34+ cells co-cultured with KD1 and those injected with CD34+ cells co-cultured with CTRL.

We further analysed multilineage development from input CD34+ populations. Flow cytometric analysis of human grafts in a representative graft mouse from each group is shown in Fig. 4d. We observed significantly lower percentage of CD45+ cells in KD1 group compared to percentage in the CTRL group. Similarly, compared to CTRL group, KD1 group also had significantly lower percentages of CD45+CD34+ cells (Fig. 4c). Observed multilineage development from input CD34+
populations co-cultured with KD1 cells coincided with expansion and LTC-IC assays in vitro. However, percentages of erythroid cells, including CD45⁺CD36⁺ and CD36⁻GPA⁺ populations in the KD1 group whose transplants of CD34⁺ cells had been co-cultured with KD1 cells had a tendency, although this was not significant, to be lower than that of mice receiving transplants of CD34⁺ cells co-cultured with CTRL. These results confirmed our in vitro data and suggest that CD29 in hUCMSCs played an important role in the ability of hUCMSCs to maintain multipotency of CB CD34⁺ cells in vitro.

Discussion

Self-renewal and differentiation of HSCs rely on the specified stem cell-niche interaction. Previous studies have demonstrated that human MSCs from human umbilical cord (hUC) are able to support haematopoiesis (17,18). Some results suggest that MSCs can improve therapeutic effects of HSC transplantation in clinical treatment and haematopoietic engraftment in NOD/SCID mice (22,23). However, mechanisms involved in controlling the fate of HSCs by hUCMSCs remained largely unknown.

In this study, first we identified that hUCMSCs supported CB CD34⁺ cell expansion in vitro and highly expressed CD29; this is consistent with previous work (10,11,18,20,21). CD29 is an adhesion molecule; it has been used to isolate murine HSCs and other somatic stem cells (21,22), and is known to be important for HSC-niche functions. Interestingly, addition of soluble anti-CD29 antibody to co-cultures significantly blocked expansion of CB CD34⁺ cells caused by hUCMSCs, specifically CD34⁺CD38⁻ populations (Fig. 2a); this is similar to our previous data resulting from FBMOB.
CB CD34+ cells co-cultured with control hUCMSCs had higher engraftment and differentiation potential than CB CD34+ cells co-cultured with CD29-deficient hUCMSCs immediately after transplant. Functional impairment of hUCMSCs to support HSC expansion (21,24), CD29 was possibly also involved in the ability of hUCMSCs to support HSC expansion. Mechanisms of engraftment have revealed a host of contributing factors, including adhesion molecules, such as integrins and CXCR4, an important homing factor for HSCs (26–28). Compared to CB CD34+ cells co-cultured with CTRL hUCMSCs, it is possible that CB CD34+ cells co-cultured with CD29-deficient hUCMSCs expressed lower levels of VCAM; this leads to looser interaction between engrafted human cells and the niche. Results from BM also rule out the idea that CB CD34+ cells co-cultured with CD29-deficient hUCMSCs had impaired capacity to home and proliferate in the marrow, consistent with the data from engraftment of CD49fHSC (25). These all demonstrate that CB CD34+ cells co-cultured with CD29-deficient hUCMSCs gave rise to all major haematopoietic lineages, but failed to engraft long term, indicating that after CD29 knockdown, ability of hUCMSCs to long term support HSC expansion is reduced. On the basis of our results and previous reports, we offer the concept that adhesive molecular constructs of hUCMSCs play an important role during haematopoietic development.

In summary, our data suggest the potential contribution of CD29 in hUCMSCs in expansion of haematopoiesis. Our study demonstrated that, in addition to being important for mediating HSC-niche interactions, CD29 is also possibly involved in the ability of hUCMSCs to support HSC expansion.

Acknowledgements

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References