Platelet Precursor Cells Can Be Generated from Cultured Human CD34+ Progenitor Cells But Display Recirculation into Hematopoietic Tissue upon Transfusion in Mice

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Keywords

CD34 · CD41 · CD61 · HSC ·
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Summary

Background: Whereas ex vivo expanded megakaryocytic progenitor cells have been investigated for their ability to support platelet regeneration, the question whether more mature platelet-like particles expanded from hematopoietic progenitor cells may be useful for transfusion purposes remains largely elusive. **Methods:** Human peripheral blood progenitor cells (PBPCs) were enriched using surface expression of CD34 by immunoselection. CD34+ enriched PBPCs were expanded ex vivo in serum-free medium supplemented with cytokines. As a proof-of-principle, distribution of expanded CD61+ particles was analyzed after transfusion into Non-Obese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice. **Results:** Highest ex vivo expansion for CD41+/CD61+ cells was achieved when medium was supplemented with SCF, TPO and IL-3. During expansion culture, CD34 marker expression decreased from 85 to 2–8%, while megakaryocytic cells appeared and CD41 and CD61 expression increased from 3 to about 30%. After transfusion of the expanded cells in NOD/SCID mice, CD61+ cells located mainly to bone marrow and to a lesser degree to spleen, but also circulated in blood. **Conclusion:** Platelet-like particles using cytokine-substituted serum-free medium can be generated efficiently from CD34+ expansion cultures, but mainly home to hematopoietic tissue.

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Schlüsselwörter

CD34 · CD41 · CD61 · HSC ·
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Zusammenfassung

Hintergrund: Während die Fähigkeit ex vivo expandierter megakaryozytärer Vorläuferzellen auf ihre Fähigkeit die Thrombozytenregeneration zu unterstützen untersucht wurde, fehlen Daten zur Zirkulationsfähigkeit und Verteilung thrombozytenähnlichen Partikeln unmittelbar nach Transfusion. **Methoden:** Humane periphere Blutvorläuferzellen (PBPCs) wurden mittels der Oberflächenexpression von CD34 durch Immunselektion angereichert. Die CD34+ angereicherten PBPCs wurden ex vivo in serumfreiem Medium mit Zytokinen expandiert und in vivo nach Transfusion in Non-Obese Diabetic/Severe Combined Immunodeficiency(NOD/SCID)-Mäuse analysiert. **Ergebnisse:** Die höchste Ex-vivo-Expansion von CD41+/CD61+ Thrombozytenvorläuferzellen und thrombozytenähnlichen Zellen konnte erreicht werden, wenn das Medium mit SCF, TPO und IL-3 substituiert wurde. Während der Expansionskultur nahm die CD34-Markerepression von 85 auf 2–8% ab, während megakaryozytäre Zellen erschienen und die CD41- und CD61-Expression von 3 auf 30% anstieg. Nach Transfusion der expandierten Thrombozytenvorläuferzellen und thrombozytenähnlichen Zellen in NOD/SCID-Mäuse wurden CD61+-Zellen hauptsächlich im Knochenmark und geringerem Grad in der Milz gefunden, aber zirkulierten ebenfalls im Blut. **Schlussfolgerungen:** Thrombozytenähnliche Partikel können in einem zytokinsupplementierten, serumfreien Medium effizient aus CD34+ Zellen generiert werden und wandern in hämatopoetische Gewebe ein.

Introduction

Platelets are produced at a scale of 1×10^{11} cells/day in a healthy human adult. As an alternative to platelets isolated by platelet apheresis or concentration from whole blood donations used for therapeutic platelet substitution [1–3], in vitro generation of platelets or megakaryocytically committed progenitors from hematopoietic stem and progenitors cells (HPCs) in liquid cultures has been proposed [4, 5]. Cytokines which have been investigated for this purpose include thrombopoietin (TPO), stem cell factor (SCF), interleukins (IL-3, IL-11) and fetal liver tyrosine kinase 3 ligand (FLT3L) [6, 7]. In addition to its specific role in promoting megakaryocyte proliferation and differentiation, TPO has been known to be involved in stem cell survival, self-renewal and expansion. The presence of this cytokine is not absolutely required in megakaryocyte formation, but it strongly increases the density of platelet-specific markers and accelerates platelet formation [8–10]. The megakaryocytic differentiation process in vivo can be monitored by the surface presence of the megakaryocytic markers CD41 (GPIIb / α IIa integrin) and CD61 (GPIIIa / β_3 integrin) [11]. Their presence increases during the differentiation from stem cell megakaryocytic progenitors into platelets.

We here investigated the effect of five different cytokine combinations, all including TPO and SCF, on the potential to generate CD41+ and CD61+ megakaryocytic and platelet-like cells from G-CSF-mobilized peripheral blood progenitor cells (PBPCs). We show that the cells can be generated in serum-free cultures, resulting in morphologically recognizable full megakaryocytic maturation. We injected the cells from these cultures intravenously into immunodeficient mice. We found that the human CD61+ cells generated in the cultures display a similar homing behavior in mice as human platelets.

Material and Methods

Chemicals and Antibodies

Albumin from bovine serum was purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Iscove's modified Dulbecco's medium (IMDM) supplemented with L-glutamin and 25 mmol/l HEPES as well as calcium- and magnesium-free phosphate-buffered saline (PBS) were obtained from GIBCO/Invitrogen GmbH (Karlsruhe, Germany). Fc-receptor blocking reagent was obtained from Miltenyi Biotec (Bergisch Gladbach, Germany). The phycoerythrin(PE)-labeled antibodies against human CD34 (clone 581), CD41 (clone HIP8), CD61 (clone VI-PL2) and IgG1 isotype (clone MOPC-21) were all purchased from BD Pharmingen (Heidelberg, Germany). Antibody against PE coupled to microbeads for cell isolation was obtained from Miltenyi Biotec. Human cytokines stromal cell-derived factor-1 α (SDF-1 α), FLT3L, SCF, TPO and IL-3 were all obtained from PeproTech Inc. (Rocky Hill, NY, USA). Cyto-morphology was assessed using DiffQuik Stain Kit from Fisher Scientific (Schwerte, Germany) after centrifugation on microcopic glass slides.

Isolation and ex vivo Expansion of CD34+ HSC/HPC

Cells were isolated from aliquots from peripheral blood stem cell harvests of G-CSF-treated patients undergoing autologous stem cell transplantation for a diagnosis of multiple myeloma or malignant lymphoma accord-

ing to a vote by the local ethics committee after informed consent. Enrichment was performed using magnetic beads technology (Miltenyi Biotec) according to the manufacturer's protocol. Briefly, $1\text{--}4 \times 10^8$ freshly isolated cells were labeled with PE-conjugated 25 μ g/ml anti-CD34 as primary antibody per 10^7 cells, incubated for 30 min at 4 °C, washed with PBS and subsequently incubated with 20 μ l beads-conjugated secondary antibody anti-PE per 10^7 cells. Incubation was performed for 30 min at 4 °C followed by passage over an LS selection column, and purity was assessed by flow cytometry and found to range from 70–80%. Subsequently, enriched CD34+ cells were inoculated at 3×10^4 cells/ml in serum-free X-vivo 20 medium (Gibco, Paisley, UK) in 24-well plates (Nunc, Wiesbaden, Germany) and cultured at 37 °C in the presence of 20 ng/ml each of the human cytokines. Half medium changes were performed twice a week. Wells were plated in replicates, and individual cultures were terminated at different time points for the assessment of morphology and expression of cell surface markers. Cell viability was determined using trypan blue dye exclusion and found to be >90%.

Flow-Cytometric Analysis of Adhesion Molecule Expression

For analysis of surface molecule expression, approximately 10^5 cells were incubated in PBS. FcR blocking reagent was added first with 1:50 dilutions to avoid unspecific binding of antibodies. Subsequently cells were stained at 1:50 of antibodies for 30 min at 4 °C in the dark using PE-conjugated anti-CD34, anti-CD41 and anti-CD61 or IgG controls. After incubation cells were washed twice in PBS and analyzed using an Epics XL-MCL flow cytometer (Beckman Coulter, Krefeld, Germany). Data analysis was performed using System II Version 3.0 (Beckman Coulter).

Morphological Analysis of ex vivo Expanded CD34+ Cells

Three times 10^5 cells were diluted in 300 μ l PBS and coated on a microscope slide using cytopins at $800 \times g$ for 10 min. Subsequently cells on slides were fixed in 99% methanol for 2 min and stained consecutively for 10 s in DiffQuik Stain Solution I and for 10 s in DiffQuik Stain Solution II. Stained slides were washed in aqua bi.dest and assessed using light microscopy.

Homing Assay of Expanded CD34+ Cells and Whole Blood-Derived Platelets in Mice

Non-Obese Diabetic/Severe Combined Immunodeficiency (NOD/SCID) mice aged 6–12 weeks were obtained from Charles River Laboratories (Sulzfeld, Germany) and kept with food and water ad libitum in the Central Animal Facility of Frankfurt University Hospital. Animal experimentation was done under a protocol approved by the local animal welfare committee. Cells from cultures or aliquots from whole blood-derived platelet concentrates in additive solution were washed once by centrifugation and addition of PBS and kept at room temperature for up to 30 min. Cells were resuspended in 200 μ l PBS and injected i.v. into NOD/SCID mice. After 24 h, mice were sacrificed by cervical dislocation, and tissues were prepared and minced with a 100 μ m Cell Strainer (BD Pharmingen) as previously described [12–14]. Single cell suspensions were kept on ice and analyzed within the next 3 h by flow cytometry. 100,000 cells were acquired in pre-set gates for human CD61+ cells. For determination of absolute cell numbers in the target tissues, frequencies of homed cells were corrected by subtraction of events in non-transplanted mice and adjusted with correction factors for the fraction of the total tissue analyzed as described [12, 13].

Results

Proliferation of CD34+ in Culture with Different Cytokines

Various studies have previously investigated the potential of hematopoietic growth factors to generate megakaryocytic

Fig. 1. Amplification of total nucleated cells from CD34+ cells in the presence of different growth factors. Cells (2×10^4) were inoculated into media containing 20 ng/ml each of SCF, TPO (black squares) and different combinations of IL-3 (grey squares), FLT3L (white triangles), IL-3 + FLT3L (black circles) and SDF-1 (grey triangles). Total cell numbers were determined from aliquots of the culture. Values significantly different from media without IL-3 are denoted by asterisks; * = $p < 0.05$. Values and error bars represent means $\pm$ SEM; $n = 3$.

cells in culture. Our study focused on the role SCF and TPO as basic supplementation, and in addition IL-3, FLT3L and SDF-1. Analysis of total cell numbers in the cultures revealed a maximum 48-fold increase of total nucleated cells with a combination of SCF, TPO and IL-3 over the culture period of 16 days (fig. 1). The further addition of FLT3L did not increase total cell numbers until day 13. Under the other investigated conditions, the increase in cell numbers was limited to a change of cell numbers by a factor of 8–13 (fig. 1).

Expression of CD34 and Platelet Markers on Expanded Cells

To determine the induction of the differentiation process in these cultures, we evaluated expression of cell surface markers. Aliquots of cultivated cells were stained for CD34 and platelet markers CD41 and CD61 at different time points during 13 days of culture. Cell surface expression of CD34 decreased in all tested cytokine combinations from 85% to less than 8% (fig. 2). At the same time, expression of platelet markers increased and was highest in media containing SCF, TPO with or without IL-3, rising from 3 to 29–30% for CD41 and 24–28% for CD61 expression. In contrast, addition of FLT3L without IL-3 resulted in a much lower increase of cells with platelet markers, with only 8% for CD41 and to 7% for CD61 expression. Thus, media including SCF and TPO $\pm$ IL-3 showed the highest potential for ex vivo megakaryocytic differentiation.

Morphology of Differentiating CD34+ Cells

Endonucleation of cultivated cells was also observed during this expansion period. For this, cells were fixed on glass slides using cytopsins, and nuclear staining was performed by Pap-

Fig. 2. Flow-cytometric analysis of stem cell and megakaryocytic marker expression of CD34+ cultivated cells. Percentages of CD34, CD41 and CD61 expression of cell cultures was followed for 13 days in media with different cytokine combinations. Values and error bars represent means $\pm$ SD; $n = 3$.

penheim's stain. During the culture, platelet precursor cells emerged (fig. 3A). A ploidy analyses of CD61+ cells of this culture using propidium iodide showed cells containing polyploid DNA content (fig. 3B).

Circulation Behavior of Transfused Freshly Isolated and Cytokine-Expanded Cells in Mice

To analyze the circulation behavior of the ex vivo generated CD41+ and CD61+ cells, a proof-of principle transfusion experiment was performed. Ex vivo expanded CD34+ cells and, as positive control, mature platelets were transfused into non-preconditioned immunodeficient NOD/SCID mice. After 24 h cell suspensions were prepared from different tissues, and the frequency of homed cells was analyzed using antibodies against human CD61. The CD61+ cells were also gated for low forward scatter. Flow-cytometric detection of platelets from human whole blood-derived platelet concentrates included as positive control indicated presence in the circulation of the transfused platelets in blood, but also in the spleen, in the lungs and, to some degree, in bone marrow (fig. 4). CD61+ cells derived from cytokine-expanded cells were also detected in blood and bone marrow (fig. 4).

Fig. 3. Morphological analysis of differentiation. Freshly isolated CD34⁺ cells were cultivated in SCF and TPO. **A** At different intervals during the culture, cells were collected, spun on glass slides and stained. Bi- and multinuclear cells were obtained after 6 days in culture, fully developed platelet precursor cells were detectable after 9 days in culture; n = 6. Magnification, 400-fold. **B** Ploidy was measured flow-cytometrically by propidium iodide staining on CD61⁺ gated cells. Cell populations containing 2, 4 and 8 chromosomes are distinguishable.

Discussion

This study investigated the potential of the cytokines IL-3, FLT3L and SCF to support the growth- and differentiation-promoting effects of TPO and SCF on the ex vivo generation of megakaryocytic cells from human CD34⁺ PBPC. Moreover, the study shows that the culture-expanded megakaryocytic cells, derived from cultures grown in the most effective growth factor combination SCF + TPO + IL-3, circulate in blood upon injection into mice.

In our serum-free cytokine-expanded cultures, we obtained highest numbers of megakaryocytic cells using a growth factor combination of IL-3 in addition to TPO and SCF. This corresponds to earlier findings using SCF- and TPO-based growth factor combinations [9]. Interestingly, addition of FLT3L did not induce higher output of megakaryocytic cells until day 13 of culture. Moreover, cultures which were supplemented with FLT3L and which lacked IL-3 also did not prove more efficient in stimulating megakaryopoiesis than SCF and TPO alone. Compared with the results from

Williams et al. [7], the total nucleated cell expansion was superior in our IL-3-containing cultures compared with their multi-growth factor combination including SCF, TPO, IL-1, IL-6 and IL-11 [6]. All cultures, except for the low proliferating cultures supplemented with FLT3L and lacking IL-3, developed substantial megakaryopoietic differentiation, including the production of platelet-like CD41⁺ particles from megakaryocytes in the final stages. These cell particles were quantified using flow cytometry with human-specific CD41 and CD61 antibodies and were found to constitute up to one third of all cells. However, it is difficult to formally prove that these particles are indeed mature, functional platelets. In a first approach we analyzed the total cells from matured cultures using laminar flow chambers. We observed the formation of platelet aggregates when platelets from whole blood were flushed over fibrinogen-coated surfaces at 0.5 dyn/cm² (data not shown). This process was antagonized efficiently by preincubation of the platelets with an anti-GpIIb/IIIa antibody or prostaglandin E2. We failed to induce any visible aggregation from the culture-derived platelet-like particles, and

Fig. 4. Homing of culture expanded megakaryocytic cells after injection into NOD/SCID mice. 5×10^6 cells from cultures expanded in SCF, TPO and IL-3 from culture day 9, or 3×10^8 platelets from whole blood-derived platelet concentrates were intravenously injected into non-preirradiated NOD/SCID mice. **A** Flow-cytometric analysis of samples of mice that received mature platelets from a platelet concentrate or expanded cells are shown. Cells were gated after staining with human-specific anti-CD61+ antibody. **B** Quantitative evaluation of homed whole blood-derived platelets (closed circles), and SCF + TPO + IL-3 – expanded cells (open circles) in spleen, bone marrow lungs and blood 2 and 24h after application to the mice. Values are means + SD; n = 5–6.

only very few of the culture-derived particles were able to interact with a preformed cell aggregate. This points to a functional deficit of the culture-generated CD41+/CD61+ particles. It is possible that, in part, the non-aggregating particles are disintegrating parts of megakaryocyte cytoplasm in cell culture. Likely, the CD41+/CD61+ particles generated during megakaryocytic differentiation do not find the correct survival and developmental stimuli in the cytokine expansion cultures, which lack specific microenvironments for platelets such as endothelial or stromal cells and which also contain activated macrophages which could even eliminate nascent platelets. In addition, the freshly formed platelets may be-

come activated once they are generated by the surrounding cells and by soluble stimuli.

Since our expansion cultures contained up to 35% CD41+/CD61+ cells, we studied the circulation behavior of these culture-derived platelet-like and megakaryocytic cells after transfusion in mice. For identification of the transfused platelets, we selected human-specific antibody against GpIIa/IIIb, which has also been shown to be efficient in identifying human platelets in immune NOD/SCID mice in a recent study by Newman et al. [15]. We traced a frequency of around 1% of normal human platelets at 24 h after injecting approximately 7×10^7 platelets, which is roughly comparable to the

7% detected by Newman and colleagues after 200 µl of platelet-rich plasma which corresponds to a dose of $2-3 \times 10^8$ platelets per mouse. In contrast to previous studies, which looked at platelet levels on day 3 after transfusion as the earliest time point [16], we analyzed such cells 24 h after transfusion. At least at this time point, we observed that CD61+ cells (with forward scatter profile of platelets) localized preferentially to the bone marrow and spleen, but also circulated. Previous work has shown that expanded CD34+ HPC cells can contribute significantly to platelet formation in immunodeficient mice [4, 5, 8, 10, 16, 17]. These groups did however not investigate the homing behavior of their progenitors. Our study, analyzing all (so, mostly the mature) megakaryocytic cells in hematopoietic tissues, shows that these cells are found mainly in the bone marrow. Only few of these cells were detectable in the lungs [18]. Some groups also used cultured cells in addition to fresh cells, showing a synergistic effect: The contribution in the earlier phase after transfusion (around days 7–14) is associated with application of the expanded progenitors,

whereas at the later stage (around weeks 2 and 3) these are likely derived from the non-expanded progenitors [16], but again data on earlier time points have not been investigated or reported. Taken together it remains to be investigated which type of culture-expanded cells is capable to contribute to counteract bleeding and therefore would be most useful in the first days after transfusion.

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Disclosure

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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