Flow Cytometric Assessment of Haematopoietic Stem and Progenitor Cells

Durchflusszytometrische Charakterisierung hämatopoetischer Stamm- und Progenitorzellen

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Summary: The quantitative determination of CD34-expressing cells in peripheral blood, bone marrow or cord blood has become the generally accepted method for the assessment of the haematopoietic quality of autologous or allogeneic stem and progenitor cell grafts. At present, the rare cells (0.1 to 0.5 % of nucleated cells) are identified based on expression densities of the antigens CD34 and CD45 and light scatter characteristics in a complex gating strategy, which is still under debate. Single-platform counting allows the direct determination of absolute cell numbers by flow cytometry, based on lyse/no-wash procedures and calibration through reference beads or volumetric measurements. In addition to being associated with a number of pitfalls, the current assays do not answer important diagnostic questions in haematopoietic stem cell transplantation. First, the CD34+ antigen does not precisely identify cells responsible for either the early rise in platelets and neutrophils or the long-term stable engraftment following transplantation. Second, different subsets of CD34+ cells seem to represent better indicators for the early or late haematopoietic engraftment quality. Moreover, T-cell and dendritic cell populations seem to be independent determinants of immune reconstitution and the graft-versus-tumour response in allogeneic settings. The detection of minimal residual disease in stem cell grafts in the case of leukaemias and lymphomas still represents a technological challenge. Finally, in view of new applications of stem cell therapy and the upcoming new methods for the manipulation of grafts, a further development of the diagnostic methodology available is urgently needed and is on the horizon.

Keywords: CD34; haematopoietic stem and progenitor cells; stem cell transplantation; flow cytometry; absolute counting; single-platform counting.

Zusammenfassung: Die quantitative Zählung von CD34 exprimierenden Zellen in peripherem Blut, Knochenmark oder Nabelschnurblut ist zur allgemein akzeptierten Methode für die Beurteilung der hämatopoetischen Qualität von Stamm- und Progenitorzelltransplantaten geworden. Gegenwärtig werden die seltenen Zellen (0,1 bis 0,5 %) über die Expressionsdichten der Antigene CD34 und CD45 sowie Seitlichtstreuungseigenschaften in einer komplexen Strategie identifiziert. Trotz des routinediagnostischen Einsatzes bei der autologen oder allogenen hämatopoetischen Stammzelltransplantation ist die Methodik weiterhin umstritten. Ein-Gerät-Verfahren erlauben die direkte Bestimmung von absoluten Zellzahlen über waschfreie Lyse-Prozeduren und eine Kalibration über Referenzpartikel oder volumetrische Messung. Vor kurzem wurden die Verfahren international standardisiert, dennoch bleiben viele diagnostische und prognostische Fragen bei der hämatopoetischen Stammzelltransplantation weitgehend unbeantwortet. So identifiziert das CD34-Antigen weder genau diejenigen Zellen, die für den frühen Anstieg von Thrombozyten und Neutrophilen, noch die, die für die langzeitstabile Rekonstitution nach Transplantation verantwortlich sind. Des weiteren sind unterschiedliche Subpopulationen von CD34+-Zellen mit der hämatopoetischen Qualität von Knochenmarks- oder mobilisierten peripheren Stammzelltransplantaten assoziiert. Außerdem scheinen T-Lymphozyten und dendritische Zellen wichtige Determinanten der Immunrekonstitution und der *graft-versus-tumor* Antwort bei allogenen Transplantationen zu sein. Darüber hinaus stellt die Detektion von minimaler Resterkrankung in Transplantaten im Falle von Leukämien und Lymphomen eine technische Herausforderung dar. Aufgrund der zunehmenden klinischen Anwendungen der Stammzelltherapie und der Verfügbarkeit neuer Techniken der Transplantat-Manipulation ist eine Weiterentwicklung der diagnostischen Methodik dringend erforderlich.

Schlüsselwörter: CD34; hämatopoetische Stamm- und Progenitorzellen; Stammzelltransplantation; Durchflusszytometrie; Absolutzellzählung; single-platform counting.

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History of haematopoietic stem cell therapy

The analysis of the pathobiology of the irradiation response in animal models in the early 1950s led to the observation that toxicity to the haematopoietic organs was dose limiting. By the injection of bone marrow, irradiation injury could be reduced in mice and guinea pigs [1]. More than 10 years later, allogeneic bone marrow transplantation in humans was first introduced as a potential cure for inborn disorders of the haematopoietic system, such as congenital immune deficiencies [2, 3]. It was not until 1978 that the transplantation of cryopreserved autologous bone marrow was detected to hasten haematopoietic recovery in man following high-dose chemotherapy. This ultimately led to the concept of intensified (high-dose) chemotherapy with autologous stem cell support [4].

While initially bone marrow served as the major source of haematopoietic stem cells, after a first report on the successful transplantation of autologous, granulocyte colony stimulating factor (G-CSF)-mobilized peripheral blood stem cells in 1990 [5], peripheral blood replaced bone marrow as the preferred source of autologous stem cells [6]. Subsequently, haematopoietic stem and progenitor cells mobilized by G-CSF or a combination of chemotherapy and growth factors, also have been used for allogeneic transplantation [7]. As compared to bone marrow, peripheral blood transplants lead to faster haematopoietic recovery. In the allogeneic setting, the infusion of much larger numbers of donor lymphocytes may well be a double-edged sword. While the faster immunological recovery may increase graft-versus-tumour effects, a concomitant increase in chronic graft-versus-host disease has been reported (reviewed in [6, 8]). Therefore, the isolation of pure populations of stem cells has been suggested as an improvement of peripheral blood leukapheresis, which may both reduce graft-versus-host disease in allogeneic transplantation as well as the tumour load in autologous transplantation.

In the era of bone marrow as the source of haematopoietic stem and progenitor cells, the amount of cells transplanted in the autologous or allogeneic setting was based on the number of mononuclear cells infused per kilogram body weight. The higher heterogeneity of mobilized peripheral blood grafts, however, led to the need to find a diagnostic correlate of the haematopoietic quality of these stem and progenitor cell preparations, both as an estimate of the number of stem and progenitor cells to be transplanted and as a means to monitor the timing of apheresis. This proved to be particularly important in the often-delayed mobilization kinetics in pretreated patients or those with lymphomas.

Clinical indicators of the haematopoietic quality of stem and progenitor cell preparations

Three clinical end points can be used to describe the dose-limited effects of the transplantation of autologous haematopoietic stem and progenitor cells [9]:

- The early engraftment quality typically is estimated based on the time to the rise of peripheral blood neutrophil granulocyte concentrations to $0.5 \times 10^9/\text{L}$ and the rise of platelet concentrations to $50 \times 10^9/\text{L}$, also manifested as independency of platelet transfusions
- The primary stable haematopoietic repopulation is defined as the independency of platelet or erythrocyte transfusion within 3 months after transplantation as well as the rise of leukocytes to more than $3.0 \times 10^9/\text{L}$ within the same time period
- The secondary transplant deficiency is defined as a decrease of peripheral blood cells to subnormal values for at least one lineage and at least 1 month following initial full function of the transplant (haemoglobin $>7 \text{ mmol/L}$, leukocytes $>4.0 \times 10^9/\text{L}$, platelets $>100 \times 10^9/\text{L}$)

Analytical assessment of the haematopoietic potential of stem and progenitor cell preparations

Different analytical approaches have been used to assess the quality of stem cell preparations with regard to the above-described end points. Reconstitution in animal models such as immunodeficient mice represents the gold standard. Stem cell quality in these models is assessed as the multilineage long-term reconstitution in competitive repopulation assays, restoration of haematopoiesis in irradiated animals, or the formation of day 12 colony-forming unit (CFU) spleen.

The classical assays used to predict the reconstitution and the haematopoietic potential, including culture-dependent *in vitro* models such as the initiation of long-term haematopoiesis on irradiated stroma cells (long-term culture initiating cells or cobblestone area-forming cell assays), the formation of myeloid multilineage colonies, and the formation of lineage-specific colonies (e.g. colony-forming unit granulocyte/macrophage, CFU-GM), are only of a limited analytical value as they are only able to assess later differentiation stages of progenitor cells. At the same time, it is unclear whether these laborious assays such as the CFU-GM assay, which are often used for the routine assessment of the quality of therapeutic stem cell preparations, add any clinically important information to the flow cytometric quantification of stem and progenitor cells ([10]; reviewed in [6]). Owing to the extended incubation and culture time required, these assays, furthermore, cannot be used for the assessment of the apheresis time point in the framework of a peripheral blood mobilization scheme.

Until the identification of the sialomucin CD34 as a determinant of the haematopoietic stem and progenitor cell fraction, different approaches, such as the flow cytometric analysis of cells with a low staining intensity for the mitochondrial membrane potential stain Rhodamine 123 or the determination of cells with a lack of expression of lineage-specific antigens for T cells, B cells, NK cells, monocytes, neutrophils or erythroid cells, have been followed. The CD34 antigen has been the best-known marker of haematopoietic stem cells since the successful haematopoietic reconstitution in baboons with CD34+ selected bone marrow cells was described in 1988 ([11]; reviewed in [12]). Using peripheral blood stem and progenitor cell preparations, transplantation of at least 2.0 to $2.5 \times 10^6/\text{kg}$ autologous CD34+ cells will almost invariably lead to an early engraftment of neutrophils as defined above within 9 to 11 days (reviewed in [6]). Decreased amounts of 1.0 to $2.5 \times 10^6/\text{kg}$ CD34+ cells or increased amounts of $>5.0 \times 10^6/\text{kg}$ CD34+ cells primarily affect the kinetics of platelet recovery without a significant effect on the quality of neutrophil recovery. For allogeneic transplantation, usually higher concentrations of $>4.0 \times 10^6/\text{kg}$ CD34+ cells are required and recommended.

Biology of the CD34 antigen

In its cellular expression characteristics, the CD34 antigen, a highly O-glycosylated type 1 transmembrane protein, does not show specificity for early lymphohematopoietic progenitor cells but is also expressed on endothelial cells within small vessels, embryonic fibroblasts and selected single cells in foetal or adult neural tissue as well as vascular tumours, e.g. Kaposi sarcoma [13, 14]. It is speculated that CD34 has a function in cell-cell adhesion and the inhibition of haematopoietic differentiation. CD34(−/−) mice, however, show normal development and are characterized by a reduction in CFUs, which is of uncertain relevance, and a normal radiation sensitivity. On a molecular level, L selectin has been defined as a ligand for CD34, which is expressed by high-venule endothelial cells. Of note, L selectin does not bind to haematopoietic cells expressing the CD34 antigen, suggesting different conformational expression.

Principles of the flow cytometric analysis of CD34+ cells

In flow cytometry, the quantitation of CD34+ cells was technically difficult and highly irreproducible until recently improved assays became available, which are currently standardized. This is due to the complexity of determining a rare cell fraction, which only comprises 0.1 to 0.5 % of the nucleated cells at the decision point for apheresis in the mobilization of peripheral blood [15–18]. According to current consensus, CD34+ cells

are identified in a complex gating strategy as cells with a dim expression of the pan-leukocyte antigen CD45 and side and forward light scatter characteristics similar to lymphocytes (Fig. 1). Typically, a bright fluorochrome such as R-phycoerythrin and CD34 antibodies that show a broad reactivity independent of the carbohydrate moieties or terminal sialic acids of the antigen (class II or class III) are used for the detection of CD34+ cells.

Initially CD34+ counts were determined in peripheral blood samples or apheresis products in a so-called 'dual-platform strategy', in which the percentage of CD34+ cells among leukocytes was determined by flow cytometry, while the absolute counts were calculated

Figure 1 Flow cytometric characterization of CD34+ cells. Analysis of stabilized cord blood as a reference sample. Identification of CD34+ cells with low side scatter and forward scatter similar to lymphocytes (a) and high expression of CD34 (b). Backgrounding on CD45 reveals low expression similar to neutrophils (c). An analysis gate on CD34+ cells and lymphocytes in scatter (d) is used for the analysis of CD34+ subpopulations. The CD34-lymphocyte population and CD34+ cells show similar background fluorescence in fluorescence 2 (e). AC133 (CD133) selectively stains a subpopulation of CD34+ cells.

using the total counts derived from a haematology analyser. This strategy typically included the washing of samples after staining and erythrocyte lysis. The combination of these two procedures led to selective cell losses during sample preparation. Further differences resulted from the erroneous counting of normoblasts and nonviable cells by the two technologies, which led to a large analytical error. Single-platform counting, in contrast, is a technology that allows direct determination of absolute counts based on either the simultaneous counting of beads, which are added as calibrators to the sample during preparation, or alternatively, the use of flow cytometers with a volumetric calibration [19]. Only few instruments with such calibration are, however, currently available. Sample preparation is minimized in single-platform counting, as only erythrocyte lysis is performed after incubation with the monoclonal antibodies without any washing before analysis. This contributes to a smaller analytical error in the direct determination of e.g. CD4 or CD34 counts [20, 21]. Introduction of bead-based single-platform counting with a standardized assay and targeted training of laboratory performance has also strongly improved the intra- and interlaboratory comparability of results in multicentre studies [22]. In contrast to the conventional consensus approach, which is still used in many external quality-control schemes, stabilized test material produced from spiked samples has been introduced as a control in these multicentre studies. Specific problems of these recently introduced bead-based single-platform technologies, however, have only recently been detected. Thus, dilution of samples, e.g. in the case of leukapheresis products, with a concentrated nucleated cell count, may reduce the protein concentration of the sample to a critical value, which leads to clumping of the counting beads, so that erroneously high numbers of cells are calculated [23]. This common problem can be avoided by supplementation of a sufficient protein concentration in the dilution buffer. Despite these recent improvements in CD34+ counting, however, a number of problems still remain unresolved. Thus, CD34 counts in apheresis products are typically determined before freezing and thawing. Samples prepared for transplantation may differ significantly in the pre- and post-freezing counts of viable CD34+ cells due to differences in the cryopreservation procedure or cryoprotectants used. To differentiate viable cells from nonviable CD34+ cells, nonviable cells can be counterstained and simply detected by flow cytometry. An important improvement for further transplantation strategies, the correlation of CD34+ cell counts in the product prepared for transplantation with the clinical end points described above, should be addressed systematically.

Supportive laboratory diagnostics in the context of stem cell transplantation

A number of further analytical questions need to be addressed in cellular diagnostics in the context of the mobilization of stem cells for apheresis, the analytical characterization of the apheresis product, as well as the monitoring of the post-transplantation process. The lack of the routine availability of rapid and user-independent nonflow cytometric methods for the determination of CD34+ cells has led to the search for surrogate parameters, which may allow the prediction of the mobilization of these cells in the peripheral blood. However, none of the technologies developed on the currently available haematology analysers, including the determination of immature cell fractions based on scatter or diffraction with or without cytochemistry, has been shown to be superior to the determination of total white blood cell counts as a surrogate marker of CD34+ counts in peripheral blood during the mobilization process.

In the characterization of the apheresis product, the determination of minimal residual disease by flow cytometry seems to be a clinically interesting approach with the potential to discriminate patients who seem to benefit from a modified treatment [24]. Particularly in the case of acute myeloid leukaemia, the procedure is technically difficult to standardize and, therefore, no controlled studies with relevance to the clinical outcome are available yet. Similarly, the analysis of telomere length dynamics in peripheral blood has been shown to be a useful parameter for the characterization of stem cell-associated disorders such as aplastic anaemia [25]. The same assays, therefore, could also be employed as predictive parameters of *in vitro* expanded CD34 cells with an impaired haematopoietic quality.

Currently, no reliable parameters are available to indicate engraftment in the early post-transplantation phase. The flow cytometric determination of reticulated platelets seems to represent the earliest parameter of engraftment and may thus be systematically evaluated to monitor the post-transplantation phase [26]. This technique has the potential to improve the prediction of platelet kinetics, which besides its application in engraftment monitoring should allow a more directed therapy with platelet concentrates.

In the case of allogeneic transplantation, further analytical questions arise in the context of the immunological compatibility of the graft, the prediction and monitoring of the immunological reconstitution, and graft-versus-host disease following transplantation. In contrast to classical HLA typing for analysis of immunological compatibility, flow cytometry can be used as a more sensitive method for the analysis of tissue compatibility, also allowing the detection of antibodies that do not activate complement [27]. In several protocols, T cell and NK-cell counts are determined and selectively reduced in allogeneic apheresis products in order to adjust the kinetics of immune reconstitution correlated

with viral infections, but also with graft-versus-leukaemia and graft-versus-host effects. Some of these protocols are based on the independent administration of T cells (minitransplantation) to allow graft-versus-leukaemia effects but to reduce unwanted graft-versus-host effects. Moreover, the content of dendritic cell fractions of either myeloid or lymphoid origin in different types of transplants has been suggested to correlate with the proinflammatory potential of these grafts and may thus influence graft-versus-leukaemia and graft-versus-host reactions [28]. As a means of monitoring the risk of infectious complications, the HLA-tetramer-based enumeration of HLA-restricted, cytomegalovirus-specific CD8⁺ T cells in the grafts and monitoring of these T cells after stem cell transplantation has been suggested as a rapid and sensitive tool to identify patients at risk of developing cytomegalovirus disease [29]. Similarly, HLA-tetramer technology has been used to determine minor histocompatibility antigen-specific cytotoxic T lymphocytes in patients with graft-versus-host disease [30]. Furthermore, the cytokine-based determination of alloreactive T cells should allow broadening this approach [31].

Analytical assessment of haematopoietic stem and progenitor cells beyond CD34

Differences in the engraftment kinetics and quality of transplants derived from bone marrow, mobilized peripheral blood and cord blood have stimulated the search for cells that may more precisely correlate with the early and late transplantation success than total CD34⁺ cell counts. In this context, subpopulations of CD34⁺ cells, which express antigens correlated with myeloid differentiation such as CD33, megakaryocytic differentiation such as CD41, and adhesion antigens such as CD62L, have been shown to be better predictors of the early recovery of neutrophils and platelets than the total CD34 count [9, 32]. Transplantation studies in animal models continuously allow the identification of further human haematopoietic cell populations with selective early engraftment characteristics [33]. The analysis of these subpopulations, however, represents a major technical problem regarding the standardization of the determination of the fraction of positive cells in the presence of a continuum of expression densities, such as in the case of CD38. Despite the reduction of adhesion in the presence of EDTA [34], the nonspecific adhesion of platelets leads to analytical problems. Recently, cellular standards have been introduced that will allow a more controlled quantitation of antigen coexpression in future studies [35].

Similarly, a number of antigens such as c-mpl, CD90, CD133 and vascular endothelial growth factor receptor-2 (KDR) have been suggested to allow the determination of the subfraction of CD34⁺ cells representing the early haematopoietic cells responsible for the quality of long-term engraftment [36–39]. Until now,

however, none of these subsets has been unequivocally shown to be a predictor of engraftment in a clinical setting.

A major controversy finally exists regarding the significance of CD34⁺ stem cells both in mice and in men (reviewed in [12]). Thus, *Osawa et al.* reported that only the CD34⁺ fraction of bone marrow cells of normal adult mice are capable of inducing long-term haematopoietic reconstitution [40]. Similarly, *Goodell et al.* observed that side population cells, which are defined by two-colour flow cytometry with Hoechst 33342 staining, are highly enriched for haematopoietic stem cells which are negative or only weakly positive for CD34 [41]. Since in mobilized murine blood, in contrast to bone marrow, stem cells seem to reside in the CD34⁺ expressing fraction [42], it is conceivable, that the expression of CD34 may reflect the activation and/or the kinetic state of stem cells in a reversible manner [43]. However, the observation of an increased rate of secondary bone marrow failure after transplantation with CD34⁺ enriched peripheral blood preparations devoid of the CD34⁺ fraction indicates a selective loss of stem cells with long-term engraftment capability by these procedures and emphasizes the clinical relevance of the CD34⁺ fraction for haematopoietic reconstitution. As a novel means to better characterize the CD34⁺ fraction, expression of the ABC transporter breast cancer resistance protein, or ABCG2, was identified as the molecular determinant of the side population in a variety of species [44]; ABCG2, therefore, represents a candidate for the pluripotent selection of stem cells from adults.

Outlook

The analysis of CD34⁺ cells currently still represents the gold standard for the assessment of the timing of apheresis and the amount of stem and progenitor cells transplanted. Recently, pluripotent stem cells have been reported to be present in several organs including foetal haematopoietic tissue such as liver and spleen but also in the myocardium. Conversely, pluripotent stem cells have also been described in haematopoietic tissues giving rise to differentiated cell types of several organs and tissues. The true plasticity of these stem cells, however, still awaits independent confirmation [45, 46]. In consequence, peripheral blood or bone marrow progenitor cell transplantation has been suggested as a generally applicable strategy to treat systemic diseases involving nonhaematopoietic tissues [47]. Despite these restraints, the recent detection of stem cells with pluripotent characteristics and multiorgan potential in haematopoietic tissues will promote the increasing role of the analysis of these cells in the near future.

Finally, the precise flow cytometric analysis of stem and lineage-committed progenitor cells may also attain diagnostic applications outside of the classic field of haematopoietic stem cell transplantation. Since the low

quantitative precision of the determination of endothelial progenitor cells in colony-forming assays recently allowed establishing the number of these cells as a surrogate biological marker for insufficient endothelial repair and increased cardiovascular risk [48], the advantage of quantitative and multiparameter flow cytometric assays is likely to provide a rapid and reproducible assay system to monitor general tissue repair and regeneration processes.

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