

REVIEW

CD34⁺ or CD34⁻: which is the more primitive?

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Remarkable progress has been achieved in the characterization and isolation of primitive hematopoietic stem cells (HSC). HSC represent a very small subset of hematopoietic cells and provide self-renewal, possess differentiation capacity and allow a constant supply of the entire hematopoietic cell spectrum. Until recently, CD34 has been used as a convenient marker for HSC, since CD34⁺ cells have been shown to possess colony-forming potential in short-term assays, maintain long-term colony-forming potential in *in vitro* cultures and allow the expression and differentiation of blood cells from different hematopoietic lineages in *in vivo* models. Clinical and experimental protocols have targeted CD34⁺ cells enriched by a variety of selection models and have readily used these for transplantation, purging and gene therapies and targets for future organ replacement. Recent studies in murine and human models, however, have indicated that CD34⁻ HSC exist as well, which possess engraftment potential and distinct HSC characteristics. These studies challenge the dogma that HSC are uniformly found in the CD34⁺ subset, and question whether primitive HSC are CD34⁺ or CD34⁻. In this review, results on murine and human CD34⁺ and CD34⁻ HSC, differences between them and their possible interactions are examined.

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Introduction

Among billions of bone marrow (BM) cells a small fraction of primitive hematopoietic stem cells (HSC) exists that provide self-renewal, differentiation capacity and a constant supply of the whole cell spectrum of all hematopoietic lineages throughout our life. HSC are an ideal target for autologous and allogeneic transplantation, purging strategies, gene transfer and – with recent results on the plasticity of HSC – for organ replacement therapies. The understanding of mechanisms underlying their extensive proliferative capacity, their multilineage differentiation and self-renewal and their further characterization are keenly pursued. Since specific markers of the unique functional properties of HSC have not yet been identified, their detection and enumeration require the use of retrospective assays.

The cell surface sialomucin-like adhesion molecule CD34 has been used as a convenient marker for HSC.^{1–3} The CD34 antigen is expressed on 1–5% of mononuclear BM cells, on a subpopulation of hematopoietic cells, both HSC and early committed progenitors.³ CD34⁺ cells have been shown to possess colony-forming potential in short-term assays,¹ maintain long-term colony forming potential in *in vitro* cultures⁴ and allow the differentiation of blood cell lineages in immunocompromised mice.⁵

Apart from cell surface markers for isolation of HSC,

numerous other assays that measure the stem cell activity have been exploited.^{6–25} Clonal analyses using limiting dilution assays in Dexter-like cultures have been used (quality analysis), however, these are time-consuming due to the number of flasks that have to be analyzed.^{26,27} In the past, HSC have been characterized with respect to their ability to form spleen colonies in irradiated mice (CFU-S). Within various CFU-S types, most primitive HSC show marrow repopulating ability (MRA), resistance to 5-FU, low retention of the supravital fluorochrome rhodamine-123 (Rh-123) and are defined as pre-CFU-S.^{26,28} HSC have also been isolated by means of stroma-dependent long-term bone marrow cultures (LTBMC), either by weekly assessment of cobblestone area-forming cells (CAFC)^{26,29,30} or clonogenic cell output (determined by assessment of 5–8-week-old cultures; LTC-IC).³¹ In these frequency analyses, early CAFC were found to be highly sensitive to 5-FU, whereas week 5–8 CAFC were observed in the CD34⁺ Rh-123^{dull} HLA-DR^{low} fraction.^{26,29,30}

More recently, *in vivo* long-term marrow repopulating assays have been exploited in which the capacity of cells to reconstitute the hematopoietic system of host animals are measured. Of these, the sheep *in utero* transplantation system is used, in which human HSC are transferred into the sheep fetus *in utero* and are followed for the presence or absence of human blood cells after the sheep is born.³² Nevertheless, the human sheep xenograft model has limitations: it is not readily accessible, laborious, restricted to few institutions and therefore has not been useful clinically. A popular alternative for investigating the phenotype of human HSC is the nonobese diabetic mice with severe combined immunodeficiency disease (NOD/SCID) assay.³³ This model is more accessible and allows detection of myeloid and lymphoid progeny within 6 weeks and at high efficiency. Limiting dilution analyses in this model have shown that the human cell engraftment is quantitative, independent of exogenous cytokine administration, and attributable almost exclusively to the CD38⁻ subset of CD34⁺ cells.^{5,34,35} Nevertheless, CD34⁻ human HSC capable of repopulating NOD/SCID mice^{34,36,37} and fetal sheep³⁸ have also been detected.

Historical data and results from CD34 positive hematopoietic stem cell research

From early experimental results it became apparent that BM can be used to rescue lethally irradiated mice, and that BM-derived HSC form splenic nodules in irradiated recipients and allow hematopoietic reconstitution after radiation-induced hematopoietic failures.^{27,39}

HSC were later discovered to represent only a fraction of hematopoietic cells of BM, mobilized peripheral blood (mPB) or umbilical cord blood (CB). Initial characterization of HSC was done on the basis of Rh-123 exclusion^{26,27} or lectin affinity and showed that CFU-S-8, CFU-S-12 and marrow repopul-

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ating cells or pre-CFU-S were physically largely separable.^{26–29} In addition, HSC were characterized using their expression of specific surface markers, such as in the mouse with the complete absence of hematopoietic lineage markers, the expression of the stem cell antigen (Sca-1) and low expression of Thy-1.^{6–13}

After the successful hematopoietic reconstitution of baboons with selected CD34⁺ BM, CD34⁺ cells became the hallmark of murine and human HSC.⁴⁰ Donnelly *et al*⁴¹ supported this, demonstrating that murine CD34⁺ long-term repopulating cells (LRC) are more than 100 times more abundant than CD34⁻ LRC, and that CD34⁺ cells, not CD34⁻ LRC could be maintained in suspension culture. Although Lin⁻CD34⁺ and Lin⁻CD34⁻ cells contained LRC, he postulated that both constitute two functionally distinct populations, where in competitive repopulation experiments Lin⁻CD34⁺ cells could provide both short- and long-term engraftment, whereas Lin⁻CD34⁻ cells were only capable of long-term engraftment.⁴¹

Human HSC are currently defined as Lin⁻CD34⁺DR⁻ based on results of LTC-IC and various other assays.^{42–46} With use of the human fetal sheep xenogenic transplantation model, CD34⁺, Lin⁻CD34⁺CD38⁻ and CD34⁺Thy1⁺Rh123^{low} cells of BM and fetal liver (FL) have been shown to establish multilineage hematopoietic engraftment and can be transferred to secondary and tertiary recipients.^{5,24,42,47} Directly comparing CD34⁺ and CD34⁻ cells, Gao *et al* have demonstrated that human CD34⁺ rather than CD34⁻ cells provide the major contribution to hematopoietic engraftment in NOD/SCID mice (Table 1).⁴⁸

CD34 negative hematopoietic stem cells

Recent data reveal the presence of highly purified Lin⁻CD34⁻ subpopulations and suggest the absence of long-term reconstitution potential in the CD34⁺ fraction. This contrasts with former results on CD34⁺ HSC, the use of CD34 as a marker for HSC and the long-term effects of CD34⁺ selection in human transplant settings.^{49–53}

Recently, selection of cells based on the rapid efflux of the fluorescent DNA-binding dye Hoechst 33342 (HO) has allowed the identification of a distinct cell population with long-term repopulating activity termed side-population (SP).^{53,54} The SP phenotype is due to the selective expression of ABC-G2/BCRP on HSC, which like the MDR/P-glycoprotein is a verapamil-sensitive member of the ABC transporter family. SP cells have long-term repopulating activity in mice, and largely lack CD34 expression.^{53,54} In an attempt to characterize SP cells further, we and others found that human SP cells are mostly CD34⁻ and CD38⁻, as well as negative for CD3, CD14, glycoporphin A, AC133 or CD90. In CFU assays, we did not observe highly increased colony-forming potential.⁵⁵ In this context, Liu and Verfaillie⁵⁶ has reported that CB SP cells are depleted of colony-forming cells (CFC) as compared with CD34⁺ non-SP cells. Of interest is that with further subfractioning, CB CD34⁺ SP cells have been shown to have a high clonogenic potential similar to that of CB Lin⁻ cells, whereas Lin⁻CD34⁻ SP cells are nearly depleted of a CFU potential.⁵⁷ It has also been demonstrated that common lymphocyte progenitors are present within the CB SP population.⁵⁷ These may dilute the CFC in the human SP population, thereby leading

Table 1 Selected results on CD34⁺ and CD34⁻ HSC

Author	Species	Cells	Results on stem cell features	Ref.
Berenson	Baboon	BM CD34 ⁺	Hematopoietic reconstitution in baboons	40
Morel	Murine	BM CD34 ⁺	Long-term marrow repopulation (LTR) in mice	12
Morel	Murine	BM Thy-1 ^{low} Lin ⁻ low Sca-1 ⁺	LTR cells are equally distributed between CD34 ⁺ and CD34 ⁻ subsets	61
Donnelly	Murine	BM CD34 ⁺ Lin ⁻ , CD34 ⁻ Lin ⁻	Both contain LTR, in CD34 ⁺ Lin ⁻ 100x increased	41
Osawa	Murine	BM CD34 ^{low} Lin ⁻ Sca ⁺	Single CD34 ^{low} Lin ⁻ Sca ⁺ cells engraft in 20% recipient mice	60
Goodell	Murine	BM SP	SP cells are CD34 ^{low/-} and become CD34 ⁺ after stromal culture	53
Sato	Murine	BM Lin ⁻ c-kit ⁺ Sca-1 ⁺ CD34 ⁻	5-FU results in activated CD34 ⁺ cells; reversion of CD34 ⁺ to CD34 ⁻ is possible	62
DiGiusto	Human	BM CD34 ⁺	Early progenitors are found within the CD34 ⁺ population	42
Civin	Human	BM CD34 ⁺	Highly purified CD34 ⁺ reconstitute hematopoiesis	24
Bhatia	Human	CB CD34 ⁺ CD38 ⁻	Repopulation capacity in NOD/SCID	5
Hogan	Human	CB CD34 ⁺	Engraftment into NOD/LtSz-scid/scid mice	44
Sutherland	Human	BM CD34 ⁺ Thy-1 ⁺ Lin ⁻	Regeneration of multilineage hematopoiesis in fetal sheep model	47
Bhatia	Human	CB CD34 ⁻ Lin ⁻	Engraftment in mice	36
Gallacher	Human	CB CD34 ⁻ CD133 ⁺ CD7 ⁻	Engraftment in NOD/SCID	14
Zanjani	Human	BM CD34 ⁻ T ⁻	Engraftment in fetal sheep	38
Gao	Human	CB, BM, PB CD34 ⁺ vs CD34 ⁻	CD34 ⁻ cells provide only minor contribution to hematopoietic engraftment	48

to the low CFU readout. The most likely reason for the low CFU potential, however, is the immature SP phenotype, which does not allow these cells to grow in methylcellulose without prior differentiation in short-term suspension culture. Suspension culture leads to the maturation of less primitive progenitors,⁵⁴ and with day 5 and day 7 expanded cells to an increased CFU potential.^{36,55} For LTC-IC, we observed the highest enrichment with apheresis (AP) specimens compared with CB or BM SP cells,⁵⁵ in line with the finding that the LTC-IC potential of unsorted AP cells is higher as compared with CB or BM.⁵⁸ Storms *et al*⁵⁷ have also reported that the CD34⁺ SP fraction in CB was highly enriched for LTC-IC progenitors as compared with unsorted cells, whereas the CD34⁻ SP population contained only a low proportion of LTC-IC. Of interest, FL SP cells have recently been shown to include all transplantable human HSC activity detectable in NOD/SCID mice. The HSC activity was confined to the CD34⁺CD38⁻SP⁺ population.⁵⁹ These data suggest that the property of HSC within human SP cells, at least when assayed by LTC-IC and the NOD/SCID assays, is mostly confined to the CD34⁺ SP fraction, nevertheless does not exclude CD34⁻ cells as candidate HSC.

For murine cells, the most direct evidence of CD34⁻ HSC came from a study by Osawa *et al*,⁶⁰ who reported that single CD34⁻c-kit⁺Sca-1⁺Lin⁻ BM cells can reconstitute the lymphohematopoietic system for at least 10 months in 21% of lethally irradiated mice, whereas the majority of CFCs, CFU-S and progenitors, able to provide early but unsustainable multilineage engraftment, were recovered among the CD34⁺ subset. Thus, while CD34⁻ HSC were unable to provide short-term engraftment, they sustained long-term multilineage engraftment. To reconcile this observation, Morel *et al*⁶¹ separated murine Thy-1^{low}Lin^{-/low}Sca-1⁺ (TLS) into CD34⁺ and CD34⁻ HSC and demonstrated that CD34⁺ TLS are highly enriched for CFC, CFU-S, cobblestone area forming cells (CAFC) and radioprotective cells and contain half of the CRU in TLS marrow. CD34⁻ TLS – although depleted of progenitor cells – were highly enriched in HSC with competitive long-term repopulating potential. Although mice transplanted with CD34⁺ TLS recovered hematopoiesis faster compared with CD34⁻ TLS, both populations were capable of sustaining long-term multilineage hematopoiesis for at least 6 months in primary and 5 months in secondary recipients.⁶¹

The issue supporting the existence of long-term repopulating cells only in the CD34⁻ cell fraction, as opposed to long-term repopulating cells in both CD34⁺ and CD34⁻ fractions,^{12,14,41,61} has become even more complex with initial reports on murine CD34⁻ HSC that could be induced to express CD34 and increase their engraftment capacities following exposure to 5-FU or cytokines.^{62,63} Sato *et al*⁶² found that long-term engraftment cells reside in the CD34⁻ population. He hypothesized that murine CD34⁻ HSC exist in an extremely quiescent state, and that activation is required to cause an induction of cells, thereby making the latter more readily able to engraft in mice (Table 1).

For human cells, Zanjani *et al*⁶⁸ demonstrated *in vivo* engraftment potential of human CD34⁻Lin⁻ BM cells in fetal sheep in primary and secondary recipients, and Bathia *et al*⁶⁶ showed engraftment of human CB CD34⁻Lin⁻ cells in NOD/SCID mice and the appearance of CD34⁺ cells in animals transplanted with CD34⁻ cells, suggesting the latter to be more primitive than the former. Dick's group thereby showed that CD34⁻ cells have minimal clonogenic and long-term culture initiating cell activity, but possess *in vivo* repopulating activity in NOD/SCID mice, and that CD34⁻ SCID repopulat-

ing cells (SRC) expand in suspension culture, whereas CD34⁺ SRC may expand but lose their repopulating ability.^{5,37} Moreover, CB Lin⁻CD34⁻ cells have been shown to generate CD34⁺ HSC when cultured in the presence of a murine BM stroma cell line which suggest that Lin⁻CD34⁻ cells possess extensive potential for the generation of CD34⁺ HSC *in vitro* and that these CD34⁺ cells produce CFU, LTC-IC and SRC with multilineage differentiation potential, all of which are characteristic features of HSC.⁶⁵

Potential reasons for diverse results on CD34⁻ and CD34⁺ hematopoietic stem cells

In contrast to primitive human BM HSC which are predominantly CD38^{-/low}, long-term reconstituting murine stem cells express high levels of CD38, which suggests differences of human and murine cells in the expression of cell surface markers. Also, the coexpression of subpopulation with other markers, such as Sca-1 and c-kit,^{61–64} as compared with Lin⁻CD34⁺/CD34⁻ cells does not seem to identify the same long-term repopulating capability cell population.^{13,41,48,64,65} Early discrepancies with respect to results on CD34⁺ and CD34⁻ cells may also be due to the low but detectable levels of CD34 which can cause overlap in purified subsets.^{12,14,41,60–68}

Differences in the sensitivity of the *in vivo* engraftment assays may also be related to the lack of administration of accessory cells, administration of cytokines to the transplanted mice, experimental manipulation of the HSC cell preparation and/or myeloablative treatment of mice.^{36,38–41,60–63} In addition, differences in the long-term repopulation ability of CD34⁺ and CD34⁻ cells may result from antibody staining irregularities so that clear guidelines for analyzing CD34⁻ vs CD34⁺ cells seem appropriate.^{36,38–41,60–63}

Moreover, if Lin⁻CD34⁻ cells are not isolated with utmost care and contain one single or very few CD34⁺ cells, the latter may account for the HSC activity within the CD34⁻ population. These variations underscore the necessity that further work is needed to characterize murine and human HSC, and to most ideally find a marker for positive selection of CD34⁻ HSC.

Discussion and conclusions

Although evidence that CD34⁻ cells represent HSC still appears immature, xenogenic human sheep *in utero* transplantation,³⁸ murine studies by Osawa *et al*,⁶⁰ demonstration of CD34⁻ HSC in NOD/SCID^{36,61,62} and development of CD34⁺ cells with culture of initially CD34⁻ HSC,^{62–64,66} suggest that HSC activity exists within the murine and human Lin⁻CD34⁻ population. This redirects us to consider which cell population to select. If HSC exist within the CD34⁻ population, the enrichment of CD34⁺ cells – current practice in stem cell transplantation and to date one of the most efficient and effective cell separation methods – may be of concern, since this may result in a loss of at least a portion of HSC. On the other hand, thousands of successful autologous and allogeneic transplantations with use of CD34⁺ selected cells makes the use of CD34⁺ HSC still valid.

One reason among others of the apparent CD34⁺ vs CD34⁻ controversies – at least in the murine system – seems related to their activation and/or cycling state.^{65–71} Accepting recent work that indicates that CD34⁻ HSC indeed exist,^{36–38,53,54}

these seem likely to be quiescent, convert to CD34⁺ phenotype upon activation by 5-FU or after culture, and after transplantation, revert to a CD34⁻ phenotype again.^{62–67} Nevertheless, since murine studies on CD34⁻ and CD34⁺ HSC suggest that both are freely interconvertible,⁶² and if this applies to human HSC also, the CD34⁺ selection may be appropriate to distinguish potent HSC from quiescent stem cells or mesenchymal precursors. Thus, if the human CD34 expression pattern mirrors that in the murine system, quiescent CD34⁻ HSC should be found in the BM which will, as their CD34⁺ counterpart, be activated with regard to proliferation and differentiation. Recent results on G₀ and G₁ CB CD34⁺ cells have revealed a more than 1000-fold difference in GM-CFC, 250-fold higher number of BFU-E and 600-fold higher expansion potential of G₀ compared to G₁ cells. This suggests that HSC reside preferentially in the G₀ phase of the cell cycle.⁶⁸ This has also been convincingly shown by Glimm *et al*,⁶⁹ who demonstrated that human CB cells transiting the S/G₂/M phases of the cell cycle after growth factor stimulation – due to a reversible silencing of the engraftment potential of HSC present in these populations – do not reenter G₀ and do not repopulate the BM of irradiated NOD/SCID mice.

Finally, results demonstrating a variation in the engraftment potential of CD34⁺ and Lin⁻CD34⁻ cells in fetal sheep recipients with respect to the source of progenitors are of interest: when serial transplants are performed in sheep fetus, the engraftment occurs earlier with mPB CD34⁺ than BM and is lower with PB CD34⁺ and Lin⁻CD34⁻ than BM cells.⁷⁰ Whereas comparable CFC, LTC-IC or cobblestone area forming cells per CD34⁺ cells are similar in mPB and BM, generation of secondary CFC from an individual LTC-IC in mPB is significantly lower than that of BM. Previous studies have distinguished distinct classes of HSC with different engraftment properties: of these, long-term repopulating cells (LTRC) have a lifelong ability to produce all blood cells and generate progeny that display similar potentialities upon transfer to secondary and tertiary recipients. Other cells with similar differentiation potentialities may reconstitute both myeloid and lymphoid compartments, but typically for less than 4 months. Additional types of short-term repopulating cells (STRC), that are either myeloid or lymphoid restricted have also been identified.^{34,71–75} In order to distinguish these distinct HSC classes, transplants in NOD/SCID- β 2 microglobulin-null (NOD/SCID- β 2m^{-/-}) mice showed that repopulation of three distinct HSC takes place: one of myeloid restriction (STRC-M) and one with dual myeloid and lymphoid-repopulating ability (STRC-ML), neither of which efficiently engrafted NOD/SCID mice.³⁴ In addition, NOD/SCID- β 2m^{-/-} were engrafted after 6–8 weeks by more primitive LTRC-ML that engraft NOD/SCID mice equally efficiently. Of interest was that in mPB both STRC-M and STRC-ML activities were markedly elevated as compared with BM or CB. This relatively elevated STRC content of mPB seems to explain the apparently faster rates of hematopoietic recovery.³⁴ Although the frequency of HSC with primary long-term repopulation may thus be lower in mPB than BM, supported by serial transplantation experiments which have exhausted the repopulating ability of Lin⁻CD34⁺ cells from PB faster than from BM,⁷⁰ in the clinical setting, where large doses of PB MNC or CD34⁺ cells are given, these have been shown to contain sufficient cells with long-term engraftment potential.

Perspective

Transplants of hematopoietic cells have assumed an important role in the treatment of many malignancies. They hold much promise for the clinical application involving gene therapy, tolerance induction to facilitate allogeneic or xenogenic organ transplants and other modalities, where HSC purification and/or expansion before transplantation is desirable. Recently, exciting data have demonstrated that the potential of HSC is almost unlimited to generate whole organ systems.^{76–79} Therefore, the potential of CD34⁻ and CD34⁺ HSC also lies within their apparent capacity of transdifferentiation to cells of widely diverse tissues, such as BM-derived mesenchymal cells, already used to treat osteogenesis imperfecta in children,⁷⁹ as a source of hepatic oval cells,⁸⁰ for cardiocytes,⁸¹ neural cells^{82,83} and vice versa.⁸⁴ Although the frequency of HSC, with respect to SP, Lin⁻CD34⁺CD38⁻ or Lin⁻CD34⁻ cells may be low, their isolation and use as targets for gene therapy, stem cell expansion, transplantation and/or regeneration of injured tissue, as shown for ischemic cardiac muscle,^{77,81} may verify their clinical relevance.

Deciding which BM, PB or CB population to select is an important (pre)clinical issue. Current results to date, as summarized in this paper, suggest only performing CD34 selections for transplant purposes when alternative methods are not available.

Much energy still needs to be placed in the CD34⁻ isolation to best ensure that the latter is as pure as possible. This and the characterization of HSC will need further extensive investigation to finally clarify the value of CD34⁻ cells.

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