

Defining Progenitors Based on Their Expression of Aldehyde Dehydrogenase

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Umbilical Cord Blood Transplantation (UCBT)

Bone marrow transplantation (BMT) provides a profound and rich history for newly emerging cellular therapies. Over the past 30 years one of the most significant advances in BMT has been the use of allogeneic umbilical cord blood (UCB) as an alternative hematopoietic graft.¹⁻³ Historically, UCB was considered as a discarded by-product from childbirth but now it is routinely banked for transplantation.^{4,5} UCB offers multiple practical advantages, which include the following:

- (1) an ease of procurement;
- (2) no risk to the graft donor;
- (3) no donor attrition; and
- (4) a reduced likelihood of transmitting infections.

Furthermore, UCBT apparently offers a reduced risk of severe (Grade III/IV) Graft-versus-Host disease; therefore, the criteria for HLA matching

are less stringent.^{6–8} This in itself greatly increases the probability of donor–recipient matches. UCBT provides a particularly appealing alternative for recipients who do not have a matched, related or unrelated hematopoietic stem cell (HSC) donor.

In spite of all its advantages, the principal problem with UCBT is that engraftment of all the hematopoietic cell lineages is delayed. This raises central questions surrounding the graft itself. The speed of hematopoietic engraftment correlates most strongly with the total dose of mononuclear UCB cells delivered to the patient. Unfortunately, UCB is collected in a single harvest and there is no opportunity to add to the graft at a later time. More recent studies correlate patient survival with the graft's total content of CD34⁺ progenitor cells.⁸ Thus, increasing the total dose of hematopoietic progenitors in a graft might provide a means to improve clinical outcome. It is well established that HSC can provide long-term hematopoietic recovery;^{9,10} however, other progenitors replenish the blood in the period immediately after a transplant.^{11,12} Strategies to enhance cord blood grafts would be greatly strengthened by a more complete understanding of the total progenitor content of UCB. Toward that end, over the past several years we have developed alternative strategies to explore the fundamental physiology of the HSC and progenitor compartment. One promising strategy has been to define progenitors based on their expression of aldehyde dehydrogenase (ALDH).

Early hematopoietic development

Based on over 20 years of experimental modeling, normal hematopoietic development has been carefully dissected to define various progenitor compartments (Fig. 1).^{9,10} Murine progenitors, in particular, have been rigorously defined based not only on their cell surface antigen expression, but more importantly, also by their function.^{9–11} In the broadest terms, hematopoietic progenitors can be classified based on their capacity for self-renewal and their capacity for myeloid and/or lymphoid development. As a therapeutic endpoint, hematopoietic progenitors are also frequently stratified based on their transplantability. HSC are the most primitive hematopoietic cell subset. These are transplantable cells that can repopulate all the blood cell lineages for the lifespan of the recipient and are therefore frequently referred to as long-term HSC (LT-HSC). Their permanence infers that the cells must divide while maintaining their full developmental potential. In experimental animal models, this can be confirmed by performing

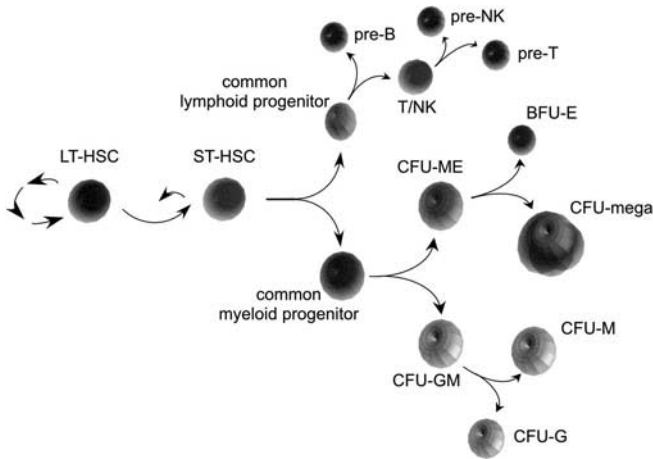


Fig. 1. Hematopoietic development. Stable and permanent hematopoiesis is established from long-term hematopoietic stem cells (LT-HSC). These give rise to short-term (ST)-HSC. Both the LT- and ST-HSC have the capacity to differentiate into all of the hematopoietic cell lineages. The fundamental distinction between the LT- and ST-HSC is that the LT-HSC self-renew while maintaining their entire hematopoietic potential. As their capacity for lineage development becomes more restricted, the ST-HSC give rise to progenitors that specify either myeloid or lymphoid cell lineages.

secondary bone marrow transplants. This inherent capacity for self-renewal distinguishes LT-HSC from all other progenitors. Other transplantable cells establish themselves only transiently.^{12,13} Specifically, as LT-HSC differentiate they give rise to short-term HSC (ST-HSC). Similar to LT-HSC, the ST-HSC are transplantable progenitors that engraft to develop into all of the hematopoietic cell lineages; however, unlike LT-HSC, they persist in the transplant recipient for only a limited time. As ST-HSC differentiate they give rise to progenitors that can specify either the entire lymphoid compartment or the entire myeloid compartment.^{14,15} These are referred to as the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP), respectively. The CLP and CMP give rise to progenitors that are increasingly lineage-restricted. This is particularly evident within the myeloid compartment since those progenitor cells form distinctive colonies based on their lineage potential. These clonogenic cells demonstrate that they are active progenitors and not mature cells. Thus, in total, hematopoietic differentiation can be viewed as a process where the stem cells first lose their capacity for self-renewal and then become progressively restricted in their capacity for lineage development.

Early human hematopoietic development has been most extensively defined within the CD34⁺ cell subset, a cell fraction that is both heterogeneous and diverse.^{16–25} Human CD34⁺ cells clearly contain transplantable cells with a capacity for multilineage development,^{16–20} yet also comprise cells with more limited potentials.^{21–25} To discriminate cells with distinct functions, CD34⁺ cells have been fractionated based on their expression of other antigens. As an example, CD34⁺ CD38^{neg} cells contain the highest frequency of NOD/SCID repopulating cells (SRC) (1 SRC/600 cells), a population of primitive myelo-lymphoid progenitors.¹⁶ As CD34⁺ cells differentiate they begin to express CD38,²¹ however, the expression of CD38 is common to many hematopoietic progenitors and by itself it cannot be used to distinguish between myeloid- and lymphoid-specific progenitors. Therefore, other antigens must be used to separate these two compartments. For example, human CD34⁺ lymphoid-specific progenitors may express either CD7 or CD10.^{22–25}

Physiological Parameters that Define Hematopoietic Progenitors

While CD antigens have been considered as extremely useful tools to fractionate cells, these antigens are frequently not linked with any specific physiological context or consequence. Several years ago we initiated a series of strategies to define hematopoietic progenitors based on their physiology.^{26–29} These studies have focused primarily on mechanisms for drug resistance and were based largely on the clinical experience that autologous HSC sometimes survive chemotherapies. Resistance to chemotherapies may be mediated by a variety of mechanisms. In some cases, membrane-associated pumps actively rid cells of toxic agents.^{26,30,31} Efflux pumps are not specific for a single drug, but rather recognize classes of drugs that share structure. Their activity can be measured by monitoring the loss of fluorescent dyes. This strategy has gained popularity most recently through a Hoechst 33342 dye efflux assay originally described by Goodell *et al.*³⁰ However, a wide variety of similar assays have been described previously.³¹ Moreover, although these assays rely primarily on efflux, each of these assays may also depend on secondary physiological properties, such as the cell's degree of mitochondrial activation.³¹ Thus, characterizing progenitors based on the capacity to efflux dyes is a strategy that should perhaps be explored more deeply.

As part of our studies we have developed and characterized an alternative strategy for identifying hematopoietic progenitors based on their expression of ALDH,^{32–34} an intracellular enzyme that specifically confers resistance to the nitrogen mustard cyclophosphamide. In general, this cell isolation strategy relies on the use of non-polar fluorescent aldehyde substrates that can traverse the cell membrane (Fig. 2).^{27,35} In the presence of ALDH the aldehydes are oxidized, become polar and are retained by the cell membrane. Thus, fluorescence increases in the cells that express ALDH. The first formal tests for this strategy were performed at the Johns Hopkins University using murine BMT assays.³⁶ In those studies, hematopoietic progenitors were initially fractionated by counter-current elutriation, a process that enriches small, primitive progenitors (Fr25 cells). Cells that expressed high levels of ALDH were then purified by their conversion of the fluorescent substrate dansyl aminoacetaldehyde. The Fr25 ALDH^{br} cells contained progenitors that were capable of transplanting themselves into secondary hosts, even when as few as ten cells were assayed per transplant. To achieve re-transplantation, the purified cells must have been expanded in the primary transplant recipient while maintaining a capacity to repopulate hematopoiesis. Therefore, these studies provided strong evidence

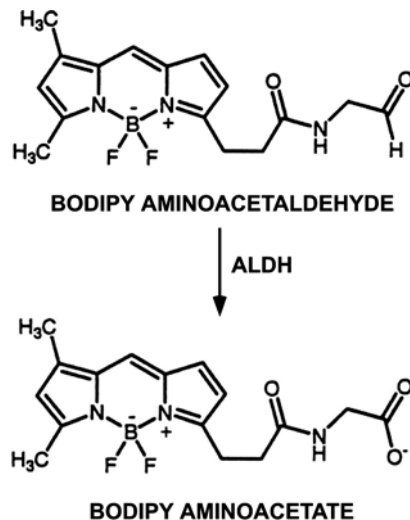


Fig. 2. Fluorescent substrates for ALDH. Fluorescent ALDH substrates (such as BODIPY aminoacetaldehyde) are non-polar and traverse the membrane. Upon exposure to ALDH these dyes acquire charge and become cell impermeant, and thus cell fluorescence increases (modified from Storms *et al.*²⁷).

that LT-HSC expressed ALDH. In addition, the Fr25ALDH^{br} cell population was equally notable for the absence of specific progenitors. The Fr25 ALDH^{br} cells did not form foci in the spleen, a measurement for short-term progenitors with a capacity for both myeloid and lymphoid development. Thus, the expression of ALDH discriminated LT-HSC from transplantable short-term progenitors.

The work in murine transplantation model was provocative and exciting; however, the cell purification itself required exposure to a potentially harmful ultraviolet laser. To eliminate exposure to ultraviolet light and as a first step toward transferring this work to a clinical setting, a second-generation aldehyde substrate was synthesized using BODIPY-FL fluorochrome.²⁷ Using BODIPY aminoacetaldehyde (BAAA; Aldefluor[®]), cells that comprised slightly less than 1% of the total UCB could be readily identified based on their low orthogonal light scatter and their high ALDH expression (SSC^{lo} ALDH^{br} cells, see Fig. 3).^{27,29} These cells did not express

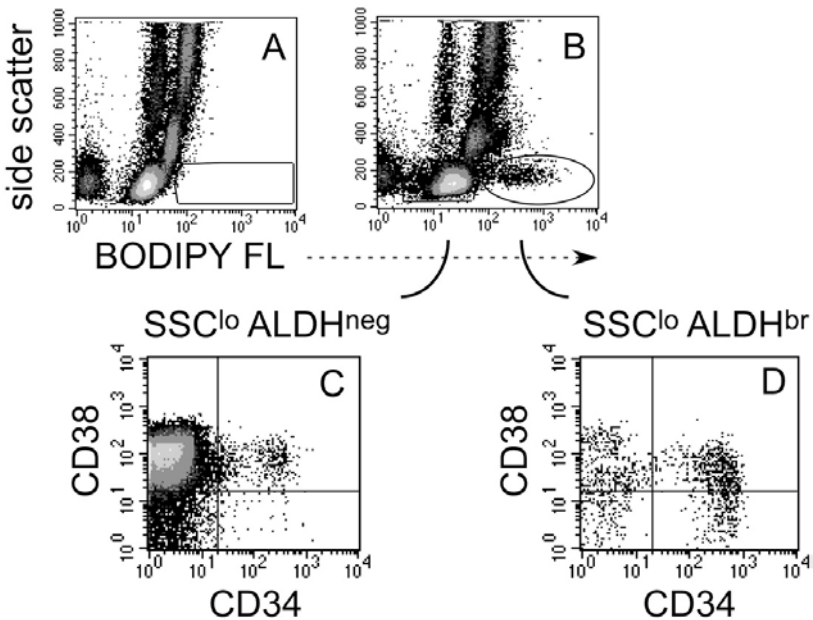


Fig. 3. Defining ALDH^{br} progenitors. For our studies, cells were stained with 1 μ M BODIPY aminoacetaldehyde. SSC^{lo} ALDH^{br} and SSC^{lo} ALDH^{neg} cells were defined (B) based on the background fluorescence established using enzyme-inhibited controls (A). On average, approximately 50% of SSC^{lo} ALDH^{br} cells expressed CD34⁺ (D) and these cells were highly enriched with CD34⁺ CD38^{neg} cells (modified from Storms *et al.*²⁹).

lineage-specific antigens and, more importantly, they were capable of eliminating the fluorescent dye by a mechanism that apparently involved a verapamil-sensitive efflux pump.²⁷ From a clinical perspective, this was significant because the dye would not linger inside progenitor cells and any potential cytotoxic effects from foreign agents would decrease. From a biological perspective, this was significant because a single lin^{neg} cell population appeared to simultaneously express two physiological mechanisms for drug resistance. This phenotype — lin^{neg} efflux^+ ALDH^{br} — strongly suggested a cell with progenitor function. Indeed, when placed into developmental assays, the SSC^{lo} ALDH^{br} cell fraction contained clonogenic myeloid progenitors (CFU) as well as more primitive cells with a capacity to initiate long-term cultures (LTC). Subsequent studies indicated that SSC^{lo} ALDH^{br} UCB cells were also highly enriched with transplantable cells that engraft NOD/SCID mice (SRC).^{29,33,37} Therefore, by both phenotype and function, the SSC^{lo} ALDH^{br} cell fraction rapidly enriched progenitors.

In the studies on human UCB, this simple isolation strategy enriched a mixture of progenitors that included clonogenic cells committed to the myeloid lineage as well as transplantable cells with a potential for both myeloid and lymphoid development. This was unlike the ALDH^{br} cells that had been isolated from murine bone marrow, which appeared to represent a nearly pure population of long-term repopulating stem cells. The primary distinction between the two studies resides with the use of counter-current elutriation to carefully fractionate the murine bone marrow based on cell size. Indeed, this fractionation had been used previously to enrich transplantable cells.³⁴ However, we note that the progenitors that were enriched from SSC^{lo} ALDH^{br} UCB were entirely consistent with bone-marrow-derived progenitors that display resistance to cyclophosphamide.^{38–40}

Defining CD34^+ Progenitors Based on Their Expression of ALDH

Preliminary studies from multiple different groups had all noted that the SSC^{lo} ALDH^{br} cells were enriched with CD34^+ cells, and that the ALDH^{br} CD34^+ cells included a high frequency of CD34^+ CD38^{neg} cells (Fig. 3).^{27,28,37} However, while approximately 60% of the SSC^{lo} CD34^+ lin^{neg} express ALDH, at least some CD34^+ cells did not. This provided

an opportunity to evaluate the association of ALDH within the context of well-described CD34⁺ progenitors.

Primitive CD34⁺ cells express ALDH

The NOD/SCID xenograft model is commonly used to monitor transplantable human progenitors.¹⁶ In those studies, human hematopoietic engraftment to the bone marrow can be readily assayed by the presence of human CD45⁺ cells. In addition, a progenitor's capacity for multilineage development is typically confirmed by the presence of human B lymphoid cells as well as shorter-lived myeloid cells in the marrow. Finally, in some mice, human CD34⁺ progenitors persist in the marrow even 18–20 weeks post-transplant.²⁹ This suggests that human hematopoiesis is still actively evolving, even at relatively late time points.

When CD34⁺ progenitors were fractionated as ALDH^{br} and ALDH^{neg}, the two populations were strikingly dissimilar in their capacity to engraft NOD/SCID marrow.²⁹ In both short- and long-term transplantation assays, 1000 to 3000 ALDH^{br} CD34⁺ cells were sufficient to reliably engraft the marrow of NOD/SCID mice. Limiting dilution analyses estimated that approximately 1 in 4700 ALDH^{br} CD34⁺ cells were SRC (95% confidence interval [CI]: 1/3700 to 1/6900). In contrast, SRC were too rare within the ALDH^{neg} CD34⁺ cell fraction for their frequency to be reliably estimated. A similar observation was described recently when lin^{neg} ALDH^{br} UCB cells were fractionated based on their expression of CD133.³² In those studies the highest frequency of SRC was found within cells that co-expressed CD133 and ALDH. Although a subset of these cells was CD34^{neg}, it was noteworthy that a majority of CD133⁺ ALDH^{br} cells also expressed CD34. While it remains to be formally tested, collectively these data suggest that the cells that co-express the constellation of CD34, CD133 and ALDH might be the cells that most efficiently achieve engraftment, at least as monitored in the NOD/SCID transplantation model. At least part of the transplantability of these cells may lie in an inherent capacity for the cells to rapidly establish themselves within the marrow.³²

Primitive progenitors can also be monitored by their growth in LTC assays. After five weeks in culture, LTC maintain clonogenic myeloid progenitors, committed lymphoid progenitors as well as cells that can re-initiate secondary LTC cultures.²⁹ Therefore, cells that initiate LTC are myelo-lymphoid progenitors; however, the culture assays themselves do not carry the more stringent requirement for cell homing that is present in

transplantation assays. LTC have been established with ALDH^{br} CD34⁺ and ALDH^{neg} CD34⁺ UCB cells as an independent measurement for primitive progenitors. As had been observed with SRC, the two cell populations were quite dissimilar in their relative content of cells that could initiate secondary LTC.²⁹ After ten weeks in culture, the ALDH^{br} CD34⁺ cells yielded approximately 100-fold greater CFU when compared with ALDH^{neg} CD34⁺ cells. However, the more critical comparison came from limiting dilution analyses which estimated that 1 in 43 ALDH^{br} CD34⁺ cells would initiate secondary LTC whereas only 1 in 1130 ALDH^{neg} CD34⁺ cells initiated secondary LTC. Therefore, based on their performance in LTC, primitive progenitors were 25-fold more frequent within the ALDH^{br} CD34⁺ cell fraction.

ALDH^{br} and ALDH^{neg} CD34⁺ cells display different responses in short-term culture

Progenitor cells with more limited developmental potentials also contribute to hematopoiesis. Unlike what had been observed in studies on primitive progenitors, the ALDH^{br} and ALDH^{neg} CD34⁺ cell fractions displayed more subtle differences in their contents of short-term progenitors.²⁹

Short-term myeloid progenitors are most commonly discriminated by their capacity to form colonies in hematopoietic progenitor colony assays (HPCA). Both ALDH^{br} CD34⁺ and ALDH^{neg} CD34⁺ UCB cell fractions contained clonogenic progenitors; however, myeloid CFU were 2.5-fold more frequent within the ALDH^{br} CD34⁺ subset (Fig. 4). This was consistent with previous observations that the SSC^{lo} ALDH^{br} cell fraction was enriched with myeloid progenitors. In complementary studies, the growth and differentiation of ALDH^{br} and ALDH^{neg} CD34⁺ progenitors were monitored in short-term cultures that contained recombinant human IL-3, IL-7 and IL-15 (Fig. 4). These conditions specifically encourage the rapid and robust development of NK cells. However, even though the ALDH^{br} CD34⁺ cells expanded extensively under these conditions, they predominantly gave rise to CD13⁺ myeloid cells. In paired cultures, the ALDH^{neg} CD34⁺ cells also gave rise to myeloid progeny although to a significantly lesser degree. The total myeloid output was approximately seven-fold greater in cultures initiated with ALDH^{br} CD34⁺ cells. This was presumably measuring a response to IL-3, and was consistent with their clonogenic potential as monitored in HPCA. In total, these data indicated that short-term myeloid progenitors were more frequent among the

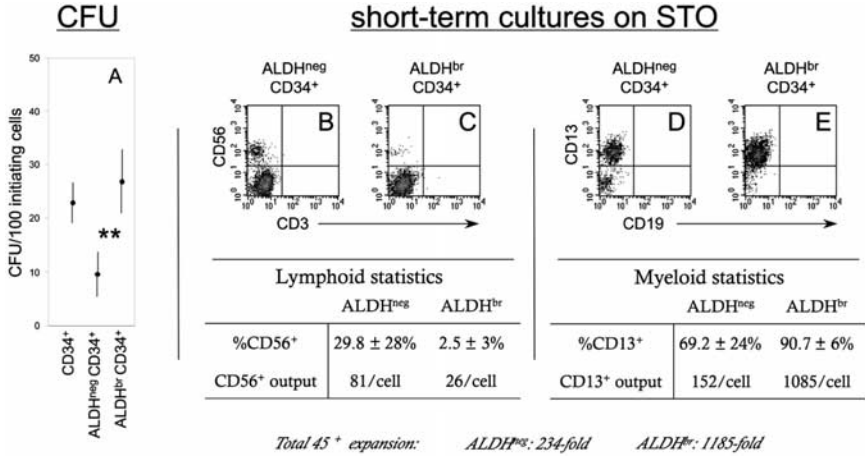


Fig. 4. Defining short-term progenitors. (A) ALDH^{br} CD34⁺ and ALDH^{neg} CD34⁺ cells were monitored for clonogenic myeloid progenitors and the total number of myeloid colonies were normalized per 100 cells that initiated each culture ($n = 7$). **Statistical comparisons were drawn between ALDH^{br} and ALDH^{neg} cells using paired non-parametric analysis (Wilcoxon signed rank test; ** $P = 0.06$). (B–E) Short-term progenitors were cultured on STO fibroblast feeder stroma in the presence of IL-3, IL-7 and IL-15 under the conditions that were designed to support NK development ($n = 10$). The progeny of ALDH^{br} CD34⁺ and ALDH^{neg} CD34⁺ cells were compared their relative expression of the lymphoid antigen CD56 (B and C) or the myeloid antigen CD13 (D and E). Average percentages (\pm SD) and the total output of (modified from Storms *et al.*²⁹).

ALDH^{br} CD34⁺ cells. These data might also suggest that both cell fractions contain cells that are irreversibly committed to myeloid differentiation.

As mentioned above, NK development is rapid and robust in response to IL-3, IL-7 and IL-15. This was most clearly evident in cultures initiated with ALDH^{neg} CD34⁺ cells, which consistently gave rise to high percentages of CD56⁺ lymphoid cells (Fig. 4). In contrast, even with vigorous growth, the ALDH^{br} CD34⁺ cells exhibited only a limited capacity toward NK development. In total, the ALDH^{neg} CD34⁺ cells produced fewer total cells yet gave rise to three-fold more NK progeny than did the ALDH^{br} CD34⁺ cells. These differences are consistent with their expression of the early lymphoid antigenic determinants CD7 and CD10 (Fig. 5). These data suggest that the expression of CD7 was distributed across the ALDH^{neg} and a ALDH^{dim} CD34⁺ cell subset; however, CD34⁺ CD10⁺ cells were nearly exclusively ALDH^{neg}.

To briefly summarize these data, the ALDH^{br} CD34⁺ phenotype was associated with primitive hematopoietic progenitors and with short-term myeloid progenitors. In contrast, the ALDH^{neg} CD34⁺ cells may be a reservoir of committed lymphoid progenitors. Based on data with regard to cell phenotypes, it is tempting to stratify the CD34⁺ cell compartment based on their expression of ALDH and CD38. On going studies are attempting to define whether distinct CD34⁺ developmental compartments can be defined based on three primary phenotypes (Fig. 5):

- (1) ALDH^{br} CD34⁺ CD38^{neg} cells should be enriched with primitive cells, such as SRC;
- (2) ALDH^{dim/br} CD34⁺ CD38^{dim/br} cells should be enriched with myeloid progenitors; and
- (3) ALDH^{neg} CD34⁺ CD38^{br} cells should be enriched with lymphoid progenitors.

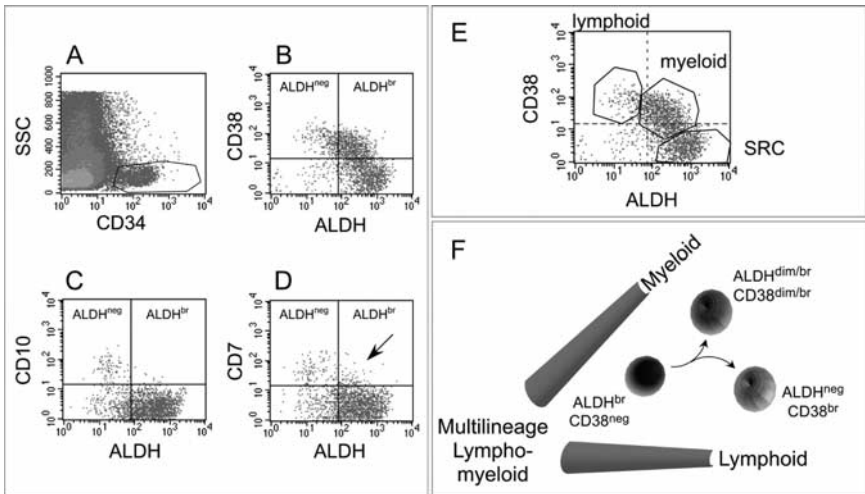


Fig. 5. Defining human hematopoietic progenitors. SSC^{lo} CD34⁺ cells were defined in UCB (A) to examine the expression of CD38, CD10 and CD7 relative to the expression of ALDH (B,C,D). The CD34⁺ cells with the brightest ALDH expression exhibited the lowest CD38 expression (B). In contrast, the expression of CD10 and CD7 were predominantly found within CD34⁺ cells that do not express ALDH (C and D). Some CD7⁺ cells were evident within an ALDH^{dim} cell fraction (indicated by an arrow). Based on the data regarding their developmental potentials, it is tempting to speculate that the relative expression of ALDH, CD34, and CD38 might be used to discriminate functional subsets of CD34⁺ cells (E) A model for lineage-specific development by CD34⁺ cells is presented (F).

Clinical Endpoints

This cell isolation strategy was intended not only to increase our understanding of basic progenitor physiology, but hopefully to also translate that knowledge into the clinic. To date, this simple assay has been used within the context of clinical monitoring. In two studies the expression of ALDH has been used to evaluate the quality of human hematopoietic transplant grafts.^{28,41} Both studies suggest that the content of SSC^{lo} ALDH^{br} cells within the graft is a positive clinical indicator for successful engraftment. One study suggests a correlation between these cells and the graft's total content of clonogenic progenitors. CFU content is routinely monitored in clinical transplantation studies as an indicator of the progenitor content of the graft. These data are consistent with our understanding of the total progenitor content on the ALDH^{br} cell fraction.

ALDH in Other Tissues

The expression of ALDH has been most extensively characterized within the context of early hematopoiesis. In addition, although most studies have focused primarily on normal hematopoiesis, recent studies suggest that ALDH may be a part of the phenotypic signature for at least some human leukemic stem cells.⁴² One question that remains is whether ALDH participates within the early ontogeny of other cell types. ALDH is highly expressed in neural progenitors identified from the fetal rat brain.⁴³ Furthermore, when treated with a specific inhibitor of ALDH, zebrafish embryos suffer a total loss of hindbrain development,⁴⁴ a phenotype that mimics a known mutant in a retinoic acid receptor. This strong phenotype suggests that ALDH may play a pivotal role during early neurogenesis. Other tissues have not been explored in detail. We have noted subpopulations of adipose-derived mesodermal progenitors that express ALDH;⁴⁵ however, a careful examination of the developmental potential of those cells has yet to be performed.

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