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Aldehyde dehydrogenase activity as a marker for the quality of hematopoietic stem cell transplants

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Summary:

Taking advantage of fluorescent substrates for their metabolic marker aldehyde dehydrogenase (ALDH), hematopoietic stem cells (HSC) were defined as *SSC^{lo}ALDH^{br}* – reflecting their low orthogonal light scattering and bright fluorescence intensity in flow cytometry. Based thereon, we investigated the usefulness of ALDH activity for characterizing HSC graft quality, particularly under stress conditions. We first compared the expression of ALDH *vs* CD34 in bone marrow and peripheral blood stem cell (PBSC) samples over 7 days. We noted that (i) only ALDH activity but not CD34 expression strongly reflected colony-forming ability over time, and that (ii) PBSC grafts stored at room temperature lost most of their progenitor cells within just 48 h. We then retrospectively related ALDH and CD34 expression as well as granulocyte–macrophage colony-forming units (CFU-GM) potential for 19 cryopreserved allogeneic PBSC grafts to engraftment data. Strikingly, in all six patients who received markedly decreased numbers of SSC^{lo}ALDH^{br} cells, this was associated not only with almost complete loss of CFU-GM potential but also with delayed establishment/permanent absence of full hematopoietic donor cell chimerism, whereas all other patients showed early complete donor chimerism. In conclusion, we suggest to measure ALDH activity as a surrogate marker for HSC activity, and to transport and store PBSC under controlled cooling conditions.
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High enzymatic activity of aldehyde dehydrogenase (ALDH) has been shown to be a characteristic feature of primitive hematopoietic progenitor cells in mice[1] and humans.[2] To overcome technical limitations applying to the detection of intracellular enzyme activity in the life state, Jones *et al*[3] developed a fluorescent substrate for ALDH (dansyl-aminoacetaldehyde, DAAA), which enables assessment of ALDH activity using fluorescence-activated cell sorting (FACS).[3] Further, a second substrate (BODIPY aminoacetaldehyde, BAAA) has been described[4] and commercialized (ALDEFLUOR™). By the use of FACS, a specific cell fraction from human cord blood (CB) characterized by low orthogonal light scattering and bright fluorescence intensity (SSC^{lo}ALDH^{br}) was shown to be highly enriched for hematopoietic stem cells (HSC) and progenitor cells.[4] In a recent publication, Hess *et al*[5] characterized highly purified ALDH^{hi}Lin^{-} cells from human CB both *in vitro* and *in vivo*. Importantly, they showed that this cell population exclusively contains the NOD/SCID-repopulating activity of CB.[5] This is very well in agreement with data from Fallon *et al*,[6] who showed that the numbers of SSC^{lo}ALDH^{br} but not CD34^{+} cells in autologous transplants do correlate with both neutrophil and platelet engraftment.[6]

The use of functional rather than cell surface markers to identify and isolate stem cells may have several advantages.[4–6] For instance, the human CD34 antigen broadly and successfully used in clinical hematopoietic stem cell transplantation (HSCT) to characterize and enrich early progenitors is a very stable molecule, which may be present on cell membranes for very long periods of time.[7] Consequently, a possible dissociation between the phenotype as established on the base of surface markers (eg CD34) and the true repopulating capability of putative HSC and progenitor cells has been supposed.[4,5] Since such a phenomenon would have important consequences for clinical HSCT, we compared expression kinetics of CD34 *vs* ALDH in various bone marrow (BM) and leukapheresis samples during a 7-day storage period (reflecting stress conditions) in relation to their viability and colony-forming ability. In a retrospective analysis, we also compared SSC^{lo}ALDH^{br} and CD34^{+} numbers in HSC grafts cryopreserved between 2000 and 2003 for allogeneic stem cell transplantation (we used pilot tubes frozen in parallel) and related the results to clinical engraftment data of the respective patients.

Patients and methods

In vitro storage experiments were performed in a prospective way using samples from 13 different BM and 11

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different PBSC grafts donated in 2004 at the Hamburg BMT center. In the retrospective study, we analyzed 19 consecutive allogeneic PBSC grafts, which had to be frozen due to medical and/or logistic reasons.[8] All patients were transplanted at the BMT center Hamburg between 2000 and 2003. They were nursed in laminar air-flow isolation rooms. Relevant patient data are summarized in Table 1.

Expression kinetics of CD34 and ALDH under different transplant storage conditions

Cell probes directly taken from whole grafts (after informed consent) were first analyzed on the day of harvest (day 0) using flow cytometry essentially as described.[7,9] Briefly, CD34 expression was examined according to ISHAGE guidelines. $SSC^{lo}ALDH^{br}$ were identified using ALDEFLUOR® (Becton Dickinson, Heidelberg, Germany), and cell viability was determined with 7-amino actinomycin D (7-AAD) dye[10] (Beckman-Coulter, Krefeld, Germany). FACS data were acquired and analyzed on an Epics XL-MLC (Beckman-Coulter). After initial analysis, cell samples were aliquoted and stored at either $+4°C$ or at room temperature without dilution to best resemble usual conditions during transport of clinical transplants. CD34 and ALDH levels were determined at days 1, 2, 3 and 7. Individual parameters obtained at day 0 were set at 100% and data obtained on subsequent days were calculated relative to these.

Determination of granulocyte–macrophage colony-forming units (CFU-GM)

In parallel to the FACS analyses, CFU-GM assays were carried out on days 0, 2 and 7 essentially as described.[11] In brief, 1×10^5 cells were seeded in 1 ml methyl cellulose (MC) supplemented with growth factors (Methocult®, Stem Cell Technologies, St Katharinen, Germany) to define numbers of CFU-GM in the analyzed samples. CFU-GM were counted after 11–14 days. Colony numbers determined for day 0 were set at 100% and relative numbers were calculated for each group/time point, respectively.

Results

Number of $SSC^{lo}ALDH^{br}$ but not of $CD34^+$ cells correlate with CFU-GM potential

We first measured the relative numbers of $SSC^{lo}ALDH^{br}$ and $CD34^+$ cells in fresh BM ($n = 13$) and PBSC ($n = 11$) grafts by flow cytometry. As expected,[5,6] these values were almost identical for both BM (CD34: $1.22 \pm 0.18\%$; $SSC^{lo}ALDH^{br}$: $1.26 \pm 0.26\%$) and PBSC (CD34: $0.79 \pm 0.29\%$; $SSC^{lo}ALDH^{br}$: $0.77 \pm 0.30\%$) grafts confirming coexpression of the two markers in a significant amount of progenitor cells. In fact, double staining for both markers revealed that >98% of the $SSC^{lo}ALDH^{br}$ cells also expressed CD34 in all samples analyzed (data not shown).

These figures changed with storage time (Figure 1). While stable total leukocyte counts were observed (not shown), a growing discordance between CD34 expression and ALDH activity was seen with time, which was particularly evident in those probes stored at room temperature (Figure 1b and d). After day 2, the observed differences were significant ($P < 0.05$) even at 4°C for both BM and PBSC (Figure 1). For four of the BM and three of the PBSC samples, we generated additional CFU-GM data for each time point. Importantly, relative numbers of $SSC^{lo}ALDH^{br}$ cells were always in very strong accordance with the colony-forming capability (CFU-GM) of a given sample, whereas no correlation was seen between CFU-GM activity and CD34 expression after more than 48 h storage (Figure 1).

Early dramatic loss of progenitor cells in PBSC transplants stored at room temperature

Besides the above-described findings regarding the utility of different markers for quality control of stem cell grafts, our data summarized in Figure 1 clearly show that both BM and PBSC preparations can be transported and stored for up to 48 h at $+4°C$ without losing more than 20% of their progenitor (CFU-GM) capacity. In fact, according to viability, ALDH activity and importantly also CFU-GM data, BM grafts are quite stable even at room temperature for at least 48 h. In striking contrast, when stored at room temperature, PBSC grafts already lose >20% of their progenitors within 1 day as evident from both Aldefluor and viability analysis. After another 24 h, only about one-fifth of the initially present progenitors had retained their colony-forming capability. Notably, under these conditions, loss of ALDH activity strongly correlated with the decrease of CD34 cell viability as measured by 7-AAD dye exclusion (Figure 1).

Indications for impaired engraftment with decreased numbers of $SSC^{lo}ALDH^{br}$ cells

To investigate the influence of another strong stress factor on the numbers of infused $SSC^{lo}ALDH^{br}$ and CD34 cells, we finally initiated a retrospective analysis for all available allogeneic transplants applied after freezing/thawing between 2000 and 2003 (Table 1). Since cryopreservation is not a standard procedure with allogeneic transplants, the number of available samples was limited to 19. We made use of pilot tubes, which were frozen at the Institute for Transfusion Medicine of our hospital at the time of PBSC harvest under the same conditions as the later infused grafts. Cells were thawed and subjected to FACS analysis and CFU-GM assays as described above. Data are summarized in Table 1. As expected, relative numbers of $CD34^+$ (0.23–1.17%) as well as $SSC^{lo}ALDH^{br}$ cells (0.15–1.09%) showed great variability in individual probings. Overall, mean percentages of $SSC^{lo}ALDH^{br}$ (0.36%) were slightly lower than those of $CD34^+$ cells (0.52%), leading to a decreased mean ratio between the two values of 0.73 (range 0.27–1.60). Relative $SSC^{lo}ALDH^{br}$ numbers were particularly low (0.16%; range 0.15–0.19%) for six of the 19 probings (Figure 2a, group II). At the same time, mean $CD34^+$ percentages in these six probings did not differ significantly from the others (Figure 2a). This resulted in

Table 1 Stem cell numbers determined in allogeneic PBSC grafts by $CD34^+$ and ALDH expression in relation to clinical transplantation data

No. (pilot tube)	*After freezing/thawing*					*Calculated $CD34^+$ cells ($\times 10^6$/kg)*[a]	*Transplanted viable $CD34^+$ cells ($\times 10^6$/kg)*[b]	*Transplanted $ALDH^+$ cells ($\times 10^6$/kg)*[b]	*Diagnosis*	*Conditioning*	*Hematopoietic donor chimerism (day)*
	$CD34^+$ viable (%)	*CD34 (%)*	*$SSC^{lo}ALDH^{br}$ (%)*	*ALDH/CD34*	*CFU-GM*						
1[c]	96	0.35	0.28	0.80	11	No[c]	No[c]	No[c]	NHL	NA	NA
2	**83**	**0.56**	**0.15**	**0.27**	**1**	**8.8**	**3.0**	**1.0**	**AML**	**Reduced**	**<95% (45–110)[d]**
3	91	0.41	0.36	0.88	13	4.0	3.5	3.3	MM	Reduced	Full (18)
4[e]	96	0.33	0.27	0.82	21	5.2	3.4	4.3	CML	Standard	Full (26)
5[e]	97	0.38	0.61	1.60	47						
6[c]	92	0.26	0.24	0.92	9	No[c]	No[c]	No[c]	MM	NA	NA
7	90	0.23	0.22	0.96	8	4.9	2.6	2.8	MM	Reduced	Full (25)
8	**75**	**0.42**	**0.16**	**0.38**	**2**	**9.1**	**4.1**	**2.1**	**ALL**	**Standard**	**Full (43)**
9	97	0.50	0.51	1.02	11	6.9	8.4	8.7	ALL	Standard	Full (16)
10	94	0.88	0.59	0.67	25	15.5	6.9	6.0	OMF	Standard	Full (28)
11	**68**	**0.54**	**0.15**	**0.28**	**0**	**13.7**	**5.6**	**2.3**	**MM**	**Reduced**	**<90% (22)[d]**
12	98	0.64	0.44	0.69	38	8.7	5.5	3.9	MDS	Standard	Full (21)
13	96	1.17	1.09	0.93	17	12.3	10.0	9.7	AML	Reduced	Full (22)
14	**81**	**0.56**	**0.18**	**0.32**	**3**	**9.7**	**5.8**	**2.3**	**AML**	**Standard**	**No (27)[f]**
15	96	0.59	0.58	0.98	20	19.5	9.6	9.7	AML	Reduced	Full (29)
16	**77**	**0.71**	**0.19**	**0.27**	**0**	**27.7**	**9.8**	**3.4**	**ALL**	**Standard**	**<95% (43), full (64)**
17	**84**	**0.45**	**0.15**	**0.33**	**0**	**2.2**	**3.2**	**1.3**	**AML**	**Reduced**	**<50% (14–100)[d]**
18	93	0.51	0.48	0.94	22	11.4	4.6	4.7	CML	Standard	Full (22)
19[c]	96	0.35	0.28	0.80	11	No[c]	No[c]	No[c]	NHL	NA	NA

Patients with low $SSC^{lo}ALDH^{br}$/CD34 ratios are highlighted in bold (compare group 2 in Figure 2).
NA = not applicable.
[a]Calculated based on CD34 counts before freezing.
[b]As measured/calculated after thawing (viable CD34 were determined using the ISHAGE protocol).
[c]Three of the 18 patients included into this study did not proceed to transplantation.
[d]Did not reach full donor chimerism.
[e]Two stem cell harvests were performed for one patient.
[f]Engrafted only after second transplant.

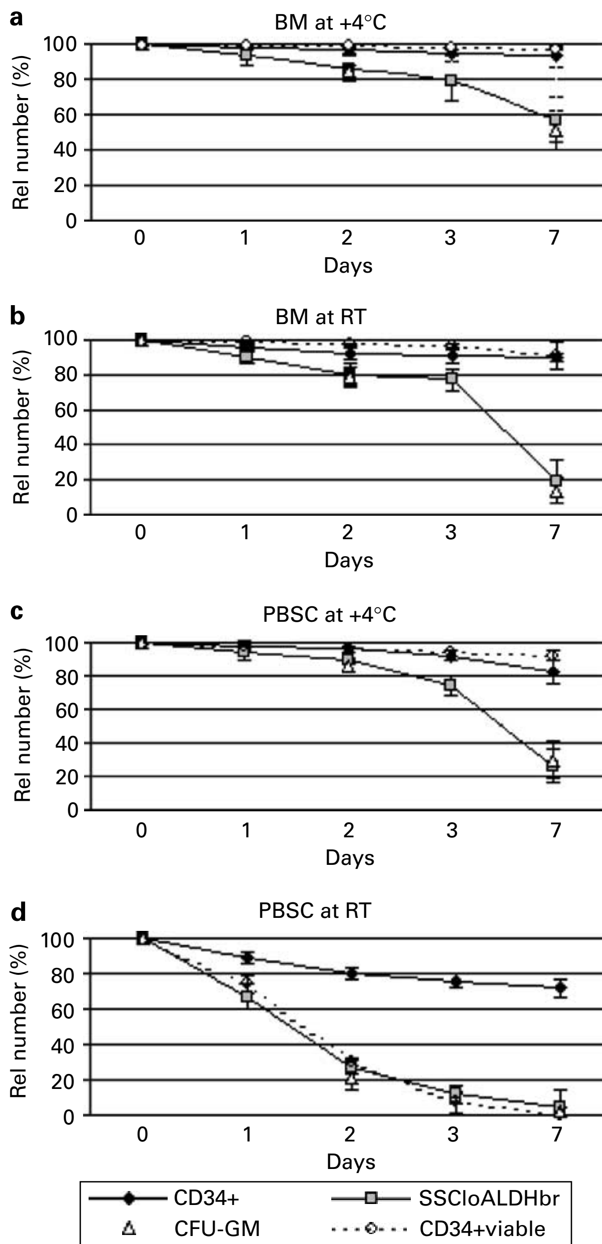

Figure 1 Kinetics of CD34 *vs* ALDH expression, CD34 viability and CFU-GM capabilities of mobilized (PBSC) and BM-derived HSC under different storage conditions (4°C *vs* room temperature). Samples obtained from whole BM (**a** and **b**) or PBSC (**c** and **d**) grafts were stored without previous modification at 4°C (**a** and **c**) or room temperature (**b** and **d**), thus mimicking the actual conditions during transport of clinical transplants from unrelated donors. CD34 positivity (black rhombuses), viability of CD34+ cells (white circles) and ALDH expression (gray quadrates) were measured by flow cytometry on days 0, 1, 2, 3 and 7. Mean values from 13 different BM and 11 different PBSC grafts are shown. Kinetics of corresponding CFU-GM numbers (triangles) were determined in MC assays for a limited number of randomly chosen samples – four with BM and three with PBSC (each time in duplicates). Importantly, if analyzed separately, kinetics of CD34 and ALDH expression as well as viability of CD34+ cells for those randomly selected samples almost exactly reflected the summarizing data illustrated here. All data were set in relation to the corresponding numbers obtained for the respective freshly harvested cells.

very low $SSC^{lo}ALDH^{br}/CD34^+$ ratios (mean 0.30, range 0.27–0.38) for these six samples, whereas the mean ratio in the other 13 transplants (group I) was 0.92 (0.67–1.60) (Figure 2b). We next compared the numbers of $CD34^+$ *vs* $ALDH^+$ cells actually transplanted per kg in the two groups. CD34 counts had initially been measured before cryopreservation (Table 1, Figure 2c) – there was almost no difference between the two groups (group I: $9.4 \pm 5.7 \times 10^6$/kg *vs* group II: $11.9 \pm 8.6 \times 10^6$/kg). Furthermore, no significant difference in CD34 numbers was observed after thawing (Table 1, Figure 2c), even when dead cells were excluded using 7-AAD staining in accordance with the ISHAGE protocol (group I: $6.1 \pm 2.8 \times 10^6$/kg *vs* group II: $5.3 \pm 2.5 \times 10^6$/kg). However, when we finally calculated numbers of $SSC^{lo}ALDH^{br}$ cells transplanted per kg (Table 1, Figure 2c), we found a significant decrease in group II as compared to group I (group I: $5.9 \pm 2.8 \times 10^6$/kg *vs* group II: $2.0 \pm 0.9 \times 10^6$/kg; $P < 0.01$). Importantly, lower numbers of $SSC^{lo}ALDH^{br}$ cells in the six transplants from group II were associated with almost complete loss of CFU-GM potential (Figure 2d), confirming the above data with fresh grafts.

It is noteworthy that of the 15 patients transplanted, all six patients who received PBSC with decreased numbers of $SSC^{lo}ALDH^{br}$ (group II) experienced prolonged periods of mixed chimerism (we were in no case able to detect full donor chimerism before day 40) or never reached full chimerism (four out of six), whereas all other nine patients showed full donor chimerism after 16–29 days (mean: 23).

Discussion

Based on the recently observed high enzymatic activity of ALDH in HSC[1,2] and the development of fluorescent substrates for this enzyme,[3,4] FACS-based approaches have been established to identify and quantify early blood progenitor cells.[3–6] We utilized flow cytometry to measure the numbers of $SSC^{lo}ALDH^{br}$ cells, a population shown to contain most of the blood-forming capacity,[4] in freshly collected BM and PBSC grafts. As shown before by others,[5] numbers of $SSC^{lo}ALDH^{br}$ were in very good agreement with $CD34^+$ counts, the standard marker for HSC in HSCT. We also confirmed data[6] that this is due to the coexpression of ALDH and CD34 by almost all $SSC^{lo}ALDH^{br}$ cells. We then subjected BM and PBSC graft samples to storage at either room temperature or 4°C. This matches conditions during transport of allogeneic transplants over long distances. To expose HSC to maximum stress, we extended the storage period up to 7 days. Importantly, long-term storage of BM and PBSC samples revealed clear differences between $SSC^{lo}ALDH^{br}$ and $CD34^+$ numbers at both temperature conditions obviously reflecting a dissociation between a conserved surface marker phenotype and the actual functional activity of the cells.[5] This may be due to a loss of viability of $CD34^+$ cells as shown for PBSCT samples stored at room temperature by further FACS analyses with the inclusion of 7-AAD, a fluorescent DNA-binding agent. However, we have also (eg at 4°C) seen impaired ALDH function with retained viability of $CD34^+$ cells, indicating a loss of progenitor potential with a conserved cell surface ($CD34^+$) phenotype. To finally verify this question, we carried out CFU-GM assays at different time points of the storage

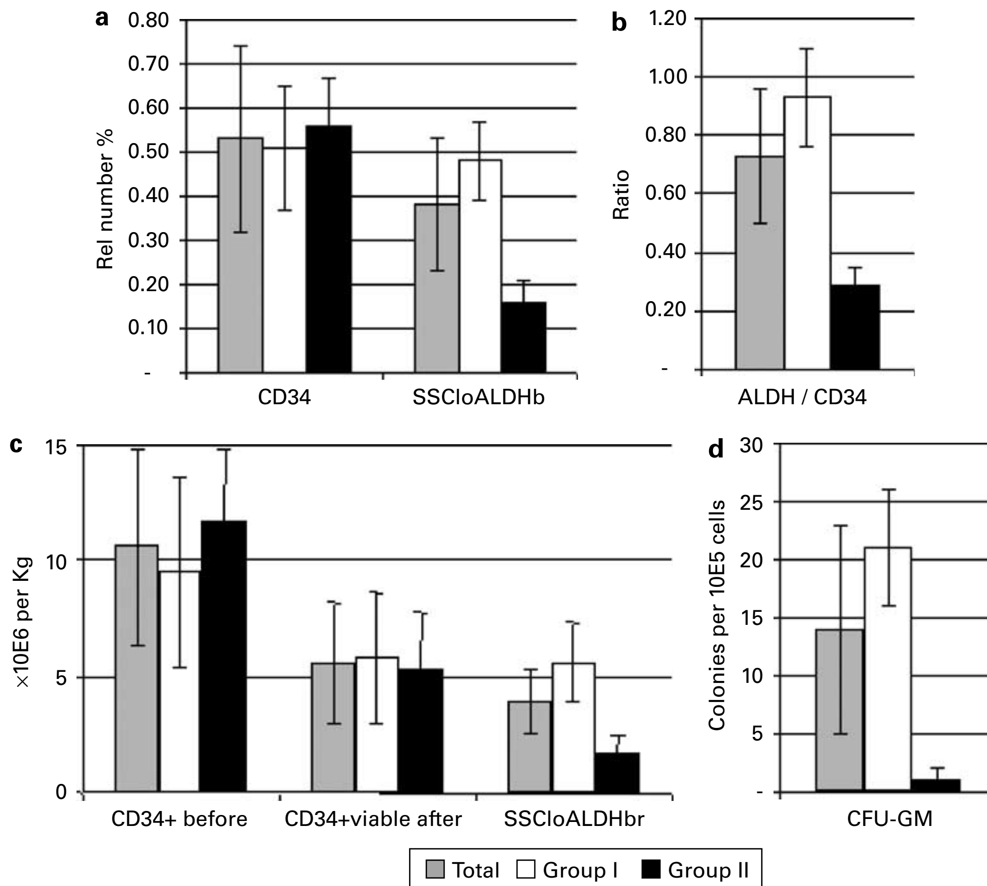

Figure 2 Retrospective analysis of frozen/thawed PBSC transplanted between 2000 and 2003. In accordance with the ratio of $SSC^{lo}ALDH^{br}$ to $CD34^+$ cells, the 19 grafts were divided into two groups representing those with 'normal' ratios (>0.5, group I) and those with decreased ratios (<0.5, group II) (compare Table 1). In both groups, very similar relative numbers of $CD34^+$ cells were detected, whereas the numbers of $SSC^{lo}ALDH^{br}$ were significantly lower in group II as compared to group I (**a**). This resulted in the already mentioned strong difference in the $SSC^{lo}ALDH^{br}$ to $CD34^+$ ratio (**b**). When we compared numbers of $CD34^+$ per kg as calculated before cryopreservation and numbers of actually transplanted (after thawing) viable $CD34^+$ cells, we found no significant differences between groups I and II. In contrast, numbers of $SSC^{lo}ALDH^{br}$ transplanted per kg were strongly and significantly ($P<0.01$) reduced in group II as compared to group I (**c**). Notably, almost no CFU-GM potential was seen in group II (**d**). These data were in good correlation with clinical outcomes in the two different groups (see Table 1).

period. At all conditions and time points, relative numbers of colony-forming units turned out to be in very good correlation with numbers of $SSC^{lo}ALDH^{br}$ cells but not with $CD34^+$ counts as estimated according to the ISHAGE protocol.

A freezing/thawing cycle is another type of stress HSC grafts are frequently exposed to, particularly in the autologous setting. Allogeneic PBSC grafts cryopreserved due to medical and/or logistic reasons have also been successfully used for transplantation.[8] In a retrospective study, we analyzed ALDH activity *vs* CD34 positivity in 19 consecutive allogeneic PBSC grafts frozen in our hospital between 2000 and 2003. Clinical data including chimerism kinetics were obtained for 15 patients transplanted with those grafts. The results of this retrospective analysis are in strong agreement with our *in vitro* data. Indeed, in six out of six cases decreased $SSC^{lo}ALDH^{br}$ as compared to $CD34^+$ cell numbers (ratio below 0.4) were associated with delayed establishment or permanent absence (four out of six) of full hematopoietic donor chimerism. Absolute numbers of transplanted ALDH-expressing cells for these six patients were significantly decreased as compared to the other nine transplant recipients, who earlier displayed full hematopoietic chimerism. At the same time, no differences in mean $CD34^+$ cell numbers as determined before cryopreservation and after thawing were observed. These results are in line with recent data from Fallon *et al*,[6] who showed correlation between engraftment and $SSC^{lo}ALDH^{br}$, but not $CD34^+$ cell numbers in fresh autologous PBSC transplants. Since the numbers of both autologous[6] and allogeneic patients investigated so far are still limited, we suppose that these data urgently need to be confirmed in larger prospective studies.

Taken together, our *in vitro* and clinical data strongly indicate that measuring CD34 expression at least in 'stressed' samples may overestimate the quality of a given transplant. They furthermore imply that ALDH would be a much better marker for quality control.

Another important result of the given study relates to the stability of PBSC *vs* BM transplants at different

temperature conditions. These data confirm that both BM and PBSC may be stored for several days at $+4°C$ without significant decreases in their repopulating capacity.[12,13] On the contrary, at room temperature PBSC grafts lost $>80\%$ of their CFU potential within 48 h, whereas numbers of progenitor cells were quite stable in BM transplants for up to 72 h. This observation strongly indicates the need for controlled cooling conditions during transport of PBSC. In fact, although 4–8°C is often recommended for the transportation and storage of PBSC, common standards are still to be established. Moreover, usual transport systems do not guarantee maintenance of constant temperatures within this range.[14]

The observed difference in stem cell stability may be due to several reasons, for example, the presence of supportive stroma cells in BM transplants, the low amount of plasma in PBSC transplants and/or increased metabolic activity of mobilized as compared to BM stem cells.

In conclusion, we have confirmed that ALDH expression is a very useful marker for stem and progenitor cell activity in HSCT. Based on our results, we suppose that (i) ALDH activity as measured by flow cytometry may be well suited for quality control of HSC grafts, especially under stress conditions such as long transportation or a freezing/thawing cycle and (ii) stable temperature conditions should be ensured during storage as well as transport of PBSC transplants.

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