CD26 expression in donor stem cell harvest and its correlation with engraftment in human haematopoietic stem cell transplantation: potential predictor of early engraftment

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Background: The efficiency of haematopoietic stem and progenitor cells (HSPCs) is important when donor cell numbers are limiting. Stable white blood cell (WBC) and platelet engraftment is crucial for the outcome of haematopoietic stem cell transplantation (HSCT).

Design: This article evaluates CD26/dipeptidyl peptidase-IV expression on mobilised peripheral blood stem cell (PBSC) harvest of donors and its correlation with engraftment in HSCT. We have analysed CD26 expression on cells in various gates, that is, lymphocytes, monocytes, neutrophils and all populations using flow cytometry tool.

Results: Ours is the first study on human mobilised PBSC harvest from cancer patients or allogeneic related donors (n = 28) to demonstrate that increased CD26 expression leads to early engraftment in transplanted cancer patients. Correlation of CD26 expression with WBC engraftment was statistically significant (lymphocyte gate: P < 0.00001; monocyte gate: P < 0.00001; neutrophil gate: P < 0.00001; all populations: P < 0.00001). CD34 expression is a known predictor of engraftment. Nevertheless, there was no correlation between CD34 and CD26 expression in these cases.

Conclusions: This study has given important leads indicating that CD26 expression may be an independent predictor of engraftment. Further study with large number of patients as well as study on circulatory CD26 may add valuable information towards improving current knowledge on CD26.

Key words: CD26 expression, flow cytometry, haematopoietic stem cells, PBSC transplantation, predictor of engraftment

CD26/dipeptidylpeptidase-IV is a widely expressed cell surface peptidase that exhibits a complex biology encompassing cell membrane-associated activation of intracellular signal transduction pathways, cell–cell interaction and enzymic activity exhibited by both the membrane-anchored and the soluble forms of the enzyme [1, 2]. CD26 is expressed constitutively on many haematopoietic cell populations, including activated B and T lymphocytes and natural killer cells, endothelial cells, fibroblasts, and epithelial cells [1, 3, 4], that are involved in migration. The enzyme activity of CD26 is capable of cleaving N-terminal dipeptides from polypeptides with either proline or alanine residues in the penultimate position. CD26-mediated truncation of natural substrates has drastic effects on the biological activity or function. CD26 may also potentially modulate immune responses by directly regulating lymphocytes [5]. On human T cells, CD26 exhibits a costimulatory function. It is known to mediate signalling by direct interaction with cytoplasmic domain of CD45. It was reported that CD26 was involved in interactions with extracellular matrix proteins, collagen and fibronectin.

The role of CD26 expression in haematopoietic stem cell transplantation (H SCT) and modulation of stem cell homing and engraftment has been demonstrated in mouse model using human cord blood haematopoietic stem and progenitor cells (HSPCs) [6] and mouse HSPCs in utero [7]. It was demonstrated that inhibition or deletion of CD26 on donor cells enhanced short-term engraftment, competitive repopulation, secondary transplantation and mouse survival. Animal studies have proven that CD26 is an essential component of normal granulocyte colony-stimulating factor...
patients and methods

Human G-CSF-mobilised leukapheresis samples were collected from cancer patients (n = 21) selected for autologous clinical transplantation or from their allogeneic related healthy donors (n = 11). Stem cell harvest or leukapheresis samples were obtained after routine peripheral blood stem cell (PBSC) collection. These samples were obtained after written informed consent of relatives and patients. The procedure for collection of this material was in accordance with procedures approved by the human ethics committee of Tata Memorial Centre. The characteristics, clinical history and treatment record of patients who underwent transplant and who were considered for correlation study (autologous, n = 17; allogeneic, n = 11) are summarised in Table 1.

Mobilisation was carried out by daily subcutaneous injection of G-CSF. After mobilisation chemotherapy, PBSC counting started when the leucocyte nadir was passed and the absolute leucocyte concentration was >0.5 x 10⁹/l. Cell viability was examined by light microscopy using trypan blue staining (Invitrogen, Grand Island, NY).

CD34 and CD26 expression by flow cytometry

After red cell lysis, nucleated cells from stem cell harvest were suspended in FACS buffer (phosphate-buffered saline with 1% fetal calf serum and 0.02% sodium azide) and stained with anti-human CD26 conjugated to fluorescein isothiocyanate (FITC; BD Pharmingen, San Jose, CA). Cells were stained with isotype mouse IgG1-FITC, which served as control. The presence of human CD34+ cells was identified by anti-CD45-FITC (Becton Dickinson, San Jose, CA) antibodies. The light-scattering properties and the fluorescence of cells stained with FITC were measured on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, USA). For the analysis of antigen levels, the fluorescence signals corresponding to debris and cell aggregates were first gated out using the forward scatter (FSC) and side scatter (SSC) display. Positively stained cells were gated on various gates. Figure 1A depicts the schematic representation of gated population, while Figure 1B shows gated population on a dot plot of representative stem cell harvest. Area with low SSC and FSC properties was marked as ‘lymphocyte’ (L) gate, that with medium FSC and SSC as ‘monocyte’ (M) gate, and that with high FSC/SSC as ‘neutrophil’ (N) gate. Area including all gates was marked as ‘all populations’ gate. Data were collected, stored and analysed with CELLQuest software (Becton Dickinson). CD34+ cells were defined as described earlier in the International Society for Hematotherapy and Graft Engineering protocol for CD34 analysis [11].

engraftment studies

WBC engraftment was considered to have occurred on the first day of 3 consecutive days in which the absolute count exceeded 1.0 x 10⁹/l. Platelet engraftment was considered to have occurred on the first day of 7 consecutive days in which platelet count exceeded 20 x 10⁹/l. Whole blood was analysed for long-term donor engraftment.
CD26 expression on L-gated cells were 53.7 ± 3.7% (range 16.76%–86.92%) and 20.1 ± 4.8% (range 0.46%–42.13%) for patients and donors, respectively. The difference was statistically highly significant ($P = 0.000007$). Similarly, the increase in CD26 expression in patients compared with healthy donors was statistically significant in case of M gate ($P = 0.00005$), N gate ($P = 0.0011$) and all populations gate ($P = 0.00045$; Figures 1 and 3B).

Figure 2. Laser confocal microscopy images of expression of CD34/CD45 (A) and CD34/CD26 (B) antigens on HSPC and colocalisation. Upper row of panel A shows (serially from left to right) expression of CD45 (green fluorescence), CD34 (red fluorescence), CD34–CD45 expression (yellow fluorescence indicates colocalisation) and CD34–CD45 along with transmission image and only bright-field microscopy image. Horizontal bar at lower right corner of each image indicates scale bar (5 μm). The lower figure in panel A depicts distribution of the colour intensity of CD45 expression on x-axis and CD34 expression on y-axis. The upper right quadrant in this figure indicates intensity of CD34–CD45 expression on HSPC. Panel B from left to right indicates expression of CD34 (red fluorescence), expression of CD26 (green fluorescence) and dual expression (yellow fluorescence). HSPC, haematopoietic stem and progenitor cell.

WBC engraftment was achieved in all patients ($n = 17$) with a mean of 10.1 ± 0.2 days (range 8–11 days) in autologous cases and 13.2 ± 0.3 days (range 11–15 days) in allogeneic transplantation ($n = 11$) cases (Supplemental Figure S1, available at Annals of Oncology online). The faster WBC engraftment seen in autologous cases as compared with allogeneic cases was statistically extremely significant.
Engraftment of platelets was achieved with a mean of 11.5 ± 0.9 days (range 4–19 days) in autologous cases and 10.9 ± 0.9 days (range 7–16 days) in allogeneic transplantation cases (Supplemental Figure S1, available at Annals of Oncology online). It was observed that the difference between days of platelet engraftment in autologous and allogeneic cases was statistically nonsignificant. All patients showed virtually 100% engraftment of donor lymphoid cells, without any evidence of residual recipient cells.

The correlation study was carried out on patients demonstrating complete remission on transplantation day +30. The CD34 and CD26 expression was correlated with days of WBC and platelet engraftment in 28 patients who underwent autologous (n = 17) and allogeneic (n = 11) transplantation. CD34 expression showed statistically significantly negative correlation with WBC (Figure 4A panel a; P = 0.037) and platelet engraftment (Figure 4A panel b; P = 0.0006) in autologous transplantation cases. In autologous + allogeneic cases (n = 28), correlation of CD34 expression with platelet engraftment was statistically significant (Figure 4A panel d; P = 0.008). In allogeneic transplantation cases, correlation was not statistically significant (supplemental Figure S2, available at Annals of Oncology online).

It was observed that the CD26 expression showed negative Pearson correlation with days of WBC engraftment. As evident in Figure 4B, correlation was extremely statistically significant in autologous + allogeneic cases (L gate: P < 0.00001; M gate: P < 0.00001; N gate: P < 0.00001; all populations: P < 0.00001; Figure 4B panels a–d). Correlation with WBC engraftment was statistically significant in autologous cases (L gate: P = 0.011; M gate: P = 0.017; N gate: P = 0.019; all populations: P = 0.037;

**Figure 3.** (A) Scatter plot of percent expression of CD34<sup>hi</sup>CD45<sup>low</sup> positive nucleated cells obtained from PBSC harvest of cancer patients (n = 21) and healthy related donors (n = 11). The International Society for Hematotherapy and Graft Engineering gating method was followed during flow cytometry analysis. Horizontal bar and adjacent values indicate mean ± standard error values. (B) Scatter plot of percent cells expressing CD26. Horizontal bar and adjacent values indicate mean ± standard error values. 'Auto' and 'allo' indicate PBSC from cancer patients (n = 21) and healthy related donors (n = 11), respectively. P values indicate statistical significance analysed by paired t-test for comparison between auto and allo sets within each gate, i.e. from left to right, L-, M-, N- and ALL gates denote lymphocyte, monocyte, neutrophil and all populations gated, respectively. PBSC, peripheral blood stem cell.
Figure 4. This figure represents correlation graphs. (A) Correlation of CD34 expression with days of WBC (a, c) and platelet (b, d) engraftment in autologous transplantation ($n = 21$; a, b) and autologous + allogeneic transplantation ($n = 28$; c, d). (B) Correlation graphs of percent CD26 expression on cells gated on lymphocyte (a, e, i), monocyte (b, f, j), neutrophil (c, g, k) and all populations gates (d, h, l) against days of WBC engraftment in autologous + allogeneic (a–d, $n = 28$), autologous (e–h, $n = 17$) and allogeneic cases (i–l, $n = 11$). Values top right of each section show Pearson’s correlation coefficient $r$ and $P$ value of statistical significance by two-tailed $t$-test. WBC, white blood cell.

Figure 4B panels e–h) and allogeneic cases (L gate: $P = 0.042$; M gate: $P = 0.042$; N gate: $P = 0.019$; all populations: $P = 0.011$; Figure 4B panels i–l).

Although the association of CD26 expression with platelet engraftment was found to be as negative Pearson correlation, the data were not statistically significant (Supplemental Figure S3, available at Annals of Oncology online).

To evaluate the association of CD26 with CD34 expression on cells of stem cell harvest in all transplantation cases, Pearson’s correlation test was applied. It was interesting to note that there was no correlation between CD34 and CD26 expression (Figure 5 panels a–d).

discussion

CD26 is a transmembrane glycoprotein with intrinsic dipeptidyl peptidase-IV activity that is expressed on numerous cell types and has a multitude of biological functions. An important aspect of CD26 biology is its peptidase activity and its functional and physical association with molecules with key roles in various cellular pathways and biological programs. Role of CD26 in immune regulation has been extensively characterised, with recent findings elucidating its linkage with signalling pathways and structures involved in

Figure 5. Association between percent CD34 and CD26 expression (panel a: L-gated cells; panel b: M-gated cells, panel c: N-gated cells and panel d: all-gated population) in nucleated cells of PBSC harvest ($n = 28$). The Pearson’s correlation coefficient $r$ for all graphs was 0. L-, M-, N- and ALL gates denote lymphocyte, monocyte, neutrophil and all populations gated, respectively. PBSC, peripheral blood stem cell.

T-lymphocyte activation as well as antigen-presenting cell–T-cell interaction [12].

In this study, we have analysed CD26 expression in human mobilised PBSC harvest from cancer patients and allogeneic
related donors. Most of the earlier reported studies on CD26 expression on stem cells were carried out in mice. Earlier studies reported by others showed CD26 expression on CD34+ cord blood cells [13] or Sca-1+ c-kit+ cells in mobilised mice peripheral blood [8] but have studied its correlation with allogeneic HSCT in mice. Bonig et al. [10] have observed low expression of CD26 on enriched CD34+ cells from mobilised peripheral blood than bone marrow samples. Nevertheless, they have not given absolute numbers or percentages of CD26 expression. We observed high expression of CD26 on CD34+ cells (80%–90%; Supplemental Figure S4, available at Annals of Oncology online). We hypothesised that since complete leukapheresis product is used during transplantation, it is ideal to profile CD26 expression in all cell populations from harvest instead of looking only at CD34+ cells [14] or c-kit+ cells [7] as reported by previous authors.

It is reported that G-CSF up-regulates CD26 expression [14]. In line with this observation, in our study, cells in stem cell harvest post-G-CSF mobilisation of cancer patients showed increased percent expression of CD26 peptidease. Cells in stem cell harvest of allogeneic donors exhibited decreased expression of CD26. The mechanism underlying the decrease in CD26 expression of cells from healthy related donors remains to be investigated. Our study forms the first report that profiles CD26 expression on all cell populations in stem cell harvest. Since soluble CD26 also demonstrates similar activity as membrane-anchored form [11], investigations of kinetics of soluble CD26 levels in transplanted patients may add valuable information in knowledge towards increasing efficiency of transplantation. Circulating CD26 levels have not been reported earlier in transplantation studies. Nevertheless, in arthritis, decreased circulating CD26 levels have been shown to influence CD26-mediated regulation of stromal derived factor 1 (SDF1)- CXCR4 signaling axis [5]. In our study too, circulating CD26 levels remain to be investigated.

CD26 cleaves a large number of chemokines and peptide hormones in vitro, but comparatively fewer peptides have been identified as endogenous physiological substrates for CD26 in vivo [1]. One of the substrates identified is chemokine SDF1, which plays a crucial role in homing/mobilisation of HSPCs to and from bone marrow to blood and tissues [13, 15]. CD26 plays a crucial role in control of immune function, inflammatory responses and behaviour [1]. Circulatory CD26 has been implicated in modulation of inflammatory response in experimental arthritis [5]. Increased levels of SDF1 and its receptor CXCR4 have been observed in antigen-induced arthritis in CD26 knockout mice. The severity of rheumatoid arthritis has been reported to be negatively associated with CD26 activity and plasma levels [5]. Furthermore, the reduction in natural killer cell antitumour activity [16] and CD4+ T/NKT cell numbers [17], and reduced plasma levels of cytokines or immunoglobulins and other dysfunction in immune response [18] were observed in CD26 knockout animal models. Bonig et al. [10] observed no effect of CD26 inhibition on homing of mobilised peripheral blood cells. Therefore, CD26 inhibition as reported earlier by others [6–9] may not be a viable alternative for increasing HSCT efficiency as it may lead to immune imbalance. Instead, our study forms the first report underpinning the inverse correlation of CD26 expression with day of engraftment, i.e. higher expression leads to faster engraftment.

CD34 expression is a known predictor of engraftment. In our study, autologous cases showed good correlation with WBC and platelet engraftment. Nevertheless, in allogeneic cases, CD34 expression failed to exhibit statistical correlation with WBC or platelet engraftment. In both autologous and allogeneic cases, CD34 expression failed to show correlation with WBC but showed significant correlation with platelet engraftment. It was also observed that transplanted CD34+ cell dose did not obtain a close correlation with day of engraftment (data not shown), a phenomenon in agreement with other reports [19]. Other receptors like CD133 in humans [19] or CD49e+ and CD43+ in mice [20] have been implicated as potential markers of engraftment; nevertheless, evidence is needed to prove the results. In this study, we have evaluated CD26 as a potential predictor of engraftment. It was observed that CD26 expression had a strong negative correlation with day of WBC engraftment in autologous transplant, allogeneic transplant and autologous + allogeneic transplant cases. The data corroborated well with day of WBC engraftment and CD26 expression in autologous and allogeneic cases. WBC engraftment for autologous cases was significantly ($P = < 0.0000001$; Supplemental Figure S1, available at Annals of Oncology online) faster than that of allogeneic cases. It was observed that CD26 expression level was significantly lower ($P < 0.001$) in allogeneic cases as compared with autologous cases (Figure 3B). Thus, higher CD26 expression in autologous cases leads to faster WBC engraftment, while lower CD26 expression in allogeneic cases leads to slower WBC engraftment. Similar trend of association was also noted in allogeneic cases regarding the association of CD26 expression with platelet engraftment; nevertheless, it was not statistically significant. It was interesting to note that CD34 and CD26 expression exhibited negligible correlation. These data strongly indicate that CD26 may be considered as an independent predictor of engraftment.

In a parallel study, we have observed that SDF1-manipulated HSPC leads to up-regulation of CD26 along with increased ex vivo expansion of progenitor cells (Supplemental Figure S5, available at Annals of Oncology online). These data support earlier observation by Christopherson et al. [14] that growth factors up-regulate CD26 expression. When nucleated cells from mobilised peripheral blood were incubated in the presence of a mixture of growth factors (with or without SDF1), CD26 expression was significantly up-regulated on these cells.

It was observed that CD26 expression on G-CSF-mobilised peripheral blood cells of healthy donor cases was significantly lower than that on cells from cancer patients. This phenomenon has not been reported anywhere previously and the underlying mechanism needs to be explored.

To summarise, we can say that this study has provided important leads that CD26 peptidase is expressed significantly on cells from mobilised peripheral blood harvest and it shows negative correlation with days of WBC engraftment in transplanted patients. As it failed to show correlation with CD34 expression, which is a known predictor of engraftment, it could be considered as a potential independent predictor of engraftment. We agree that the sample size is very small and
a more detailed study with large number of patients and investigation of mechanism underlying the phenomenon is warranted. It would also be important to correlate circulating levels of CD26 with engraftment before we prove association of CD26 expression with engraftment.

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References