[Frontiers in Bioscience 13, 1795-1805, January 1, 2008]

CD26 inhibition and hematopoiesis: a novel approach to enhance transplantation

Timothy B. Campbell, Hal E. Broxmeyer

Department of Microbiology and Immunology, and the Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202 and the Walther Cancer Institute, Indianapolis, IN 46208

TABLE OF CONTENTS

1. ABSTRACT

Dipeptidylpeptidase IV/CD26 is expressed on the surface of various cell types. Through its enzymatic activity, its major function is to cleave the N-terminal dipeptide from diverse molecules including members of the chemokine family of cytokines. The N-terminus of these chemokines is important for activation of and binding to seven-transmembrane G-protein linked chemokine receptors, and early studies showed truncation by CD26 physiologically alters these properties of select chemokines resulting in diverse functional outcomes. Stromal-derived factor-1 (SDF-1/CXCL12), a chemokine involved in hematopoietic cell chemotaxis, homing, mobilization and survival, is cleaved by CD26 producing a form that is inactive in CXCR4 signaling and has some antagonistic properties *in vitro*. Recent studies have shown that the inhibition of cell-surface CD26 peptidase activity on hematopoietic stem/progenitor cell (HSC/HPC) populations increases their SDF-1/CXCL12 directed chemotaxis *in vitro*, and *in vivo* homing and engraftment. CD26 inhibition may, therefore, represent a novel approach to increasing the efficacy and success of HSC/HPC transplantation, especially under conditions of limiting donor cell yield.

2. INTRODUCTION

Hematopoiesis is a highly coordinated and regulated process involving self-renewal and proliferation of hematopoietic stem cells (HSCs), differentiation of HSCs to hematopoietic progenitor cells (HPCs) and mature blood cells, and trafficking of HSCs/HPCs and more mature blood cells throughout the body in response to injury, inflammation and other stresses. Small protein mediators such as growth factors and chemokines play important roles in many of these processes (1). Chemokines are proteins which signal and cause the directed movement, i.e. chemotaxis, of blood cells to and from various places in the body. The interaction of chemokines and seven-transmembrane G-protein linked chemokine receptors is an important component of hematopoietic functions including T-cell migration to lymph nodes and to sites of inflammation, antigen presenting cell recruitment, and HSC/HPC homing and retention in the bone marrow (2-6). Chemokine signaling is a powerful mechanism to help control hematopoiesis and inflammation, and regulation of this process is necessary for normal physiological responses. The N-terminus of various chemokines is important for normal function and signaling (3, 7, 8).

Enzymes which cleave the N-terminus of these proteins would be expected to have important roles in modulating hematopoiesis.

Dipeptidylpeptidase IV/CD26 is a cell surface dipeptidase expressed widely throughout the body (9). The structure of CD26 is a 110-kDA glycoprotein with a small cytoplasmic region, a transmembrane section and an extracellular section containing the enzymatic activity (9). The dipeptidylpeptidase region of CD26 cleaves the N-terminal dipeptide from various substrates including neuropeptides, hormones and chemokines at the penultimate proline or alanine residue (9-11). CD26 cleavage of chemokines is physiologically relevant and alters the function of many of these proteins. It is clear that the action of CD26 in particular on the chemokine stromal-derived factor- 1 (SDF-1/CXCL12) has biological consequences, especially regarding hematopoietic stem cell chemotaxis (12). This review will focus on earlier work done on CD26 and chemokines, with emphasis on the effect of CD26 on SDF-1/CXCL12 and the most recent work on its role in HSC/HPC biology.

3. EARLY WORK ON CD26 AND CHEMOKINES

It has been known for over a decade that CD26 is capable of cleaving different chemokines, with the result in most cases being an alteration of the function of that chemokine. Some chemokines shown to be cleaved by CD26 include regulated on activation, normal T cell expressed and secreted (RANTES/CCL5); granulocyte chemotactic protein-2 (GCP-2/CXCL6); macrophage inflammatory protein-1β (MIP-1β/CCL4); macrophage-derived chemokine (MDC/CCL22); the CXCR3 ligands monokine induced by IFN-γ (Mig/CXCL9), IFN-γ-inducible protein-10 (IP-10/CXCL10) and IFN-inducible T-cell α-chemoattractant (I-TAC/CXCL11); eotaxin/CCL11; macrophage inflammatory protein-1α (MIP-1α/LD78β/CCL3-L1) and stromal derived factor- 1 (SDF-1/CXCL12) (13-27). One of the first substrates shown to be physiologically cleaved by CD26 was RANTES/CCL5, and the studies on this chemokine illustrate the powerful effect CD26 can have on the function of these substrates (13-15).

3.1. The ability of RANTES/CCL5 to signal and receptor preference are altered by CD26 truncation

RANTES/CCL5 is a chemokine involved in cellular migration during inflammation, through its action on three receptors: CCR1, CCR3 and CCR5. Two major studies in 1997 and 1998 by Oravecz *et al* (13)and Proost *et al* (14), respectively, investigated the role of CD26 in the N-terminal dipeptide cleavage of RANTES/CCL5 and the effect of this cleavage on monocyte cell biology. Both groups showed that soluble CD26 could indeed cleave the N-terminal dipeptide from RANTES/CCL5. This cleavage was physiologically relevant since the cleaved form was isolated from cell cultures (14). The truncated form of RANTES/CCL5 was unable to stimulate migration of a monocyte cell line, THP-1, in chemotaxis assays and was greatly reduced in its ability to stimulate calcium release in both primary monocytes and a cell line, indicating a failure to fully activate the receptor (14). Proost *et al* (14) showed further that pre-treating THP-1 cells with truncated RANTES/CCL5 was able to desensitize the response of the cells, as measured by intracellular calcium release, to full-length RANTES/CCL5 as well as inhibit the migration of the cells to the full-length molecule. However, this desensitization was seen using much higher concentrations of truncated RANTES/CCL5 compared to full-length, again indicating an inability of the cleaved form to fully bind to and/or signal through the receptor. Oravecz *et al* (13) found interestingly that only immature monocytes, but not monocytes matured using M-CSF (M-CSF-derived macrophages), had a defective signaling response to the cleaved form of RANTES/CCL5, indicating a difference between these two cell types. Through a series of mechanistic experiments, they were able to show that truncated RANTES/CCL5 was unable to signal significantly through CCR1 (on monocytes and M-CSF-derived macrophages), but was able to signal normally through CCR5 (only on M-CSF-derived macrophages) (13). Receptor selectivity was changed by CD26 cleavage, explaining the different responses in these two cell types. Both groups showed that cleaved RANTES/CCL5 (aa 3-68) had an equal or enhanced ability to inhibit HIV infection in *in vitro* models presumably by either steric hindrance or down-modulation of the CCR5 receptor, a co-receptor for M-tropic HIV strains. Further work by Struyf *et al* showed that truncated RANTES/CCL5 had significantly decreased signaling through its third chemokine receptor CCR3 (15). These studies demonstrate that the simple act of CD26 cleaving a dipeptide from a chemokine can have drastic effects on its biology, including skewing the receptor preference. The importance of the loss of the N-terminus in determining this preference had been shown in a previous study by Gong *et al* (28) using synthetic RANTES/CCL5 mutants.

3.2. CD26 cleaves other chemokines resulting in varied functional outcomes

In addition to RANTES/CCL5, CD26 cleaves other chemokines, including MIP-1β/CCL4 (16, 17); MDC/CCL22 (18, 19); the CXCR3 ligands Mig/CXCL9, IP-10/CXCL10 and I-TAC/CXCL11 (21, 22); eotaxin/CCL11 (20), MIP-1α/LD78β/CCL3 (23), and GCP-2/CXCL6 (14) illustrating the wide repertoire available to this peptidase.

3.2.1. Receptor specificity of MIP1β is altered by DPPIV cleavage

The cleaved form of MIP1β is able to signal normally through CCR5, the cognate receptor for the full-length molecule, and is able also to signal through two additional receptors: CCR1 and CCR2b, indicating a change in receptor specificity due to CD26 processing (17). Truncated MIP-1β/CCL4, resulting from CD26 cleavage, can be isolated from lymphocyte cultures suggesting a physiological role for this molecule (16).

3.2.2. DPPIV cleavage of MDC/CCL22 abrogates CCR4 signaling

Cleavage of MDC/CCL22 by CD26 produces a truncated MDC molecule significantly impaired in CCR4

signaling and CCR4-induced chemotaxis in lymphocytes. However, truncated MDC/CCL22 induces chemotaxis of monocytes, binds to monocytes normally and inhibits the HIV infection of monocytes as well or better than full-length MDC/CCL22, suggesting that truncated MDC/CCL22 has altered receptor specificity by losing the ability to signal through CCR4, but maintaining the ability to signal through an unidentified receptor found on monocytes but not on lymphocytes (18, 19). Interestingly, CD26 cleaves not only the first two N-terminal residues of MDC/CCL22 but, in a subsequent step, cleaves after the fourth residue (glycine) to produce MDC/CCL22 (aa 5-69) (18). MDC/CCL22 is an exception to the selectivity of CD26 cleavage.

3.2.3. CD26 cleaves the CXCR3 ligands IP-10/CXCL10, I-TAC/CXCL11 and Mig/CXCL9 resulting in functional changes

A physiologically-relevant concentration of recombinant CD26 cleaves the N-terminus from IP-10/CXCL10, I-TAC/CXCL11 and Mig/CXCL9 (21). All three of these cleaved chemokines are ineffective at inducing chemotaxis in CXCR3-transfected cell lines; cleaved IP-10 and I-TAC are also unable to stimulate chemotaxis of primary lymphocytes (21, 22). The signaling ability of cleaved IP-10 and I-TAC chemokines, as measured by calcium release assays, is greatly reduced; however, their receptor binding is only modestly decreased (21, 22). Cleaved Mig, in contrast, does not have significantly decreased binding to nor activation of CXCR3 (21). Both truncated IP-10 and I-TAC are able to act as antagonists to the full-length chemokines in signaling assays as well as chemotaxis assays (21, 22). Interestingly, cleaved I-TAC has some ability alone to downregulate the surface expression of CXCR3, although not as effectively as the full-length molecule (22). This ability to downregulate CXCR3 is likely by an alternative signaling pathway since no detectable calcium release is caused by truncated I-TAC.

3.2.4. Cleaved eotaxin/CCL11 is defective in CCR3 signaling but not in binding to CCR3

Eotaxin/CCL11 is truncated by CD26, producing a form that is greatly impaired in signaling through its receptor CCR3 but is only partially impaired in binding (20). The result is a protein which cannot induce chemotaxis of eosinophils but can act as an antagonist of chemotaxis towards full-length eotaxin/CCL11 and can desensitize the cells to stimulation by the full-length molecule. In addition, the truncated form inhibits HIV infection in an eosinophil model as effectively as the full-length molecule. The truncation of eotaxin/CCL11 produces a protein which interferes with the normal functioning of the receptor presumably by steric hindrance mechanisms (20).

3.2.5. MIP-1α/LD78β/CCL3-L1 is activated by DPPIV cleavage

The effect of CD26 cleavage on MIP-1α/LD78β/CCL3-L1, an isoform of MIP1α/LD78α/CCL3, is different than most other chemokines summarized. Instead of diminishing the activity or altering the receptor specificity, truncation of MIP-1α/LD78β/CCL3-L1 produces a form which is more potent in signaling through two out of three of its cognate receptors, CCR1 and CCR5, than the full-length molecule. The cleaved protein is able to stimulate chemotaxis of monocytes and lymphocytes better than full-length MIP-1α/LD78β/CCL3-L1 (23). The cleavage of MIP-1α/LD78β/CCL3-L1 by CD26 is physiologically relevant since the truncated form can be isolated from monocyte conditioned medium (23). The full-length isoform MIP-1α/LD78β/CCL3-L1 can signal through CCR3, an additional receptor not utilized by the MIP1α/LD78α/CCL3 isoform (24). Cleavage by CD26 decreases this ability to signal through CCR3, in contrast its effects on signaling through CCR1 and CCR5 (24).

3.2.6. GCP-2/CXCL6 activity is unaffected by DPPIV cleavage

Truncated GCP-2/CXCL6 can be isolated from cell cultures, and purified CD26 can cleave the full-length molecule *ex vivo*, indicating this form may have a physiological role. However, CD26 cleavage has no effect on the signaling of GCP-2/CXCL6 in peripheral blood neutrophils. Truncated GCP-2/CXCL6 can signal as effectively as full-length in calcium release assays in these cells (14). Chemotaxis and other functional assays have not been reported using truncated GCP-2. Until these studies are performed, the possibility that cleaved GCP-2 has functional changes not reflected by calcium assays is open.

3.3. CD26 cleaves SDF-1/CXCL12 resulting in loss of signaling and gain of antagonistic properties

SDF-1/CXCL12 plays an important role in hematopoietic cell chemotaxis, mobilization, homing, and engraftment (29-31). SDF-1/CXCL12 signals through the CXCR4 receptor, which up until recently was thought to be the only receptor involved. However, a series of studies have also identified a role for CXCR7 as a receptor for SDF-1/CXCL12 (32-36). SDF-1/CXCL12 acts as a competitive inhibitor of T-tropic HIV viral binding, since this virus uses CXCR4 on lymphocytes as a co-receptor (25, 26). Like many chemokines studied to date, structure-function experiments have shown that the N-terminus of SDF-1/CXCL12 is important for CXCR4 receptor binding and activation, with activation being highly dependent on the first two residues. In fact, loss of the first two residues, resulting in the form SDF-1/CXCL12 (aa 3-68), abolishes CXCR4 activation but only modestly impairs CXCR4 binding (about a 10-fold decreased binding affinity) (7). The role of CD26 in the physiological cleavage of SDF-1/CXCL12 was established by a series of studies published by Proost *et al (*25), Shioda *et al* (26)and Ohtsuki *et al (*27). Similar to the studies on RANTES/CCL5, these groups examined the effect of CD26-mediated SDF-1/CXCL12 cleavage on chemotaxis, CXCR4 activation and HIV inhibition in both cell lines and primary peripheral blood cells. These studies demonstrated that CD26 could indeed cleave SDF-1/CXCL12 under physiological conditions in transfected cell lines overexpressing CD26; and recombinant soluble CD26 could cleave SDF-1/CXCL12 *in vitro*. Truncation of SDF-1/CXCL12 by recombinant CD26 produced SDF-1/CXCL12 (aa 3-68), a truncated molecule which lost its ability to induce chemotaxis - similar to the

Table 1. CD26-truncated chemokines

CD26-Truncated Chemokine	Functional Changes	References
RANTES/CCL5 (aa 3-68)	Change in receptor selectivity Decreased CCR1 activity Decreased CCR3 activity Equal or Increased CCR5 activity	13, 14, 15
MIP1β/CCL4 (aa 3-69)	Change in receptor specificity Addition of CCR1 and CCR2b activity No change in cognate CCR5 activity	16, 17
MDC/CCL22 (aa 3-69 and aa 5-69)	Decreased activation of CCR4 Possible activity via unidentified receptor on monocytes	18, 19
Eotaxin/CCL11 (aa 3-74)	Minimal decrease in CCR3 binding Loss of CCR3 activation	20
MIP1α/LD78β/CCL3-L1 (aa 3-70)	Increased activity through receptors CCR5 and CCR1 Decreased activity through CCR3	23
GCP-2/CXCL6 (aa 3-77)	No changes in intracellular calcium release from neutrophils	14
IP-10/CXCL10 (aa 3-77)	CXCR3 binding and activation are significantly reduced	21
I-TAC/CXCL11 (aa 3-73)	Binding to CXCR3 is modestly reduced CXCR3 intracellular calcium release is significantly reduced CXCR3 internalization is modestly reduced	21, 22
Mig/CXCL9 (aa 3-103)	No significant decrease in CXCR3 binding nor intracellular calcium release	21
SDF-1/CXCL12 (aa 3-68)	Substantially reduced activation of CXCR4 Binding to CXCR4 is modestly reduced	7, 25, 26, 27

situation with other chemokines summarized thus far (e.g. RANTES/CCL5, eotaxin/CCL11, and MDC/CCL22). In contrast to the full-length chemokine, SDF-1/CXCL12 (aa 3-68) had very poor ability to inhibit HIV infection *in vitro* (25, 26). In intracellular calcium release assays by Proost *et al* (25), the truncated SDF-1/CXCL12 (aa 3-68) had some ability to desensitize subsequent signals from full-length SDF-1/CXCL12, but this was at significantly higher concentrations of the cleaved form compared to full-length. Importantly, even at these higher concentrations, the cleaved form could not stimulate calcium release and thus was not activating CXCR4. This desensitization can be explained by a mechanism in which SDF-1/CXCL12 (3-68) cannot signal through the receptor but can bind, albeit less efficiently, to CXCR4. This would result in some ability to block the response to full-length SDF-1/CXCL12, similar to the case with eotaxin/CCL11, presumably by steric hindrance mechanisms. This model is supported by the structure-function study performed on N-terminal truncated SDF-1/CXCL12 molecules, which showed a strong correlation between their ability to bind to CXCR4 and antagonistic behavior but less of a correlation between their ability (or lack thereof) to activate receptor signaling and antagonism (7). We should note that Proost *et al* (25) found this effect of cleaved SDF-1/CXCL12 on desensitization, but Shioda *et al* (26) stated they did not. An alternative explanation for the desensitizing effect of cleaved SDF-1 (aa 3-68) could be an ability to downmodulate CXCR4 by activating an alternative signaling pathway not involving, and therefore not measured by, calcium release. The existence of such a pathway is supported by the data summarized earlier on I-TAC/CXCL11 (22). Table 1 summarizes the data on the functional changes of CD26 truncated chemokines. For further information on CD26 cleavage of various substrates, including chemokines, the interested reader can consult detailed reviews, broader in scope than our current review, written by many of the researchers who initially described these effects: references (37-40).

All of these examples illustrate two major concepts: (1) the N-terminus plays an important role in determining chemokine binding, activation and specificity, and (2) CD26 can affect the *in vitro* function of these chemokines by N-terminus cleavage. For SDF-1/CXCL12 specifically, the chemotactic and anti-HIV functions are highly dependent on the N-terminus. Cleavage by CD26 abolishes these functions and produces a molecule with possible antagonistic properties towards full-length SDF-1/CXCL12. Since SDF-1/CXCL12 helps control multiple steps in hematopoiesis, our laboratory began work on studying the role of CD26-mediated cleavage in these processes.

4. INHIBITION OF CD26 AFFECTS MOUSE HSC HOMING AND ENGRAFTMENT

The functional qualities of cleaved SDF-1/CXCL12 suggested that it may not be very useful in anti-HIV treatment, and little work has been done on this relationship since the early papers. The fact that CD26 can cleave SDF-1/CXCL12 to a chemotactically-null molecule was of interest to our lab since part of our research has dealt with SDF-1/CXCL12 chemotaxis, signaling and related effects on HSCs/HPCs. Kent Christopherson II, a former post-doctoral member of the Broxmeyer laboratory, with other members in the laboratory, performed the seminal work on CD26 and SDF-1/CXCL12, especially with regard to chemotaxis, homing and engraftment of hematopoietic cells.

Christopherson and colleagues in the Broxmeyer laboratory showed that immature HPCs/HSCs from both human cord blood (CB) and mouse bone marrow (BM) expressed CD26 on their surface (12, 41, 42). Purified $CD34^+$ cells, a population enriched for HPCs/HSCs, from human CB were positive for surface expression of CD26 (about 8% of total population) (12). Furthermore, a higher percentage of $CD34^+CD38^-$ cells from human CB, a population enriched for HSCs compared to HPCs, expressed CD26 as compared to the more mature population $CD34^+CD38^+$ (43). CD26 was present on the surface of 70% of mouse HSCs, phenotypically defined by their expression of c-kit and sca-1 antigens and lack of

lineage antigens (c-kit$^+$sca1$^+$lin$^-$) (41). Both human and mouse hematopoietic CD26$^+$ cells have functional CD26 peptidase activity measured by an *in vitro* enzymatic assay utilizing a chromogenic reaction. The fact that both mouse and human HSCs/HPCs expressed functional CD26 suggested that it may play a role in SDF-1/CXCL12 mediated functions such as chemotaxis.

HSCs/HPCs from both mouse and human sources express CXCR4, the receptor for SDF-1/CXCL12, and can exhibit chemotaxis towards a positive gradient of SDF-1/CXCL12. CD26, expressed on the surface of these cells, could be expected to negatively affect chemotaxis by cleaving full-length SDF-1/CXCL12 to a form that cannot induce chemotaxis. Thus, by using a selective inhibitor of CD26, such as Diprotin A or Val-Pyr (41, 44), one could predict that chemotaxis could be enhanced by protecting the full-length SDF-1/CXCL12 from truncation and allowing it to signal through CXCR4. Pre-treating human CB CD34$^+$ cells with the CD26 inhibitor Diprotin A significantly increased the percentage of cells able to migrate to SDF-1/CXCL12 (12). Furthermore, FACS sorted CD26$^-$ cells, taken from the total CD34$^+$ population, had increased chemotaxis compared to unsorted cells and were unaffected by Diprotin A, as expected. This suggested that the CD26$^+$ fraction had a negative effect on the chemotaxis of the CD34$^+$ CB cells.

In studies on mouse HSCs/HPCs, Christopherson and members of the Broxmeyer laboratory found that Diprotin A pre-treatment of these cells again enhanced SDF-1/CXCL12 chemotaxis of these cells *in vitro* (41, 42). In addition, purified truncated SDF-1/CXCL12 (aa 3-68) was unable to stimulate chemotaxis; however, pre-incubation of these cells with the truncated form decreased chemotaxis to full-length SDF-1/CXCL12 (41). In these experiments, similar concentrations of truncated and full-length SDF-1/CXCL12 were used, which is in contrast to some of the early papers showing that truncated SDF-1/CXCL12 could only desensitize CXCR4 at very high concentrations. This desensitization can be explained by a mechanism in which cleaved SDF-1/CXCL12 (aa 3-68) can bind to but cannot activate CXCR4, thus sterically inhibiting binding of the full-length chemokine.

The effect of CD26 on SDF-1/CXCL12 induced *in vitro* chemotaxis was clear from these studies. However, SDF-1/CXCL12 and CXCR4 are known to play important roles in *in vivo* processes such as mobilization, homing and engraftment of HSCs/HPCs. The role of CD26 in these clinically relevant processes in the mouse was established by the Broxmeyer lab through Christopherson's continued work.

Mobilization refers to the ability of HSC/HPCs to be released from the bone marrow into the peripheral blood stream usually in response to drugs and/or growth factors (41, 45, 46). G-CSF is one growth factor that can cause mobilization of mouse HSCs/HPCs by mechanisms that are just now beginning to be understood (45).The SDF-1:CXCR4 axis is known to play an important role in mobilization; disruption of this axis by competitive inhibitors of SDF-1/CXCL12 binding to CXCR4, such as AMD3100, can lead to mobilization (29, 30). Because it can cleave SDF-1/CXCL12, the possible role of CD26 in mediating *in vivo* G-CSF mobilization was studied. Two different strains of mice (C57BL/6 and DBA/2) were treated with two CD26 inhibitors, Diprotin A and Val-Pyr, during G-CSF induced mobilization and the extent of mobilization of HPCs was measured by colony-forming assays using peripheral blood cells. Both CD26 inhibitors reduced the numbers of G-CSF mobilized colony-forming cells (= HPCs) in the periphery in both strains of mice (41). In further studies using CD26 knockout mice, the absence of CD26 substantially decreased normal G-CSF induced mobilization when compared to wild-type mice (46). Thus, CD26 was shown to have a role in the mechanism of G-CSF induced mobilization. One explanation for these results is a reduced ability of CD26 null or inhibited cells to cleave SDF-1/CXCL12 and break the SDF-1:CXCR4 anchor holding HSC/HPCs in the BM.

The implication of these studies was that CD26 plays an important role in regulating the response of HSCs/HPCs to SDF-1/CXCL12 both *in vitro* and *in vivo*. However, the most important question was what inhibition of CD26 would do to homing and engraftment of HSCs/HPCs *in vivo*. This was especially relevant considering the important role that the SDF-1:CXCR4 interaction plays in homing of HSCs to the bone marrow (31). Similar to mobilization, it has been shown that manipulation of this axis can negatively affect the ability of HSCs to engraft BM (31). The *in vitro* effects of inhibiting CD26 activity on chemotaxis, in fact, predicted the *in vivo* effects on homing and engraftment. Pre-treating donor HSC/HPCs in a congenic mouse transplant assay, i.e. mouse donor cells into mouse recipients, with Diprotin A or Val-Pyr for short periods before transplant significantly and dramatically increased the short-term homing, long-term engraftment, competitive repopulation of the donor cells, and secondary repopulation of donor cells, a measure of the self-renewal ability of donor HSCs (42). As a control, CD26-/- mouse donor HSCs were transplanted and found to have increased homing and engraftment compared to wild-type (42). The effect of CD26 on engraftment and repopulation of donor cells was seen under conditions of limiting cell numbers; that is, in these mouse models CD26 inhibition can lower the threshold needed to get adequate repopulation and survival of lethally irradiated recipient mice (42).

The mechanism explaining these observations that short-term CD26 inhibition on donor cells in congenic mouse models leads to enhanced long-term engraftment likely involves a primary effect on homing of the cells. Homing refers to a process in which the freshly transplanted donor cells move out of the circulation and into the bone marrow niche to take up residence (47, 48). It is thought to occur shortly after injection of donor cells into a recipient, and is usually measured less than 48 hours after transplantation. This process is thought to precede and is a requirement for proliferation and differentiation of HSCs/HPCs in the BM niche. Since Diprotin A and Val-Pyr are short-term reversible inhibitors which maintain

maximal inhibition of CD26 up to four hours after removal and then begin to slowly decrease in inhibition (42), it is likely that the homing and/or retention process is targeted and responsible for the increases in long-term engraftment found in these experiments. As mentioned above, an increase in homing of the CD26 inhibited or CD26-/- donor cells was indeed observed (42). Although other explanations are feasible and have not been ruled out, the effect on homing is one of the most logical at this time.

The effect seen on engraftment in mouse transplants has been reproduced in two studies by independent laboratories. The first group, Tian *et al*, showed that inhibition of CD26 could enhance engraftment of limiting numbers of virally-transduced hematopoietic cells expressing a recombinant allogeneic MHC class I molecule (49). The authors showed that the enhanced level of engraftment seen in animals transplanted with Diprotin A pre-treated cells correlated with decreased rejection of an allogeneic skin graft (49). The second group to confirm and expand our laboratory's findings was Peranteau *et al* (50). This group showed that CD26 inhibition significantly increased homing and engraftment in the context of non-ablative, allogeneic *in utero* hematopoietic-cell transplantation (IUHCT). Specifically, they were able to show that short-term homing (4 hours or 48 hours) of transplanted whole BM and purified HSCs pre-treated with Diprotin A was enhanced, the number of mice with positive engraftment was increased, the level of stable engraftment was increased up to 6 months post-transplant, and the Diprotin A treated cells could dramatically outcompete non-treated congenic donor cells in competitive IUHCT experiments (50).

5. INHIBITION OF CD26 AFFECTS HUMAN CD34$^+$ CELL ENGRAFTMENT *IN VIVO*

The studies published by Christopherson while in the Broxmeyer laboratory focused primarily on the role of inhibiting CD26 in a congenic mouse transplant model using mouse donor cells and mouse recipients. Before this strategy advanced further towards translational clinical utility, it was necessary to investigate the role of CD26 in a more clinically relevant model. Therefore our laboratory decided to study CD26 inhibition using the NOD/SCID xenogeneic model. The NOD/SCID model is a widely accepted tool for assessing human HSC engraftment *in vivo* (51, 52). It involves sub-lethally irradiating recipient mice and injecting various numbers of CD34$^+$ purified human HSCs/HPCs. The effect of short pre-treatment of Diprotin A on the engraftment of human CB CD34$^+$ cells, a population containing both HSCs and HPCs (53), was tested using this model. Human CB is a clinically relevant HSC/HPC source to study (54), and given that one of the major limitations to expanded CB use is the low numbers of cells obtained, it is a perfect candidate for therapies like CD26 inhibition which attempt to increase homing. In fact, CB transplantation has benefits over other sources of cells including lower graft vs. host disease, lower risk of viral transmission and no harm to the donor (54, 55). Using the NOD/SCID model, CD26 inhibitor pre-treatment significantly enhanced the CD34$^+$ cell engraftment, similar to the previous mouse congenic transplant studies (43). In addition, pre-treatment of an impure population of HSC/HPCs (less than 40% CD34$^+$) led to significant enhancement of engraftment, a situation which is more clinically relevant since pure CD34$^+$ cells are not used in CB transplants. This effect on CD34$^+$ cell engraftment seemed to be related to the level of engraftment attained in the recipient mice using control treated cells. For example, when very low, almost immeasurable engraftment was attained with control cells, Diprotin A did not seem to have an effect. Similarly, when high levels of engraftment were reached with control cells, Diprotin A treatment did not seem to enhance nor reduce the engraftment. The effect of Diprotin A was most prominent when control cell engraftment was easily measurable, but still less than 20% chimerism. Hematopoietic cell homing and engraftment are complex processes and it may be the case that in the human system CD26 inhibition cannot overcome barriers to engraftment (seen in the very low transplants) and likewise cannot enhance an already robust or maximal transplant. The differentiation, as measured by expression of phenotypic surface molecules, of the human cells once engrafted in the NOD/SCID animals was not significantly affected by Diprotin A pre-treatment, suggesting that this treatment does not push the cells towards one lineage as opposed to others (43). The effect of this treatment on T-cells, however, cannot be evaluated at this time since T-cell differentiation of transplanted human cells in the NOD/SCID mice is inconsistent (56, 57). Better models, including immunodeficient mouse strains which allow robust T-cell differentiation will help to fully answer any concerns about negative effects of Diprotin A on lineage differentiation.

Studies by two independent laboratories published at the same time as our own confirmed the effect of inhibition of CD26 on engraftment of human HSCs (58, 59). The first, published by Christopherson *et al* (58) showed that pre-treatment of either CD34$^+$-purified or lineage-depleted human CB cells with Diprotin A resulted in enhanced engraftment of NOD/SCIDβ2m^{null} mice. The NOD/SCIDβ2m^{null} model is an enhancement of the NOD/SCID strain by deletion of the β_2-microglobulin gene, leading to fewer natural killer (NK) cells and better engraftment of the human cells (60, 61). The effect of Diprotin A in this study was more dramatic in the lineage-negative CB population, which had 7.5 fold increased engraftment compared to a 4 fold increase in highly purified CD34$^+$ cell treated with Diprotin A. The percent of CD26$^+$ cells was higher in the lineage-negative population compared to the CD34$^+$ population as was the CD26 activity, while only about 30% of the lineage-negative population was CD34$^+$. This suggests that CD34$^-$CD26$^+$ accessory cells negatively affect engraftment of the repopulating HSCs and that inhibiting these cells as well as the CD34$^+$CD26$^+$ cells leads to dramatic increases in cell engraftment. The second study on human HSCs and CD26 was published by Kawai *et al* (59) and showed that *in vivo* treatment of NOD/SCID animals with Diprotin A significantly enhanced the engraftment of G-CSF mobilized peripheral blood (MPB) CD34$^+$ cells. In addition, this treatment could enhance the engraftment of retrovirally

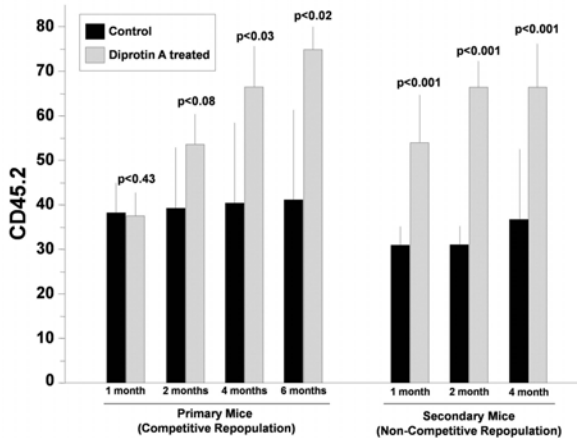

Figure 1. *In vivo* treatment of primary lethally-irradiated recipient mice with Diprotin A enhances primary competitive repopulating and secondary non-competitive repopulating capacity of untreated donor mouse bone marrow stem cells. This Figure is modified from that of Figure 4 in a previous publication (29) with the addition of new data for the 2 and 4 month time points for secondary mice. The experimental design and interpretations can be found in (29).

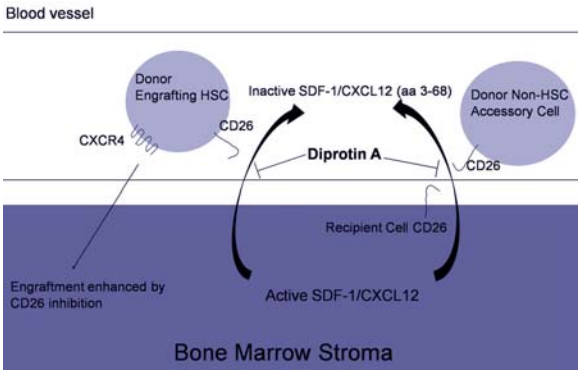

Figure 2. Proposed mechanism of CD26 action *in vivo*. Transplanted HSCs migrate into the recipient BM due to various mechanisms, one of which is SDF-1/CXCL12 signaling. CD26, expressed on donor HSCs, donor accessory cells (non-HSCs) and recipient BM cells are proposed to cleave SDF-1/CXCL12 *in vivo* to an inactive, truncated form which negatively affects the engraftment of the donor HSCs. In the presence of CD26 inhibitors such as Diprotin A, this cleavage of SDF-1 is blocked and the HSCs are enhanced in migration into and retention within the BM.

transduced human $CD34^+$ MPB cells. This group found no effect of *ex vivo* Diprotin A pre-treatment on the engraftment of these cells, despite the fact that the cells expressed CD26 and exhibited CD26 activity after an *ex vivo* culture period but no CD26 expression before the culture period. The lack of an *ex vivo* effect of Diprotin A pre-treatment in this study could reflect a difference between the HSCs derived from G-CSF MPB and those from CB used in ours (43) and Christopherson's (58) studies. Our group has shown that *in vivo* treatment of recipient mice with Diprotin A enhances the primary competitive and secondary non-competitive repopulating capacity of untreated congenic mouse bone marrow donor HSCs ((29); and Figure 1).

6. SUMMARY AND PERSPECTIVE: THE FUTURE OF CD26 AND HEMATOPOIETIC STEM CELL BIOLOGY

The ability of CD26 to cleave chemokines can result in dramatic functional alterations, with some truncated chemokines exhibiting reduced binding and activation of cognate receptors, others exhibiting reduced activation but only slightly reduced binding and still others exhibiting increased activation or alteration of receptor selectivity. CD26 can cleave SDF-1/CXCL12, a chemokine with great importance in hematopoietic cell biology. This cleavage of SDF-1/CXCL12 produces a molecule with dramatically reduced ability to signal through its receptor, CXCR4, but with only a modest decrease in binding ability (7). The truncated SDF-1/CXCL12 (aa 3-68) is ineffective at inducing chemotaxis of hematopoietic cells, and furthermore is able to block normal chemotaxis to full-length SDF-1/CXCL12 (antagonistic properties). The presence of cell surface CD26 negatively affects chemotaxis towards SDF-1/CXCL12 *in vitro*; inhibiting CD26 leads to enhanced chemotaxis. CD26 is also involved in the *in vivo* functions of mouse and human HSCs. Inhibition of CD26 or deletion by knockout leads to increased short-term homing, long-term engraftment and competitive repopulation and secondary repopulation in a non-competitive assay of mouse hematopoietic cells. For human HSCs, inhibiting CD26 leads to increased engraftment in mouse xenogeneic models.

The exact mechanism explaining the *in vivo* effects we and other laboratories have observed due to inhibition of CD26 has not been fully elucidated. There is ample evidence suggesting that CD26 is acting through its ability to cleave SDF-1/CXCL12 to an inactive form, thus negatively affecting the homing and engraftment of HSCs *in vivo*. First, we know that CD26 acts *in vitro* to cleave SDF-1/CXCL12 and that this form negatively affects the chemotaxis of the cells. Second, we know that the peptidase activity of CD26 modulates the *in vivo* effects seen, since Diprotin A acts by competitively inhibiting this property of the enzyme (44). Third, the increased short-term homing observed by our laboratory in mouse congenic transplants using Diprotin A pre-treated or CD26-/- cells could be reversed by treatment with AMD3100, a selective CXCR4 antagonist, suggesting that the effects are mediated through the SDF-1:CXCR4 axis (42). Figure 2 shows a diagram of the proposed mechanism of action of CD26 *in vivo* based on evidence to this point and emphasizing its effect on SDF-1/CXCL12. However, there are likely additional processes occurring which account for some of the mechanism. First, we have summarized here the wide effect CD26 has on many different chemokines. It is possible that *in vivo* CD26 could be cleaving other chemokines, in addition to SDF-1/CXCL12, that are affecting the homing of HSCs and/or accessory cells

leading to effects on establishment of the hematopoietic niche. Furthermore, in addition to its well-established peptidase activity, CD26 has been shown to bind to components of the extracellular matrix (ECM) as well as interact with cell-surface signaling proteins. CD26 can bind, outside of its active site, to the ECM molecules fibronectin and collagen, as well as cell surface signaling proteins such as adenosine deaminase (ADA) and CD45 (9, 11). The physiological relevance of these interactions is not well known, but they illustrate the point that CD26 is more than a simple dipeptidase enzyme. There is some signaling associated with CD26 as well, as studies using cross-linking anti-CD26 antibodies have shown multiple tyrosine phosphorylation events occur downstream (11). This is probably not a direct effect of CD26, since the cytoplasmic region is very short, but is likely mediated by a CD26 binding partner. Inhibiting the peptidase activity of CD26 could conceivably have an effect on any or all of these non-enzymatic functions and could be part of the CD26 mechanism of action *in vivo*. Finally, there is evidence that CD26 can functionally bind to CXCR4, and this CD26:CXCR4 complex can be internalized by SDF-1/CXCL12 stimulation of lymphocytes (62).

The potential clinical use of this strategy will undoubtedly require more work with animal models, such as canine and primate, using CB as well as other transplantable sources such as MPB and BM. It is important to note that in the human to mouse xenogeneic experiments cited here, it is not clear whether treating the recipient or the donor cells or some combination will be most effective at increasing engraftment. For MPB donor cells, treatment of the cells alone was not effective at increasing engraftment, but for CB donor cells this pre-treatment did enhance engraftment. Treatment of the cells alone would be preferred to treatment of the recipient in order to avoid any possible side-effects of the inhibitor. But, it may be that a combination of treating the cells and the recipients is best given the fact that CD26 is expressed in many tissue beds and found in a soluble form in the plasma (9). Our laboratory has published preliminary data showing that treating only the recipient mice with Diprotin A can increase the chimerism of donor cells in a mouse congenic competitive repopulation assay (29). An update of our original figure (29) following the recipient animals for longer periods post secondary non-competitive transplants is shown in Figure 1.

CD26 inhibitors are currently in clinical use for the treatment of diabetes. CD26 can cleave hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP), both of which can stimulate insulin release in the post-prandial response to foods (63, 64). CD26 cleavage of these hormones has a negative effect on glucose metabolism, and inactivation of CD26 activity is thought to extend their half-life leading to better blood glucose responses in diabetics. The fact that CD26 plays an important role in glucose metabolism led various companies to develop multiple CD26 inhibitors, with a least one, Sitagliptin (Merck) having FDA approval (65). These compounds vary in their structures, mechanism of action (competitive vs. non-competitive), and duration (long-lasting vs. short-lasting). The ability of these newer, clinical grade CD26 inhibitors to affect HSC biology should be tested, first in animal models, to determine if they can work in these contexts. The results of clinical trials on diabetes to date have shown minimal toxicity due to these compounds (63). However, it is important to note that long-term follow up has not taken place and firm conclusions about the safety of these inhibitors cannot be made at this time. In addition, there are safety factors to consider in transplant patients who are immunosuppressed and have hematologic pathologies; the fact that these drugs are safe in one population does not guarantee that they will be safe in another. There is also a concern that inhibiting CD26 in a human transplant recipient could lead to HSCs seeding other tissue beds, besides BM, in the body that produce SDF-1/CXCL12 gradients (e.g. lung and lymphoid organs). Whether the HSCs in these non-BM environments could thrive, proliferate and possibly harm the patient may be unlikely but is still a concern. In fact, there is evidence that bone marrow-derived cells can home to non-hematopoietic organs (66).

It is our goal that the ability to increase homing due to inhibition of CD26 activity will be able to be, alone or combined with other strategies such as *ex vivo* expansion and immunotherapy, to extend the potential therapeutic uses of limiting hematopoietic cell populations, such as those from human CB. This therapy may provide more patients with the best chance of a successful outcome after hematopoietic cell transplantation.

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Key Words: CD26, SDF-1, CXCL12, CXCR4, Hematopoietic Stem Cells, Transplantation, Review

Send correspondence to: Hal E. Broxmeyer, Ph.D., Department of Microbiology and Immunology, and the Walther Oncology Center, Indiana University School of Medicine, 950 West Walnut Street, R2-302, Indianapolis, IN 46202, Tel: 317-274-7510, Fax: 317-274-7592, E-mail: hbroxmey@iupui.edu

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