Modulation of Hematopoietic Chemokine Effects In Vitro and In Vivo by DPP-4/CD26

Hal E. Broxmeyer, Maegan Capitano, Timothy B. Campbell,* Giao Hangoc, and Scott Cooper

Dipeptidyl peptidase 4 (DPP4)/CD26 truncates certain proteins, and this posttranslational modification can influence their activity. Truncated (T) colony-stimulating factors (CSFs) are decreased in potency for stimulating proliferation of hematopoietic progenitor cells (HPCs). T-CXCL12, a modified chemokine, is inactive as an HPC chemotactic, survival, and enhancing factor for replating or ex-vivo expansion of HPCs. Moreover, T-CSFs and T-CXCL12 specifically downmodulates the positively acting effects of their own full-length molecule. Other chemokines have DPP4 truncation sites. In the present study, we evaluated effects of DPP4 inhibition (by Diprotin A) or gene deletion of HPC on chemokine inhibition of multicytokine-stimulated HPC, and on chemokine-enhancing effects on single CSF-stimulated HPC proliferation, as well as effects of DPP4 treatment of a number of chemokines. Myelosuppressive effects of chemokines with, but not without, a DPP4 truncation site were greatly enhanced in inhibitory potency by pretreating target bone marrow (BM) cells with Diprotin A, or by assaying their activity on *dpp4/cd26*^{-/-} BM cells. DPP4 treatment of myelosuppressive chemokines containing a DPP4 truncation site produced a nonmyelosuppressive molecule, but one which had the capacity to block suppression by that unmodified chemokine both in vitro and in vivo. Additionally, DPP4 treatment ablated the single cytokine-stimulated HPC-enhancing activity of CCL3/MIP-1 α and CCL4/MIP-1 β , and blocked the enhancing activity of each unmodified molecule, in vitro and in vivo. These results highlight the functional posttranslational modulating effects of DPP4 on chemokine activities, and information offering additional biological insight into chemokine regulation of hematopoiesis.

Introduction

H_{EMATOPOIESIS} IS CONTROLLED by microenvironmental cell interactions, which in part are mediated by cytokines and chemokines released by these cells [1,2]. While insight is increasing into how cytokines/chemokines stimulate, enhance and/or suppress proliferation of hematopoietic progenitor cells (HPCs), less effort has gone into how posttranslational modification of cytokines and chemokines influences their functional activities. Such information is of significance and relevance for a more precise understanding of the regulation of normal hematopoiesis, and how to better control hematopoiesis during stress- and disease-induced situations.

Studies of how posttranslational modification of proteins by enzymes influences the functional activity of cytokines and chemokines are under reported. Dipeptidyl peptidase 4 (DPP4) [3,4] is present as CD26 on the surface of many cells, including hematopoietic stem cells (HSCs), HPCs, and mature cells, and is found within cells, plasma, and serum [5–7]. DPP4 cleaves dipeptides from the N-terminus usually after a penultimate proline or an alanine and with less efficiency after other penultimate amino acids such as serine. We pre-

viously demonstrated that DPP4 treatment of stromal-derived factor-1 (SDF-1; CXCL12) produced truncated CXCL12 that was inactive as a chemotactic agent, and that could block chemotaxis induced by full-length (FL) CXCL12 [5]. Inhibition of DPP4 activity on target cells by small peptides such as Diprotin A (ILE-PRO-ILE) or VAL-PYR, or functional deletion of *dpp4* in cells from *cd26*^{-/-} mice prevented dpp4-induced truncation of CXCL12 and allowed for enhanced chemotactic and homing activities of CXCL12 [6,7], as well as for enhanced engrafting capacity of limiting the number of mouse bone marrow (BM) cells into lethally irradiated congenic mice in competitive and noncompetitive short- and long-term HSC assays in vivo [6], and of engraftment of low numbers of human cord blood (CB) CD34⁺ cells injected into sublethally irradiated immunodeficient mice [8–10].

CXCL12 also has survival-enhancing activity for HSC and HPC, enhances growth factor-induced replating of HSC in vitro, a measure of the limited self-renewal capacity of HPC, and enhances ex-vivo expansion of CD34⁺ CB cells [7,11–13]. Preventing DPP4 truncation of CXCL12 significantly increased these CXCL12 activities [5,7]. Colony-stimulating factors (CSFs), including human and murine granulocyte-macrophage colony-stimulating factor

Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, Indiana.

*Current affiliation: Department of Medicine, University of California, San Francisco, California.

(GM-CSF), granulocyte colony-stimulating factor (G-CSF), interleukin (IL)-3, and EPO, have DPP4 truncation sites, and inhibiting DPP4 truncation with Diprotin A greatly enhanced the stimulating activities of these CSFs in vitro and in vivo [7]. In addition, in vivo inhibition of DPP4 in mice by injecting Diprotin A into mice, or by oral administration of the FDA-approved small molecular weight DPP4 inhibitor, sitagliptin, greatly enhanced the engrafting capabilities of mouse BM HSC, as well as the recovery of phenotypically defined HSC and HPC, and functionally defined HPC after nonlethal doses of radiation, or after chemotherapeutic agents such as 5-fluorouracil and cyclophosphamide [6,7].

Hence, inhibition of DPP4 or deletion of *cd26/dpp4* increases the positively acting effects of a chemokine, CXCL12, and of selected CSFs [5,7]. It is now apparent that a large number of cytokines and chemokines have putative DPP4 truncation sites [7,14,15]. The fact that DPP4 actually truncates chemokines with DPP4 truncation sites has been reported [16–18]. Since a number of chemokines, including CCL3/MIP-1 α , CXCL4/PF-4, CXCL5/ENA-74, CXCL6/GCP-2, CXCL8/IL-8, CXCL9/MIG, and CXCL10/IP-10 with DPP4 truncation sites [14], have colony-inhibiting effects on multi-cytokine-stimulated colony formation in vitro, and myelosuppressive effects in vivo in mice [19–42], and the chemokines CCL3/MIP1 α and CCL4/MIP-1 β (also with a DPP4 truncation site) have enhancing effects on single CSF-stimulated colony formation in vitro [21,36,43], we questioned what effects DPP4 truncation would have on the in vitro and in vivo functions of these chemokines. We now report that inhibition of DPP4 on target cells in vitro and deletion of *cd26/dpp4* in mice enhances the activities of these chemokines, and that DPP4 posttranslational modification of selected chemokines by truncation modifies their functional activities.

Materials and Methods

Mice and cells

BM cells were from male and female C57Bl/6 and *cd26^{-/-}/dpp4* mice (on a C57Bl/6 mouse strain background) at 6–10 weeks old, as previously described [6,7]. The Indiana University Committee on Use and Care of Animals approved all the animal studies (IACUC Study No: 10985 MD/R/E/AR). Cells were unseparated and either left untreated or were preincubated with Diprotin A to inhibit DPP4 enzymatic activity, and washed before the addition of CSFs $\pm$ SCF, $\pm$ chemokines [3–5]. CCR5 $^{-/-}$ and CCR5 $^{+/+}$ mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Chemokines and cytokines

All recombinant human chemokines and mouse cytokines were purchased from R&D Systems (Minneapolis, MN), and had previously been tested for activity [21,24,25,29–43].

DPP4 pretreatment of chemokines

This was done as previously described [7]. Before their use, Diprotin A was added to chemokine preparations to inactivate the DPP4 before the chemokine was assayed for activity, as noted in text and figure legends.

In vitro assays

Multi-cytokine-stimulated and single CSF-stimulated BM colony formation, which respectively represent immature and more mature HPC, was performed as previously reported [7,21].

In vivo assays

These were done as previously reported [22,30,33,40], and are described within the text and figure legends.

Statistical analysis

Significant differences were determined by two-tailed Student's *t*-test.

Results

Inhibition of *dpp4* enzymatic activity or deletion of *dpp4* genes in mouse BM cells enhances selected chemokine-mediated myelosuppression of HPC proliferation in vitro

Chemokine structures and receptors have been well described [44–48]. Some, but not all, chemokines suppress HPC proliferation in vitro and in vivo [49,50]. Of the myelosuppressive chemokines [49,50], a number have DPP4 truncation sites [14,16–18]. Since chemokine inhibition is manifest on target BM cell populations that express CD26, a cell surface DPP4, we reasoned that this DPP4 could possibly modify the actions of myelosuppressive chemokines. We chose representative myelosuppressive chemokines with DPP4 truncation sites (Table 1) and assessed their suppressive activity on multi-cytokine [recombinant murine (rm)-GM-CSF and rm-stem cell factor]-stimulated granulocyte macrophage progenitor cell (CFU-GM) colony formation of C57Bl/6 mouse BM cells that were either preincubated with control (phosphate-buffered saline) medium or with Diprotin A (5 mM; ILE-PRO-ILE; a specific DPP4 inhibitor) for 1 h before washing, or with untreated BM cells from *cd26^{-/-}* mice (on a Bl/6 mouse strain background) (Fig. 1A; results are expressed as percent inhibition of CFU-GM colony formation). Multi-cytokine-stimulated

TABLE 1. DIPEPTIDYL PEPTIDASE 4 CLEAVAGE SITES ON HUMAN CHEMOKINES

Chemokines	N-terminus
CCL2/MCP-1	QPDAINAPVTCCYNFT
CCL3/MIP1 α	A PLAADTPTACCFSTY
CCL4/MIP-1 β	APMGSDPPTACCFSTY
CCL5/RANTES	S PYSSDTTPCCFAYIAR
CXCL4/PF-4	E AEEEDGDLQCLCVKT
CXCL5/ENA-74	A AVLRELRCVCLQTTQ
CXCL5/ENA-78 (non-cleavable)	AGPAAAVLRELRCVCL
CXCL6/GCP-2	G PVSAVLTELRCCTCLR
CXCL8/IL-8	S AKELRCQCIKTYSKP
CXCL8/IL-8 (non-cleavable)	AVLPRSAKELRCQCIK
CXCL9/MIG	T PVVRKGRCSISTNQ
CXCL10/IP-10	V PLSRTVRCTCISISNQ
XCL1/Lymphotactin	VGSEVSDKRTCIVSLTT

Bold letters denote the DPP4-truncation site.

FIG. 1. Influence of DPP4 inhibition or *cd26/dpp4* deletion on chemokine suppression of mouse BM CFU-GM colony formation in vitro. BM cells from control mice (BI/6) preincubated with control medium for 1 h followed by washing of cells, control mouse cells preincubated with 5 mM Diprotin A for 1 h and washed, or *cd26/dpp4*^{-/-} cells were plated at 5×10^4 cells/mL in the presence of rmGM-CSF (10 ng/mL) and rmSCF (50 ng/mL) minus and plus various concentrations of chemokines as indicated. Results are expressed as percent inhibition compared to control medium. For (A), control colony numbers were 98 ± 4 , 98 ± 3 , and 84 ± 4 , respectively, and for (B) were 71 ± 6 , 69 ± 5 , and 76 ± 2 (mean $\pm$ 1SD) for BI/6, BI/6 with Diprotin A, and *cd26*^{-/-} cells. *Significant difference, $P < 0.05$ compared to control medium within each experiment. DPP4, dipeptidyl peptidase 4; BM, bone marrow; CFU, colony-forming unit; GM, granulocyte macrophage; GM-CSF, granulocyte-macrophage colony-stimulating factor; rm, recombinant murine; SCF, stem cell factor; SD, standard deviation.

HPC are more immature subsets of HPC than those stimulated by a single CSF [1]. The five chemokines (CCL3, CXCL6, CXCL9, CXCL10, and CXCL4) tested suppressed colony formation of C57BL/6 BM cells pretreated with control medium, consistent with results in previous studies using untreated BM. Maximal suppression ($\sim 50\%$ inhibition), previously shown to relate to percent of progenitor cells in S-phase of the cell cycle at the time the chemokines are added, was detected at 100 ng/mL chemokine, with greatly reduced, but still significant suppression at 10 ng/mL chemokine. In contrast, suppressive activity was detected at 10- to 100-fold lower concentrations (0.01–1.0 ng/mL) when target BM population were first pulsed with Diprotin A. Enhanced suppressive activity at low concentrations of chemokine was confirmed against BM cells from *cd26*^{-/-} mice.

Three chemokines that have a DPP4 truncation site, but are not inhibitory (CXCL12, CCL4, and CCL5) had no myelosuppressive activity when assayed at 100, 10, 1.0, 0.1, or 0.01 ng/mL on either control BM, BM cells preincubated with Diprotin A, or *cd26*^{-/-} BM (0–10% inhibition, $P > 0.05$; data not shown). We compared myelosuppressive effects of

CXCL5 (a known suppressive chemokine with a DPP4 truncation site) with that of CXCL5 produced without a DPP4 cleavage site (Table 1), and also of CXCL8 (another known suppressive chemokine with a DPP4 truncation site) to that of CXCL8 produced without a DPP4 cleavage site (Table 1). As seen in Fig. 1B, CXCL5 and CXCL8 were equally inhibitory within the range previously reported for these chemokines (significant suppression to 10 ng/mL) on control BM cells. In contrast to FL forms of CXCL5 and CXCL8, which manifested significant suppressive activity on BI/6 BM cells pretreated with Diprotin A or on *cd26*^{-/-} BM cells at concentrations as low as 0.01 ng/mL, CXCL5 and CXCL8 without DPP4 truncation sites, were only slightly or not at all enhanced in suppressive activity on DPP4-inhibited or *cd26*^{-/-} BM cells compared to control BM cells. XCL1, a myelosuppressive chemokine without a DPP4 truncation site, was not enhanced in activity at lower chemokine concentrations when target BM cells were *cd26*^{-/-} (Fig. 2). Thus, myelosuppressive chemokines with, but not without, a DPP4 truncation site are significantly enhanced in myelosuppressive activity at lower chemokine concentrations when DPP4 is either inhibited or deficient in target BM cells.

FIG. 2. Influence of DPP4 treatment of chemokines on their suppressive activity on control (Bl/6) and *cd26*^{-/-} mouse BM CFU-GM colony formation in vitro and their effects on the suppressive activity of their own FL chemokine molecule. BM cells were stimulated as in the legend to Fig. 1, and results expressed as mean colonies $\pm$ 1SD per 5×10^4 cells plated. **P* < 0.05 compared to control. T, DPP4-treated molecule after pre-incubation with soluble DPP4; NT, molecule without DPP4 truncation site that was pre-treated with soluble DPP4. FL, full length.

DPP4-treated chemokines are inactive in vitro as myelosuppressive proteins, and block the inhibitory activity of the FL form of that specific chemokine

To test effects of DPP4 treatment on myelosuppressive activities of chemokines, we studied CCL3, CXCL9, and CCL2/MCP-1, each with a DPP4 truncation site, and XCL1 without a DPP4 truncation site (Table 1) for inhibition of colony formation by Bl/6 or *cd26*^{-/-} BM cells (Fig. 2; data expressed as colonies per 5×10^4 cells plated). Lower concentrations (0.1–1.0 ng/mL) of CCL3, CXCL9, and CCL2, but not of XCL1 (no DPP4 truncation site), were myelosuppressive on *cd26*^{-/-} compared to control Bl/6 BM (see black bars). DPP4 treatment of chemokines before assay, followed by addition of Diprotin A to inactivate the DPP4 before use in the assay resulted in loss of myelosuppressive activity for CCL3, CXCL9, and CCL2, but not for XCL1, on control and *cd26*^{-/-} BM cells (see gray bars). DPP4-treated CCL3, CXCL9, and CCL2 at a 1:1 ratio of 100 ng DPP4-treated:100 ng untreated chemokine blocked myelosuppression of their respective FL chemokines. DPP4-treated XCL1, which lacks a DPP4 truncation site, did not

block activity of the FL form of XCL1 at a 1:1 ratio of 100 ng:100 ng (see crosshatched bars toward bottom of Fig. 2). Thus, DPP4-treated chemokines with DPP4 truncation sites do not manifest myelosuppressive activity, but they block inhibition of their FL form.

We next used *cd26*^{-/-} mouse BM cells to determine what ratio of DPP4-treated to FL chemokine was required for DPP4-treated chemokine to block myelosuppression by FL chemokine, and if DPP4-treated chemokine of one type could block the FL form of another type of chemokine (Fig. 3A, B; with results expressed as colony numbers per 5×10^4 cells/mL). As shown in Fig. 3A, DPP4-treated CCL3, CXCL9, and CCL2, but not DPP4-treated XCL1, were inactive as suppressor molecules (gray bars). DPP4-treated CCL3, CXCL9, and CCL2 were able at a ratio of 1:1 (100 ng:100 ng), but not at a 100 ng:10 ng or 1 ng ratio of DPP4-treated to FL molecule to block suppression of FL chemokine of that type (crosshatched bars). DPP4-treated CXCL9 and CCL2, or XCL1 were not able at 1:1 (100 ng:100 ng) ratios to block the suppressive activity of FL CCL3; DPP4-treated CCL3 and CCL2, and XCL1 were not able to block suppression by FL CXCL9; DPP4-treated CCL3, CXCL9, and XCL1 were not able to block the suppressive

FIG. 3. Influence of DPP4 treatment of chemokines on the suppressive activity of *cd26^{-/-}* mouse BM CFU-GM colony formation in vitro. (A and B) designate 2 separate experiments. Cells were plated as stated in Fig. 1, and results expressed as mean colonies $\pm$ 1SD per 5×10^4 cells plated. **P* < 0.05 compared to control. T and NT are as designated in legend to Fig. 2.

activity of FL CCL2, and neither DPP4-treated XCL1 nor the DPP4-treated CCL3, CXCL9, or CCL2 were able to block the activity of XCL1 (see crosshatched bars, Fig. 3). Thus, DPP4-treated forms of one chemokine were not able to block myelosuppressive activities of another chemokine molecule. Lack of cross-blocking activity is not unexpected as these chemokines bind different chemokine receptors. As noted in

our previous studies, blocking effects of truncated GM-CSF on the activity of FL GM-CSF were mediated at the receptor level [7].

Myelosuppressive effects of certain chemokines are mediated through specific receptors (eg, for CXCL8/IL-8 [30] and CCL2/MCP-1 [32,35]). However, the receptor-mediating myelosuppression by CCL3/MIP-1 α is not known. CCL3/

MIP-1 α has two main receptors: *CCR1* and *CCR5* [44–48]. Inhibitory effects of CCL3/MIP-1 α are not mediated through *ccr1* [36]. Therefore, we used *ccr5*^{−/−} mice to see if CCL3/MIP-1 α was active as a myelosuppressive agent on these cells in vitro and in vivo. CCL3/MIP-1 α was as inhibitory on *ccr5*^{−/−} BM cells in vitro (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd) and in vivo (Supplementary Fig. S2) as on control *ccr5*^{+/+} BM. Thus, *ccr5* does not appear to be the receptor mediating the myelosuppressive effects of CCL3/MIP-1 α . This receptor still remains to be identified.

To determine the concentration of DPP4-treated chemokine necessary to block the inhibitory activity of their own FL chemokine, we used amounts of DPP4-treated molecules that were 1/2, 1/4, 1/8, or 1/16 that of the FL molecule. As shown in Fig. 3B (crosshatched bars), 50 ng/mL of DPP4-treated CCL3, CXCL9, and CCL2, respectively, completely blocked the suppressive activity of 100 ng/mL of their own FL chemokine; 25 ng/mL of each DPP4-treated chemokine partially blocked

suppression of 100 ng/mL of their own chemokine. Thus, DPP4-treated chemokine could at one-fourth to one-half the concentration of its own FL chemokine partially or fully block myelosuppression. This is consistent with our previous report that lower amounts of truncated GM-CSF outcompeted higher amounts of FL GM-CSF for receptor binding [7]. As an additional control, CXCL12, a nonmyelosuppressive chemokine, which has a DPP4 truncation site, was not myelosuppressive in either FL or DPP4-treated form, and the combination of FL and DPP4-treated CXCL12 was not myelosuppressive.

DPP4-treated chemokines are inactive in vivo as myelosuppressive proteins, and block the in vivo inhibition of multicytokine-responsive HPC numbers and cycling by the FL form of that specific chemokine

To determine if effects noted in vitro for FL and DPP4-treated chemokines (Figs. 1–3) are also manifested in vivo. FL or DPP4-treated CCL3 (Fig. 4A), CXCL8 (Fig. 4B), and

FIG. 4. Influence of s.c. injection of FL, DPP4-treated, and the combination of DPP4-treated plus FL chemokine (each given at a different site) to C57Bl/6 mice on absolute numbers and cycling status of BM hematopoietic progenitor cells. Results are shown as mean numbers $\pm$ 1 SEM for three mice per group with each mouse assessed individually. Experiments for groups (A–C) mice were each done on different days. BM cells were pre-treated $\pm$ high specific activity tritiated thymidine, washed and plated at 5×10^4 cells/mL in erythropoietin (EPO) (1 U/mL), pokeweed mitogen spleen cell conditioned medium (PWMSCM) (5% v/v), and SCF (50 ng/mL) with 0.1 mM Hemin. *Indicates significant difference, $P < 0.05$, compared to control, DPP4 truncated (T), or full length (F) plus T chemokines. s.c., subcutaneous; SEM, standard error of the mean.

FIG. 5. Effects of DPP4-treated CCL3 and CCL4 on in vitro colony formation of GM-CSF-stimulated CFU-GM (A, B) and of M-CSF-stimulated CFU-M (C, D). For (A) and (B), black bars denote BM cells pretreated with control medium before addition of chemokines, and gray bars denote BM cells pulse pretreated with Diprotin A before addition of chemokines. The BM cells for (C, D) were not pretreated with Diprotin A. Results in (A–D) are given as mean $\pm$ 1SEM and represent two different experiments each. The significance values shown compare results to the control medium (no chemokine; black bars compared to black bars, and gray bars compared to gray bars) for BM cells stimulated by 10 ng/mL GM-CSF (A) or for 10, 1, and 0.1 ng GM-CSF (B), and the significance values compare results to control medium (no chemokine for BM cells stimulated by 100 ng/mL M-CSF (C) or for 100 and 10 ng/mL M-CSF (D). We also used 1 ng M-CSF, but no colonies formed with or without chemokines in this group. M, macrophage; NS, not significant; $P > 0.05$.

CXCL9 (Fig. 4C) were injected subcutaneously (s.c.) into BL/6 mice each alone, or in combination (FL and DPP4-treated molecules were injected s.c. separately at different sites in the mice). Mice were sacrificed and analyzed 24 h later, a time shown by us previously to detect significant inhibition of numbers and cycling status of HPC [22,33]. FL chemokines suppressed numbers and cycling of HPC, DPP4-treated chemokines were not suppressive, and each DPP4-treated chemokine blocked the activity of their own FL chemokine (Fig. 4). Thus, *in vivo* effects of FL and DPP4-treated chemokines mimic their *in vitro* actions.

DPP4-treated CCL3 and CCL4 are inactive as enhancing proteins for proliferation of single cytokine-responsive HPC *in vitro*, and block the enhancing activity of their FL proteins

CCL3 and CCL4/MIP-1 β enhance colony formation of CFU-GM and CFU-macrophage (M) stimulated, respectively by GM-CSF alone and M-CSF alone *in vitro* [21,36,43]. CCL3, but not CCL4, acts as a suppressive agent for proliferation of multicytokine-stimulated colony formation of HPC, and since DPP4-treatment of CCL3 changes it to noninhibitory molecule against multicytokine-stimulated proliferation of HPC, but does block this suppression by FL CCL3, we tested whether DPP4 treatment of CCL3 and CCL4 would influence their enhancing activity for proliferation of single cytokine-stimulated HPC. Single cytokine-responsive HPC are a more mature progenitor cell population than multicytokine-responsive HPC [1]. As shown in Fig. 5A and B for GM-CSF-stimulated CFU-GM and Fig. 5C and D for M-CSF-stimulated CFU-M on BM cells not pretreated with Diprotin A (black bars in Fig. 5A–D), DPP4-treated CCL3 and CCL4 were inactive as enhancing agents for GM-CSF-responsive CFU-GM and for M-CSF-responsive CFU-M. Moreover, DPP4-treated CCL3 and CCL4, respectively, blocked enhancement of their FL counterparts. Since CCL3 and CCL4 mediate their enhancing activities through a common chemokine receptor, CCR1, we evaluated the capacity of DPP4-treated CCL3 and CCL4 to reciprocally block the enhancing activity of the other FL chemokine. DPP4-treated CCL3 blocked the enhancing effects of FL CCL4, and DPP4-treated CCL4 blocked the enhancing effects of FL CCL3 (Fig. 5A, C).

We comparatively evaluated the enhancing effects of CCL3 and CCL4 on colony formation with or without first pretreating them with Diprotin A to inhibit DPP4 (Fig. 5A, B). As reported previously, GM-CSF had greater CSF activity on Diprotin A-pretreated than control BM cells [7]. However, neither FL CCL3 nor CCL4 demonstrated enhancing effects on GM-CSF stimulation of Diprotin A-pretreated cells, with colony formation of GM-CSF-stimulated Diprotin A-pretreated cells being comparable to that of GM-CSF-stimulated colony formation of control (C57BL/6) BM cells plated in the presence of FL CCL3 or CCL4. Of interest, DPP4-treated CCL3 and CCL4, while having no enhancing activity on GM-CSF-stimulated control BM cells, had an inhibitory activity on GM-CSF-stimulated Diprotin A-pretreated BM cells. This highlights the complexity of cytokine regulation of hematopoiesis when the action of DPP4 and its effects on chemokine action are taken into consideration.

M-CSF does not have a DPP4 truncation site, and inhibition of CD26 on target cells does not enhance M-CSF-

FIG. 6. Effects of CCL3 *in vivo* in C57BL/6 mice (not pretreated) on absolute numbers and cycling status of GM-CSF-responsive BM CFU-GM (A, B) and M-CSF-responsive BM CFU-M (C, D). Mice were injected s.c. with 10 μ g CCL3, 10 μ g DPP4-treated CCL3, or with a combination of 10 μ g CCL3 and separately into another injection sites (s.c.) with DPP4-treated CCL3. Results are shown as mean $\pm$ SEM for three mice per group with each mouse assessed separately. Significance values compare CCL3-injected mice with those of control mice (injected with control diluent). F equals non-DPP4-treated full-length CCL3. T, DPP4-treated CCL3.

stimulated colony formation [7]. Thus, we did not pretreat target cells with Diprotin A for effects of CCL3 and CCL4 on M-CSF-stimulated colony formation (Fig. 5C, D), FL-CCL3, and CCL4-enhanced M-CSF-stimulated colonies, but DPP4-treated CCL3 and CCL4 were without enhancing activity, and blocked the effects of the FL CCL3 and CCL4.

Administration of CCL3 to mice *in vivo* results in enhanced proliferation of GM-CSF- and M-CSF-responsive HPC, whereas DPP4-treated CCL3 is not active in this action and counteracts the effect *in vivo* of administrated FL CCL3

We had not previously assessed the *in vivo* activity of a chemokine on numbers and proliferation of single cytokine-responsive HPC, which in context of CCL3 or CCL4-enhancing activity would allow us to compare their *in vitro* and *in vivo* effects. We now report effects of *in vivo* administration s.c. of CCL3, DPP4-treated CCL3, and the combination of CCL3 and DPP4-treated CCL3 (each injected

s.c. into different sites) on absolute numbers and cycling status (% HPC in S-Phase of the cell cycle) of CFU-GM responsive to stimulation by GM-CSF, and of CFU-M responsive to stimulation by M-CSF. As noted in Fig. 6, CCL3 administration enhanced absolute numbers and cycling status of GM-CSF-responsive CFU-GM and M-CSF-responsive CFU-M. DPP4-treated CCL3 had no enhancing effects on absolute numbers or cycling status of GM-CSF-responsive CFU-GM and blocked the enhancing effects of non-DPP4-treated FL CCL3 on these actions. DPP4-treated CCL3 had no enhancing effect on absolute numbers of M-CSF-responsive CFU-M, and blocked the enhancing effect of non-DPP4-treated CCL3 on this effect. In contrast to effects of DPP4-treated CCL3 on cycling of GM-CSF-responsive CFU-GM, DPP4-treated CCL3 decreased cycling of M-CSF-responsive CFU-M in the absence and presence of administration of FL CCL3. Hence, in vitro enhancing effects of CCL3 and of DPP4-treated CCL3 on single cytokine-stimulated HPC are confirmed in vivo.

Discussion

Chemokines are important members of the cytokine family of biological regulators [1,2,44–48]. Posttranslational modification of cytokines may change their biological activities. DPP4 truncation of the CSFs: GM-CSF, G-CSF, IL-3, and EPO, and of SDF-1/CXCL12 results in a less efficient CSF for stimulation of colony formation, and an inactive SDF-1/CXCL12 for chemotaxis and survival of HPCs [5–7]. Moreover, these truncated molecules downmodulate the activities of the FL forms of their molecules. Since regulation of hematopoiesis reflects on both positively and negatively acting cytokines and chemokines [1], and a number of chemokines that have been implicated in regulation of hematopoiesis in vitro and in vivo [48–50] have DPP4 truncation sites [14], we assessed the biological effects in vitro and in vivo of DPP4-treated myelosuppressive chemokines (CCL2, CCL3, CXCL4, CXCL5, CXCL6, CXCL8, CXCL9, and CXCL10) on immature subsets of HPCs that in vitro respond to stimulation by multiple cytokines, and of DPP4-treated enhancing chemokines (CCL3 and CCL4) that act on single CSF (GM-CSF or M-CSF)-stimulated more mature subsets of HPCs.

It is clear that DPP4 treatment of these members of the CC and CXC family of chemokines modify their biological activities. Myelosuppressive chemokines lose their suppressive activities when treated with DPP4, and these DPP4-treated chemokines compete with their FL forms to blunt the inhibitor activity manifested by the FL forms. Enhancing activity of the two members of the CC family, CCL3 and CCL4, that manifest this activity is lost upon DPP4 treatment of these chemokines and DPP4-treated CCL3 and CCL4 reciprocally block enhancement by their FL forms, likely through their receptor-binding capacity to the same receptor, CCR1, which is involved in the enhancing activities of these chemokines [32,36]. The in vitro activities of both the myelosuppressive and enhancing chemokines studied in this report, was confirmed in vivo in mice, suggesting that DPP4 truncation of these chemokines may be of physiological relevance. Of interest, is that CCL3, but not CCL4, can both suppress (on multi-CSF-stimulated immature subsets of HPC) and enhance (on single CSF stimulation of more mature subsets of CFU-GM) colony formation in vitro and progenitor cell proliferation in vivo.

While the enhancement of CCL3 is manifested through CCR1, as is that of CCL4, it is not yet clear what receptor mediates the myelosuppressive activity of CCL3. CCR1 does not mediate suppression through CCL3 [36] and it is now clear that CCR5, another major receptor for CCL3, does not mediate suppression by CCL3 in vitro or in vivo. Thus, a search is still on for the receptor mediating the myelosuppressive effects of CCL3.

We still have much to learn about cytokine- and chemokine-mediated regulation of hematopoiesis, and whether posttranslational modifications of these proteins is likely playing a role, perhaps even more than a blocking activity on the FL forms of these proteins. Future studies may define whether or not DPP4-truncated molecules have qualitative, in addition to quantitative, signaling and biological effects different from their FL forms. We find it intriguing that DPP4-treated CCL3 and CCL4 not only did not have the enhancing activity on GM-CSF-stimulated colony formation when the target cells were pretreated with Diprotin A to inhibit cellular DPP4 activity, but also in this situation, the DPP4-treated chemokines manifested suppressive activity on more mature subsets of CFU-GM. We have not previously found any of the FL chemokines to be suppressive on single cytokine-stimulated colony formation by CFU-GM [49,50].

It may be that DPP4 truncation of cytokines and chemokines is most relevant in a stressed situation, when the hematopoietic system is recovering from radiation or toxic drugs, or perhaps during disease. DPP4 does not seem to be as obviously involved with hematopoiesis during nonstressed steady-state conditions, where hematopoiesis seems relatively similar in control mice versus *cd26/dpp4*^{-/-} mice or in mice receiving a DPP4 inhibitor such as Diprotin A or sitagliptin. Sitagliptin is currently in clinical trial to accelerate recovery of single CB transplantation for treatment of patients with leukemia and lymphomas [51,52]. DPP4 seems to be mainly active during recovery from radiation or chemotherapy drugs, such as 5-fluorouracil. This is where inhibition of DPP4 activity shows effects, such as accelerated hematopoietic recovery [7], although this does not rule out effects of DPP4 activity in steady-state conditions.

Further studies on the role of DPP4 in cytokine/chemokine-mediated hematopoietic, and perhaps other tissue regulatory events, may help to better define normal physiological cell and tissue activities, and abnormalities during stress and disease. It may even be possible to use truncated molecules or prevention of DPP4 truncation to treat various disease states.

Acknowledgments

These studies were supported by the US Health Service National Institutes of Health (NIH) grants R01 HL056416, R01 HL067284, R01 HL112669, and a Center of Excellence in Molecular Hematology grant P01 DK090948 (and U54 DK106846) to H.E.B. M.C. was supported as a post-doc on NIH T32 DK07549 to H.E.B. T.B.C., currently an assistant professor at UCSF, was supported as a pre-doc on NIH T32 DK07519 during some of the beginning studies reported in this article.

Author Disclosure Statement

No competing financial interests exist.

References

1. Shaheen M and HE Broxmeyer. (2011). Hematopoietic cytokines and growth factors. In: *Cord Blood Biology, Transplantation, Banking, and Regulation*. Broxmeyer HE, ed. AABB Press, Bethesda, MD, pp 35–74.
2. Shaheen M and HE Broxmeyer. (2013). Principles of cytokine signaling. In: *Hematology: Basic Principles and Practice*, 6th edn., Chapter 14. Hoffman R, EJ Benz, Jr., LE Silberstein, H Heslop, JI Weitz, J Anastasi, eds. Elsevier Saunders, Philadelphia, PA, pp 136–146.
3. Rasmussen HB, S Branner, FC Wiberg and N Wagtmann. (2003). Crystal structure of human dipeptidyl peptidase IV/CD26 in complex with a substrate analog. *Nat Struct Biol* 10:19–25.
4. Aertgeerts K, S Ye, MG Tennant, ML Kraus, J Rogers, BC Sang, RJ Skene, DR Webb and GS Prasad. (2004). Crystal structure of human dipeptidyl peptidase IV in complex with a decapeptide reveals details on substrate specificity and tetrahedral intermediate formation. *Protein Sci* 13:412–421.
5. Christopherson KW, G Hangoc and HE Broxmeyer. (2002). Cell surface peptidase CD26/dipeptidylpeptidase IV regulates CXCL12/stromal cell-derived factor-1 alpha-mediated chemotaxis of human cord blood CD34+ progenitor cells. *J Immunol* 169:7000–7008.
6. Christopherson KW, G Hangoc, CR Mantel and HE Broxmeyer. (2004). Modulation of hematopoietic stem cell homing and engraftment by CD26. *Science* 305:1000–1003.
7. Broxmeyer HE, J Hoggatt, HA O'Leary, C Mantel, BR Chitteti, S Cooper, S Messina-Graham, G Hangoc, S Farag, et al. (2012). Dipeptidylpeptidase 4 negatively regulates colony-stimulating factor activity and stress hematopoiesis. *Nat Med* 18:1786–1796.
8. Campbell TB, G Hangoc, Y Liu, K Pollok and HE Broxmeyer. (2007). Inhibition of CD26 in human cord blood CD34+ cells enhances their engraftment of nonobese diabetic/severe combined immunodeficiency mice. *Stem Cells Dev* 16:347–354.
9. Christopherson KW, LA Paganessi, S Napier and NK Porecha. (2007). CD26 inhibition on CD34+ or lineage-human umbilical cord blood donor hematopoietic stem cells/hematopoietic progenitor cells improves long-term engraftment into NOD/SCID/Beta2null immunodeficient mice. *Stem Cells Dev* 16:355–360.
10. Porecha NK, K English, G Hangoc, HE Broxmeyer and KW Christopherson. (2006). Enhanced functional response to CXCL12/SDF-1 through retroviral overexpression of CXCR4 on M07e cells: implications for hematopoietic stem cell transplantation. *Stem Cells Dev* 15:325–333.
11. Broxmeyer HE, S Cooper, L Kohli, G Hangoc, Y Lee, C Mantel, DW Clapp and CH Kim. (2003). Transgenic expression of stromal cell-derived factor-1/CXC chemokine ligand 12 enhances myeloid progenitor cell survival/apoptosis in vitro in response to growth factor withdrawal and enhances myelopoiesis in vivo. *J Immunol* 170:421–429.
12. Broxmeyer HE, L Kohli, CH Kim, Y Lee, C Mantel, S Cooper, G Hangoc, M Shaheen, X Li and DW Clapp. (2003). Stromal cell-derived factor-1/CXCL12 directly enhances survival/apoptosis of myeloid progenitor cells through CXCR4 and G(alpha)i proteins and enhances engraftment of competitive, repopulating stem cells. *J Leukoc Biol* 73:630–638.
13. Broxmeyer HE, JA Mejia, G Hangoc, C Barese, M Dinuer and S Cooper. (2007). SDF-1/CXCL12 enhances in vitro replating capacity of murine and human multipotential and macrophage progenitor cells. *Stem Cells Dev* 16:589–596.
14. Ou X, HA O'Leary and HE Broxmeyer. (2013). Implications of DPP4 modification of proteins that regulate stem/progenitor and more mature cell types. *Blood* 122:161–169.
15. O'Leary H, X Ou and HE Broxmeyer. (2013). The role of dipeptidyl peptidase 4 in hematopoiesis and transplantation. *Curr Opin Hematol* 20:314–319.
16. Proost P, P Menten, S Struyf, E Schutyser, I De Meester and J Van Damme. (2000). Cleavage by CD26/dipeptidyl peptidase IV converts the chemokine LD78beta into a most efficient monocyte attractant and CCR1 agonist. *Blood* 96:1674–1680.
17. De Meester I, C Durinx, G Bal, P Proost, S Struyf, F Goossens, K Augustyns and S Scharpe. (2000). Natural substrates of dipeptidyl peptidase IV. *Adv Exp Med Biol* 477:67–87.
18. De Meester I, AM Lambeir, P Proost and S Scharpe. (2003). Dipeptidyl peptidase IV substrates. An update on in vitro peptide hydrolysis by human DPPIV. *Adv Exp Med Biol* 524:3–17.
19. Gewirtz AM, B Calabretta, B Rucinski, S Niewiarowski and WY Xu. (1989). Inhibition of human megakaryocytopoiesis in vitro by platelet factor 4 (PF4) and a synthetic COOH-terminal PF4 peptide. *J Clin Invest* 83:1477–1486.
20. Graham GJ, EG Wright, R Hewick, SD Wolpe, NM Wilkie, D Donaldson, S Lorimore and IB Pragnell. (1990). Identification and characterization of an inhibitor of haemopoietic stem cell proliferation. *Nature* 344:442–444.
21. Broxmeyer HE, B Sherry, L Lu, S Cooper, KO Oh, P Tekamp-Olson, BS Kwon and A Cerami. (1990). Enhancing and suppressing effects of recombinant murine macrophage inflammatory proteins on colony formation in vitro by bone marrow myeloid progenitor cells. *Blood* 76:1110–1116.
22. Maze R, B Sherry, BS Kwon, A Cerami and HE Broxmeyer. (1992). Myelosuppressive effects in vivo of purified recombinant murine macrophage inflammatory protein-1 alpha. *J Immunol* 149:1004–1009.
23. Dunlop DJ, EG Wright, S Lorimore, GJ Graham, T Holyoake, DJ Kerr, SD Wolpe and IB Pragnell. (1992). Demonstration of stem cell inhibition and myeloprotective effects of SCI/rhMIP1 alpha in vivo. *Blood* 79:2221–2225.
24. Broxmeyer HE, B Sherry, S Cooper, L Lu, R Maze, MP Beckmann, A Cerami and P Ralph. (1993). Comparative analysis of the human macrophage inflammatory protein family of cytokines (chemokines) on proliferation of human myeloid progenitor cells. Interacting effects involving suppression, synergistic suppression, and blocking of suppression. *J Immunol* 150:3448–3458.
25. Lu L, M Xiao, S Grigsby, WX Wang, B Wu, RN Shen and HE Broxmeyer. (1993). Comparative effects of suppressive cytokines on isolated single CD34(3+) stem/progenitor cells from human bone marrow and umbilical cord blood plated with and without serum. *Exp Hematol* 21:1442–1446.
26. Keller JR, SH Bartelmez, E Sitnicka, FW Ruscetti, M Ortiz, JM Gooya and SE Jacobsen. (1994). Distinct and overlapping direct effects of macrophage inflammatory protein-1 alpha and transforming growth factor beta on hematopoietic progenitor/stem cell growth. *Blood* 84:2175–2181.
27. Mayani H, MT Little, W Dragowska, G Thornbury and PM Lansdorp. (1995). Differential effects of the hematopoietic inhibitors MIP-1 alpha, TGF-beta, and TNF-alpha on cytokine-induced proliferation of subpopulations of CD34+ cells purified from cord blood and fetal liver. *Exp Hematol* 23:422–427.
28. Gewirtz AM, J Zhang, J Ratajczak, M Ratajczak, KS Park, C Li, Z Yan and M Poncz. (1995). Chemokine regulation of human megakaryocytopoiesis. *Blood* 86:2559–2567.

29. Broxmeyer HE, S Cooper, N Hague, L Benninger, A Sarris, K Cornetta, S Vadhnan-Raj, P Hendrie and C Mantel. (1995). Human chemokines: enhancement of specific activity and effects in vitro on normal and leukemic progenitors and a factor-dependent cell line and in vivo in mice. *Ann Hematol* 71:235–246.
30. Broxmeyer HE, S Cooper, G Cacalano, NL Hague, E Bailish and MW Moore. (1996). Involvement of interleukin (IL) 8 receptor in negative regulation of myeloid progenitor cells in vivo: evidence from mice lacking the murine IL-8 receptor homologue. *J Exp Med* 184:1825–1832.
31. Gao JL, TA Wynn, Y Chang, EJ Lee, HE Broxmeyer, S Cooper, HL Tiffany, H Westphal, J Kwon-Chung and PM Murphy. (1997). Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J Exp Med* 185:1959–1968.
32. Boring L, J Gosling, SW Chensue, SL Kunkel, RV Farese, Jr., HE Broxmeyer and IF Charo. (1997). Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J Clin Invest* 100:2552–2561.
33. Broxmeyer HE, A Orazi, NL Hague, GW Sledge, Jr., H Rasmussen and MS Gordon. (1998). Myeloid progenitor cell proliferation and mobilization effects of BB10010, a genetically engineered variant of human macrophage inflammatory protein-1 α , in a phase I clinical trial in patients with relapsed/refractory breast cancer. *Blood Cells Mol Dis* 24:14–30.
34. Broxmeyer HE, CH Kim, SH Cooper, G Hangoc, R Hromas and LM Pelus. (1999). Effects of CC, CXC, C, and CX3C chemokines on proliferation of myeloid progenitor cells, and insights into SDF-1-induced chemotaxis of progenitors. *Ann N Y Acad Sci* 872:142–162.
35. Reid S, A Ritchie, L Boring, J Gosling, S Cooper, G Hangoc, IF Charo and HE Broxmeyer. (1999). Enhanced myeloid progenitor cell cycling and apoptosis in mice lacking the chemokine receptor, CCR2. *Blood* 93:1524–1533.
36. Broxmeyer HE, S Cooper, G Hangoc, JL Gao and PM Murphy. (1999). Dominant myelopoietic effector functions mediated by chemokine receptor CCR1. *J Exp Med* 189:1987–1992.
37. Kim CH, CK Qu, G Hangoc, S Cooper, N Anzai, GS Feng and HE Broxmeyer. (1999). Abnormal chemokine-induced responses of immature and mature hematopoietic cells from motheaten mice implicate the protein tyrosine phosphatase SHP-1 in chemokine responses. *J Exp Med* 190:681–690.
38. Kim CH, G Hangoc, S Cooper, CD Helgason, S Yew, RK Humphries, G Krystal and HE Broxmeyer. (1999). Altered responsiveness to chemokines due to targeted disruption of SHIP. *J Clin Invest* 104:1751–1759.
39. Sarris AH, HE Broxmeyer, U Wirthmueller, N Karasavvas, S Cooper, L Lu, J Krueger and JV Ravetch. (1993). Human interferon-inducible protein 10: expression and purification of recombinant protein demonstrate inhibition of early human hematopoietic progenitors. *J Exp Med* 178:1127–1132.
40. Daly TJ, GJ LaRosa, S Dolich, TE Maione, S Cooper and HE Broxmeyer. (1995). High activity suppression of myeloid progenitor proliferation by chimeric mutants of interleukin 8 and platelet factor 4. *J Biol Chem* 270:23282–23292.
41. Hromas R, CH Kim, M Klemsz, M Krathwohl, K Fife, S Cooper, C Schnizlein-Bick and HE Broxmeyer. (1997). Isolation and characterization of exodus-2, a novel C-C chemokine with a unique 37-amino acid carboxyl-terminal extension. *J Immunol* 159:2554–2558.
42. Broxmeyer HE, S Cooper, G Hangoc and CH Kim. (2005). Stromal cell-derived factor-1/CXCL12 selectively counteracts inhibitory effects of myelosuppressive chemokines on hematopoietic progenitor cell proliferation in vitro. *Stem Cells Dev* 14:199–203.
43. Broxmeyer HE, B Sherry, L Lu, S Cooper, C Carow, SD Wolpe and A Cerami. (1989). Myelopoietic enhancing effects of murine macrophage inflammatory proteins 1 and 2 on colony formation in vitro by murine and human bone marrow granulocyte/macrophage progenitor cells. *J Exp Med* 170:1583–1594.
44. Bacon K, M Baggiolini, H Broxmeyer, R Horuk, I Lindley, A Mantovani, K Matsushima, P Murphy, H Nomiyama, et al. (2002). Chemokine/chemokine receptor nomenclature. *J Immunol Methods* 262:1–3.
45. Zlotnik A and O Yoshie. (2012). The chemokine superfamily revisited. *Immunity* 36:705–716.
46. Allen SJ, SE Crown and TM Handel. (2007). Chemokine: receptor structure, interactions, and antagonism. *Annu Rev Immunol* 25:787–820.
47. Bachelier F, A Ben-Baruch, AM Burkhardt, C Combadiere, JM Farber, GJ Graham, R Horuk, AH Sparre-Ulrich, M Locati, et al. (2014). International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors. *Pharmacol Rev* 66:1–79.
48. Youn BS, C Mantel and HE Broxmeyer. (2000). Chemokines, chemokine receptors and hematopoiesis. *Immunol Rev* 177:150–174.
49. Broxmeyer HE and CH Kim. (1999). Regulation of hematopoiesis in a sea of chemokine family members with a plethora of redundant activities. *Exp Hematol* 27:1113–1123.
50. Broxmeyer HE. (2001). Regulation of hematopoiesis by chemokine family members. *Int J Hematol* 74:9–17.
51. Farag SS, S Srivastava, S Messina-Graham, J Schwartz, MJ Robertson, R Abonour, K Cornetta, L Wood, A Secrest, et al. (2013). In vivo DPP-4 inhibition to enhance engraftment of single-unit cord blood transplants in adults with hematological malignancies. *Stem Cells Dev* 22:1007–1015.
52. Velez de Mendizabal N, RM Strother, SS Farag, HE Broxmeyer, S Messina-Graham, SD Chitnis and RR Bies. (2014). Modelling the sitagliptin effect on dipeptidyl peptidase-4 activity in adults with haematological malignancies after umbilical cord blood haematopoietic cell transplantation. *Clin Pharmacokinet* 53:247–259.

Address correspondence to:

Hal E. Broxmeyer, PhD

Department of Microbiology and Immunology

Indiana University School of Medicine

950 West Walnut Street, R2-302

Indianapolis, IN 46202-5181

E-mail: hbroxmey@iupui.edu

Received for publication January 27, 2016

Accepted after revision March 2, 2016

Prepublished on Liebert Instant Online March 4, 2016