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The bone marrow microenvironment and leukemia: biology and therapeutic targeting

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Abstract

Multiple studies have demonstrated that interaction with the bone marrow stromal microenvironment contributes to the survival of leukemia cells. One explanation for this phenomenon is the interaction between the cell surface receptors CXCR4 and CXCL12. Through CXCL12/CXCR4-mediated chemotaxis, leukemia cells migrate to microscopic niches within the bone marrow, which leads to increased proliferation and survival. Several studies have suggested that increased CXCR4 expression may portend a poor prognosis in various types of leukemia, possibly due to increased protection of leukemia cells by bone marrow stroma. A potential therapeutic strategy to overcome this stromal-mediated survival advantage is to target CXCR4. Inhibition of CXCR4 may allow leukemia cells to be released from bone marrow niches that confer resistance to chemotherapy and negate the survival benefit imparted by bone marrow stroma.

Keywords

ALL; AMD3100; AML; BKT140; CXCR4; CXCL12; microenvironment; plerixafor; SDF-1; TN14003

Overview of bone marrow microenvironment

Cellular interactions in bone marrow microenvironment

Hematopoiesis occurs through a complex interplay between hematopoietic stem and progenitor cells, and supporting cells present in the bone marrow. The bone marrow contains hematopoietic stem cells (HSCs), endothelial cells, osteoblasts, osteoclasts and mesenchymal stem cells (MSCs), which give rise to stromal cells including adipocytes, chondrocytes, fibroblasts and myocytes [1]. Researchers have suggested that the bone marrow can be subdivided into compartments, or niches, and that the growth and development of HSCs may be affected by supporting cells residing within each niche. The most commonly used niche concepts are the osteoblastic [2] or endosteal niche [3], which contains osteoblasts and stromal cells in close proximity to the bone, and the vascular niche [4], which is composed of endothelial cells in close proximity to vascular structures.

Supporting cells within each bone marrow niche produce soluble factors, molecules and ligands that interact with molecules and ligands on the surface of HSCs and hematopoietic progenitor cells (HPCs). These interactions contribute to the proliferation, differentiation, quiescence, migration and egress of HSCs and HPCs. For example, very late adhesion

molecule-4 (VLA-4) ligand, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin are molecules expressed by bone marrow endothelial cells [1]. Through ligand–receptor interactions, these molecules help to mediate adhesion of HSCs to the endothelium, and therefore help to retain HSCs within the bone marrow. Other cell–cell interactions, including the contact between Notch-1 on primitive HSCs and Jagged-1 on stromal cells, help to promote proliferation of HSCs [5]. On the other hand, binding of the receptor tyrosine kinase Tie2 on HSCs to angiopoietin-1 on osteoblasts induces quiescence [6]. Soluble factors, such as stem cell factor, bind to receptors on the surface of HSCs, such as c-kit, to induce their growth and proliferation [7]. CXCL12, which is constitutively produced by bone marrow stromal and endothelial cells, helps to mediate the migration, growth and differentiation of HSCs through its receptor CXCR4 [8].

Multiple studies have demonstrated that the interaction between CXCR4 and CXCL12 plays a critical role in the regulation of hematopoiesis. Newer studies have also suggested that this chemokine/receptor relationship is an important pathway in hematologic malignancies. This article will focus primarily on the interaction between CXCL12 and CXCR4, and its role in leukemia.

Overview of bone marrow microenvironment & hematopoietic malignancies

Recently, researchers have focused on the bone marrow microenvironment and its role in cancer. Multiple studies have demonstrated that interaction with bone marrow stromal cells contributes to the survival of leukemia cells both *in vitro* and *in vivo*. Additional studies have suggested that the leukemic state induces changes in the bone marrow that affect both hematopoiesis and bone marrow architecture.

Several studies have demonstrated that the survival of primary leukemic blasts can be supported by bone marrow stroma mono layers. One such study found that blasts from patients with B-lineage acute lymphoblastic leukemia (ALL) had an enormous survival advantage when cocultured with stroma versus when cultured in media alone [9]. Further experiments demonstrated that this survival advantage was contact-dependent, as blasts cultured in physical contact with stroma had higher rates of proliferation than blasts grown in permeable cell culture inserts hanging in a stroma-containing well or blasts grown in media alone [10]. These experiments suggest that both soluble factors and direct cell-cell interactions are critical in leukemia cell survival.

Stroma can also protect malignant cells from chemotherapy-induced death. For example, a mainstay of pediatric ALL therapy is treatment with asparaginase, which induces apoptosis because of low asparagine synthetase expression in ALL cells. When ALL cells are grown in co-culture with bone marrow stroma cells, they are protected from asparaginase-induced apoptosis due to high asparagine synthetase expression by stroma cells [11].

Several groups have shown that patients with acute leukemia and preleukemic diseases, such as myelodysplastic syndromes and myeloproliferative neoplasms, have higher levels of bone marrow vascularization and angiogenesis [12]. One study that compared core bone marrow biopsies from children with ALL to healthy controls found that leukemic bone marrow biopsies had distinct microvascular patterns, including increased density of micro-vessels [13]. Another group demonstrated that culturing bone marrow stromal cells grown from CD133⁺/CD34⁺ stem cells from acute leukemia patients can lead to the formation of capillary-like structures on a basement membrane matrix [14]. In these experiments, long-term culture of CD133⁺/CD34⁺ cells, which were also CD45[−], resulted in the development of a variety of bone marrow stromal cells, including fibroblasts and endothelial cells. The ability to induce angiogenesis may therefore be a mechanism to enhance survival of leukemia cells in the bone marrow.

Growth factors may also help to modulate the survival of leukemic blasts in the bone marrow microenvironment. VEGF is expressed by both acute myeloid leukemia (AML) cell lines and blasts [15], and a single-institution study suggested that VEGF levels are a prognostic indicator in patients with AML [16]. Evaluation of a panel of angiogenic growth factors in adults with newly diagnosed AML suggested that elevated levels of angiopoietin-2 correlated with poor initial response to therapy [17]. Levels of urinary basic FGF were elevated in patients with pediatric ALL [13]. Furthermore, bone marrow stromal cells from patients with acute leukemia appear to produce more IGF-1 and CXCL12 than control stromal cells [14].

Evidence also suggests that leukemia cells can overtake mechanisms that regulate the growth and development of HSCs. A series of elegant experiments utilizing *in vivo* imaging demonstrated that leukemic cells specifically disrupt the niches of normal HSCs [18]. Mouse transplant experiments showed that both CD34⁺ HSCs and NALM-6, a pre-B cell ALL cell line, preferentially localize to perivascular niches that are high in CXCL12. However, when CD34⁺ HSCs and NALM-6 were transplanted together, NALM-6 outcompeted HSCs for the preferred CXCL12-high niches. Because NALM-6 cells homed to the CXCL12-high niches, CD34⁺ HSCs were forced to home to less desirable niches within the bone marrow. This altered homing resulted in an overall decrease in CD34⁺ cells, as well as a consequent inability of CD34⁺ cells to mobilize in response to cytokines. A mouse model of Notch1-induced leukemia found that the development of leukemia had different effects on hematopoietic cell compartments [19]. In these leukemic mice, HSCs were quiescent but were able to proliferate and differentiate when transplanted to non-leukemic recipient mice. On the other hand, HPCs in leukemic mice exhibited increased proliferation and subsequent exhaustion. These experiments offer evidence that leukemia causes significant disruption of normal hematopoiesis.

A recent study demonstrated that an abnormal bone marrow stromal microenvironment by itself can lead to dysfunctional hematopoiesis and even leukemia [20]. In this study of murine hematopoiesis, *Dicer1* was deleted in osteoprogenitor cells. *Dicer1*-deleted mice had abnormal differentiation of osteoprogenitors into mature osteoblasts, and this ultimately led to myelodysplasia. Transplant of hematopoietic cells from *Dicer1*-deleted mice into wild-type *Dicer1* mice led to robust engraftment and no evidence of myelodysplasia. However, transplant of normal hematopoietic cells from wild-type *Dicer1* mice into *Dicer1*-deleted mice led to myelodysplasia. Interestingly, a small number of *Dicer1*-deleted mice also developed myeloid sarcoma and AML. These experiments suggest that the bone marrow stromal microenvironment plays a significant role in the development of myelodysplasia and hematologic malignancy.

CXCR4 & CXCL12

History of CXCR4 & CXCL12 with HIV & later in hematopoiesis

The interaction between CXCR4 and CXCL12 is now known to mediate the migration and retention of HSCs within niches in the bone marrow microenvironment [21]. CXCR4, a member of the CXC chemokine receptor family, was first described in 1994 as a seven-transmembrane domain, GTP-binding protein coupled-receptor present on the surface of white blood cells [22]. It was later identified as a necessary cofactor for HIV-1 [23,24] and HIV-2 [25] to enter the cell. Further investigation revealed that CXCR4 is present on the surface of lymphocytes, neutrophils, monocytes and HSCs. Additional exploration found that CXCR4 is expressed on a higher percentage of immature T lymphocytes compared with more mature T lymphocytes and that the cell surface expression of CXCR4 is higher on immature T lymphocytes [26].

CXCL12 is a chemokine that induces the migration of various white blood cells, including pro-B and pre-B lymphocytes, T lymphocytes, monocytes, and neutrophils [27-29]. CXCL12 was first cloned in 1993 [30,31] and was initially described as an attractant of lymphocytes [32]. CXCL12 has two forms, α and β , which are products of alternative gene splicing [33]. Early studies found that CXCL12 induces lymphocyte chemotaxis through binding to CXCR4. Further experiments demonstrated additional functional effects of CXCL12 binding to CXCR4, as exposure to CXCL12 blocked CXCR4-mediated entry of HIV-1 into peripheral blood mononuclear cells, HeLa-CD4 cells, and CXCR4 transfected cells [34]. Production of CXCL12 is regulated by the sympathetic nervous system and is governed by circadian rhythms [35].

Additional examination of CXCR4 and CXCL12 found that their interaction is important in normal hematopoiesis [36]. Specifically, *CXCR4*-deficient mice demonstrate a marked decrease in B lymphopoiesis and myelopoiesis, and die in the perinatal period [37], while *CXCL12*-deficient mice have defective myelopoiesis [38]. CXCL12 and CXCR4 also play important roles in bone marrow engraftment. In an experiment in which human severe combined immunodeficient (SCID) repopulating human stem cells were transplanted into SCID and nonobese diabetic (NOD)/SCID mice, *in vitro* migration of human stem cells toward a gradient of CXCL12 correlated with the ability of the cells to engraft *in vivo* [39]. Furthermore, treatment of the stem cells with an antibody against CXCR4 prior to transplant led to failure of engraftment. Recent research has also identified a subset of perivascular, CXCL12-producing MSCs as important components of the bone marrow microenvironment [40]. These MSCs express nestin, are in close association with the bone marrow vasculature, and are innervated by the sympathetic nervous system. Murine transplant experiments have demonstrated that HSCs home to niches rich in nestin-expressing MSCs.

Several studies have also demonstrated that chemokines, including CXCL12, can interact with integrins in order to mediate both cell rolling and cessation of movement [41]. For example, exposure to CXCL12 leads to enhanced affinity of VLA-4 to VCAM-1 in lymphocytes [42], monocytes [43], neutrophils [44] and CD34⁺ cells [45,46]. In addition, the interaction between the CXCL12/CXCR4 axis and the integrins in HSC homing and engraftment was demonstrated in a series of notable experiments [46]. *In vitro* experiments using CD34⁺ cells found that CXCL12/CXCR4 binding causes activation of VLA-4 and lymphocyte function-associated antigen (LFA)-1, which then leads to VLA-4 and LFA-1-dependent adhesion to VCAM-1 and intracellular adhesion molecule-1, respectively. CXCL12 was also found to mediate VLA-4 and LFA-1-dependent migration through a vascular endothelial cell layer. *In vivo* transplant experiments found that CD34⁺ cells treated with anti-VLA-4, anti-VLA-5 or anti-LFA-1 antibodies prior to transplantation into NOD/SCID mice led to significantly lower levels of engraftment than transplantation of CD34⁺ cells pretreated with an anti-CD34 antibody. Another group found that simultaneous blockade of $\alpha 4$ integrin and CXCR4 led to mobilization of HSCs and HPCs, again suggesting prominent roles for VLA-4 and CXCR4 in the retention of hematopoietic cells within the bone marrow microenvironment [47].

Mechanism of CXCR4/CXCL12 interaction

CXCR4 is activated after binding of extracellular CXCL12. Activation of CXCR4 results in phosphorylation and endocytosis via clathrin-coated pits. After endocytosis, CXCR4 can either be ubiquitinated, which targets the receptor for lysosomal degradation [48], or recycled back to the cell surface [49,50]. While cell surface localization of CXCR4 is required for its activation, leukocytes have significant amounts of intracellular stores of CXCR4 [50]. Once CXCR4 is activated, both G protein-dependent and G protein-independent signaling occurs [51]. The Src family of tyrosine kinases, as well as phospholipase C- β and PI3K, are activated in a G protein-dependent manner. On the other

hand, the JAK/STAT pathway is activated in a G protein-independent manner [52]. CXCR4 activation through CXCL12 also results in an increase in intracellular calcium [53]. The overall result of CXCR4 activation is chemotaxis toward CXCL12 [27]. A recent study reported that exposure to CXCL12 promotes quiescence of CXCR4-expressing HSCs, while HSCs that lack CXCR4 proliferate in response to CXCL12 [54].

CXCR4 transcription is mainly regulated by two transcription factors. Nuclear respiratory factor-1 is a positively regulating transcription factor, while Yin-Yang 1 is a negatively regulating transcription factor [55,56]. Multiple external factors can also influence the expression of surface CXCR4. Cytokines, including TGF-1 β , IL-2, IL-4, IL-6, IL-7, IL-10 and IL-15, and growth factors, such as EGF, VEGF, basic FGF and stem cell factor, have all been shown to induce upregulation of CXCR4 [49,51]. Stimulation of peripheral blood mononuclear cells with phytohemagglutinin and IL-2 causes upregulation of CXCR4 and subsequent increased chemotaxis toward CXCL12 [57]. Contact with CXCL12, phorbol esters, pertussis toxin and inflammatory cytokines, including TNF- α and IFN- γ , all cause downregulation of surface CXCR4 [49,51,53]. Hypoxia causes upregulation of CXCR4 via hypoxia-inducible factor 1- α , which in turn leads to increased chemotaxis in response to CXCL12 [58]. Furthermore, hypoxia-inducible factor 1- α also leads to increased CXCL12 gene expression in endothelial cells under hypoxic conditions [59].

Role in leukemia

CXCR4 is commonly expressed by a variety of leukemia cells, both lymphoid and myeloid, and the CXCL12/CXCR4 interaction has been shown to be critical in the retention of both HSCs and leukemia cells in the bone marrow [60]. In acute leukemias, surface CXCR4 levels are highest in acute pro myelocytic leukemia (French–American–British [FAB] subtype M3), myelomonocytic AML (FAB subtypes M4 and M5) and B-lineage ALL [61]. Variability in surface CXCR4 expression on AML blasts appears to have functional relevance, as one study demonstrated that higher cell surface density of CXCR4 correlated with increased pseudoemperipolesis, or the migration of cells beneath a layer of bone marrow stromal cells [62]. Pseudoemperipolesis is at least partially mediated by CXCR4 in AML [63], and is mediated by CXCR4 and CXCL12 in chronic lymphocytic leukemia (CLL) [64]. A number of normal hematopoietic cells, including lymphocytes, HPCs and HSCs, exhibit pseudoemperipolesis [62,64]. This phenomenon is thought to mimic the manner in which HPCs and HSCs access supportive microscopic niches in the bone marrow, and it is conceivable that malignant cells would act in the same manner in order to secure their own survival.

Interestingly, infiltration of nonhematopoietic tissues such as gingiva or skin is most often observed in high CXCR4-expressing AML (monocytic differentiation or FAB M4 and M5) [65]. It is therefore possible that CXCL12-induced migration may play a role in the development of extramedullary leukemia.

On a molecular level, CXCL12 and CXCR4 act through a number of pathways to promote leukemia progression. For example, binding of CXCL12 to CXCR4 leads to activation of the PI3K/Akt and MAPK pathways, which help to mediate the survival and proliferation of leukemia cells [66]. The NF- κ B pathway is also activated by CXCL12, which induces the production of soluble factors, such as matrix metalloproteinases, IL-8 and VEGF, that can help to degrade the extracellular matrix and induce blood vessel formation [66,67]. Exposure to CXCL12 also leads to increased affinity of blasts to niches in the bone marrow microenvironment [68], presumably through CXCL12-induced increases in the affinity of integrins, such as VLA-4 and VLA-5.

Effects of anticancer drugs on CXCR4 expression in hematologic malignancies

Various anticancer compounds have been shown to affect CXCR4 expression. For example, studies have suggested that histone deacetylase inhibitors can modulate levels of CXCR4, implying that CXCR4 expression can be epigenetically regulated [69]. Valproic acid induces increases in both surface and mRNA CXCR4 expression in CD34⁺ cells, including cord blood HSCs and the AML cell lines KG-1a and KG-1 [70]. These changes result in a functional advantage, as valproic acid-treated cells had increased chemotaxis toward a CXCL12 gradient when compared with untreated cells. Further investigation demonstrated that the increase in CXCR4 mRNA correlated with the acetylation status of histone H4.

A subsequent study by the same group suggested that the effects of valproic acid on CXCR4 are dependent on the maturation status of the cells, as defined by CD34 expression [71]. Treatment of the CD34⁺ AML cell lines HL-60 (promyelocytic) and THP-1 (monocytic) with valproic acid resulted in decreased surface CXCR4 expression. Treatment of primary AML samples with valproic acid resulted in an upregulation of CXCR4 in immature, highly CD34⁺ samples and downregulation of CXCR4 in more differentiated CD34⁺ samples (FAB subtypes M3, M4, treatment-related AML). These changes resulted in increased and decreased chemotaxis to a CXCL12 gradient, respectively.

Treatment of CLL primary samples with suberoylanilide hydroxamic acid not only induced apoptosis, but also resulted in a downregulation of surface and mRNA CXCR4 expression [72]. Downregulation of CXCR4 resulted in impaired chemotaxis toward a CXCL12 gradient and impaired pseudoemperiopolesis.

Tyrosine kinase inhibition can also affect levels of CXCR4. Treatment of KBM-5 and K562 cell lines, as well as primary chronic myelogenous leukemia (CML) samples, with imatinib resulted in increased expression of CXCR4 [73]. This, in turn, caused increased leukemia cell chemotaxis toward stroma and induction of cell cycle arrest/quiescence. Treatment of the *BCR-ABL*-positive BV-173 ALL line with imatinib and nilotinib was shown to cause an upregulation of surface CXCR4 [74]. Furthermore, the use of low-dose dasatinib in *BCR-ABL*-positive ALL cell lines induced an increase in surface CXCR4 expression [75]. By contrast, another study found that *in vitro* treatment of blasts taken from patients in CML blast crisis with imatinib induced downregulation of both surface and mRNA CXCR4 expression [76].

Thus, dynamic upregulation of CXCR4 in leukemia cells can occur in response to anticancer therapy. Functionally, this might result in enhanced interaction between leukemia cells and the bone marrow microenvironment, and a subsequent survival advantage. It would follow that baseline expression and/or dynamic upregulation of CXCR4 by leukemia cells could represent a mechanism of therapeutic resistance. Therefore, targeting CXCR4 may be an effective therapeutic strategy to prevent/overcome resistance and enhance/restore sensitivity to anticancer agents.

CXCR4 inhibitors

Peptide antagonists

The major categories of CXCR4 inhibitors under clinical investigation include small peptide antagonists, nonpeptide antagonists and antibodies [60]. The peptide-based CXCR4 antagonists were derived from the naturally occurring substances tachyplesin and polyphemusin, which were isolated from the Japanese and American horseshoe crabs, respectively [77]. Tachyplesin and polyphemusin were able to inhibit replication of HIV *in vitro* and synthetic analogs of the compounds were subsequently synthesized. T22 is an 18-residue peptide that is a synthetic analog of polyphemusin. Initial studies of T22

demonstrated both low cytotoxicity and effective anti-HIV activity, and T22 was subsequently shown to be an inhibitor of CXCR4 [78]. This established T22 both as a CXCR4 inhibitor and as an agent with an achievable therapeutic window. Other peptide-based CXCR4 antagonists were then developed, including T134 and T140, which are 14-residue peptides that were synthesized based on the structure of T22 [79]. While T140 was able to very potently inhibit both CXCR4 and entry of HIV-1, the compound lacked stability in serum. Thus, analogs of T140 were developed, including TC14012, TN14003 and TZ14001 [80]. All were found to be stable in serum during preclinical testing, and both TC14012 and TN14003 were both effective at inhibiting CXCR4. TN14003 has been tested preclinically in a variety of solid tumors and was able to inhibit the invasion and migration of breast cancer [81] and pancreatic cancer [82] cell lines, thereby suggesting CXCR4 inhibition as a means to prevent the development of metastatic disease. In addition, TN14003 was able to induce HPC and HSC mobilization [83], and improve post-HSC transplant bone marrow recovery in murine experiments [84]. TN14003, now known as BKT140, is currently under investigation in a Phase I/II trial in patients with multiple myeloma [201].

Nonpeptide antagonists

Nonpeptide antagonists of CXCR4 include plerixafor (Mozobil™, formerly AMD3100) and AMD3465. Plerixafor is a bicyclam that is a reversible inhibitor of CXCR4 [85], while AMD3465 is a reversible *N*-pyridinylmethylene monocyclam antagonist of CXCR4 [86]. In preclinical studies as an anti-HIV agent, plerixafor was able to block the *in vitro* infection of human peripheral blood mononuclear cells by T-tropic strains of HIV [87]. A clinical trial of plerixafor in patients with HIV, however, did not result in decreases in HIV RNA levels, but all patients developed leukocytosis [88]. The possible utility of plerixafor in hematology and oncology was then evaluated and plerixafor was studied as a HSC-mobilizing agent. A randomized Phase III clinical trial found that patients who underwent HSC mobilization with plerixafor and granulocyte colony-stimulating factor (G-CSF) had a significantly higher CD34⁺ cell count than those who were mobilized with G-CSF alone [89]. Plerixafor was recently approved by the US FDA for use in combination with G-CSF for mobilization of HSCs in adults with non-Hodgkin's lymphoma or multiple myeloma.

Monoclonal antibodies

MDX-1338, also known as BMS-936564, is a fully human anti-CXCR4 antibody. Preclinical experiments demonstrated that MDX-1338 blocks the binding of CXCL12 to CXCR4-expressing cells, which in turn inhibits CXCL12-induced intracellular calcium surge and chemotaxis [90]. Furthermore, MDX-1338 was able to cause apoptosis in several CXCR4-expressing cell lines, as well as inhibit the growth of AML and Burkitt's lymphoma in murine xenograft models. MDX-1338 is being investigated in a Phase I trial of adults with relapsed or refractory AML [202].

Preclinical data using CXCR4 inhibitors

Because the CXCL12/CXCR4 connection is important in keeping leukemia cells within the protective bone marrow microenvironment, it would be reasonable to attempt to target that interaction. Inhibition of CXCR4 could allow leukemia cells to be released from bone marrow niches that confer resistance to chemotherapy and lead to chemotherapy-induced death. CXCR4 inhibitors have been investigated as single agents, in combination with G-CSF, and in combination with anticancer agents.

Acute myeloid leukemia

A study using the AML cell line U937 demonstrated that the use of AMD3100 decreased proliferation, induced differentiation and induced death of leukemic cells [91]. Another study found that AMD3100 dampened pseudoemperipolesis of AML blasts through human umbilical vein endothelial cell and marrow stromal monolayers [92].

The combination of AMD3100 and cytarabine in a mouse model of acute promyelocytic leukemia resulted in decreased tumor burden and improved overall survival compared with mice treated with cytarabine alone [93]. A recent study combining CXCR4 inhibition with an HDAC inhibitor resulted in increased apoptosis of both AML cell lines and primary samples [94]. In this study, treatment of AML cell lines and patient samples with the HDAC inhibitor panobinostat alone resulted in decreased CXCR4 mRNA and protein levels. Further investigation revealed that treatment with panobinostat or the Hsp90 inhibitor AU922 led to proteasomal degradation of CXCR4 by inhibiting its interaction with Hsp90.

Two recent studies suggest that CXCR4 inhibition may be useful in patients with Flt3 mutations. Flt3 is a receptor tyrosine kinase found on a variety of hematopoietic progenitor cells as well as leukemia cells. Flt3–internal tandem duplications (ITDs) are found in acute myeloid leukemias and portend a poor prognosis [95]. One study demonstrated a proliferative advantage of Flt3–ITD AML blasts compared with Flt3 wild-type AML blasts when grown in co-culture with bone marrow stroma [96]. While this proliferative advantage was negated by the addition of AMD3100, growth of Flt3 wild-type AML blasts was not affected by AMD3100. Interestingly, physical blast–stroma contact was necessary for the proliferative advantage, as neither CXCL12-supplemented media nor exposure to stroma-produced soluble factors led to a significant improvement in proliferation compared with Flt3 wild-type cells. Another study found that treatment of Flt3–ITD AML blasts with AMD3465 in combination with either cytarabine or the tyrosine kinase inhibitor sorafenib resulted in increased apoptosis *in vitro* and decreased leukemic burden *in vivo* [97].

Acute lymphoblastic leukemia

In vitro experiments using T140, TC140012, T134 and AMD3100 on pre-B cell ALL cells resulted in inhibition of CXCL12-induced chemotaxis and migration into bone marrow stromal layers [98]. A study examining mouse models of childhood ALL suggested that the use of CXCR4 inhibitors can mobilize leukemic cells into the peripheral blood and inhibit metastasis to extramedullary sites; extended use of CXCR4 antagonists led to a reduction in leukemic cells in the peripheral blood and spleen [99].

CXCR4 inhibition may also be useful in the treatment of high-risk ALL. Cases of infant ALL that harbor a myeloid/lymphoid or mixed-lineage leukemia (*MLL*) gene rearrangement are quite difficult to treat and patients often have a dismal outcome [100]. A xenograft mouse model of infant *MLL*-rearranged ALL found that treatment with the combination of a Flt3 tyrosine kinase inhibitor (CEP-701), and AMD3100 and G-CSF as mobilizing agents resulted in a lower leukemic burden than treatment with either CEP-701 or the mobilizing agents alone [101]. The presence of the BCR–ABL fusion protein in ALL also confers a poor prognosis [102], and combined inhibition of CXCR4 and the BCR–ABL tyrosine kinase may be a promising therapeutic strategy. The addition of AMD3100 to dasatinib resulted in increased dasatinib-induced cell death of BCR–ABL-positive ALL cell lines [75]. Co-culture of these cell lines with bone marrow stroma and treatment with low-dose dasatinib resulted in the development of dasatinib-resistant populations. Further investigation found that low-dose dasatinib induced an increase in surface CXCR4 expression.

Chronic myelogenous leukemia

Treatment of the BCR–ABL-positive BV-173 cell line with imatinib and nilotinib resulted in an upregulation of surface CXCR4 [74]. Cells grown in co-culture with stroma were protected from imatinib- or nilotinib-induced apoptosis, and the addition of AMD3100 negated this protective effect. Similarly, treatment of KBM5, K562 and primary CML cells with imatinib led to upregulation of CXCR4 [73]. Co-culture with stromal cells protected KBM-5 cells from imatinib-induced apoptosis, and this protective effect was reversed by treatment with AMD3465.

Co-culture of BV-173 or primary CML patient samples with mesenchymal bone marrow stromal cells protected cells from imatinib-induced apoptosis; treatment with AMD3100 restored sensitivity to imatinib [103]. The ability of imatinib-treated BV-173 cells to engraft in NOD/SCID mice also appeared to be affected by stromal cell interactions. Specifically, imatinib-treated BV-173 cells co-cultured with mesenchymal bone marrow stromal cells had a higher level of engraftment in NOD/SCID mice when compared with imatinib-treated BV-173 cells cultured in media alone, suggesting that bone marrow stromal cells protect leukemia-initiating cells from imatinib-induced cell death.

Chronic lymphocytic leukemia

T140 and its analogs have been shown to abrogate CXCL12-induced responses of CLL cells [104]. For example, *in vitro* inhibition of CXCR4 in primary samples of CLL caused decreased actin polymerization, which in turn resulted in decreased chemotaxis and pseudoemperipolesis. In particular, treatment with TC14012 and TN14003 decreased the survival advantages conferred by both synthetic CXCL12 and stromal cells. Stromal cells also conferred protection from fludarabine-induced apoptosis, and this protective effect was neutralized by the addition of CXCR4 antagonists. Another study found that CLL cells were protected from apoptosis caused by the monoclonal antibodies alemtuzumab and rituximab when co-cultured with stroma; the addition of TN14003 counteracted the protective effect of stroma [105].

These experiments collectively demonstrate the importance of the bone marrow microenvironment in the survival of a variety of leukemic cell types. Blockade of the interaction between CXCR4 and CXCL12 appears to diminish the protective effect of bone marrow stroma, and the incorporation of CXCR4 inhibitors as a new class of anticancer agents may be effective in the treatment of leukemia.

Clinical data on the importance of CXCR4

Prognostic factors in leukemia

Several studies have suggested that increased CXCR4 expression may be a prognostic indicator in childhood ALL [106], adult AML [107] and B-cell CLL [108]. In pediatric ALL, surface CXCR4 expression has been shown to be higher in patients with extramedullary disease [106]. Another study found that blasts from children with relapsed ALL with bone marrow and extramedullary disease in the testicles or CNS had a significantly lower CXCR4 level than blasts from children with relapsed disease that was isolated to the bone marrow [109], suggesting that blasts were not tightly retained in the bone marrow and then allowed to metastasize. In AML, several studies have indicated that high expression of CXCR4 can confer a poorer prognosis [110,111].

Interaction with tyrosine kinases

CXCR4 signaling appears to interact with important tyrosine kinases found in leukemias. An interaction between Flt3–ITD and CXCR4 has been demonstrated in several papers. One

study showed that leukemia cell lines expressing Flt3-ITD had significantly increased chemotaxis toward CXCL12 when compared with Flt3 wild-type cell lines [112]. Furthermore, incubation with Flt3 ligand initially enhanced migration toward CXCL12, but prolonged incubation resulted in decreased migration through downregulation of CXCR4 expression and inhibition of its down-stream signaling pathway. Another study by the same group demonstrated that incubation with Flt3 ligand resulted in decreased bone marrow homing of murine marrow cells transduced with Flt3-ITD *in vivo*, in spite of increased migration to CXCL12 *in vitro* [113]. In addition, Flt3-ITD cells demonstrated increased ability to generate colony-forming unit-granulocyte/macrophage when exposed to CXCL12, compared with cells transduced with Flt3 wild-type or empty vector.

The Philadelphia chromosome, or the fusion of breakpoint cluster region (*BCR*) on chromosome 22 and v-abl Abelson murine leukemia viral oncogene homolog (*ABL*) on chromosome 9, is the hallmark of CML and occurs in a subset of children and adults with ALL [114]. The presence of *BCR-ABL* fusion leads to an altered chemotactic response of HSCs to CXCL12 [115]. *BCR-ABL* appears to increase expression of the β -integrin LFA-1 and disrupt CXCL12/CXCR4 signaling pathways [116]. High expression of the p210 *BCR-ABL* oncoprotein in leukemia cell lines leads to down-regulation of CXCR4 through decreased transcription, while low expression of p210 *BCR-ABL* induces a CXCR4 signaling defect but no change in CXCR4 expression [76]. Patients in CML blast crisis also appear to have decreased CXCL12-induced chemotaxis.

Clinical data regarding CXCR4 inhibitors in hematologic malignancies

Several trials are attempting to define the role of CXCR4 inhibition in the treatment of hematologic malignancies. Inhibition of CXCR4 could mobilize leukemic cells, force leukemic cells to enter the cell cycle, and make leukemic cells susceptible to cytotoxic chemotherapy and/or other anticancer therapies.

An adult with relapsed AML was treated with plerixafor in combination with reinduction chemotherapy that included cytosine arabinoside and idarubicin as part of a compassionate use program in Germany [117]. One day after administration of AMD3100, the amount of CD34⁺ blasts present in the bone marrow was reduced by over 40%. While reinduction therapy resulted in the clearance of peripheral blasts, the patient had persistent bone marrow disease at the end of reinduction. This patient had FAB subtype M0 AML, which is known to have low levels of surface CXCR4 expression [61], but interestingly had significant mobilization of blasts in response to CXCR4 inhibition.

An open-label Phase I/II study of plerixafor in combination with mitoxantrone, etoposide and cytarabine in adults with relapsed or refractory AML also showed promising results [118]. Complete remission was achieved in half of patients. Plerixafor induced a mean 2.5-fold increase in AML blasts into the peripheral blood, and higher baseline CXCR4 expression correlated with increased blast mobilization in response to CXCR4 inhibition.

Plerixafor has also been evaluated in CLL and multiple myeloma. Preliminary results of a Phase I dose escalation trial combining plerixafor with rituximab in patients with relapsed CLL showed mobilization of CLL cells in response to plerixafor [119]. In addition, eight out of 14 evaluable patients had stable disease or a partial response. A Phase I study of plerixafor in combination with bortezomib in patients with relapsed or refractory multiple myeloma found that ten out of 12 evaluable patients had stable disease or better, with one complete response [120].

Other leukemia–microenvironment interactions

Recent studies have demonstrated that CXCL12 also binds to another member of the CXC chemokine receptor family CXCR7 [121]. CXCL7 was initially classified as an orphan receptor but was later found to bind CXCL12, and chemotaxis in CXCR7⁺ cells is induced by CXCL12 [122]. While the affinity of CXCL12 for CXCR7 is ten-times higher than that of CXCR4, CXCR7 also binds to interferon-inducible T-cell chemoattractant [123]. CXCR7 is expressed at low levels in normal CD34⁺ cells but highly expressed in several AML cell lines [124]. Furthermore, treatment of leukemia cell lines with interferon-inducible T-cell chemoattractant led to phosphorylation of Akt and MAPK p42/p44 and increased adhesion to human umbilical vein endothelial cell monolayers. Another study showed that CXCR7 can dimerize with CXCR4 in T lymphocytes and interfere with CXCL12-induced intracellular calcium mobilization, interactions between CXCR4 and G proteins, and chemotaxis [125].

Studies have suggested that other adhesion molecules and ligands, such as VLA-4, fibronectin, homing-associated cell adhesion molecule and LFA-3, can play a role in leukemia cell adherence to stroma and subsequent release from the bone marrow into the periphery [126,127].

Evaluation of integrin expression on HPCs, leukemic cell lines and primary AML blasts found consistent expression of VLA-4 α and VLA-5 α [128]. VLA-4 is known to bind to VCAM-1, and leukemia/stromal cell adhesion was interrupted by treatment with either anti-VLA-4 or anti-VCAM-1 antibodies. In pre-B-cell ALL, leukemia/stroma adhesion is mediated by the β 1 integrins VLA-4 and VLA-5 on leukemic cells and VCAM-1 on stroma cells [129].

One experiment showed that treatment of precursor-B ALL cells with anti-VLA-4 antibodies prior to transplant in NOD/SCID mice resulted in significantly lower homing of cells to the bone marrow [130]. In addition, treatment of NALM-6 cells with CXCL12 caused increased adhesion to fibronectin, laminin and VCAM-1 [68]. Additional experiments demonstrated that CXCL12-stimulated adhesion to fibronectin was mediated by VLA-4 and VLA-5, adhesion to laminin was mediated by VLA-6 and adhesion to VCAM-1 was mediated by VLA-4. NALM-6 cells were then pretreated with CXCL12 over a prolonged period of time in order to decrease surface CXCR4 expression; mice transplanted with CXCL12-pretreated NALM-6 cells had significantly decreased leukemic cells in the bone marrow and no evidence of extramedullary disease, presumably due to decreased responsiveness to *in vivo* CXCL12 and blunted activation of adhesion molecules, as a result of decreased surface CXCR4 expression caused by CXCL12 pretreatment.

A prospective evaluation of VLA-4 as a prognostic indicator in pediatric AML found that patients with high VLA-4 expression had a better outcome than those with low expression [131]. Conversely, a separate prospective evaluation of prognostic markers in adults with AML found that patients with high expression of CXCR4, VLA-4 and focal adhesion kinase had a significantly shorter overall survival [132].

Interestingly, a study of primary AML, ALL and CLL samples found that a high number of cases expressed VCAM-1 [133]. In addition, patients with AML and high expression of VCAM-1 had leukemic skin infiltrates, while patients with CLL and high expression of VCAM-1 had advanced Rai stages. VCAM-1 expression appeared to be highest in B cells and CD56⁺ AML cells. The hypoxic environment of the bone marrow appears to cause lymphocytes to bind to mesenchymal cells via the integrin LFA-1 [134]. AML cells also bind to stroma via a β 1 and β 2 integrin-dependent mechanism [135], and pseudoemperipolesis appears to be mediated by CXCR4 and α 4 β 1 integrins [63].

Future directions

Several clinical trials of CXCR4 inhibitors in AML, multiple myeloma and HSC mobilization are actively recruiting patients, including trials of plerixafor, BKT140, MDX-1338, and the oral agents MSX-122 and TG-0054 [203]. For example, plerixafor is being combined with a variety of chemotherapeutic agents and tyrosine kinase inhibitors, such as cytarabine, daunorubicin, clofarabine, mitoxantrone, etoposide and sorafenib, in the treatment of adults with AML. In another trial, plerixafor is being used in combination with rituximab in the treatment of CLL or small lymphocytic lymphoma. CXCR4 inhibition is also being evaluated in multiple myeloma in a Phase I/II trial of BKT140.

A trial of plerixafor in the pediatric population is planned and will test the combination of plerixafor, cytarabine and etoposide in the treatment of children and young adults with relapsed or refractory AML, ALL or myelodysplastic syndrome. Plerixafor is also being evaluated as a mobilizing agent prior to allogeneic stem-cell transplant.

One potential drawback to inhibiting CXCR4 is that leukemic blasts will be released into the peripheral circulation. While CXCR4 inhibition will drive blasts out of protective bone marrow niches and likely make them more sensitive to chemotherapy, some experts have expressed concern that mobilized blasts may have the potential to infiltrate extramedullary organs. However, all of the currently open clinical trials examining CXCR4 inhibitors as chemosensitizers are using the inhibitors in combination with more than one chemotherapeutic agent, thereby increasing the probability that mobilized blasts will be killed.

Conclusion

The interaction of CXCL12 and CXCR4 is clearly critical in the regulation of normal hematopoiesis, and significant evidence exists that this axis is important in leukemia. Phase I and II trials of CXCR4 inhibitors have shown potential in several hematologic malignancies. Ongoing and future trials combining CXCR4 inhibition with chemotherapy, tyrosine kinase inhibitors, epigenetic-modifying medications and other anti-cancer agents will help to define the role of CXCR4 inhibitors in hematologic malignancies.

Expert commentary

Much research has been performed in order to uncover mechanisms that leukemia cells utilize for survival, proliferation and resistance to chemotherapy. Preclinical studies have clearly demonstrated the importance of the bone marrow stromal microenvironment in the survival of leukemia. In particular, much emphasis has been placed on the role of CXCR4 and CXCL12 as an external pathway to provide leukemia with a proliferative advantage. The interaction between CXCR4 and CXCL12 may also mediate the survival of leukemia-initiating cells, which can contribute to chemoresistance and relapse. Targeting the CXCL12/CXCR4 axis through the use of CXCR4 inhibitors can potentially alter the treatment of both pediatric and adult leukemias.

Five-year view

Future research will likely solidify the role of CXCR4 and CXCL12 as a pathway that supports the survival of malignant cells. The results of ongoing and planned clinical trials will define the role of CXCR4 inhibition in the treatment of hematologic malignancies. Additional studies may also uncover other interactions between leukemia and stromal cells that contribute to cell survival and resistance to treatment. We anticipate that the future of

leukemia therapy will focus not only on survival mechanisms that are intrinsic to the malignant cell, but also on extrinsic pathways that are mediated by the microenvironment.

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- of considerable interest

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Key issues

- CXCR4 is a seven-transmembrane domain, GTP-binding protein-coupled receptor present on a variety of normal hematopoietic cells, including lymphocytes, monocytes, neutrophils, and hematopoietic stem and progenitor cells.
- Activation of CXCR4 by CXCL12 leads to chemotaxis of CXCR4-expressing cells to microscopic niches within the bone marrow.
- High levels of CXCR4 expression have been demonstrated in subsets of acute, chronic, lymphoid and myeloid leukemias.
- CXCR4 and CXCL12 appear to modulate chemotaxis, pseudoemperipolesis and chemotherapy resistance in preclinical studies of leukemia cell lines and primary patient samples.
- Multiple CXCR4 inhibitors have been developed including peptide antagonists (T140 and its analogs TC14012 and TN14003/BKT140), nonpeptide antagonists (plerixafor/AMD3100 and AMD3465) and monoclonal antibodies (MDX-1338/BMS-936564).
- Preclinical studies have demonstrated that CXCR4 inhibition can decrease or even reverse protection from chemotherapy and/or tyrosine kinase inhibitor-induced apoptosis that is conferred by bone marrow stroma.
- Treatment strategies currently being studied in clinical trials include CXCR4 inhibitors as single agents or in combination with chemotherapy.