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Cell Trafficking in Chronic Lymphocytic Leukemia

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Abstract

Chronic lymphocytic leukemia (CLL) is an indolent lymphoproliferative disorder characterized by both circulating peripheral disease as well as involvement of the lymph nodes and bone marrow. Increasing evidence suggests that the stromal microenvironment provides anti-apoptotic and pro-survival signals to CLL cells, and may contribute significantly to resistance to a wide variety of treatments. Our understanding of the complex interactions involved in CLL cell trafficking continues to grow. Chemokines and corresponding chemokine receptors are key factors for organizing CLL cell trafficking and homing and the complex cellular interactions between CLL and accessory cells. Important chemokines include CCL3, CCL4, and CCL22, which are released by CLL cells, and CXCL12, CXCL13, CXCL9, 10, 11, CCL 19, and CCL21, which are constitutively secreted by various stromal cells. Integrins such as VLA-4 (CD49d) as well as selectins and CD44 also likely play a role in directing CLL cell migration within the tissue microenvironments. Data are also emerging that other molecules such as MMP-9 and cytoskeletal proteins also contribute to CLL cell trafficking. Though this interplay is complex, it is critical that we improve our understanding of CLL cell trafficking to facilitate the development of novel therapies that target these pathways. Several drugs in clinical development, such as CXCR4 antagonists and PI3K, Btk, and Syk inhibitors appear to modulate CLL cell trafficking and CLL-stroma interactions. Here, we review the current understanding of the molecular interactions that underlie CLL cell trafficking and we highlight some of the promising approaches underway to target these pathways therapeutically in CLL.

INTRODUCTION

Growing evidence suggests that cell trafficking plays a critical role in the pathophysiology of chronic lymphocytic leukemia (CLL). Chemokines such as CCL3, CCL4, CCL22, and IL-8 are secreted by CLL cells and recruit T cells and other accessory cells that can deliver pro-survival signals. Stromal cells in the bone marrow and lymph nodes secrete other chemokines such as CXCL12, CXCL13, CXCL 9, 10, 11, and CCL 19 and 21, which bind to a variety of corresponding receptors on the CLL cell surface (Figure 1). These interactions lead to CLL cell chemotaxis into the tissue microenvironments, where the malignant cells are then subject to survival and proliferation signals through the B cell

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receptor and other pathways. Once in the tissues, CLL cells are surrounded by a supportive microenvironment that includes cells expressing CD40L, fibronectin, and VCAM-1, all of which provide additional survival and anti-apoptotic signals to the CLL cells. Here, we will discuss the underlying biology of CLL cell trafficking, review what is known about the complex interactions of CLL cells with stroma, and highlight novel strategies being undertaken to disrupt stroma-mediated survival signals as part of a new therapeutic approach in CLL.

CHRONIC LYMPHOCYTIC LEUKEMIA

CLL is the most common leukemia in the Western hemisphere, with an incidence of 4.2 per 100,000 or about 15,000 new cases diagnosed in the United States each year [1]. Although there have been recent improvements in prolonging survival with combination chemoimmunotherapy regimens, the disease remains incurable with conventional therapy, and about 4,400 men and women die of CLL in the U.S. each year. A common clinical observation has been that, although therapies are often effective at killing CLL cells in the peripheral blood, residual disease remains in the bone marrow and lymph nodes. It is likely that these malignant cells sequestered in the tissue receive protection from a wide variety of treatments through pro-survival signals and inhibition of apoptosis fostered by the stromal microenvironment. The complex biology underlying how these CLL cells are recruited, maintained, and released from the stroma is an area of active investigation.

CHEMOKINES

Chemokines Secreted by CLL cells

Biology—Recent studies suggest that chemokines secreted by CLL cells may play a key role in creating a supportive microenvironment via attraction of accessory cells, such as T cells and monocytes. Two such chemokines are CCL3 and CCL4, which normally play a role in the adaptive immune response, but have also been found to be highly expressed and secreted by activated CLL cells [2]. CCL3/4 have also been shown to be elevated in the plasma of CLL patients, and to be strongly associated with poorer prognosis [3]. The adaptive advantages gained by CLL cells secreting CCL3/4 remains unclear. There are some data to suggest that these chemokines may play a role in recruiting CCR5+ regulatory T cells into closer proximity with B cells, which may allow these T cells to more efficiently provide survival signals to the malignant B cells in CLL [4]. Recent in vitro work by us modeled the interactions of the lymph node microenvironment by showing that CLL cells secrete CCL3/4 when co-cultured with nurse-like cells (NLCs) [5]. Interestingly, CLL cells isolated from the lymph nodes displayed similar gene expression profiles (GEP) as CLL cells co-cultured with NLCs, with robust up-regulation of CCL3/4 [6]. Zucchetto and colleagues also recently showed that CD38 and CD49d may play a key role in stimulating CCL3/4 production, and ultimately to improving survival of CLL cells [7].

Similar to CCL3/4, CCL22 may play a role in recruiting T cells into the tissues where they can interact with CLL cells. It is likely that CCL22 acts more as a secondary T cell recruitment signal rather than a primary signal, as evidenced by the fact that only after CD40 ligation does CCL22 get secreted by CLL cells [8]. Interestingly, peripheral blood-derived CLL cells do not appear to secrete CCL22, whereas lymph node or bone marrow-derived CLL cells do, presumably because these latter cells have been exposed to CD40 ligation. The main receptor for CCL22 is CCR4, which is highly expressed on FoxP3+ regulatory T cells (Tregs). The immunosuppressive function of Tregs has been well-characterized, but the specific mechanism of how these cells would provide survival signals to CLL cells is less well understood. One hypothesis is that after CD40 ligation, secreted CCL22 itself may lead to upregulation of anti-apoptotic proteins (e.g. BCL-2 and MCL-1) increasedthrough

increased phosphorylation of IKKα/β [9]. By dichotomizing CLL patients into groups that were either responsive or unresponsive to CD40 ligation, Scielzo and colleagues recently showed that unresponsive patients had a shorter time to progression and also found that CLL cells from unresponsive patients were able to proliferate without as much need for stromal support [9].

When stimulated with CD40 or CD74, interleukin-8 (IL-8) has also been found to be secreted by CLL cells, which through an autocrine or paracrine mechanism may provide additional survival signals to the malignant cells [10]. Another potential trigger for CLL cell IL-8 secretion may be through the nuclear factor kB (NF-kB) family, as the receptor activator of NF-kB ligand (RANKL) has been shown to lead to the release of IL-8 and to cause CLL cells in vitro to show decreased apoptosis in response to chemotherapy [11]. Plasma IL-8 levels have been shown to correlate with survival in CLL [12], and given this prognostic significance, clarifying the mechanisms underlying IL-8 mediated survival merits further investigation.

Therapeutic Targets—Inhibiting the secretion of these pro-survival chemokines by CLL cells is a promising therapeutic target. There is growing evidence that inhibition of B cell receptor (BCR) signaling, such as with a spleen tyrosine kinase (Syk) inhibitor, leads to decreased secretion of CCL3/4 by CLL cells co-cultured with NLCs or after stimulation with anti-IgM [2]. The phosphoinositide 3′-kinase (PI3K) delta inhibitor CAL-101 (GS1101), which inhibits the B-cell receptor pathway downstream of Syk, was also recently shown to cause marked reductions in circulating CCL3/4 in patients treated with the drug [5]. Although direct inhibition of CCL22 or IL-8 has not yet been targeted in CLL, the reliance of the production of these chemokines on CD40L stimulation suggests that inhibition of CD40L may be a potential therapeutic target. Indeed, the novel antagonist anti-CD40 monoclonal antibody, HCD122, was shown in vitro to be a potent inhibitor of B-cell growth, and to lead to antibody-dependent cellular cytotoxicity of CLL cells [13], prompting interest in the further development of this antibody in the treatment of CLL.

Chemokines Secreted by Stromal Cells

Biology—While chemokine secretion by CLL cells may play an important role in recruitment of accessory cells, chemokines secreted by stromal cells are also critical for homing and tissue retention of CLL cells. CLL cells are thought to constantly circulate throughout the body, and powerful chemotactic signals sent from bone marrow and lymph node stromal cells attract CLL cells into the microenvironment, where they are then surrounded by a diverse array of both pro-survival and proliferative signals. Once resident in these stromal environments, CLL cells are protected from a diverse array of therapeutic interventions, and thus understanding how the stroma signals to attract these cells is of critical importance with regard to developing more effective therapies in CLL.

Perhaps the best understood of these stromal chemokines is CXCL12 (formerly called stroma-derived factor-1 (SDF-1)), originally characterized as a pre-B cell growth factor [14]. CXCL12 has at least two major effects on CLL cells—it causes migration towards stromal cells and it independently and directly provides survival signals, as shown by us in the NLC system [15]. Both of these effects are mediated through the CXCR4 receptor on the surface of the CLL cell, which is downregulated via receptor endocytosis once activated by CXCL12 [16]. Thus, CXCR4 cell surface expression can be used as a marker of CXCL12 exposure, with circulating CLL cells in the peripheral blood typically expressing high levels of surface CXCR4, and CLL cells resident in the bone marrow or lymph nodes having lower levels of surface CXCR4 [17]. Upon stimulation by CXCL12, signaling through the CXCR4 receptor has pleotropic effects on CLL cells, including activation of PI3 kinases [16], serine

phosphorylation of signal transducer and activator of transcription 3 (STAT3) [18] and p44/42 MAP kinases [15], as well as effects on calcium homeostasis.

The CXCL12/CXCR4 axis can also be modulated by a variety of other pathways in the CLL cell. For example, increased signaling through the BCR pathway may lead to enhanced chemotaxis to CXCL12 [19]. These interactions of CXCR4 and BCR signaling can be inhibited by either Syk [20] or PI3K [21] inhibition, both of which result in reduced chemotaxis and CLL cell migration. Additionally, interactions of CXCR4 with CD38 may play an important role, as CD38+ CLL cells appear to have enhanced chemotaxis, an effect which can be blocked with anti-CD38 mAbs [22]. Recent data also suggest that ZAP-70(+) CLL cells receive RAF-dependent survival signals in response to CXCL12 [23].

Another key chemokine in CLL trafficking is CXCL13 (formerly called B cell-attracting chemokine 1 (BCA-1)), which binds to the CXCR5 receptor and mediates lymphocyte homing and the positioning of lymphocytes in the follicular lymph node architecture [24]. CXCL13 is constitutively secreted by lymph node stromal cells, recruiting both normal and malignant lymphocytes into the lymph nodes [25]. There, CXCL13 further directs lymphocytes into the typical follicular arrangement where normally they would proceed from centrally-located centroblasts into more peripheral centrocytes. These effects of CXCL13 are observed in B1 B cells, which are thought to potentially represent the normal counterparts to CLL cells [26] and have been shown to have the capacity for self-renewal [27]. Since CLL cells can form pseudofollicles, it has been hypothesized that CXCL13 also directs the arrangement of the malignant cells, and may foster the development of CLL cell proliferation centers.

CXCR5 is highly expressed on the surface of CLL cells [28], and binding of CXCL13 has been shown to lead to CXCR5 receptor endocytosis and a variety of downstream effects, including increased cell migration, actin polymerization, and p44/42 MAPK pathway activation [29]. Abnormalities in the CXCR5/CXCL13 axis have also been described in a variety of autoimmune diseases, such as lupus [30]. Given the propensity of CLL patients to develop autoimmune phenomena such as autoimmune hemolytic anemia, it is tempting to speculate that alterations in CXCR5 signaling may contribute to the development of these sequellae.

CCL19 and CCL21 are two additional chemokines secreted by stromal cells, and they serve as ligands for the CCR7 receptor on the CLL cell surface. These molecules normally play an important role in the trafficking and distribution of immature normal B and T cells [31]. Increasingly, a role for CCL19/21 and CCR7 has been recognized in CLL. CCL19/21 are secreted by stroma, and after binding to CCR7 lead to migration of CLL cells across the vascular endothelium [32]. Expression of CCR7 on the CLL cell surface is typically high, and the degree of positivity has been shown to be associated with expression of ZAP-70, CD38 [33], and with the presence of lymphadenopathy [32]. The ability of CCL19/21 to induce CLL cell migration also appears to be more pronounced in ZAP-70/CD38 positive CLL cells. Recent work with primary CLL patient samples suggests that ZAP-70 may directly increase the expression of CCR7 predominantly through ERK1/2, increasing the response and migration toward CCL21 [34]. These effects are also likely mediated through a variety of downstream effectors, such as Rho kinase and PI3 kinase [35]. Another modifier of the CCL19/21-CCR7 axis is the human chemokine receptor on activated macrophages (CRAM), which is expressed on B cells in a maturation-stage dependent manner. High levels of CRAM in CLL cells were recently shown to be detrimental to efficient chemotaxis with CCL19, an effect which appears to be maintained over time [36].

The effects of the chemokines CXCL9, 10, and 11 and their receptor CXCR3 are less well-characterized in CLL. They are thought to be induced by interferon-gamma (IFN-g) and to mediate chemotaxis at sites of inflammation [37]. CXCR3 is consistently expressed on CLL cells, but the level of expression is variable [38]. One study showed overexpression of CXCR3 in patients with early stage CLL, which was then confirmed with gene expression profiling [39]. Another study found that low CXCR3 expression was associated with advanced stage and poorer prognosis in CLL patients [40]. Although these studies do suggest a potentially important role of CXCL9, 10, and 11 in the trafficking of CLL cells, further investigation will be required to more clearly define their specific roles.

Therapeutic Targets—Once resident in the stroma, CLL cells are protected from treatment with a variety of otherwise active agents [41]. Therefore, both strategies designed to mobilize CLL cells out of stroma (so-called 'chemosensitization') as well as strategies to prevent CLL cells from migrating into the stroma hold great promise. Inhibition of the CXCR4/CXCL12 axis is the furthest along in its clinical development. CXCR4 antagonists, such as the bicyclam molecule plerixafor have already been FDA-approved as stem cell mobilizing agents for autologous transplantation in non-Hodgkin lymphoma. An ongoing study of plerixafor in combination with the anti-CD20 antibody rituximab in CLL patients has shown that plerixafor efficiently mobilizes CLL cells from the stroma into the blood, where the malignant cells are considered more sensitive to treatment with rituximab [42]. There is also interest in utilizing CXCR4 antagonists to help mobilize minimally-residual disease (MRD) in CLL patients otherwise thought to be in complete remission, with the aim of eliminating residual cells in the stroma that could later lead to relapse.

A recent observation with novel agents in CLL such as the PI3K-delta inhibitor CAL-101 (GS1101) [43], the Bruton's tyrosine kinase (Btk) inhibitor PCI-32765 [44], and the Syk inhibitor fostamatinib disodium [45] has been that these agents cause transient lymphocytosis at the same time that they cause decreases in lymph node size. It is likely that inhibition of the CXCR4/CXCL12 axis play an important role in mediating this effect [20, 21, 46], which appears to contribute substantially to the promising efficacy of these agents.

Other stromal cytokines and their receptors may also have the potential for therapeutic targeting. For example, inhibitors of CXCR3 such as AMG487 have been shown to inhibit metastases in model systems of colon cancer [47] and osteosarcoma [48]. Inhibition of CXCR5 or CCR7 could also theoretically disrupt stromal survival signals, but these strategies have not yet been fully explored.

ADHESION MOLECULES

Biology

Integrins are a diverse family of cell adhesion molecules that regulate cell growth and function in the stromal microenvironment. A key integrin in CLL is very late antigen 4 (VLA-4, CD49d), which plays an important role in the homing and retention of CLL cells in the microenvironment [49]. Recent data from Binsky and colleagues suggest that expression of VLA-4 is increased in CLL through the CD74 pathway, mediated by Tap63 [50]. Blocking of VLA-4 was found to inhibit the in vivo homing of CLL cells to the bone marrow. A recent study by Brachtl and colleagues confirmed that patients with high VLA-4 expression had significantly increased CLL infiltration of the bone marrow, and that patients with high VLA-4 expression also had a poorer prognosis [51].

In a recent study of over 300 CLL patients, Gattei and colleagues showed that CD49d was an independent predictor for both time to treatment (HR=1.74) and overall survival (HR=3.52) [52]. Recent data from Majid and colleagues also demonstrate that CD49d is

associated with CXCR4 expression in CLL, suggesting a coordinated role for these molecules in the trafficking of CLL cells to lymphoid tissues [53]. CD49d cell surface expression can be assessed by flow cytometry, and, given its prognostic significance, it may be worth considering adding CD49d to the panel of routine immunophenotyping in CLL.

The integrin lymphocyte function-associated antigen-1 (LFA-1) has been shown to exhibit aberrant behavior in CLL cells compared to normal B cells [54]. One study found reduced levels of LFA-1 in CLL cells, which was postulated to have contributed to impairment of the migratory pattern of the malignant cells [55]. Further investigation into the role of LFA-1 is ongoing.

Selectins are carbohydrate-binding molecules that facilitate adhesion of malignant cells to tissue and result in the activation of integrins and release of chemokines. Aberrant selectin activity has also been postulated to regulate cell trafficking in CLL. Work by Spessoto and colleagues suggests that laminin-332 (laminin-5) is present as a reticular mesh throughout the lymph node microenvironment, and may act as a key motility-promoting factor for CLL cells [56]. Another important molecule in CLL cell migration is vascular cell adhesion molecule-1 (VCAM-1). VCAM-1 is expressed on the surface of stromal/endothelial cells, and likely can provide direct survival signals to CLL cells that contact it [7]. In addition, elevated levels of soluble VCAM-2 have been shown to be associated with CLL tumor burden [57].

Therapeutic Targets

It is likely that inhibitors of the BCR pathway have downstream inhibitory effects on integrins and selectins. For example, Syk inhibition has been shown to reduce migration of CLL cells toward VCAM-1 [58]. Targeting integrins and selectins directly also may be a promising strategy. Recent work by Mraz and colleagues using the blocking monoclonal anti-VLA-4 antibody natalizumab has demonstrated that the drug decreases B lymphocyte adherence to stroma and partially overcomes stromal protection toward rituximab and cytotoxic drugs [59]. Although natalizumab has a potential role in treating patients with lymphoid malignancies, case reports have described cases of new primary CNS lymphoma in patients treated with the drug [60]. Although it is not clear whether a true association exists [61], these cases have tempered the excitement of using this drug in CLL. An oral inhibitor of integrin (alpha)-2 expression, E7820, is also now in development, and was recently shown to be well-tolerated in patients with advanced solid malignancies [62].

OTHER PATHWAYS

There are several other molecules with emerging evidence to suggest a role in CLL cell trafficking. Matrix metalloproteinase-9 (MMP-9) has been identified on the surface of CLL cells and not normal B cells, which may allow the malignant cells to transmigrate through stroma more efficiently [63], possibly in conjunction with increased signaling through CXCR4 [64]. There has also been recent work showing that ephrin-A4 (EFNA4) may be dysfunctional in CLL cells, leading to impaired CLL cell trafficking between the blood and the tissues [65].

The role of cytoskeletal proteins in CLL cell trafficking is increasingly being recognized. One molecule under investigation is an intracellular protein hematopoietic cell-specific Lyn substrate-1 (HS1), which acts as a central regulator of the cytoskeleton, and may play an important role in CLL cell trafficking and tissue invasion [66]. Another molecule being studied is the tandem pleckstrin homology domain protein (TAPP), which likely has multiple interaction partners in the cytoskeleton, and may lead to increased CLL cell adhesion to fibronectin and laminin [67]. Interestingly, the regulation of TAPP2 has been

linked to PI3K signaling, providing a possible mechanistic link to help explain the lymphocyte mobilizing properties of the PI3K inhibitors that have been observed in the clinic.

Although these newly-discovered mechanisms may play an important role in CLL cell trafficking, the potential to exploit them therapeutically remains to be explored.

CONCLUSIONS

The last several years have led to an explosion of knowledge about CLL cell trafficking. This intricate system is driven by the release of chemokines from CLL cells themselves, as well as potent chemokines released by stroma which interact with receptors on the CLL cell surface. These migratory signals are also modulated by cell-cell contact of CLL cell surface proteins with the integrins and selectins present on stromal cells. A wide variety of other molecules such as MMPs and cytoskeletal proteins are also likely to influence these interactions. Although CLL cell trafficking is complex, our increasing understanding of this field has the potential to lead to promising new approaches to treating this disease. Through new agents that inhibit CLL cell migration and therefore reduce stroma-mediated protective signals toward CLL cells, there is great potential to improve the treatment of CLL. Though many challenges lie ahead, our increasing understanding of CLL cell trafficking has made the prospect of developing curative therapies for this disease an increasingly more realistic goal.

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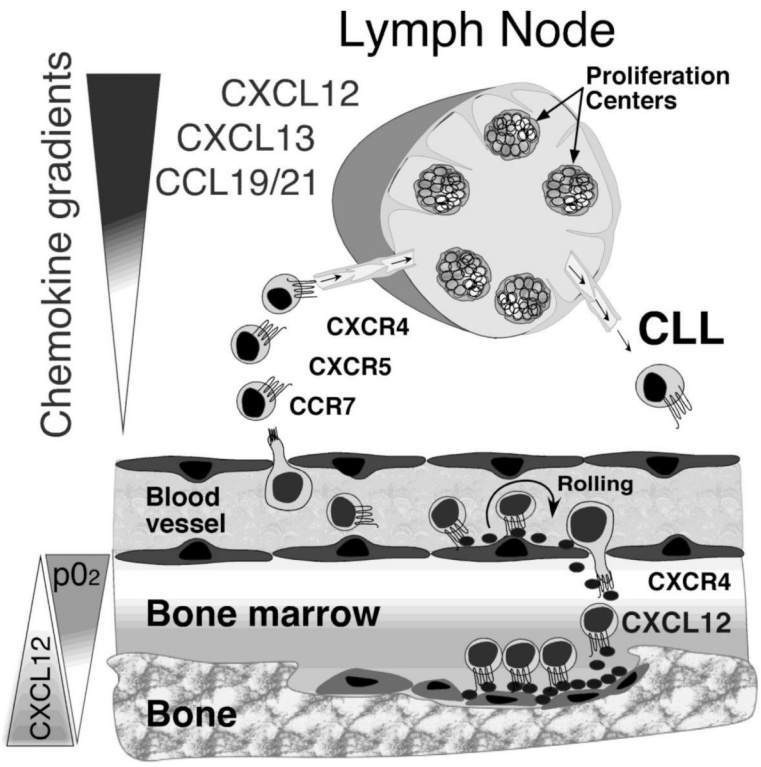

Figure 1.

CLL cell trafficking. CLL cells circulate freely in the peripheral blood, but are attracted to stromal microenvironments like the bone marrow and lymph nodes by chemokine gradients established by stromal cells. Critical chemokines such as CXCL12, CXCL13, and CCL19/21 bind to the CXCR4, CXCR5, and CCR7 receptors on CLL cells, respectively. This leads to transmigration of CLL cells into the lymph nodes and bone marrow, where they are protected by a variety of anti-apoptotic and pro-survival signals, which may ultimately contribute to treatment resistance.
(adapted after Burger JA et al, Blood 2006)