REVIEW

Stromal control of chronic lymphocytic leukemia cells

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Abstract: In the ongoing efforts to develop therapies against chronic lymphocytic leukemia (CLL), stromal factors allowing malignant cells to escape spontaneous and chemotherapy-mediated apoptosis, giving way to relapses, have been abundantly investigated. Bone marrow adherent cell types, collectively referred to as stromal cells, appear to be key players in such escape, mainly because CLL malignant cells, which rapidly undergo spontaneous apoptosis when cultured in vitro, survive, migrate, and resist cytotoxic agents in co-culture with bone marrow stromal cells. CLL displays variable clinical courses according to well-defined prognostic factors induced on malignant B-cells (CLL cells) or expressed by the transformed bone marrow stromal microenvironment. Particularly, a critical pathogenic role is played by proinflammatory factors, adhesion molecules, and signaling molecules involved in cell fate and stemness, such as Notch, Wnt, sonic Hedgehog, phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), and the B-cell CLL/lymphoma 2 (Bcl-2) family of regulator proteins. As herein discussed, these molecules probably form a complex network favoring CLL cell survival, proliferation, and chemoresistance to anticancer therapy. Characterizing the sets of signaling pathways involved in the interactions between stromal cells and CLL cells may provide new tools for CLL clinical phenotyping and for re-sensitizing chemotherapy resistant cells.

Keywords: chronic lymphoblastic leukemia, CLL, signaling pathways, cancer, stromal cells

Introduction

B-cell chronic lymphocytic leukemia (CLL), the most common lymphoproliferative disorder,[1] is characterized by the clonal expansion of mature antigen-stimulated B-cells (cluster of differentiation 19+ [CD19+]/CD5+/CD23+) in the blood and secondary hematopoietic organs, including lymphoid tissues, and bone marrow (BM), in close contact with stromal microenvironment.[1,2] The disease displays variable clinical courses according to well-defined prognostic factors induced on malignant B-cells (CLL cells) or expressed by the transformed BM stromal microenvironment.[3,4] A critical role in the pathogenesis is played by: (1) proinflammatory factors and adhesion molecules that would be involved in the migration of CLL cells into the BM stroma, including for instance the cell surface glycoprotein CD44, tumor necrosis factor-related factors, stromal cell-derived factor 1 (SDF-1) also termed as C-X-C motif chemokine 12 (CXCL12) and its receptor C-X-C motif receptor 4 (CXCR4); and (2) signaling molecules involved in fundamental cellular functions associated with tumorigenesis, including proliferation, differentiation, and apoptosis, such as phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), Notch, Wnt, sonic Hedgehog (Hh), and the B-cell CLL/lymphoma 2 (Bcl-2) family of regulator proteins.[5–8] Early and emerging experimental

evidence suggests that CLL malignant B-cells retain the capacity to respond to most of these molecules in vivo and in vitro, pointing out interesting opportunities for devising therapies for this currently incurable disease. However, the precise mechanisms of action of such molecules are not well understood, and they appear to interact in a complex fashion (Figure 1). The present review summarizes and discusses the current understanding of the molecular interactions between CLL cells and the BM stroma.

BM stromal cells support the proliferation, survival, and chemoresistance of CLL cells

BM stroma derives from mesenchymal stromal cells (MSCs), adult multipotent nonhematopoietic stem cell precursors which support the maintenance and engraftment of hematopoietic stem cells.[9,10] MSCs reside in virtually all organs containing connective tissue,[9] including lymphoid organs.[10] Co-culture with BM stromal cells allows normal B-cells to escape from spontaneous apoptosis.[11,12] Not surprisingly, early studies revealed that human BM stromal cells prevent apoptosis and support the survival of CLL cells in vitro,[13–15] and mouse stroma allowed long-term proliferation of human leukemia cells,[16] indicating a key role of factors released or induced by stromal cells in the maintenance and proliferation of CLL cells. CLL cells are also affected by these cell types and by the microenvironment in vivo. For instance, CLL cell growth is under the control of signals from T-cells and stromal cells in the pseudofollicular proliferation centers. Stromal cells would contribute to CLL cell long-term support through bidirectional interactions[17]

Figure 1 The interactions of the bone marrow stromal environment with CLL B-cells are complex. Note the crosstalk between antiapoptotic factors and angiogenic factors or proinflammatory factors.
Abbreviations: bFGF, basic fibroblast growth factor; CD44, cluster of differentiation 44; CLL, chronic lymphocytic leukemia; CXCR4, C-X-C motif receptor 4; SDF-1, stromal cell-derived factor 1; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

resulting in the activation of various survival pathways, including Wnt and Notch, as discussed further (see "Proto-oncogenic factors").

Moreover, CLL cells are also closely associated with follicular dendritic cells in both the early phase of BM involvement and in the lymph nodes.[18] Such association would play a key role in the pathogenesis of B-cell lymphoma, mainly in malignant cell proliferation and survival.[11] Furthermore, a subset of blood mononuclear cells related to BM stromal cells, that can protect CLL cells from apoptosis in vitro, was described.[11,19] Strikingly, these cells, termed as "blood-derived nurse-like cells," are rarely found in healthy donor peripheral blood,[11] indicating differences in composition and cell population ratios between normal and CLL-affected blood. These observations suggest that direct cell–cell interactions of CLL cells with accessory cells such as BM stromal cells or follicular dendritic cells create a microenvironment that protects CLL cells from apoptosis, with significant clinical implications. Also in agreement with this hypothesis, other reports have suggested that patterns and extents of malignant infiltrates correlate with CLL clinical stage and prognosis.[20,21] Notably, CLL cell co-culture with MSCs protects the first against apoptosis induced by anticancer drugs, including cyclophosphamide, fludarabine, bendamustine, prednisone, and hydrocortisone.[4,15,22–29] Thus, tissue homing of malignant blood cells has both diagnostic and therapeutic importance. Unraveling the precise mechanisms governing cell–cell interactions between the infiltrating CLL cells and resident BM stromal cells is therefore of crucial importance for designing efficient therapeutic approaches. Major reports from clinical trials and various emerging therapeutic approaches in relapsed or refractory CLL are summarized in Table 1.
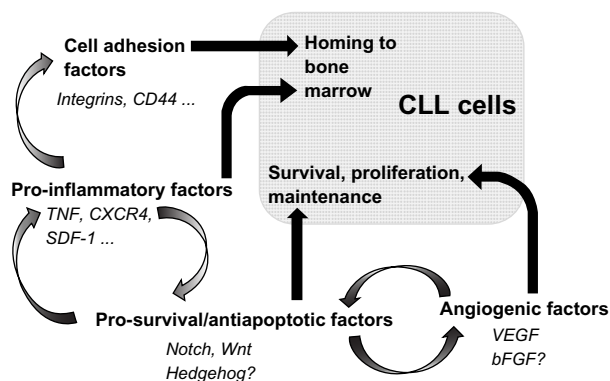
Proto-oncogenic factors
In the ongoing efforts to develop therapies against CLL, stromal factors allowing CLL cells to escape spontaneous and chemotherapy-mediated apoptosis have been abundantly investigated, among which the proto-oncogenic factors Notch, Hh, and Wnt.

Notch
The Notch family of transmembrane proteins consists of four receptors (Notch1–4) and five ligands, which are the structural homologs of *Drosophila* ligands Serrate (Jagged1 and Jagged2) and Delta (Delta-like ligand or DLL1, 3, and 4). Human BM MSCs express DLL3, 4, and Jagged1 in basal conditions.[8] Experimental data suggest Notch involvement in

Table 1 Relapsed/refractory chronic lymphocytic leukemia treatment: recent reports from clinical trials

	Mechanism of action	Preliminary/final results
Molecules		
Acadesine (AICA-riboside)	AMP-activated protein kinase activator	Acceptable safety profile but results variable probably due to the small population and the range of doses tested[102]
T-cell chimeric antigen receptors	Redirect the specificity to tumor-associated antigens	Early-phase trials are demonstrating impressive antitumor effects; multicenter trials needed[103]
Idelalisib (GS-1101, CAL-101)	Delta-specific PI3K inhibitor	Early clinical studies revealed a rapid and sustained reduction in lymphadenopathy with a transient lymphocytosis[104]
Ibrutinib (PCI-32765)	Inhibitor of BTK	High frequency of durable remissions in relapsed or refractory patients[105,106]
Perifosine (KRX-0401)	Akt inhibitor	Stabilized the disease with an Akt-independent mechanism[107]
SF1126 (LY294002/SF1101)	Pan-PI3K/mTORC inhibitor	Well tolerated; best response in patients with advanced disease[108]
Chemo/immunotherapy regimens		
OFAR2 (modified OFAR1)	Regimen: oxaliplatin, fludarabine, cytarabine, and rituximab	Significant antileukemic activity in Richter's syndrome and relapsed/refractory cases; post-remission therapy had favorable outcomes[109]
Fludarabine + cytarabine + reduced-intensity conditioning	Suggested as conditioning regimen before allogeneic hematopoietic stem cell transplantation in patients with poor-risk CLL	Early reports indicate high response rate (93%) and favorable progression-free survival and overall survival. Needs large cohort studies[110]
Combinations of chlorambucil fludarabine, rituximab, and alemtuzumab	Regimens: chlorambucil, fludarabine, fludarabine + rituximab, fludarabine + alemtuzumab, fludarabine + rituximab + alemtuzumab	Data support the use of chlorambucil as an acceptable treatment for older patients, while rituximab is beneficial regardless of age[111,112]
Lenalidomide + rituximab	Lenalidomide: immunomodulatory drug. Rituximab: chimeric monoclonal antibody targeting CD20	Active in patients with recurrent CLL; warrants further investigation in a larger cohorts[113]
Rituximab + fludarabine + total body irradiation	Suggested as conditioning regimen before allogeneic hematopoietic stem cell transplantation in advanced CLL	Early reports indicate that it is feasible, well tolerated, and allows better outcomes[114]
Rituximab + recombinant interleukin-21	Anti-CD20 antibody and recombinant human interleukin-21	Well tolerated; durable complete remissions in a small subset of patients; studies in larger cohorts needed[115]
Rituximab + bendamustine	Anti-CD20 antibody and an alkylating agent	Effective and safe in patients with previously untreated CLL[116]

Abbreviations: Akt, protein kinase B; AMP, adenosine monophosphate; BTK, Bruton's tyrosine kinase; CD20, cluster of differentiation 20; CLL, chronic lymphocytic leukemia; mTORC, mammalian target of rapamycin complex; PI3K, phosphoinositide 3-kinase.

stem cell survival and resistance to apoptosis. For instance, Notch and hypoxia-related proteins may interact to play key roles in the survival of both normal and previously irradiated human skin cells.[30] Similarly, some hypoxia-related proteins and proinflammatory molecules may induce the maintenance of hematopoietic stem cells in an undifferentiated state via Notch signaling activation.[31,32]

Besides, experimental evidence also suggests that Notch signaling plays a key role in CLL cell resistance to apoptosis. Notch receptors and ligands are constitutively expressed both on CLL cells from patients[33] and on CLL cells co-cultured with BM stromal cells.[34,35] A cohort study showed that Notch1 PEST domain mutation is of prognostic relevance in CLL patients, though in a minority of cases (5.3%).[36] In a further study, the prevalence of such Notch1 mutational activation increased with disease severity, reaching up to 31% of cases toward Richter's transformation,[37] pointing out Notch1 aberrant activation as a bad prognosis predictor. In a recent cohort study where single strand conformation

analysis was performed in 209 CLL patients, mutations of *Notch1* and *TP53* genes seem to be independent predictive markers for worse outcome in CLL patients,[38] emphasizing the contention that Notch1 mutations is a novel risk marker in CLL. Another recent study addressing Notch pathway activation in Notch1-unmutated cases revealed the existence of interplay between Notch and nuclear factor-κB (NF-κB) pathways,[39] the latter being also one of the constitutively activated pathways in CLL. Considering that both NF-κB regulators and Jagged were both unmutated even in presence of genetic lesions in the NF-κB/Notch loop, it appears that the Jagged-mediated interplay between NF-κB and Notch is independent of such lesions. Thus, therapeutic approaches targeting both Notch and NF-κB pathways simultaneously should be investigated in CLL.

Similarly, Notch signaling would also trigger other classes of signaling molecules. Evidence from genome-wide expression studies suggests the existence of a large number of Notch-regulated genes.[40] For instance, interactions between

Notch1 and tumor suppressor protein 53 (p53) signaling were reported.[41] Notch signaling ability to regulate integrin expression (eg, $\alpha_L\beta_2$, $\alpha_4\beta_1$) in CLL cells was also reported.[24] Considering that $\alpha_4\beta_1$ integrin and its ligand vascular cellular adhesion molecule 1 (VCAM1) are known to be obligatory proteins in the adhesion of B-precursors to stromal cells,[42] the present findings further supports a role for integrins in CLL pathogenesis (see also "Cell adhesion factors: cytokines"). In addition, constitutive activation of Jagged1–Notch signaling increased CLL cell survival following NF-κB activity increase and the expression of various inhibitors of apoptosis (eg, cellular inhibitor of apoptosis 2, X-linked inhibitor of apoptosis).[34] BM MSC-mediated CLL cell survival in vitro was also promoted by Notch1, 2, 4, and $\alpha_4\beta_1$ integrin signaling, as well as chemoresistance to various anticancer drugs, including the alkylating agents bendamustine and cyclophosphamide, the purine analog fludarabine, and the glucocorticoid drugs prednisone and hydrocortisone.[24,26] Such chemoresistance was at least in part p53, Bcl2, and NF-κB dependent.[43] The activation of Notch1, 2, and 4 would also trigger the release of the proinflammatory chemokine interleukin-7 (IL-7) receptor on CLL cells[24] (see also "Inflammatory factors: cytokines"). Altogether, these observations suggest that Notch signaling triggered by proto-oncogenic factors of both microenvironmental and systemic origin can be a key pathogenic event in CLL.

Of particular interest for therapy, emerging evidence indicates that compounds that interfere with Notch signaling efficiently induce apoptosis in CLL cells, including γ-secretase inhibitors I,[34,44] and Notch-signaling inhibitors acting at the transcription factor level.[45] γ-Secretase inhibitors I apoptotic activity was associated with a reduction in NF-κB and p53 activities. Another recent study showed that γ-secretase inhibitors I induce apoptosis through at least three mechanisms: proteasome inhibition, endoplasmic reticulum stress increase, and Notch downregulation.[46]

Hh

In vertebrates, the Hh family is represented by three ligands (desert Hh, Indian Hh, and sonic Hh) and two Hh receptors, which are homologs of the *Drosophila* transmembrane protein Patched (Ptch1 and Ptch2). Hh receptors are negative regulators of the signal transducer Smo. Hh family also includes the zinc finger protein Gli and some transcription factors homologs of *Drosophila* protein Cubitus interruptus.[47,48] Hh family plays various roles in cell cycle regulation and other cell fate determining events.[49] However, the role of Hh signaling in normal and malignant hematopoiesis is controversial.

Although self-renewal defects were reported in some studies,[50,51] absences of changes in hematopoietic phenotypes following Hh abrogation were also reported.[52,53]

Studies in CLL cells reveal an involvement of Hh family in the pathogenesis of the disease. Indian Hh, the most commonly expressed Hh ligands in hematopoietic stem cell progeny,[51] prolonged CLL cell survival in the stromal microenvironment.[53] Poor clinical outcome has been reported in CLL patients overexpressing Hh signaling negative regulator SUFU and zinc finger proteins Gli1 and 2.[53] Thus, defects in the intrinsic pathway, as well as increased ligand expression and probably any other change in the tumor stromal microenvironment causing Hh signaling impairment in CLL cells may negatively affect disease clinical features and therapy outcome. Interestingly, although Hh signaling targeting had no effect on normal hematopoiesis in adult mice,[54] selective inhibition of Gli proteins – the key regulators of Hh signaling – abrogated BM stromal cell-mediated CLL resistance to fludarabine-induced apoptosis.[52] Similarly, Hh signaling inhibition also increased cyclopamine-induced apoptosis in CLL cells,[52] strongly indicating that Hh signaling targeting can be a valid approach for increasing chemotherapy outcome in CLL. Thus, Hh signaling targeting should be studied further in regards to the important therapeutic implications. Considering the redundancy of survival pathways activated by stromal cells in leukemia cells, which is probably one of the major causes of the failure to develop efficient therapies for many hematologic malignancies, it would be interesting to evaluate the impact of Hh signaling targeting on CLL clinical features in vivo.

Wnt

Wnt molecules are a family of secreted proteins playing key roles in the control of cell–cell interactions, cell growth, and cell fate.[6,55] Canonical Wnt signaling pathway plays a key role in hematopoietic stem cell maintenance.[56] Following exposure to low levels of Wnt ligands, mitosis and proliferation are observed in MSCs.[57] Furthermore, human BM MSCs synthesize the Wnt pathway inhibitor Dickkopf-related protein 1, which may increase their proliferation rate.[58] A CLL-affected BM microenvironment would constitutively express various Wnt ligands, including Wnt3, 14, and 16.[59] Experimental activation of β-catenin – the main transcriptional effector of canonical Wnt signaling pathway – protected CLL cells from spontaneous apoptosis.[59] CLL cells would highly express the Wnt receptor Frizzled3, and canonical Wnt pathway targets cyclin D1 and cyclin D2.[60–62] Interestingly, the activation of these D cyclins in CD5+ B-cell lymphoproliferative disorders

would identify diagnostic groups, with cyclin D1 levels significantly higher in ζ chain-associated protein 70 CLL cases, ie, patients with worse clinical outcome.[60,63]

Besides, experimental evidence suggests an aberrant activation of canonical Wnt signaling in CLL. For example, Wnt antagonist Dickkopf-related protein 1 malfunctioning was reported;[64] and CLL cells abundantly express Wnt transcription factor lymphoid-enhancer binding factor 1, normally downregulated in mature B-cells.[65] Interestingly, unlike various cancers activating mutations of canonical Wnt signaling, including at β-catenin amino terminal, would be rare in CLL cells,[66] indicating that Wnt signaling is triggered by ligands present in the BM microenvironment. Thus, although further studies are needed, canonical Wnt signaling may represent a good target for therapy in CLL. In addition, the transcription factor/lymphoid-enhancer binding factor/β-catenin complex targets include genes regulating the transcription of various members of Notch and probably other antiapoptotic signaling pathways.[67,68] Conversely, there are also reports of Notch-mediated activation of Wnt transcription factor c-Myc,[69] and other antiapoptotic signaling pathway downstream targets, including PTEN and PI3K/Akt pathway,[70] proinflammatory cytokines,[24] and vascular endothelial growth factor (VEGF),[71] either via physical interactions with pathway downstream molecules or by transcriptional regulation. This finding suggests the possibility of developing new classes of anticancer drugs targeting many antiapoptotic signaling pathways concomitantly. Such drugs would decrease the chemoresistance of CLL cells in vivo, which is probably due to the redundancy of antiapoptotic pathways, as already mentioned.

Cell adhesion factors
Integrins
Experimental evidence indicates that integrins, which are transmembrane receptors mediating cell attachment to their surroundings, play key roles in many aspects of tissue localization of normal and malignant lymphocytes. CLL isolated from patients revealed good ability to interact with the endothelium.[72] In this study, CLL cells displayed low level bounds to unstimulated endothelium through β2/intercellular adhesion molecule, whereas endothelium stimulation allowed strong adhesion of the neoplastic population in 50% of cases, through VCAM-1, suggesting a role for endothelium activation in the initial events determining the entry of CLL cells into tissues. Notably, in another study, elevated serum levels of soluble VCAM-1 closely reflected tumor burden and other prognostic markers in a large cohort of untreated CLL

patients, and was proposed as a marker in younger early-stage patients eligible for therapeutic trials.[73]

Moreover, experimental evidence indicates that leukemia cell binding to stromal cells requires the simultaneous action of β1- and β2-integrins, though functional redundancy of cell adhesion molecules would also be present. For instance, in an in vitro study where CLL cells were co-cultured with BM stromal cells, although 30%–50% adhesion inhibition was obtained with the combination of anti-CD11a/CD18/CD29/$\alpha_4\beta_1$, attempts to inhibit CLL cell adhesion using blocking antibodies targeting integrins failed.[13] Strikingly, high levels of the antiapoptotic protein Bcl-2 were observed concomitantly and correlated positively with increases in the percentages of surviving CLL cells. Besides, blocking experiments with anti-$\alpha_4\beta_1$ integrin antibodies revealed that $\alpha_4\beta_1$ integrin confers resistance to fludarabine and cyclophosphamide by modulating Bcl-2, p53, and NF-κB in CLL cells co-cultured with BM MSCs,[24,26,74] pointing out these molecules as potential therapeutic targets.

CD44
CD44 antigen, a cell surface glycoprotein involved in cell adhesion and migration, would play a key role in malignant cell homing and fibronectin synthesis in hairy cell leukemia, a subtype of CLL.[75] However, the role of CD44 in CLL is poorly understood. Using a CLL murine model (Eµ-TCL1 transgenic mouse) and malignant cells from CLL patients, Fedorchenko et al recently investigated the functional role of CD44.[76] The study revealed that CD44 expression in CLL is mediated by the tumor microenvironment, and CD44 appeared to promote leukemogenesis by regulating stimuli of myeloid cell leukemia 1 (Mcl1) expression, as a co-receptor. More specifically, deletion of CD44 during TCL1-driven murine leukemogenesis reduced the tumor burden and increased overall survival, anti-CD44 small interfering ribonucleic acids impaired tumor cell viability, whereas engagement of CD44 by its natural ligands, hyaluronic acid or chondroitin sulfate, protected CLL cells from apoptosis. Interestingly, the inhibitory anti-CD44 antibodies IM7 and A3D8 impaired the viability of CLL cells both in vitro and in vivo, suggesting CD44 as a potential therapeutic target in CLL. CLL cell apoptosis observed in presence of the aforementioned antibodies occurred following reduction of the anti-apoptotic Bcl-2 protein Mcl1 and the activation of caspase proapoptotic pathway.

CLL cell survival was prolonged in co-cultures with immortalized follicular dendritic cells via CD44-mediated direct cell contact.[12,18] The latter was associated with Mcl1

upregulation. Interestingly, increases in Mcl1 levels and altered regulations of the *Mcl1* gene were associated with chemoresistance and worse prognosis, marked by rapid disease progression and shorter overall survival in CLL patients.[36,77–79] Furthermore, many independent groups reported an association between decreases in Mcl1 levels in CLL cells and complete remission following chemotherapy.[36,79,80] Not surprisingly, relatively recent reports pointed out the inhibition Bcl-2 family molecules as a promising approach for overcoming stroma-mediated treatment resistance in CLL.[81–83] Similarly, CLL cell targeting with a monoclonal antibody specific for CD44 was also recently reported as a potential therapeutic approach in patients with worse prognosis (CLL patients expressing ζ chain-associated protein 70).[84]

Inflammatory factors
Cytokines
BM stromal cells synthesize several cytokines, including IL-6, IL-7, and IL-10, and transforming growth factor-β, among others. These soluble factors play a role in BM stromal cell-mediated survival and proliferation of CLL cells.[85] CLL cells fail to migrate through the endothelium or stroma without cytokine stimulation in vitro, and the capacity of cytokines to modify integrin-mediated function would play a key role in the determination of the CLL clinical pattern.[72] Plander et al elegantly demonstrated the existence of crosstalk between CLL cells and BM stromal cells where the first induce the upregulation of adhesion molecules and their ligands in the latter, along with an increased cytokine production in favor of CLL cell apoptosis resistance.[86] Notably, significant increases in the survival rate of CLL cells cultured alone or co-cultured with BM MSCs were observed in presence of the proinflammatory cytokine IL-7, but not in presence of the mainly counter inflammatory cytokine IL-6.[85] Besides, a relatively recent study revealed that tumor necrosis factor-related factors (B-cell activating factor, apoptosis-inducing ligand, and CD40 L) and other proinflammatory factors are key molecules for the bidirectional crosstalk between stromal endothelial cells and CLL cells.[87] Interestingly, the abrogation of CD40 expression or suppression of B-cell activating factor and apoptosis-inducing ligand cleavases in endothelial cells reduced the survival and diversification of CLL cells, further indicating the potential of proinflammatory cytokine-dependent factors as targets for CLL therapy. Furthermore, in a recent study where CLL patients were given green tea, whose constituents (mainly epigallocatechin gallate) reported antitumor effects, circulating regulatory T-cells were decreased in CLL patients with early-stage disease. Notably, both IL-10 and transforming growth factor-β serum levels declined throughout the green tea intake period, in both patients and controls, indicating a role for these molecules in the pathogenesis of the disease.[88]

Chemokines
Stromal cells can also increase CLL cell survival by activating the homeostatic and lymphopoietic chemokine SDF-1 (CXCL12), its receptor CXCLR4, or the downstream molecules.[89] SDF-1 is constitutively produced by stromal cells in vivo,[90] and both CLL and BM stromal cells express the CXCR4 surface receptor.[31] BM stromal cells attract CLL cells via CXCR4 -mediated chemotaxis,[18,91] suggesting a key role of the first in BM infiltration by the latter.

Besides, in co-cultures of CLL cells with blood-derived nurse-like cells, the chemokine SDF-1 appeared as one of the key factors accounting for the increase of malignant cell survival.[11] Nurse-like cells also activated mitogen-activated protein kinases extracellular signal-regulated kinase 1/2, and increased the antiapoptotic protein Mcl1 in CLL cells, probably through SDF-1–CXCR4 signaling.[91] Interestingly, the disruption of the SDF-1–CXCR4 interaction by the CXCR4 inhibitor RCP168 abrogated the resistance of CLL cells to chemotherapy-induced apoptosis in vitro.[92] Chemokine receptor inhibitor effects on CLL cells should be investigated in vivo, considering the implications for therapy.

B-cell antigen receptor
In addition to the stromal secretion of cytokines, chemokines, and the resulting expression of adhesion molecules, microenvironmental regulation of CLL cell survival may also be driven by antigenic stimulation through the B-cell antigen receptor. Binder et al showed that CLL B-cell antigen receptor stimulation by stroma-derived antigens can contribute to the protective effect that the stroma exerts on CLL cells,[93] shedding new light on the complexity of the pathophysiology of the disease. Such complexity is probably due to the fact that CLL remains so far a mostly incurable disease. In addition, *immunoglobulin heavy chain variable* (*IGHV*) gene mutational status would represent a major prognostic marker in CLL, as patients with discordant mutational status may present a higher risk population.[94] This finding may probably help improve stratification in clinical trials.

Angiogenic factors
Vascularization is a determinant factor of cell and tissue fate in both normal pathological conditions. In many

hematological malignancies, aberrant neovascularization provides the neoplastic tissue with necessary substances for growth and expansion in the BM. VEGF family (VEGF-B, C, D), their receptors VEGFR1, 2, 3, and system Tie2/angiopoietins are key players in such neovascularization. Not surprisingly, serum levels of VEGF as well as other angiogenic factors, like basic fibroblast growth factor and matrix metalloproteinase 9, are elevated in the serum of CLL patients and correlate with an unfavorable prognosis;[95] BM stromal cell-mediated survival of CLL cells in vitro was also severely impaired following blockade of matrix metalloproteinase 9 activity. Furthermore, the constitutive activation of the mitogen-activated protein kinase p38 would be critical for matrix metalloproteinase 9 production on BM stromal cells, indicating new therapeutic avenues. A more recent report also suggested that hepatocyte growth factor and its receptor (c-Met) prolong the survival of CLL cells through signal transducer and activator of transcription 3 phosphorylation.[96] In this study, gene expression profile analysis revealed that soluble hepatocyte growth factor and SDF-1 were produced mainly by mesenchymal lineages, pointing out a potential role of MSCs in the disease in addition to BM stromal cells, fibroblasts, trabecular bone-derived cells, and osteoblast-like cell lines that also supported CLL survival in vitro.

Other studies of co-cultures of BM stromal cells and CLL cells revealed a marked upregulation of PI3K/NF-κB prosurvival pathway genes, which mediated a proangiogenic switch in CLL cells via upregulation of VEGF and osteopontin, and downregulation of the anti-angiogenic molecule thrombospondin-1.[97,98] However, a recent report from Gupta et al revealed that the proteasome inhibitor carfilzomib induces cytotoxicity through an atypical NF-κB response in CLL,[99] further indicating the complexity of prosurvival strategies in that malignancy. Characterizing the NF-κB role in CLL may provide new avenues for therapy. Moreover, a PI3K inhibitor (GS-1101/CAL-101) attenuated pathway signaling, induced apoptosis, overcame signals from the microenvironment,[100] and decreased mitochondrial apoptotic priming underlying stroma-mediated treatment resistance[82] in various cellular models of Hodgkin's lymphoma. Another PI3K inhibitor (ON 01910.Na) induced CLL cell death through a dual mechanism of action involving PI3K/Akt inhibition and induction of oxidative stress.[101] PI3K inhibitors should be investigated further as therapeutic agents in CLL.

Conclusion

The ongoing efforts to develop therapies against CLL have provided a better understanding of the stromal factors allowing malignant cells to escape spontaneous and chemotherapy-mediated apoptosis. Emerging experimental evidence suggests that malignant cell interaction with BM microenvironment plays a key role in CLL pathogenesis and is a determinant factor for disease poor prognosis. Infiltrating CLL cells are permanently in contact with resident stromal cells in the BM and lymph nodes. Co-culture studies have revealed that BM MSCs and other stromal cell types confer CLL cells the ability to resist both spontaneous and anticancer drug-induced apoptosis, as well as maintenance and proliferation abilities. Overall, these studies suggest the existence of a complex network of antiapoptotic and prosurvival molecules, including at least cell adhesion, proinflammatory, angiogenic, and proto-oncogenic molecules, supporting infiltrating malignant cells and neoplastic tissue maintenance in the CLL-affected BM. Interestingly, mounting evidence indicates that many prosurvival signaling pathways potentially sustaining CLL cell maintenance in the BM interact with one another. Thus, it appears that developing new classes of drugs affecting many signaling pathways simultaneously, and therefore abrogating signaling redundancy-associated chemoresistance to classical anticancer drugs, is feasible. Future studies focusing on developing such drugs would provide efficient therapeutics against CLL and related chemoresistant hematologic malignancies.

Disclosure

The authors report no conflicts of interest in this work.

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