

Chronic Lymphocytic Leukemia: Insights from Lymph Nodes & Bone Marrow and Clinical Perspectives

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ABSTRACT

B-cell chronic lymphocytic leukemia (B-CLL) is characterized by highly variable distribution of tumor mass between peripheral blood, bone marrow and lymphoid organs which is important for staging, classification and prognosis. These clinical findings with novel data about importance of B-cell receptor and its stimulation with the support of micro-environment indicate important role of tissues (lymphoid organs and bone marrow) in the pathogenesis of B-CLL. Here is presented the novel approach of simultaneous characterization of B-CLL cells from peripheral blood, bone marrow and lymph nodes by flow cytometry and immunocytochemistry, defining inter- and intraclonal diversity with respect to various molecules. These include adhesion molecules (integrins, immunoglobulins, selectins), chemokine receptors (including CXCR-4), signaling molecules and prognostic factors (CD38 and ZAP-70), proliferation and apoptosis markers (including Ki67, AgNORs with PK index, survivin, bcl-2) and therapeutic targets (CD20 and CD52) and residual hematopoietic stem cells. A number of interesting significant interactions have been discovered, pointing to the important role of neoplastic cell microenvironment. These may in addition to insights in pathogenesis and roles of different microenvironments add to diagnosis, prognosis and treatment of B-CLL patients.

Key words: B-CLL, lymph node, bone marrow, flow cytometry

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is a distinctive lymphoproliferative disorder characterized by proliferation and accumulation of small monoclonal CD5 positive B-lymphocytes. These B-CLL lymphocytes traffic between and home to a different sites resulting in variable involvement of peripheral blood, bone marrow, lymph nodes and spleen, as major lymphoid compartments¹. This is clinically translated into variable clinical presentation ranging from pure leukemic forms (with significant affection of peripheral blood and bone marrow only) to forms with significant affection of lymphoid organs only i.e. small lymphocytic lymphoma (SLL), while in majority of cases all lymphoid compartments are involved covering whole range from mostly leukemic to mostly lymphoma-like tumor distribution. The whole spectrum is recognized by World Health Organization (WHO) classification of lymphoid malignancies as the

same disease entity². Beside heterogeneity regarding tumor mass distribution and other distinctive clinical and epidemiological characteristics (such as gender and age distribution)^{3,4}, one of the most important characteristics is highly variable clinical course with numerous clinical, hematologic, serum, cellular and molecular parameters identified to be associated with prognosis^{5–9}.

Current concepts about B-CLL pathogenesis point to important role of B-cell receptor and antigen stimulation as well as supporting tissue microenvironment^{1,10}. On the other side current requirements for B-CLL diagnosis are relatively simple and reduced to demonstration of peripheral blood lymphocytosis with distinct clonal immunophenotype (CD5+, CD23+, CD20^{dim}, sIgM^{dim})¹¹. B-CLL research is also based predominantly on samples taken from peripheral blood¹¹. Well documented B-CLL intra-

clonal variability in different lymphoid compartments^{12,13} question the concept of making clear conclusions about pathogenetic processes occurring in tissue microenvironment based on characteristics of malignant clone from peripheral blood. It is also question whether documented intraclonal differences regarding receptors and signaling molecules can point to compartment specific events, cell positioning and crosstalk as well as other microenvironmental influences.

In this review we will address the importance of tissue compartments from clinical as well as biological point of view. On examples of several key B-CLL receptors and signaling molecules we will try to answer what can we learn from lymph node and bone marrow: can information obtained from lymph node and bone marrow samples provide additional knowledge about B-CLL pathogenesis and can this information add to diagnosis, prognosis and therapy?

Clinical Indicators of Lymph Node and Bone Marrow Importance

Variable affection of lymphoid compartments beside the historical importance in disease classification was early recognized to have importance for prognosis. It is in foundation of the first clinical staging system proposed by Rai et al.¹⁴ that showed strong relationship with prognosis. Its introduction revolutionized both clinical and basic research providing the measure for various novel findings. The basic idea behind this staging system is sequential affection of peripheral blood (stage 0), lymph nodes (stage I), spleen and/or liver (stage II) and bone marrow with cytopenias due to a level of infiltration (stages III and IV). Although, today we know that this concept of B-CLL progression is not valid since we deal with practically systemic disease from the beginning due to cell trafficking it has recognized two important factors associated with prognosis. First is clinically recognizable affection of lymphoid organs and second is bone marrow failure – i.e. bone marrow infiltration leading to clinically significant cytopenias.

Another major step forward was introduction of Total tumor mass (TTM) score¹⁵. This scoring system has introduced quantification of involvement of each lymphoid compartment allowing to assess and monitor total tumor burden during the course of the disease as well as to assess response to therapy. Its usefulness in prognosis and follow up was proved in numerous retrospective and prospective clinical studies and clinical trials^{16–20}. By quantifying involvement of each lymphoid compartment, i.e. peripheral blood/bone marrow, lymph node and spleen this system also allows the quantification of tumor distribution (TD) recognizing the spectrum from pure leukemia to pure lymphoma and differentiation between predominantly leukemic to predominantly lymphoma like cases⁹. It was also shown that TD has its independent prognostic significance (worse prognosis of lymphoma like cases) irrespective of total tumor burden as well as unexpected relation to gender (being more significant in

females) and some other biological parameters²¹. It is worth of noting that TTM scoring system doesn't include and it is independent of bone marrow failure.

Strong relationship of lymphoid organ and bone marrow involvement with prognosis may implicate their important role in pathogenesis of B-CLL. Quantitative and more importantly qualitative differences in lymphoid compartment affection indicate that site of B-CLL lymphocyte accumulation is important. Beside the major principle valid in B-CLL and almost all malignancies that more tumor load is associated with worse prognosis and minor principle that tumor in certain locations is associated with poor prognosis (occurring rarely in B-CLL) it can be speculated that predominant accumulation of B-CLL lymphocytes in microenvironment favoring proliferation and activation leads to a more active disease and worse prognosis. Accumulation in (or spillover to) peripheral blood which is relatively inert compartment has a lesser independent influence on prognosis. What is the biological background for these clinical observations?

B-cell Receptor and Tissue Microenvironment

Classical morphological appearance of B-CLL includes small lymphocytes accumulating and infiltrating to a variable extent to all lymphoid compartments including peripheral blood, bone marrow and lymphoid organs²². However, in lymphoid organs but also to a lesser degree in bone marrow one of the histological hallmarks specific to B-CLL are aggregates of larger cells (prolymphocytes and paraimmunoblasts) with other cells (most notably T cells) and supporting stroma^{22,23}. These aggregates form so called »pseudofollicles« resembling classical lymphoid follicles which are the sites of B-cell activation by antigen and T-cell help and proliferation. Pseudofollicles are the presumed sites of proliferation in B-CLL^{24,25} and constitute so-called »proliferation compartment« fueling so-called »accumulation compartment«. In this microenvironment there are number of interactions with other cells (T-cells, stromal cells and accessory cells like nurse-like cells, etc.) and supporting stroma that provide other important signals for B-CLL cells²⁶. There are some indications that interactions with T-cells provide signals important for proliferation, while stromal and nurse-like cells provide signals important for extended survival. A number of receptors are involved in these interactions including, CD40/CD40L, CD38, CXCR4/SDF-1, etc.^{27–32} (Figure 1).

Beside the resemblance of B-CLL proliferation centers to lymphoid follicles there is large amount of data indicating important role of immune-mediated pathways including B-cell receptor (BCR) and autoantigen stimulation as well as supporting microenvironment in the pathogenesis of B-CLL^{27,33}. Important results came out of studies of BCR including finding of highly restricted and biased gene repertoire of immunoglobulin heavy chain variable (IGHV) region compared to normal B-cell repertoire^{34,35} and recognition of two groups of patients

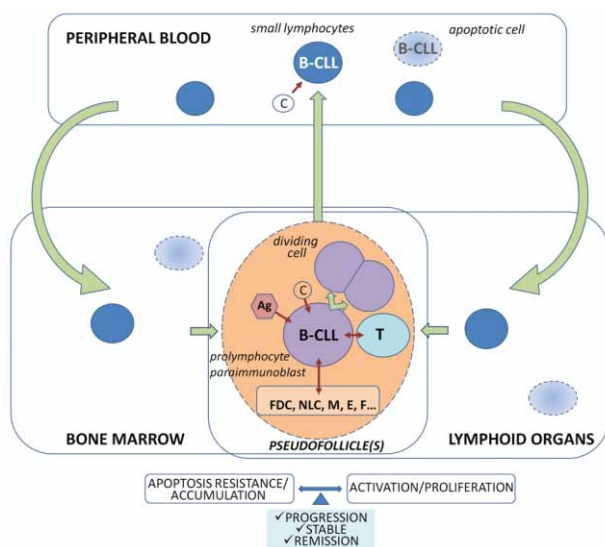


Fig. 1. Role of peripheral blood, bone marrow and lymph node microenvironment in regulation of balance between B-CLL activation/proliferation and apoptosis resistance/accumulation resulting in progressive disease, stable disease or remission with therapy. AG – antigen, C – Chemokine, cytokine milieu, T T-Cell, FDC – follicular dendritic cell, NLC – nurse-like cell, M – macrophage, E – endothelial cell, F – fibroblast.

with different prognoses based on occurrence of somatic mutations in this genes (mutated and unmutated)^{36,37}. Phenotypic³⁸ and gene expression profiling³⁹ signatures also indicate that B-CLL cells are antigen experienced cells. Signaling through BCR was found to be very important for activation, proliferation and survival. Although putative antigen stimulating BCR is unknown, B-CLL cells produce polyreactive antibodies⁴⁰ which are in a number of cases autoantibodies thus suggesting some kind of autoantigenes. All these findings are indicative that antigenic pressure may be important for selection of B-CLL cells, activation in T-cell dependent and T-cell independent manner and progression of the disease⁴¹. It is currently unknown if it is important for disease induction.

What Can We Learn from Inter- and Intraclonal Differences between Compartments?

Current knowledge about B-CLL is predominantly based on investigation of samples from peripheral blood (accumulation compartment) and *in vitro* models, while major pathogenetic processes take place in lymphoid organs and bone marrow (proliferation compartment). Intraclonal diversity, i.e. differences in B-CLL lymphocyte characteristics depending on microenvironment (peripheral blood, lymph nodes and bone marrow) in single patient and its relation to other clinical and biological characteristics may be related to pathogenetic events in B-CLL occurring in respective compartment. These events in-

clude trafficking and homing as well as activation, proliferation and apoptosis. On the other side interclonal diversity is defined as differences between different B-CLL patients. To assess compartment specific phenotypic profile and inter- and intraclonal diversities we have adopted novel approach of simultaneous investigation of samples from different microenvironments, especially lymph nodes and bone marrow compared to peripheral blood with detailed phenotypic characterization B-CLL cells with special emphasis on quantification. Major methods involved were flow cytometry and immunocytochemistry with image analysis. This approach uncovered novel data as well as proved *in vivo* some hypothesis and *in vitro* findings contributing to better characterization of molecular mechanisms of B-CLL induction and progression. Here is a brief review of our results covering adhesion molecules and chemokine receptors, important signaling molecules and prognostic factors CD38 and ZAP-70, cell kinetics, markers of proliferation and apoptosis and residual hematopoietic stem cells.

Adhesion Molecules and Chemokine Receptors

Our interest in B-CLL intraclonal diversity started from interest in underlying mechanisms for cell trafficking and homing which will provide biological basis and explanation for variable tumor mass distribution in B-CLL and its clinical significance. We have applied approach of simultaneous investigation of samples from peripheral blood, bone marrow and lymph nodes taken by fine needle aspiration and detailed phenotypic characterization of a number of molecules on B-CLL lymphocytes. Our panel consisted of extensive panel of adhesion molecules including integrins, immunoglobulins, selectins, CD44, and chemokine receptors including key CXCR-4 receptor. We have found different patterns of expression for almost all adhesion molecules and chemokine receptors between different compartments. Some of the molecules showed higher expression in lymph nodes (like CD54, β_2 integrins, CD31), some showed higher expression in bone marrow while some showed higher expression in peripheral blood (like CXCR-4, CD102, CD44)^{42–44}. Also different molecules have different relationship with a number of clinical and laboratory parameters indicating different roles in a multistep process of cell trafficking and homing, as well as linking this process to pathogenesis.

CD38 and ZAP-70

We have also evaluated CD38 and ZAP-70, key signaling molecules and prognostic markers^{45–47}. Both molecules showed similar pattern of expression with the highest expression in lymph nodes and lowest in peripheral blood^{12,48}. These findings are in line with occurrence of pseudofollicles in lymph nodes and bone marrow where B-CLL cells receive multiple activating signals.

CD20 and CD52

In recent years immunochemotherapy is gaining ground in treatment of B-CLL patients showing improval of response and survival rates^{49,50}. Two monoclonal antibodies are in wide use in the treatment of B-CLL, i.e. rituximab (targeting CD20 molecule) and alemtuzumab (targeting CD52 molecule). It is known that these antibodies have different activity in different compartments with rituximab mostly active in patients with lymphadenopathy (with 11q deletion)⁵¹ and alemtuzumab highly active in peripheral blood and bone marrow while relatively ineffective in patients with bulky lymphadenopathy^{52,53}. We have found relatively unexpected and consistent different pattern of expression of CD20 and CD52 on B-CLL cells with CD20 showing highest expression in peripheral blood and CD52 in lymph node compartment⁵⁴.

Proliferation and Apoptosis

We have studied by flow cytometry a proliferation marker Ki-67 that show higher expression in B-CLL cells from lymph nodes⁴⁴. We have also studied a number of molecules involved in cell cycle regulation and apoptosis (including survivin, bcl-2, p53) that all showed higher expression in lymph nodes⁵⁵. These results are also in line with the presence of pseudofollicles in lymph nodes and bone marrow which are presumed sites of proliferation in B-CLL.

We have also analyzed cytologic smears from peripheral blood, bone marrow and lymph nodes by DNA image cytometry and silver-stained nucleolar organizer regions (Ag-NORs). Based on these parameters proliferative-ki-

netic index (PKI) was developed showing the strong relationship with prognosis⁵⁶.

Residual Hematopoietic Stem Cells in B-CLL

Although autologous stem cell transplantation currently is not a standard treatment option for B-CLL patients it may be effective and is under investigation^{57,58}. So we have investigated residual hematopoietic stem cell compartment in B-CLL patients and also compared the peripheral blood and bone marrow stem cells. We found that older patients have fewer committed progenitors in peripheral blood and that stem cells in BM show a more evident inverse relationship with the size of the B-CLL clone⁵⁹.

Conclusions

B-CLL is systemic disease affecting all lymphoid compartments to a variable extent. In a view of well documented data suggesting importance of tissues in B-CLL, peripheral blood sampling is clearly suboptimal. Sampling of lymph nodes and bone marrow allow insight into B-CLL intraclonal heterogeneity and better describe proliferative and accumulative compartment and underlying pathogenetic process. This may lead to new diagnostic tools and may uncover novel therapeutic targets.

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KRONIČNA LIMFOCITNA LEUKEMIJA: UVIDI IZ LIMFNOG ČVORA I KOŠTANE SRŽI I KLINIČKE PERSPEKTIVE

SAŽETAK

B-stanična kronična limfocitna leukemija (B-KLL) je obilježena izrazito varijabilnom raspodjelom tumorske mase između periferne krvi, koštane srži i limfnih organa, što je značajno određivanje kliničkih stadija, klasifikaciju i prognozu. Ovi kliničke značajke zajedno s novim nalazima o značaju B-staničnog receptora i njegove stimulacije uz potporu mikrookoliša ukazuju na značajnu ulogu tkiva (limfni organi i koštana srž) u patogenezi B-KLL-a. Ovdje prikazujemo novi pristup istodobnom karakterizacijom B-KLL stanica iz periferne krvi, koštane srži i limfnih čvorova protočnom citometrijom i imunocitokemijom i definiranjem inter i intraklonalne raznolikosti obzirom na adhezijske molekule (integrine, imunoglobuline, selektine), kemokinske receptore (uključujući CXCR-4), signalne molekule i prognostičke čimbenike (CD38 i ZAP-70), biljege povezane s kinetikom (Ki67, AgNOR, PK indeks), apoptozom (survivin, bcl-2), liječenjem (CD20, CD52) te rezidualne hematopetske matične stanice. Ovakav pristup osim uvida u patogenezu može doprinijeti dijagnozi, prognozi i liječenju B-KLL bolesnika.