Cristina Scielzo
Michela Frenquelli
Marta Muzio
Maria T.S. Bertilaccio
Antonis Dagklis
Federico Caligaris-Cappio
Paolo Ghia

Unit and Laboratory of Lymphoid Malignancies, Department of Oncology, Università Vita-Salute San Raffaele and Istituto Scientifico San Raffaele, Milano, Italy

Chronic lymphocytic leukemia: an enigma for both the clinician and the immunologist

A B S T R A C T

The most recent years have witnessed a great progress in understanding the biology of chronic lymphocytic leukemia (CLL). This has allowed a parallel improvement in the definition of several biological parameters that have been validated as strong and independent prognostic markers now widely used in the setting of clinical trials. Among others, the study of the antigen receptors has been the most proficient, helping to better understand the cellular origin of CLL and especially to indicate the existence of a specific antigenic stimulation in the natural history of the disease. The presence or the absence of somatic mutations in the immunoglobulin heavy chain variable region (IGHV) gene sequence are considered the most reliable among the prognostic markers now used. Nevertheless, all this body of knowledge has not resulted in a parallel improvement in the management and treatment of CLL, which remains up to now an incurable disease. Somatic mutations though potent in predicting prognosis at cohort level, correlate with the clinical outcome and other biological prognostic markers in no more than 80% of the affected individuals, thereby hampering a real patient-tailored therapeutic approach.
All these reasons make mandatory to further study the biology of the disease and especially the IG receptor that appears to be pivotal in the onset and progression of the disease. This will be instrumental for the identification of the patient who needs a different treatment in terms of strategy and timing, for the design of biology based therapies and for patient-tailored therapeutic approaches.

Introduction

CLL is a chronic lymphoproliferative disorder characterized by the accumulation of monoclonal B lymphocytes expressing the CD5 molecule.[1] In recent years, CLL has been dissected at molecular and biological level, leading to a progressive increase in understanding the pathogenesis of the disease and to a biologically-oriented assessment of clinical prognosis.[2]

It is now clear that the natural history of CLL is characterized by initial genetic events that provide a survival advantage to the affected lymphocyte. Such a crippled cell, though being leukemic, remains responsive to physiological stimuli derived from the tumor microenvironment that play a indispensable (though not sufficient) role in the maintenance and progression of the disease.[3]

As genetic defects, fluorescence *in situ* hybridization (FISH) analysis has allowed to show that chromosomal abnormalities can be found in up to 80% of the CLL cases. Among all, the most frequent aberrations are deletions on 13q (55% of the cases), 11q (12%), 17p (8%) and trisomy of chromosome 12 (15%).[4] Genetic studies on the affected loci have allowed the identification of specific genes that may play a relevant role in the pathogenesis of CLL (e.g. microRNA genes on chromosome 13, ATM gene on chromosome 12, p53 on chromosome 17).

In particular, the finding that a cluster of microRNA (mir-15a and mir-16-1) was deleted from chromosome 13 in the cases carrying the 13q14 deletion has revealed a potentially relevant pathway in the initiation of the disease.[5] Those two miRNA appear to be down-regulated in most CLL cases even in those lacking the genetic deletion, maybe due to inefficient processing of the miRNA precursors or point mutations, though the actual mechanism has not been so far deciphered. It has been experimentally shown that mir-15a and mir-16-1 expression inversely correlates to bcl-2 expression, an anti-apoptotic protein which is over-expressed in CLL cells and it is thought to play a role in the survival of the leukemic clone. The lack of mir-15a and mir-16-1 in the majority of CLL cases would result in up-regulation of the bcl-2 protein similarly to what happens in follicular lymphoma, though in the absence of any specific translocation.[6] This finding is particularly interesting in the light of the recent and promising clinical results obtained by the use of antisense bcl-2, in randomized clinical trials in combinations with conventional chemotherapy.[7] As microRNA can be considered as natural and more effective antisense molecules, their use could be exploited in experimental settings with potential clinical applications.

Following these evidences, this (or other) genetic lesion may be important in predisposing leukemic B lymphocyte to prolonged survival and persistence *in vivo*. Cellular and molecular interactions happening in the context of the tumour microenvironment would then be critical in the propagation and progression of the leukemic clone.[8] A paradigmatic example of the importance of these *in vivo* interactions is the fact that CLL blood cells undergo apoptosis when cultured *in vitro*, unless they are exposed to stromal cells or to combinations of cytokines,[9] indicating that the in vivo accumulation of apoptosis-resistant lymphocytes is favoured by the microenvironment.

The role for antigen stimulation

As microenvironmental stimuli, several evidences strongly support the possibility that stimulation through the B-cell antigen receptor (BCR) is involved in the selection and expansion of the malignant clone.[10] CLL cells

have the phenotypic profile of B cells activated by antigen (Ag) interaction[11] and display a gene expression profile that also recalls that of antigen experienced B lymphocytes.[12] In addition, in a proportion of cases, the malignant cells can be stimulated *in vivo* through the surface immunoglobulin (IG). The remaining cases that appear to be unresponsive show the features of B cells anergized *in vivo* by an antigen.[13,14]

These phenotypic and functional evidences are paralleled by several molecular studies on the IGs expressed by CLL cells suggesting a relevant BCR-mediated selection process. The leukemic cells have a preferential IGHV gene usage, being IGHV1-69 and IGHV4-34 the most frequently used,[10] and in at least half of the cases IGHV genes carry somatic mutation,[15,16] which are taken as the hallmarks of the cellular passage through the germinal center reaction in secondary lymphoid tissues which usually follows an *in vivo* antigen encounter.[17,18] In addition, subsets of CLL patients express closely homologous if not identical (*stereotyped*) complementarity-determining region 3 (CDR3) sequences on heavy and light chains irrespective of the IGHV mutational status.[19-24] The CDR3 is the most relevant part of the IG molecule concurring to the definition of the antigen-binding groove. They also share recurrent *stereotyped* mutations or conserved sequence motifs throughout the whole IGHV region (Murray *et al.*, Blood 2007 in press). As a consequence, the remarkable sequence similarity in unrelated and geographically distinct cases implies the recognition of individual, discrete antigens or classes of structurally similar epitopes. This appears to be a CLL-unique feature as it is virtually absent among IG rearrangements from other lymphoproliferative or autoimmune disorders. On the contrary, it is a quite widespread molecular event among CLL, as to date over 100 different *stereotyped* BCR have been reported in CLL, accounting for more than 25% of the patients[25] (and Murray *et al.,* Blood, 2007, in press).

Finally, these observations are corroborated by the notion that CLL cells may express gene products associated with B-cell signalling and activation, including molecules (e.g. CD38) that influence the outcome of BCR signalling[26] or that belong to the intracellular signalling cascade originating from the antigen receptor (the z chain-associated protein of 70 kDa-ZAP70).[27] Taken together, all these evidences strongly indicate a central role of the BCR in the pathogenesis of CLL patients and raise the issue of which is the antigen(s) involved in the selection and expansion of the CLL leukemic clone. The nature of the Ag(s) is still unknown, though it has been recently suggested that, at least in a proportion of cases, the antigen involved may be a self-antigen, as witnessed by sequence similarities with rheumatoid factors or anti-cardiolipin antibodies or by the direct binding of the soluble clonal IGs to human cells.[25,28]

Clinical relevance of the biological findings

In contrast to a very homogeneous cellular phenotype characterized by low levels of CD20 and sIg, and high CD5, in the presence of CD23 expression,[29] CLL may be very heterogeneous at clinical level with some patients experiencing a real indolent course and a life expectancy similar to unaffected individuals and others having an aggressive malignancy that requires early and frequent treatments, with a median survival time of 2-3 years.[30]

The hypothesis that the BCR may be a critical molecule in the pathogenesis of the disease is further underscored by the notion that several of the above-mentioned gene products associated with B-cell signalling and activation strongly correlate with the clinical outcome of

individual patients and may help to predict patient's prognosis at the moment of initial diagnosis.[16,31]

According to this, the presence or the absence of somatic mutations in the expressed IGHV gene sequence defines two disease subtypes associated with a different clinical course.[15,16] CLL cases carrying IGHV genes with <98% similarity to the closest germline gene (*mutated cases*) generally follow a more indolent course than those with ≥98% similarity (*unmutated cases*).[15,16] Quite recently, also the presence of particular *stereotyped* receptors has been shown to correlate with a distinct clinical outcome, regardless the actual mutational status.[19,23,25] These correlations suggest that a particular antigen-binding site can be critical in determining clinical presentation and possibly also prognosis. Considering the clinical-biological associations with certain subsets, it is conceivable that future therapeutic decisions could be based not only on mutational status of IGHV genes but also on individual HCDR3 characteristics.

The presence or the absence of ZAP-70 expression[31–33] as well as of the activation marker CD38[16,34] also helps to predict at diagnosis the clinical outcome of individual patients. In particular, these markers correlate with a shorter survival time and a shorter time to progression.

All these molecules carry a strong though independent prognostic value, making it possible to predict already at the moment of the initial diagnosis the clinical outcome of individual patients.[35] That notwithstanding, discrepancies among these markers exist (between 15 and 25% depending on the series) and they still all need to be validated in prospective randomized clinical trials, thereby hampering at the moment a widespread use in a daily routine setting.[2] In addition, one has to consider that several other molecules have been described (e.g. HS1[36], LPL[37], TPO[38]) to carry a prognostic value, correlating, but not completely overlapping, with the previous ones. This wealth of factors may create confusion rather than being of help in the clinical arena, while it may allow to shed more easily light in the biological mechanisms of the disease.

Intraclonal heterogeneity in CLL

In addition to the above-mentioned heterogeneity at both clinical and biological level among different CLL patients, it is puzzling to note that each individual clone in each single patient carry a certain degree of intraclonal heterogeneity, based on the expression of intracellular or membrane molecules and depending on the anatomical site (bone marrow–BM, lymph nodes–LN, Peripheral blood–PB) examined (*intraclonal diversity*). Accordingly and irrespective of the fact that the vast majority of CLL cells present in the PB are resting, proliferating prolymphocytes and paraimmunoblasts can be identified in all patients as focal aggregates (pseudofollicles - PF) in LN and BM.[39] These cells constitute the CLL proliferating reservoir of the disease that replenishes the downstream accumulation compartment. Proliferating cells differ from circulating resting leukemic cells, in terms of expression of several molecules. Among others they express apoptosis-regulators like *Survivin*,[39] chemokines like CCL-17 and CCL-22[40], proliferation related genes like Ki67[39]. CLL PF are also infiltrated by $CD3^+$ T cells, most belonging to the $CD4^+$ subset,[41] and many among them express CD40Ligand implying that they are in an activated state.[40] These cells are thought to play an active role in sustaining the disease, by providing both survival and proliferation signals to the malignant clone. This scenario is also supported by considering that virtually all circulating CLL cells are in the G0/early G1 phase of the cell cycle.[42]

However, the investigation of telomere length and telomerase activity[43] indicates that a considerable number of cell divisions have occurred within the leukemic clone raising the question of where and to what extent CLL cells proliferate and how the proliferative compartment nourishes the accumulation compartment.

The intraclonal diversity can also be revealed by the analysis of surface markers expressed by circulating leukemic cells. The membrane expression of CD38 is an illuminating example. Beside patients whose cells are homogeneously either negative or positive, in a proportion of patients leukemic cells are characterized by the concomitant presence - within the same clone - of variable proportions of $CD38^+$ and $CD38^-$ cells (*bimodal cases*).[34] The presence within the leukemic clone of practically any amount of $CD38^+$ malignant B cells correlates with a progressive disease and poor prognosis. In addition, the $CD38^+$ subset of the clone appears to be more represented in infiltrated BM as compared to PB, suggesting that the BM environment, which is a well known privileged site of disease relapse, more easily hosts cells that are associated with adverse outcome.

Monoclonal B lymphocytosis

Several of the described external stimuli appear to be responsible for inducing changes in the functional capacity of the malignant cells. A role for microenvironmental signals can also be hypothesized for the monoclonal B lymphocytes with a CLL-phenotype circulating in the PB of otherwise healthy individuals[44,45] (monoclonal B lymphocytosis – MBL[46]). These cells are present in 3.5% of the general population and their frequency increases among the elderly and in relatives of CLL patients,[47] regardless the age.

Given the rather high frequency of such cell populations within normal individuals (approximately 100 times more frequent than CLL), it is quite unlikely that these cells might be in all cases precursors of CLL. This situation is rather reminiscent of similar situations, e.g. the detection of IGH-Bcl-2 translocations in the blood or of monoclonal immunoglobulins in the sera of healthy individuals, who will develop neither follicular lymphomas nor multiple myeloma. It is then plausible to hypothesize that a facilitating microenvironment may favour the expansion of these very tiny $CD5^+$ clonal populations and their progression into fully-fledged CLL. MBL cells might then be considered one step (maybe the first one) in the multi-step process of leukemogenesis.

Nevertheless, the fact that MBL can also involve $CD5^-$ B cells at similar frequencies,[45] suggest that B cell monoclonal expansions in healthy adults may be just a normal aspect of the aging process, leading to a typical narrowing of the B cell repertoire. It is then possible that MBL are age-linked and may develop as a result of chronic/persistent stimulations over time. The antigenic stimulation might be directly responsible for the occurrence of MBL with aging, thereby providing with high frequency a still normal cell that would become the target for subsequent transforming hits. On the contrary, it might be a promoting factor that acts upon a MBL clone that has been previously primed by a different molecular *initiation* hit, then providing a stimulus to its leukemic expansion.

Conclusions

CLL is a very complex disease with several implications in basic aspect of immunology. While making it an interesting model for both the scientist and the immunologist, it still puzzles the clinicians as it remains an incurable

disease at present even with the modern combined chemotherapeutic regimens as well as stem cell transplantation. Further dissection of the natural history of the disease and the immunological mechanisms behind its onset will allow to define innovative approaches in its treatment, tailoring a risk-adapted therapeutic strategy on the basis of the biological properties of each malignant clone.

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