ISSN 0025-7680

International Symposium
NEW DIRECTIONS IN CANCER MANAGEMENT
Academia Nacional de Medicina
Buenos Aires, 6-8 June 2007

MEDICINA (Buenos Aires) 2007; 67 (Supl. II): 41-47

CHRONIC LYMPHOCYTIC LEUKEMIA

ZAP-70 AND THE B-CELL RECEPTOR

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Abstract In addition to the important observations relating the immunoglobulin (Ig) mutation status to clinical behavior, studies on the Ig expressed in chronic lymphocytic leukemia (CLL) have revealed compelling evidence that antigen-selection contributes to the pathogenesis of this disease. CLL cells that use unmutated Ig generally can be distinguished from CLL cells that use Ig with somatic mutations by expression of the zeta-associated protein of 70 kD (ZAP-70). ZAP-70 apparently enhances the capacity of CLL cells to respond to antigen and therefore might play a causal role in the relatively aggressive clinical behavior noted for patients who have CLL cells that use unmutated Ig. Clinical surveys have found that CLL-cell expression of ZAP-70 is a stronger predictor of early disease progression than is the use by CLL cells of unmutated Ig. As such, strategies that respectively monitor or target Ig-receptor signaling in CLL might be highly useful in the risk-assessment or treatment of this disease.

Key words: immunoglobulins, ZAP-70, B-cell receptor signaling, pathogenesis

Resumen ***Leucemia linfática crónica. ZAP-70 y el receptor de la célula B***. Además de importantes observaciones relacionadas con el estado de mutaciones en inmunoglobulinas (Ig) y el comportamiento clínico, estudios sobre Ig expresadas en la leucemia linfática crónica (CLL) sugieren que la selección antigénica contribuye al desarrollo de esta enfermedad. Células de CLL que utilizan Ig no mutadas por lo general pueden ser diferenciadas de células CLL que utilizan Ig con mutaciones somáticas por la expresión de la proteína asociada a "zeta" de 70 kD (ZAP-70). ZAP-70 aparentemente aumenta la capacidad de células de CLL de responder al antígeno y por lo tanto podría jugar un papel causal del comportamiento relativamente agresivo que se observa en pacientes con células de CLL que utilizan Ig no mutadas. Estudios clínicos han determinado que la expresión de ZAP-70 por células de CLL es un marcador predictivo más fuerte de progresión temprana de la enfermedad que células CLL con Ig no mutadas. En consecuencia, estrategias que monitoreen o apunten a vías de señalización activadas por Ig en CLL podrían ser de gran utilidad en el manejo y tratamiento de esta enfermedad.

Palabras clave: inmunoglobulinas, ZAP-70, señalización por receptores de células B y patología

Immunoglobulins in CLL

Patients can be segregated into one of at least two major subgroups based upon whether or not their leukemia cells express immunoglobulin (Ig) variable region genes that have incurred somatic mutations[1]. Patients with CLL cells that express Ig heavy chain variable region genes (IgV_H genes) lacking somatic mutations tend to have an aggressive clinical course relative to that of patients who have CLL cells that express IgV_H with somatic mutations[2-4]. Generally, the Ig used by any one leukemia-cell population does not show a tendency to accumulate somatic mutations. Furthermore, patients with leukemia cells with mutated Ig receptors generally have a more indolent clinical course at diagnosis than do patients with CLL cells with unmutated Ig genes. As such, it appear certain that leukemia cells that use mutated Ig genes do not evolve from CLL cells that originally expressed unmutated Ig genes. This originally led to speculation these two subgroups patients might have distinct forms of leukemia, one form derived from memory-type B cells that had undergone Ig somatic mutation and the other form derived from naïve, or pre-germinal-center B cells.

However, examination of the Ig expressed in CLL reveals that the subgroup using unmutated Ig is not derived from naïve B cells[5]. Compared to the blood B cells of healthy adults, CLL cells apparently overuse certain Ig V_H genes. Furthermore, these genes have distinctive gene rearrangements[1, 5-7]. For example, one particular Ig V_H gene, namely V_H1-69, is used by about 15-20% of all CLL cases and typically is unmutated when expressed by CLL cells. Even though there are several alleles of

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this Ig V_H gene, which can be segregated into two subtypes based upon shared differences in the sequence encoding the second-complementarity-determining region (CDR2), only one of these two allele subtypes is used frequently in CLL. Furthermore, CLL cases that use V_H1-69 commonly use certain D segments that are not frequently used by non-neoplastic B cells, including adult blood B cells that use V_H1-69. Consequently, the amino acid sequences in the Ig heavy chain CDR3 of V_H1-69-expressing CLL cells have characteristic motifs that repeatedly are observed in CLL cells of different patients that use Ig V_H1-69. Moreover, the region encoded by the junction created by Ig V_H gene rearrangement, the CDR3, typically is significantly longer than that of B cells that express the V_H1-69 gene. Finally, among cases that use V_H1-69, there is preferential pairing of the antibody heavy chain with particular antibody light chains depending upon the sequences in the CDR3 of the antibody heavy chain. For example, Ig heavy chains encoded by unmutated VH1-69 that have the CDR3 motif GGGYDYIWGSYRPNDAFDI almost invariably are paired with kappa light chains encoded by an unmutated Ig kappa light chain variable region gene (Ig Vκ gene), designated VκA27[8]. Moreover, Ig heavy chains encoded by the unmutated V_H1-69 gene that have the CDR3 motif of YDFWSGYYPNYYYGMDV typically are paired with lambda light chains encoded by the unmutated lambda light chain gene Vλ3-9[9]. These and other examples demonstrate that the pairing of antibody heavy and light chains is non-random, but instead governed by sequences in the Ig heavy chain CDR3. This cannot be due to selection by some unidentified "superantigen", which binds to selected antibody variable regions independent of the structure of the CDR3. Instead, these examples indicate that the antibodies expressed by at least some CLL cases are highly selected for binding to some unknown self or environmental antigen(s).

Studies involving gene microarrays provided further evidence that CLL cells are derived from antigen-selected B cells. Irrespective of whether CLL cells express mutated or unmutated antibody V genes, the leukemia cells of different patients share a common gene expression profile that can distinguish them from other B cell malignancies or from normal blood B cells[10, 11]. Moreover, the gene expression profile of CLL B cells appears to fit best with that of splenic marginal zone B cells[12], suggesting that CLL cells might be derived from memory-type B cells.

ZAP-70

Although there is a common gene expression profile for CLL, leukemia cells that use unmutated Ig V genes can be distinguished from CLL cells that use mutated Ig V genes through the differential expression of a relatively small subset of genes. One of these genes encodes the ζ-associated protein of 70 kD (ZAP-70)[10]. Subsequent studies found that CLL B cells that had unmutated V genes generally expressed levels of ZAP-70 protein comparable to that of normal blood T cells, which is in contrast to most CLL B cells that had mutated V genes[13]. Moreover, CLL B cells that used mutated antibody V genes generally do not express detectable levels of ZAP-70 protein. However, the association between expression of ZAP-70 and use of unmutated Ig V genes is not absolute as there are cases that use unmutated Ig V genes that lack expression of ZAP-70[14]. Also, there are cases that use mutated IgVH that express this tyrosine kinase[14]. In a couple of informative cases of identical twins who both had CLL, the leukemia cells of each twin were found to use mutated Ig V genes, but were discordant for expression of ZAP-70[13]. Because these cases had the same genetic background, it appears that inherited factors do not control whether or not CLL cells express ZAP-70. Nevertheless, even though the association between ZAP-70 and use of unmutated Ig V genes is not absolute, ZAP-70 and/or a relatively small number of other genes, e.g. lipoprotein lipase (LPL), can be used as surrogate markers for CLL cells that use unmutated Ig[15-17].

Additional studies found that ZAP-70 has functional significance in CLL. ZAP-70 is a protein tyrosine kinase that initially was identified in T cells. It is a protein characterized by two tandem SH2 domains and a C-terminal catalytic kinase domain[18, 19]. Following ligation of the T cell receptor (TCR), a member of the Src family of protein tyrosine kinases becomes activated, which in turn phosphorylates the tyrosine-containing immunoreceptor tyrosine-based activation motifs (ITAMs) found in the cytoplasmic tails of the accessory proteins of the TCR complex[20]. ZAP-70 is recruited to these phosphorylated ITAMs and becomes activated, in turn causing activation of members of the Tec family of protein tyrosine kinases, resulting in activation of downstream signaling pathways, such as the phospholipase Cγ/Ca^{2+} signaling pathway and the Ras/mitogen activated protein kinase (MAPK) pathway[21]. B cells typically lack ZAP-70, but instead have another related protein tyrosine kinase, called *Syk*, which B cells use for signaling via the B cell receptor (BCR) complex[22]. Similar to ZAP-70, *Syk* is recruited to the phosphorylated ITAMs of the activated BCR complex where it also becomes activated[23]. Studies found that ZAP-70 could reconstitute BCR signaling in *Syk*-deficient B cells[24]. Similarly, *Syk* apparently can mediate TCR signaling in patients who were deficient in ZAP-70[25]. As such, ZAP-70 and *Syk* play similar roles in membrane antigen-receptor signaling pathways.

The B-cell Receptor Complex

The BCR complex of each B cell is comprised of surface Ig (sIg) that is noncovalently associated with CD79a and

CD79b. CD79a and CD79b are Ig accessory molecules as they allow for the transport of the assembled Ig to the B cell surface as well as the signaling events that are triggered when sIg binds to antigen. These signaling events play critical roles in stimulating B-cell proliferation and/or survival during B-cell differentiation and the immune response to antigen. As such, these signaling events are critical for selecting B cells that make Ig capable of binding antigen during the course of an immune response, allowing for differentiation of such B cells into antibody-producing plasma cells or memory B cells that can respond rapidly upon re-exposure to same antigen. Although any one of the Ig heavy-chain isotypes can serve as the B cell membrane receptor for antigen, most patients with chronic lymphocytic leukemia (CLL) have leukemia B cells that use IgM and IgD.

The cytoplasmic tails of CD79a and CD79b each contain an ITAM that can serve as a docking site for kinases and adapter proteins that participate in BCR signaling triggered by ligation of the sIg, such as when the B cell binds antigen. Upon ligation of sIg, *Syk* is recruited to the ITAMs of CD79a and CD79b, which are phosphorylated by Src kinases, such as Lyn[23] (Fig. 1). Upon phosphorylation, *Syk* can enhance the generation of second messengers that require tyrosine phosphorylation of linker proteins, such as B cell linker protein (BLNK, also known as SLP-65, BASH, or BCA)[26-28]. BLNK represents a central linker protein that bridges the B cell receptor-associated kinases with a multitude of signaling pathways that influence B cell function and development[26, 27, 29]. Immature B cells lacking BLNK experienced delayed maturation and decreased Ig kappa light chain production[29, 30]. Once phosphorylated, BLNK facilitates activation and/or phosphorylation of downstream effector molecules, such as Bruton's tyrosine kinase (btk) or phospholipase-C-gamma (PLCγ)[27, 29, 31]. Activation of PLCγ leads to an increased flux of intracellular calcium (Ca^{2++}).

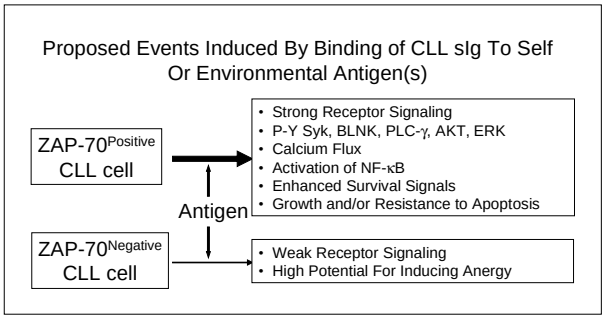

Fig. 1.– *Model For The Role Of Antigen And BCR-Signaling In Disease Progression*. Continuous or repeated exposure to self or environmental antigens capable of effecting ligation of sIg could provide a growth and/or survival stimulus to CLL B cells. CLL B cells that express ZAP-70 generally have a BCR that is more sensitive to sIg cross-linking than CLL cells that lack ZAP-70. Following exposure to antigen there may be strong receptor signaling of ZAP-70-positive CLL cells, leading to enhanced tyrosine phosphorylation (P-Y) of downstream signaling molecules (e.g. Syk, BLNK, PLC-γ, AKT, and ERK) and enhanced activation of NF-κB. Together these signals can provide stimuli for the growth and/or survival of the neoplastic B cells. On the other hand, CLL cells that lack ZAP-70 generally do not have such signaling following BCR ligation, making it less likely that such cells will be stimulated as effectively following exposure to antigen.

Providing for homeostasis are other B-cell surface molecules, such as the Fc receptor for Ig (e.g. CD32, also known as FcgRIIB). CD32 has cytoplasmic domains that contain immunoreceptor tyrosine-based inhibition motifs (ITIM), which, upon activation by Src kinases, can bind phosphatases, such as the Src homology 2-containing inositol phosphatase (or SHIP). Once recruited to the site of activation, phosphatases can de-phosphorylate activated signaling molecules and the ITAMs of the Ig accessory molecules, thereby re-setting the activation cascade that was initiated by ligation of sIg by antigen.

B-cell Receptor Signaling in CLL

The leukemia cells of different patients vary in their capacity to undergo BCR signaling. It had long been recognized that CLL B cells generally have a diminished response to sIg ligation[32-34]. The poor responsiveness of CLL B cells to sIgM cross-linking had been presumed due to the leukemia cells' relatively low-level expression of sIgM, inadequate levels or dysfunction of *Syk*[33], or expression of a mutated or splice-variant RNA encoding CD79b[35-38]. However, when the leukemia cells of different patients are compared, it became apparent that the levels to which *Syk* became phosphorylated following ligation of sIg varied between different populations of CLL cells[33, 39]. Indeed, despite expressing normal levels of *Syk*[39], the CLL cells of almost half of all patients apparently lacked the capacity to undergo *Syk* phosphorylation following BCR ligation *in vitro*. On the other hand, despite expressing relatively low-levels of sIgM, the CLL cells of some patients can respond to sIgM cross-linking in a manner comparable to that of normal, non-neoplastic B cells. Furthermore, the calcium response to sIgM ligation also varies substantially between CLL B cells of different patients, and the whole pattern of tyrosine phosphorylated proteins in response to BCR signaling appears markedly lower in low calcium responders[32, 40].

More recent studies found that leukemia cells that expressed unmutated Ig V genes[41] or ZAP-70[13] generally were more sensitive to ligation of sIgM than CLL cells that used mutated Ig or that lacked expression of this PTK. Irrespective of whether or not they express ZAP-70, CLL cells generally have similar levels of *Syk*[13], which in turn are comparable to those expressed by normal B

cells. However, those CLL cases that expressed ZAP-70 appeared more sensitive to ligation of sIgM than cases that lacked ZAP-70, regardless of whether they used mutated Ig V genes[13]. Upon ligation of sIgM, ZAP-70 becomes phosphorylated, which typically is associated with phosphorylation of *Syk*. Co-immune precipitation studies revealed that the phosphorylated ZAP-70 and phosphorylated *Syk* were recruited to the BCR complex following ligation of sIgM (Fig. 1), where they potentially could effect the phosphorylation and recruitment of downstream adapter proteins and signaling molecules that are required for subsequent BCR signaling events. The association between BCR signaling and expression of ZAP-70 suggested that this protein tyrosine kinase could enhance the signaling capacity of the BCR complex in CLL[13].

The role of ZAP-70 in BCR-signaling in CLL cells was examined in adenovirus transfection studies in which ZAP-70-negative CLL cells were made to express this protein tyrosine kinase via gene transfer. We stimulated non-transduced and transduced CLL cells with $F(ab)_2$ anti-IgM (anti-μ) and monitored for tyrosine-phosphorylation of Syk, BLNK, or PLCγ. In addition, we examined for changes in calcium flux that could be induced by ligation of sIgM. Following transfection, CLL cells made to express ZAP-70 had significantly higher levels of phosphorylated *Syk*, BLNK, and PLCγ following ligation of sIgM than did the ZAP-70-negative CLL cells that were mock transfected or transfected with a control adenovirus that did not encode ZAP-70[42]. Moreover, CLL cells transfected to express ZAP-70 experienced greater flux of intracellular calcium following sIgM ligation than did control or mock transfected CLL cells that lacked ZAP-70. Finally, CLL cells that expressed ZAP-70 were more likely to experience activation of nuclear factor kappa B (NF-κB) upon BCR ligation than CLL cells lacking this protein tyrosine kinase.

However, the kinase activity of ZAP-70 might not be the factor that is responsible for enhancing BCR signaling in CLL B cells. Indeed, CLL B cells generally express normal levels of *Syk*, a kinase that has 100-fold greater intrinsic kinase activity than ZAP-70[43]. Furthermore, normal B cells that do not express ZAP-70 generally respond to BCR-ligation better than the leukemia B cells of most patients with CLL. Finally, in contrast to ZAP-70, *Syk* can undergo stimulation in the absence of Src-related kinases, initiate immunoreceptor signaling, and promote tyrosine phosphorylation of ITAMs in the BCR complex[44]. As such, it is not certain whether ZAP-70 enhances BCR-signaling in CLL or is merely associated with other factor(s) that might typically be present in CLL with unmutated Ig genes that also enhance BCR-induced phosphorylation of *Syk*.

More recently we have performed structure-function studies to interrogate which domain(s) of ZAP-70 are required for it to enhance BCR signaling in CLL (Chen L et al, submitted). We transduced CLL cells that lacked expression of ZAP-70 with a vector encoding ZAP-70 or mutant forms of ZAP-70. These studies made the somewhat surprising finding that the tyrosine kinase function of ZAP-70 does not appear required for it to enhance BCR signaling in CLL. This also was inferred from studies using various kinase inhibitors to inhibit BCR signaling in CLL B cells[45]. As such, instead of directly effecting phosphorylation of downstream adapter proteins, it appears that ZAP-70 facilitates recruitment of Lyn and/or other Scr kinases or adaptors to the BCR complex of the CLL cell following sIg-ligation in a manner similar to how it functions in double positive thymocytes, which have limiting amounts of lck. In such cells, a kinase defective ZAP-70 still can enhance phosphorylation of the TCR-ζ chain following TCR ligation[46]. Perhaps for this reason, ZAP-70 was found more effective than Syk in promoting phosphorylation of TCR-ζ by kinases following TCR ligation[47]. With respect to it possible role in B cells that also express Syk, a recent study demonstrated that ligation of sIgM induced translocation of the BCR complex into lipid rafts on CLL cells that expressed unmutated IgV_H genes (and hence were likely ZAP-70 positive), whereas the BCR did not translocate to lipid rafts following ligation of sIgM on CLL cells that expressed mutated IgV_H genes (and hence were likely to lack expression of ZAP-70)[48]. In this regard, it is noteworthy that BCR internalization was decreased in BJAB B cells that were transfected with ZAP-70[45]. Conceivably, ZAP-70 facilitates the entry of the BCR complex into lipid rafts more effectively than Syk thereby b better able recruit Src kinases to the Ig receptor complex.

In any case, the enhanced signaling potential afforded by expression of ZAP-70 could contribute to disease progression. Intracellular calcium signaling plays an important role in variety of cell functions including growth and proliferation. Studies have suggested that calcium-activated neutral proteases could enhance the survival of leukemia B cells[49]. The transcription nuclear factor kappa (NF-κB) is another important factor for B cell survival[50-52]. Moreover, IgM engagement leading to activation of NF-κB could elicit a powerful survival program in CLL B cells[53]. This leads to a model proposing that CLL cells that express ZAP-70 are more responsive to self and/or environmental antigens that interact with the highly-selected repertoire of Ig used by CLL B cells (Fig. 1). Provided that there is repeated exposure to such antigens over time, this could allow for leukemia B cells stimulation leading to enhanced proliferation and/or resistance to apoptosis. If so, then the expression of ZAP-70 might be more closely tied to the propensity for early disease progression than the mutation status of the expressed Ig V genes.

Consistent with this notion are clinical surveys that have found CLL-cell expression of ZAP-70 to be a stronger predictor of the need for early therapy than Ig mutation status[14, 54]. Using data-driven criteria for defining a CLL

case as being positive or negative for expression of ZAP-70 or CD38 (another marker for aggressive disease in CLL), the CLL Research Consortium (CRC) conducted another study on an independent cohort of 705 CLL patients (Rassenti L, et al, submitted). As noted in earlier studies[14], this study observed a significant but not absolute association between use of unmutated IgV_H genes and expression of CD38 or ZAP-70. Although CLL-cell expression of CD38, ZAP-70, or unmutated IgV_H each was able to identify patients at significantly higher risk for requiring earlier treatment by NCI-working group criteria[55], multivariable analysis revealed that ZAP-70 was the strongest risk factor. Knowledge of the Ig V gene mutation status or CD38 did not significantly improve our ability to predict the time to first treatment for ZAP-70-positive cases. Moreover, patients who have leukemia cells that express ZAP-70 have a shorter median time from diagnosis to initial therapy than patients with leukemia cells that lack expression of ZAP-70, regardless of whether they have leukemia cells expressing unmutated Ig V genes and/or CD38.

The association between ZAP-70 and early disease progression in CLL suggests that it is a good target for development of anti-leukemia therapy. If its role in enhancing BCR signaling in CLL is responsible for this association, then targeting the kinase activity of ZAP-70 alone might not prove highly advantageous. Rather, the capacity of ZAP-70 to bind to the ITAMs of the Ig accessory molecules CD79a/b and recruit other kinases to the BCR might be responsible for its effect on BCR signaling in CLL. As such, understanding what proteins can complex with ZAP-70 in CLL cells and developing strategies that target BCR signaling in CLL *per se* might be more successful in the treatment of this disease than use of specific inhibitors of ZAP-70 kinase

In this regard, we found that activated heat-shock protein 90 (Hsp90) forms a complex with ZAP-70 in CLL cells, but not in T cells that also express ZAP-70[56]. Moreover, ZAP-70-positive CLL cells generally expressed activated heat-shock protein 90 (Hsp90) with high binding affinity for Hsp90 inhibitors, such as 17-allyl-amino-demethoxygeldanamycin (17-AAG), whereas normal lymphocytes or ZAP-70-negative CLL cells typically expressed non-activated Hsp90. Activated Hsp90 bound and stabilized ZAP-70, which behaved like an Hsp90 client protein in CLL cells, but not in T cells. Treatment with Hsp90 inhibitors, such as 17-AAG or 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG), induced ZAP-70 degradation and apoptosis in CLL cells, but not in T cells. Prior to undergoing apoptosis the treated CLL cells also had impaired BCR relative to that observed in CLL cells not treated with 17-AAG or treated with a control compound lacking the capacity to inhibit Hsp90. Transduction of ZAP-70-negative CLL cells with an adenovirus encoding ZAP-70 activated Hsp90, but not a control adenovirus, rendered the transduced CLL cells sensitive to 17-AAG. These data suggest that Hsp90 is necessary for ZAP-70 expression and activity in CLL; that ZAP-70 is unique among Hsp90 clients, in that its chaperone-dependency appears conditional on the cell type in which it is expressed; and that ZAP-70 is required for cell survival and signaling in CLL. Additionally, ZAP-70 expression in CLL cells confers markedly heightened sensitivity to 17-AAG or 17-DMAG, suggesting that these or other Hsp90 inhibitors could be effective in the therapy of patients with aggressive CLL. In any case, strategies that target Ig-receptor signaling in CLL might be useful in the treatment of this disease.

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…It is imperative in science to doubt; it is absolutely necessary, for progress in science, to have uncertainty as a fundamental part of your inner nature. To make progress in understanding, we must remain modest and allow that we do not know. Nothing is certain or proved beyond all doubt. You investigate for curiosity, because it is unknown*, not because you know the answer. And as you develop more information in the sciences, it is not that you are finding out the truth, but that you are finding out that this or that is more or less likely.*

…Dudar es imperativo en ciencia; para progresar en ciencia, es absolutamente necesario mantener la incertidumbre como parte fundamental de nuestra íntima naturaleza. Para progresar en el conocimiento, debemos ser modesto y reconocer que no sabemos. Nada es cierto o confirmado fuera de toda duda. Se investiga por curiosidad, para buscar lo *desconocido* no porque se conoce la respuesta. En las ciencias, a medida que se acumula mas información, no es que se encuentra la verdad, sino que se encuentra que esto o lo otro es más o menos probable.

Richard P. Feynman (1918-1988)

The pleasure of finding things out. Cambridge MA: Perseus Books, p 248