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Dysregulation of Apoptosis and Proliferation in CLL Cells

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1. Introduction

It is well established, that the appearance of chronic lymphocytic leukemia (CLL), the most frequent form of leukemia in adults, in the developed countries, is mainly due to the gradual accumulation of malignant clone originated from CD5/CD19/CD23 positive lymphocytes. This accumulation results from a dysregulation between proliferation and apoptosis of neoplastic cells. In normal lymphocytes these processes are in equilibrium, so that total number of these cells in the organism remains stable. It has been known for two decades that the accumulation of leukemic lymphocytes in CLL is a consequence of defects of programmed cell death, but also, to some extent, of their dysregulated proliferative activity, as shown by the blockade of certain CLL lymphocytes in G_1 cell cycle phase (Decker et al., 2002). The aim of this chapter is to discuss essential abnormalities of CLL cells apoptosis and proliferation which contribute to the development of the disease and may determine its clinical course. However it must be remembered, that significant majority of experimental data concerning survival and apoptosis of CLL cells, especially regarding cytokines, come from in vitro studies, thus it is difficult to apply them directly to in vivo situation.

Numerous studies allowed to establish, that leukemic cells both circulating in the blood and residing in lymphoid organs survive in vivo for a very long time, counted in months, due to inhibition of their programmed death, but they undergo rapid, spontaneous apoptosis in a few days when cultured in in vitro conditions (Collins et al., 1989). It is then plausible that the prolonged in vivo lifespan is due to the prosurvival influence of microenvironmental factors, in particular to the interactions of malignant lymphocytes with stromal cells (Munk-Pedersen & Reed, 2004; Deaglio & Malavasi, 2009), and probably to the B cell receptor engagement by antigens (Ghia et al., 2008; Burger et al., 2009a). The removal of CLL cells from microenvironment to in vitro culture deprives them of indispensable stimuli and leads to their rapid apoptosis. Several subpopulations of accessory stromal cells have been individualized in the connective tissue. Monocyte-derived CD68+ nurse-like cells, mesenchymal stromal cells and follicular dendritic cells seem to play a particularly important role in this process (Burger et al., 2009b).

1.1 Trafficking and homing of CLL cells in microenvironment

Interaction between chemokine receptor CXCR4 and its ligand CXCL12, formerly known as stromal cell-derived factor-1 (SDF-1), plays a crucial role in the homing of malignant

lymphocytes within host niches of the microenvironment (Burger & Kipps, 2006). Stromal cells and nurse-like cells constitutively secrete CXCL12, what is essential for retention of hematopoietic stem cells, physiologically expressing CXCR4, inside bone marrow. CLL cells, which usually strongly express CXCR4 independently from the type of the disease, make use of CXCR4/CXCL12 axis to remain in a favourable environment (Broxmeyer et al., 2005). Analogous mechanism acts through receptor CXCR5, present in high density on leukemic lymphocytes and ligand CXCL13 synthesized by nurse-like cells in lymphatic nodes and a spleen (Burkle et al., 2007). CLL cells also overexpress CCR7, a receptor interacting with chemokines CCL19 and CCL21. The intensity of this ligation is additionally regulated by atypical, non-signalling receptors CRAM and CCX-CKR (Catusse et al., 2010) and correlates with infiltration of lymphatic nodes, a process which requires a cooperation of α4 integrin (Till et al., 2002). Higher expression of CCR7 has been related to more advanced stage of the disease and the presence of lymphadenopathy (Ghobrial et al., 2004). The role of another chemokine receptor, CXCR3, is relatively poorly understood. Its expression on malignant lymphocytes considerably varies between patients but remains stable over time in individual cases, and surprisingly – lower level of CXCR3 is strongly associated with Rai stages III and IV, diffuse pattern of the bone marrow infiltration and shorter overall survival (Ocana et al., 2007). Another mechanism involved in the adhesion of CLL cells to components of microenvironment concerns integrins – glycoproteins composed of α and β subunits, mediating cell-to-cell and cell-to-matrix junction. The $α_4β_1$ integrin called VLA-4 or CD49d is variously expressed on malignant lymphocytes and acts as a receptor for fibronectin and vascular cell adhesion molecule-1 (VCAM-1 or CD106), cooperating with chemokine receptors in adhesion of these cells to stromal cells and extracellular matrix. Moreover, high expression of VLA-4 correlates with more advanced stage of the disease and shorter overall survival, revealing value as an independent negative prognostic factor (Gattei et al., 2008).

1.2 Reversible influence of CLL cells on the microenvironment

Malignant clone of CLL cells not only uses microenvironmental stimuli, but also influences neighbouring tissues in order to increase attained benefits. Communication between neoplastic lymphocytes and their microenvironment may be executed by microvesicles – detached fragments of malignant cells cytoplasm surrounded by a cell membrane, which are able to fuse nearby cells carrying there numerous proteins and lipids thus exerting impact profitable for a growth and progression of leukemia. A particular mechanism described in CLL concerns transmission of agents stimulating stromal cells to produce vascular endothelial growth factor (VEGF), what leads to enhanced angiogenesis in the bone marrow (Ghosh et al., 2010). Malignant lymphocytes can also actively attract accessory cells, particularly T lymphocytes and monocytes, thus accumulating them in microenvironment, what modifies local immune response in favour of the neoplasm progression. Main factors secreted by CLL cells for this purpose are chemokines CCL3 and CCL4, synthesized after B-cell receptor stimulation (Sivina et al., 2011), and CCL22, produced after CD40 ligation (Ghia et al., 2002).

2. Apoptosis

Processes leading to a programmed cell death can be initiated by either intracellular or extracellular signals. Accordingly, two pathways of apoptosis are distinguished: intrinsic, otherwise called "mitochondrial" and extrinsic, triggered by death receptors signalling.

A wide range of intracellular factors, like DNA damage leading to expression of p53 protein, hypoxia, or growth factors deficiency activate an intrinsic pathway influencing the transcription of Bcl-2 family proteins what leads to an increased release of cytochrome c from mitochondria to cytosol. Thereafter, cytochrome c together with Apaf-1 (apoptotic protease activating factor 1), inactive procaspase-9 and dATP form a complex called apoptosome, which activates caspase-9. This enzymatic complex launches caspase cascade, what causes nuclear condensation, DNA fragmentation, membrane blebbing and finally leads to the cell death. A protein named apoptosis inducing factor (AIF), released from mitochondrion in the same circumstances as cytochrome c, enters the nucleus and results in a cell death without cooperation of caspases. An extrinsic pathway of apoptosis is initiated by activation of several membrane receptors including Fas and TNFαR by their respective ligands. Activated receptors trigger caspase cascade via protein called Fas associated death domain (FADD), which contains domain activating procaspase-8, what leads to cell death.

2.1 Intracellular pathways of apoptosis

Human lymphocytes, as all eukaryotic cells, are equipped with a complicated machinery serving to execute an extracellular or intracellular suicide signal in response to various situations which necessitate cell its death, e.g. unrepairable DNA damage, penetration of a virus into a cell, or neoplastic transformation. Numerous anomalies disturbing this machinery were described in CLL lymphocytes. Those anomalies result in ineffective apoptosis of malignant cells and consequently in their gradual accumulation in blood and lymphoid tissue, thus influencing a clinical course of the disease.

2.1.1 Bcl-2 protein family

The Bcl-2 family is a very conservative class of proteins, detected in a wide range of eukaryotic organisms, from simple nematodes, like Caenorhabditis elegans, to mammalians. Its fundamental role is to control the mitochondrial pathway of apoptosis, by regulation of the permeability of mitochondrial membranes. Bcl-2 and Bcl-xL are principal antiapoptotic proteins of this family. They are located in the outer mitochondrial membrane where they inhibit the release of the cytochrome c from intermembrane space and the creation of the apoptosome, so that the activation of caspase-9 is impaired. As a result of prosurvival activity of Bcl-2 and Bcl-xL, caspase cascade is not activated and cells are protected from apoptosis. Mcl-1 is another important prosurvival protein in this group, structurally different from previous ones, localized predominantly in endoplasmic reticulum and nuclear membrane, interfering with other Bcl-2 agents and inhibiting the cytochrome c release. Proapoptotic members of Bcl-2 family can be divided into two subgroups, depending on number of repeated homological domains called "BH" in their structure: "multidomains" (Bax, Bak, Bok), possessing four domains called BH1, BH2, BH3, BH4, and "BH3-only" (Bim, Bad, Bid, Puma and Noxa). Those proteins can be activated by various signals, like growth factors deprivation, or p53 induced by DNA damage e.g. after radiation or cytotoxic therapy. They deactivate Bcl-2 and Bcl-xL, and support cytochrome c release, thus promoting caspase dependent programmed cells death. In some situations Bid undergoes activation by Fas receptor-induced cleavage and by caspase-8, then it promotes cytochrome c release and triggers the caspases cascade. Therefore it connects both apoptotic pathways: intrinsic and extrinsic one. (Packham & Stevenson, 2005)

Numerous abnormalities of Bcl-2 family proteins expression were observed in CLL cells and it is generally accepted, that shifted balance between different members of that family towards antiapoptotic ones plays a crucial role in prolonging of neoplastic cells in vivo survival. Relatively high expression of Bcl-2 probably because of hypomethylation of its gene were detected in cytoplasm of malignant lymphocytes (Hanada et al., 1993; Robertson et al., 1996). An elevated Bcl-2/Bax ratio was found to be related to chemoresistance and worse prognosis in this disease (Aguilar-Santelises et al., 1996; Molica et al., 1998; Thomas et al., 2000). Yet another observation proves an importance of high Bcl-2 and low Bax levels in programmed cell death inhibition: CLL cells which underwent apoptosis induced by an external factor, e.g. resveratrol, revealed remarkably decreased Bcl-2/Bax ratio (Podhorecka et al., 2011). Increased proteosomal degradation of Bax is considered as a cause of its lower expression (Agraval et al., 2008). Data concerning clinical significance of a decreased Bax level as the only disturbance are somewhat controversial, since some studies suggest its negative prognostic role (Bannerji et al., 2003), while some other ones do not confirm it (Faderl et al., 2002). Increased expression of prosurvival protein Mcl-1 was detected in approximately half of CLL cases, what is thought to inhibit apoptosis and hamper the therapeutic effect of chlorambucil as well as fludarabine (Kitada et al., 1998; Pepper et al., 2008), and rituximab (Awan et al., 2009). Moreover, low expression of MCL-1 gene was correlated with prolonged overall survival in the disease (Veronese et al., 2008). Some other observations suggest that upregulated expression of Mcl-1 plays a crucial role in a protective influence of microenvironmental factors on leukemic cells (Pedersen et al., 2002). Less is known about other Bcl-2 family members. It was shown that simultaneous deficiency of Bax and Bak proteins was related to cells resistance to majority of proapoptotic signals (Wei et al., 2001). Noxa, a protein inducing programmed cells death, is paradoxically excessively expressed in CLL lymphocytes (Mackus et al., 2005). Significance of that phenomenon remains unclear, but it was suggested, that leukemic cells in lymphatic nodes expressed low levels of Noxa, due to proliferative stimuli of microenvironment. In the absence of these signals in circulation Noxa becomes upregulated, but not strongly enough to overcome an apoptosis blockade of highly expressed Bcl-2 (Smit et al., 2007).

2.1.2 Role of p53 in activation of apoptosis

One physiological defense mechanism, aimed at the genome integrity protection, is based on induction of apoptosis when cellular DNA damage becomes irreparable. A key role in that phenomenon is played by p53, a transcription factor which expression is induced by DNA damage. This factor stimulates the expression of p21$^{Cip1/WAF1}$ – universal inhibitor of cyclin-dependent kinases – cyclin complexes, which blocks the cell cycle progression and allows the cell to repair the genetic material. When this repair cannot be completed, p53 enhances the transcription of genes encoding Bax, Puma and Noxa – proapoptotic members of Bcl-2 family, thus initiating the mitochondrial pathway of programmed cell death (Vousden & Lu, 2002). In addition, recent studies suggest, that p53 acts not only as a transcription factor, but is also able to induce apoptosis through direct binding to Bcl-2 protein, deactivating it, what subsequently activates Bax, Puma and triggers caspase cascade (Chipuk et al., 2004; Steele et al., 2008). Approximately 10% to 15% of CLL patients reveal structural aberrations or point mutations in locus 17p13, containing TP53 (gene encoding p53), what results in an improper function of this protein and defective apoptosis of leukemic cells in response to alkylating agents and purine analogues. Those disturbances

have a profound influence on the clinical picture of CLL. The presence of 17p deletion or TP53 mutations is associated with higher clinical stage of the disease, shorter treatment-free survival (Dohner et al., 2000), more aggressive clinical course, shorter progression-free and overall survival (Rossi et al., 2009). It should be mentioned, that double-strand DNA breaks activate p53 through phosphorylation and dephosphorylation of single aminoacids of its chain by ATM protein (Johnson et al., 2009). That is why the inactivation of ATM gene, located in locus 11q22.3 to 11q23.1, leads to p53 functional deficiency. Therefore ATM mutations, resulting mainly from 11q22 – q23 deletions and detected in about 20% of CLL patients, are also considered as negative prognostic factors in the disease, although of lesser importance than 17p aberrations and TP53 mutations (Dohner et al., 2000; Austen et al., 2005).

2.1.3 NF-κB signal transduction pathway

Transcription factor called nuclear factor kappa-B (NF-κB) is a homo- or heterodimeric protein composed of subunits belonging to Rel family, which contains following members identified so far: RelA, RelB, c-Rel, p50 and p52. In the inactive state NF-κB is sequestrated in the cytosol by binding to one of its specific inhibitors: IκB-α, IκB-β, IκB-γ, IκB-ε, Bcl-3, p100 or p105, called collectively "IκB" (Zheng et al., 2011). Activation of NF-κB pathway starts by the interaction of a specific ligand with a receptor activator of NF-κB (RANK), which belongs to a family of TNF-α receptors. Numerous factors can induce NK-κB: tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), osteoprotegerin, ionizing radiation, oxidative stress, or bacterial endotoxins (Vallabhapurapu & Karin, 2009). Stimulated RANK activates a group of kinases called IKK, which phosphorylate IκB liberating it from NF-κB. RANK is also able to activate NF-κB through a specific NF-κB inducing kinase (NIK). When activated, NF-κB enters the nucleus, where it induces the expression of numerous important antiapoptotic genes encoding such proteins as: prosurvival members of Bcl-2 family (Bcl-2, Bcl-xL), cellular inhibitors of apoptosis (IAP family) deactivating caspases, FLICE-like inhibitory protein (FLIP) blocking Fas-associated death domain (FADD), or TNF receptor-associated factor (TRAF), mediating antiapoptotic signals (Fan et al., 2008).

CLL malignant cells show higher constitutive activation of NF-κB than normal lymphocytes (Furman et al., 2000). The impulses such as: CD40 ligation, induction of B-cell receptor (BCR), IL-4, BAFF (B-cell activating factor) or APRIL (a proliferation inducing ligand) were reported to stimulate NF-κB in CLL cells and to antagonize physiological pathways of a programmed cell death. NF-κB expression was reported to show individual variations and may correlate with tumor burden and lymphocytes doubling count, confirming the importance of this signalling pathway in the development and progression of the disease (Hewamana et al., 2008). Currently it is generally accepted that NF-κB is one of the most important transducers of external stimuli, keeping CLL cells alive with blocked apoptosis (Cuni et al., 2004).

2.1.4 PI3K/Akt survival pathway

It is commonly acknowledged that cells need a permanent stimulation with appropriate growth factors to survive. A signalling cascade of the phosphatidylinositide 3'-OH kinase (PI3K) and Akt kinase is thought to be, at least partially, responsible for transduction of prosurvival extracellular stimuli. Their binding to membrane ligands results in displacement of PI3K to the

inner surface of a cell membrane. PI3K phosphorylates membrane phosphoinositides, which recruit Akt from cytosol to plasma membrane and change its conformation into more accessible as a substrate for specific 3-phosphoinositide-dependent protein kinases (PDK-1 and PDK-2). Thereafter PDKs activate Akt by phosphorylation. Five targets of Akt antiapoptotic action on intracellular machinery of a programmed cell death were identified. The first one is Bad – proapoptotic member of Bcl-2 family. Akt phosphorylates Bad inactivating it, thus preventing interaction between Bad and Bcl-xL. Bcl-xL liberated from Bad performs its physiological prosurvival role of blocking cytochrome c release from mitochondria. The second one is caspase-9 – an important link between apoptosome and effector caspase-3. Akt inactivates it and thus interrupts caspase cascade. The third site of Akt's influence on apoptosis is its activating action on IKKs – kinases inducing antiapoptotic pathway of NF-κB, as described above in appropriate section of this chapter. The fourth target of Akt is so called Forkhead family of transcription factors, which regulates expression of several genes important for apoptosis, including Fas ligand gene. Akt inactivates Forkhead family members by phosphorylation, thus reducing their proapoptotic effect (Datta et al., 1999). XIAP (X-linked inhibitor of apoptosis protein), one of most potent inhibitors of caspases, is the fifth target of Akt. XIAP phosphorylated by Akt becomes more resistant to ubiquitination and proteolytic degradation, therefore its prosurvival influence becomes prolonged (Dan et al., 2004).

Stimulation with microenvironmental, non-malignant, bystander cells results in a high activity of PI3K/Akt pathway in CLL lymphocytes. Those prosurvival signals reach leukemic cells through various membrane receptors, like B-cell receptor, CD40 (Cuni et al., 2004), or, described recently, CD160 – membrane protein not present in normal B lymphocytes, but expressed on leukemic ones, which has the property of activating PI3K/Akt pathway (Liu et al., 2010). It is supposed, that enhanced activity of Bcl-xL and NF-κB is the most important way of Akt's influence on cell apoptosis in CLL. Additionally, recent studies suggest that sustained activation of Akt results also in increased expression of Mcl-1 in leukemic cells, what shifts the balance between members of Bcl-2 family towards the prosurvival ones (Longo et al., 2008).

2.1.5 Ambiguous role of JNK in apoptosis

The c-Jun N-terminal protein kinase (JNK) belongs to the family of the mitogen activated protein kinase (MAPK) and is involved in a regulation of cellular apoptosis, responding to a variety of extracellular signals. Despite extensive studies published so far, the exact role of JNK in apoptosis remains unclear. Some studies suggested its proapoptotic function (Davis, 2000), some other showed its antiapoptotic activity (Yu et al., 2004), and other ones did not prove any impact at all of this factor on the programmed cell death (Lin, 2003). Probably a real effect of JNK on apoptosis depends on the type of investigated cells and stimuli tested. FasL and TNF-α may activate JNK and lead to the suppression of Bcl-2 and subsequently inhibition of the apoptosis. Some studies suggest that prior inhibition of NF-κB may be required for this antiapoptotic action of JNK (Liu & Lin, 2005). Moreover, studies performed on pro-B hematopoietic cells displayed a suppression of a programmed cell death via inactivation of Bad – proapoptotic member of Bcl-2 family – through its phosphorylation by JNK in response to interleukin-3 stimulation (Yu et al., 2004). As it was presented in one study, B-cell receptor stimulation probably did not reveal any effect on the activity of JNK pathway in CLL cells (Petlickovski et al., 2005).

2.1.6 Caspase cascade

Majority of pathways transducing extra- and intracellular proapoptotic signals converge toward caspases, a family of cysteine proteases, main executors of apoptotic processes. These proteins localize in cytosol as inactive zymogens and after induction of apoptosis they form a proteolytic chain of consecutively activated enzymes, which is called a caspase cascade. Generally, two classes of caspases are distinguished: initiator and effector ones. Initiator caspases (caspase-8, 9, 10 and 12) transduce signals from apoptotic pathways, cleave and activate effector ones (caspase-3, 6 and 7) (Riedl & Shi, 2004). The intrinsic pathway of apoptosis leads to the formation of apoptosome, which is, as already mentioned, a complex containing Apaf-1, cytochrome c liberated from mitochondria, procaspase-9 and dATP. Apoptosome activates caspase-9 which subsequently activates effector caspase-3 and caspase-6. Induction of the extrinsic pathway results in caspase-8 and caspase-10 activation through FADD, thereafter both initiator caspases mentioned above activate the effector caspase-3. Afterwards caspase-3 activates downstream effector caspase-7. Finally, main effector caspase-3, in cooperation with caspase-6 and 7, cleaves a variety of proteins, like laminA, actin, gas2, what causes cell shrinkage and membrane blebbing. Additionally, caspase-3 inactivates ICAD (inhibitor of CAD), what liberates CAD (caspase activated DNAse) and results in DNA fragmentation and nuclear chromatin condensation. All these processes finally lead to cell death (Logue & Martin, 2008).

The function of caspase cascade is controlled by a group of cysteine proteases, called IAP (inhibitor of apoptosis), containing XIAP, IAP1, IAP2, survivin and livin. They bind and potently inhibit caspase-3, 7 and 9, stopping the cascade regardless of pathway of induction – intrinsic or extrinsic one (Deveraux & Reed, 1999). The activity of IAP family proteins may increase in response to stimulation by various antiapoptotic signals which serve as effectors of specific pathways. For example, one of antiapoptotic activities of Akt is mediated through XIAP, since Akt phosphorylates XIAP, making it more resistant to proteasome-mediated degradation (Dan et al., 2004). FLIP (FLICE-like inhibitory protein), existing in two variants: c-FLIP$_S$ and c-FLIP$_L$, represents another control point of caspases activation. It contains a fragment interacting with death domain motif of FADD and simultaneously prevents activation of caspase-8 and 10, thus blocking Fas receptor signalling pathway and inhibiting programmed cell death (Irmler et al., 1997). However a physiological function of c-FLIP$_L$ is not fully explained, since recent reports suggested its role in activation of caspase-8 (Boatright et al., 2004).

CLL cells do not differ significantly from normal lymphocytes regarding to the expression of caspase family proteins. Nevertheless, as apoptosis inhibition is thought to be principal mechanism of malignant lymphocytes accumulation, so efforts to induce caspase-dependent programmed cell death are evident therapeutic direction. Indeed, caspase activation may be used as a surrogate biomarker of successful induction of apoptosis in leukemic cells by various chemotherapeutic drugs. A choice of caspase-3 activity assessment for this purpose is quite obvious, in view of central effector role of this protein in execution of death signals deriving from variety of pathways. Starting from the oldest drugs, chlorambucil is thought to induce expression of caspase-3 and apoptosis in CLL cells (Brajuskovic et al., 2004). The same phenomenon is observed for newer chemotherapeutics, like fludarabine (Stoetzer et al., 1999) and a monoclonal antibody anti-CD20 – rituximab (Byrd et al., 2002). Alemtuzumab, a monoclonal antibody anti-CD52, another immunochemotherapeutic agent

used in CLL treatment, was not reported to involve caspases pathway, but induces apoptosis through a non-classical, caspase-independent pathway (Mone et al., 2006). The latter mechanism may also represent another possible mode of action of rituximab (Stanglmaier et al., 2004).

2.1.7 Caspase-independent programmed cell death

More than ten years ago an observation was published that cells were capable to undergo apoptosis even when caspases expression was suppressed. This finding pointed out to the existence of caspase-independent mechanisms leading to a programmed cell death (Susin et al., 2000). However regardless of numerous studies, caspase-independent cell death still remains poorly understood. Currently apoptosis is classified into three subtypes. Type I, named "classical apoptosis", is the best explored one and covers all processes triggering caspase cascade, therefore it is often called "caspase-dependent". Each signalling pathway described earlier in this chapter belongs to type I of apoptosis. Type II of programmed cell death is related to increased permeability of mitochondrial membrane, analogically to intrinsic pathway of classical apoptosis activation (Kim et al., 2005). Proteins released from mitochondrial intermembrane space activate proapoptotic factors other than caspases, like calpains, cathepsins and other proteases (Constantinou et al., 2009). AIF (apoptosis inducing factor) is the best known among them, it is released from mitochondrion, then enters nucleus and initiates chromatin condensation and DNA fragmentation. Morphologically this type of apoptosis is characterized by large vacuolization of cytoplasm due to appearance of autophagosomes (Tait & Green, 2008). Type III of apoptosis is less explored; it resembles cellular necrosis and is defined strictly morphologically, with absence of visible nuclear chromatin condensation (Bras et al., 2007).

There are only single reports concerning caspase-independent apoptosis observed in CLL lymphocytes. The mechanism reported so far is triggered by membrane glycoprotein CD47, thrombospondin-1-binding member of the immunoglobulin superfamily. Activation of CD47 by appropriate ligand leads to activation of serpases which afterwards damage cytoskeletal protein called F-actin. Improper function of F-actin results in cell shrinkage secondary to cytoskeletal damage, and in translocation of Drp1 (dynamin related protein-1) from cytosol to mitochondria, where it disrupts the electron transport chain, therefore lowering ATP levels (Barbier et al., 2009). As a result of described mechanisms, disturbances in cell architecture and mitochondrial function, but no pronounced chromatin condensation are detected in cells undergoing the caspase-independent apoptosis. CLL lymphocytes can undergo the caspase-independent programmed cell death even when the classical apoptosis is disrupted. It raises hope for discovering new agents able to overcome chemoresistancy to classical drugs. Further studies on that phenomenon are thus very promising from a clinical point of view.

2.2 Membrane receptors

All metazoan cells receive numerous external stimuli determining their fate depending on momentary requirements of physiological balance in the organism, keeping them alive, or pushing onto a path of a programmed death. These signals are transmitted into cells through a multitude of receptors, among which a superfamily of TNF (tumor necrosis

factor) receptor is one of the most important. Depending on structure and signalling properties, members of TNF receptors family are generally classified into three large groups (Dempsey et al., 2003).

The first one contains: Fas receptor (FasR or CD95), TNF-α receptor 1 (TNF-R1 or CD120a), death receptor 3 binding to TWEAK (DR3, TRAMP or LARD), death receptors 4 and 5 binding to TRAIL (DR4 and DR5). All these proteins possess a characteristic death domain in their cytoplasmic tail. After activation of receptors by external ligands their death domains interact with corresponding transmitter proteins – FasR, DR4 and DR5 with Fas-associated death domain (FADD), while TNF-R1 and DR3 with TNFR-associated death domain (TRADD). In the next step the caspase cascade is triggered through a caspase-8 activation and the cell undergoes apoptosis (Kischkel et al., 2000).

The second group of TNF receptors superfamily contains: TNF-α receptor 2 (TNF-R2 or CD120b), CD40, CD27, CD30, B-cell activating factor receptor (BAFFR), TACI and BCMA (receptors recognizing both: BAFF and APRIL – a proliferation inducing ligand), lymphotoxin-β receptor (LT-βR or CD18), OX40 (CD134), TNF-α receptor 2 related protein (TNFR2-RP or TNFRIII), receptor activator of NF-κB (RANK), receptor expressed in lymphoid tissues (RELT), herpes virus entry mediator (HVEM), and others, not detected on B lymphocytes, like LIGHT receptor (LIGHTR), TROY/Taj, p75 neurotrophin receptor (p75NGFR), ectodysplasin-A receptor (EDAR), fibroblast growth factor inducible 14 (Fn14), or glucocorticoid-induced tumor necrosis factor receptor (GITR) (Darnay et al., 1999). Cytoplasmic tails of these receptors contain various numbers of TIM (TRAF interacting motifs) – protein sequences reacting with members of TRAF family (TNF receptor-associated factor). Activated TRAFs form expanded complexes with TNF receptors, IAPs and RIPs (the death domain kinase receptor interacting protein) mediating antiapoptotic signals through induction of numerous prosurvival pathways, like NF-κB, PI3K/Akt, JNK, ERK (extracellular signal regulated kinase) and others (Xie et al., 2008). Therefore activation of TNF family receptors of the second group induces inhibition of apoptosis, what brings us to an interesting conclusion, that TNF-α can act in two ways – not only proapoptotically, through TNF-R1, but also antiapoptotically, through TNF-R2 (Ihnatko & Kubes, 2008).

A class of proteins unable to transduce stimuli into intracellular signalling pathways forms the third group of TNF receptor family members. Decoy receptor 1 (DcR1 or TRAIL-R3), decoy receptor 2 (DcR2 or TRAIL-R4), decoy receptor 3 (DcR3) and TNF receptor superfamily members 22 and 23 (TNFRSF22 and TNFRSF23) belong to that group. They probably compete with other TNF receptors for their ligands, therefore impeding their activation and induction of intracellular signalling pathways (Falschlehner et al., 2007).

Available data concerning aberrations of the TNF receptors superfamily expression and function in CLL lymphocytes are scanty, but some interesting observations were published. Fas receptor is distinctly downregulated on leukemic cells (Laytragoon-Lewin et al., 1998) and attempts of its upregulation by various factors in vitro are not as efficient as in normal B cells (De Fanis et al., 2003). Nevertheless, this is unlikely to be the cause of their resistance to Fas-mediated apoptosis, because eliciting high FasR expression on a surface of CLL lymphocytes does not restore their susceptibility to that way of a programmed cell death (Romano et al., 2005). Moreover it seems that the expression of FasR on leukemic cells does not have prognostic significance to clinical course of the disease (Hjalmar et al., 2002). CD40

is strongly expressed both on CLL cells and normal B lymphocytes, without significant difference between them. Activation of CD40 on leukemic cells by its specific ligand CD40L (otherwise called CD154) induces expression of proapoptotic FasR, but at the same time it strongly activates prosurvival NF-κB pathway. As a result, antiapoptotic effect of CD40 activation prevails in CLL cells (von Bergwelt-Baildon et al., 2004). In addition it has been observed that ligation of CD40 reduces the efficacy of apoptosis induction by fludarabine in CLL lymphocytes in vitro (Romano et al., 1998). CD27 is considered as a marker of memory B cells and, when activated by CD70, it leads to plasma cell differentiation (Agematsu et al., 2000). Its expression on a surface of CLL cells does not differ significantly from normal lymphocytes, but serum levels of soluble CD27 are higher in CLL patients than control healthy subjects and correlate with some unfavourable prognostic factors, like high lymphocyte count, advanced clinical stage or high serum levels of β_2-microglobulin (Molica et al., 1998). Antigen CD30 is typical of Hodgkin lymphoma and hairy cell leukemia variant, but in contrast to normal lymphocytes, it is also detectable at low density on CLL cells. TNF-R1 is expressed neither on malignant nor on normal B lymphocytes, while TNF-R2 is detected on both, although without significant differences between them (Trentin et al., 1997).

Not only TNF superfamily receptors regulate the survival of malignant lymphocytes. CD38 is a glycoprotein mediating cell to cell interactions and acting as an adhesion molecule, with a reliable negative prognostic value for CLL patients. In vitro observations show that activation of CD38 by its ligand CD31 induces proliferation and differentiation of CLL cells and impairs their apoptosis by influence on the expression of numerous proteins of Bcl-2 family, like Bax, Bim, Puma or Mcl-1 (Deaglio et al., 2010). Similar effect is exerted by CD100 activation with plexin-B1. Since nurse-like cells from lymphoid tissue produce both ligands – CD31 and plexin B1, this phenomenon evidences the importance of environmental factors for CLL cells viability (Deaglio et al., 2005).

2.3 Influence of chemokines on the survival of CLL cells

Trafficking and homing of leukemic cells in a favourable microenvironment gives them an opportunity to benefit from a set of prosurvival factors secreted there. CXCL12, belonging to CXC chemokines and improving leukemic lymphocytes viability through induction of mitogen-activated protein kinases (MAPK or ERK 1/2) is one of them (Burger et al., 2000). However survival of CLL cells cultured in vitro together with nurse-like cells is significantly longer than those cultured only with a solution of CXCL12 (Burger et al., 2000), so it is supposed, that other substances produced by nurse-like cells influences the viability of malignant lymphocytes. Currently it is thought, that this role is played by two members of TNF superfamily: APRIL (a proliferation inducing ligand) and BAFF (B-cell activating factor of a TNF family), otherwise called BLyS (B lymphocyte stimulator). They are important survival and maturation factors of normal B lymphocytes (Mackay et al., 2003), probably influencing the expression of Bcl-2 family members (Craxton et al., 2005). After secretion by nurse-like cells, they support CLL cells survival in a paracrine manner, independently from CXCL12, through activation of NF-κB pathway, inhibiting both: spontaneous and drug-induced apoptosis (Nishio et al., 2005). Moreover, neoplastic lymphocytes also express BAFF and APRIL, probably enhancing their own viability in an autocrine way (Kern et al., 2004).

A number of studies conducted in vitro showed an influence of interleukins on a programmed cell death and survival of malignant CLL cells. Interleukin 1, nonspecific inflammatory mediator and lymphocytes activating factor, protects leukemic lymphocytes from apoptosis, spontaneous as well as induced by glucocorticosteroids (Jewell et al., 1995). Interleukin 2, the principal growth factor for T lymphocytes, inhibits the apoptosis of CLL cells by enhancing Mcl-1, Bcl-xL and survivin expression. Activated lymphocytes respond to this interleukin stronger then resting ones. Interestingly, at the same time interleukin 2 reduces the expression of Bcl-2, but global result of its activity on CLL lymphocytes remains prosurvival (Decker et al., 2010). Interleukin 4, produced by T helper cells, activates normal B lymphocytes and suppresses the apoptosis of leukemic cells through upregulation of Bcl-2 expression (Panayiotidis et al., 1993). Interleukin 5, a growth factor involved in hematopoiesis, which principal function is to stimulate the eosinophils maturation, increases spontaneous apoptosis rate of malignant lymphocytes in vitro in an unknown way, without influence on Bcl-2 expression (Mainou-Fowler et al., 1994). Interleukin 6 is an important factor of growth and differentiation of B lymphocytes. It is thought to inhibit the programmed CLL cells death by increasing the Bcl-2 levels. Moreover, higher expression of interleukin 6 correlates with more advanced stage of the disease and higher serum concentration of β_2-microglobulin (Lai et al., 2002). Physiological function of interleukin 8 is the induction of chemotaxis. In malignant lymphocytes it upregulates expression of Bcl-2, thus preventing their apoptosis. It is produced mainly by macrophages, but also CLL cells release it into the serum, thus exerting regulatory function on their own clone in an autocrine manner. Approximately a quarter of all CLL patients express abnormally high levels of interleukin 8, what correlates with a higher risk of the disease progression independently from an initial tumor burden (Molica et al., 1999). Interleukin 10 is overexpressed in malignant cells of some CLL patients and correlates with an aggressive course of the disease and short overall survival (Fayad et al., 2001). This probably results from its impact on neoplastic lymphocytes cell cycle, because inhibition of interleukin 10 transcription leads to the enhanced apoptosis of the cells of a murine CLL model (Yen Chong et al., 2001). Interleukin 13, another cytokine involved in B lymphocytes activation, impedes leukemic cells apoptosis induced by interleukin 2 in vitro (Chaouchi et al., 1996). Interleukin 24 triggers apoptosis in CLL cells recruited to the cell cycle, by the inactivation of STAT3 kinase thus stabilizing expression of p53 (Sainz-Perez et al., 2008).

3. Cell proliferation

As mentioned at the beginning, CLL is traditionally considered as a result of inhibition of in vivo apoptosis. A wide variety of disturbances in CLL lymphocytes apoptosis was a subject of earlier sections of this chapter. There are numerous additional evidences supporting this opinion through demonstration of a weak proliferative potential of CLL cells. Low DNA content assessed by flow cytometry, low expression of Ki-67 and PCNA (proliferating cell nuclear antigen) – proteins associated with a nuclear proliferation, finally low rates of BrdU (bromodeoxyuridine) or 3H-thymidine incorporation – assays estimating the extent of DNA synthesis, are similar as in quiescent lymphocytes, what suggests arrest of leukemic cells in G_0 phase of a cell cycle (Caligaris-Cappio & Hamblin, 1999). However there have been several studies published in recent years, supporting the hypothesis, that malignant clone of CLL comprises cells which are recruited to a proliferation cycle but arrested in its G_1 phase (Damle et al., 2010), and that a small but significant fraction of all leukemic cells proliferates with measurable birth rates (Chiorazzi, 2007).

3.1 Proliferation centers

Numerous studies showed that proliferation rate of CLL lymphocytes is not the same in each organ and compartment, but cells with higher birth rate accumulate in specific structures of a bone marrow and lymphatic nodes called pseudofollicles or proliferation centers, composed of lymphocytes, prolymphocytes and paraimmunoblasts of a neoplastic clone, with accompanying follicular dendritic cells, mesenchymal stromal cells and CD4-positive T lymphocytes, where CLL cells have optimal microenvironmental conditions for growth and dividing (Caligaris-Cappio & Ghia, 2008). Malignant cells in those areas are characterized by a higher expression of Ki-67, CD71, CD38, MUM1/IRF-4 and coexpression of survivin and Bcl-2, factors typically associated with proliferation (Soma et al., 2006). Features of proliferation centers have a clear influence on the course of the disease. Patients with larger, confluent pseudofollicles estimated histopathologically in lymphatic nodes, with higher mitotic index and higher Ki-67 expression measured in these areas, more often suffer from the aggressive form of the disease and have significantly shorter overall survival (Gine et al., 2010). Furthermore it is suggested, that pseudofollicles accumulate CLL cells with genetic alterations (Balogh et al., 2011). Estimation of proliferation centers in bone marrow is possible rather in early stages of the disease, because in more advanced stages trephine biopsy often reveals diffuse pattern of a bone marrow infiltration, another well known negative prognostic factor in CLL, with faded structure of pseudofollicles (Mauro et al., 1994).

3.2 Cell cycle regulatory proteins

The important evidences in favour of CLL cells recruitment to a cell cycle were obtained from investigations concerning family of serine-threonine kinases called cyclin dependent kinases (cdk). Their appearance in cytosol and activation in precisely fixed phases of a cell-division cycle by junction with regulatory subunits called cyclins is crucial for a proper course of DNA replication and mitosis. In the beginning of G_1 phase cdk4 and cdk6 bind to cyclin D and phosphorylate the retinoblastoma protein (pRb), what activates transcription factors of E2F family and initiates transcription of proteins participating in DNA replication. Thereafter the association of cdk2 with cyclin E is fundamental for beginning of S phase (Sanchez & Dynlacht, 2005). It is reported, that significant number of malignant lymphocytes express several cyclins and cdks normally present in early G_1 cell cycle phase. The increased levels of cdk4 and cyclin E were observed in CLL cells (Wołowiec et al., 1995; Korz et al., 2002) and higher expression of cdk4 was associated with presence of 17p or 11q deletions (Winkler et al., 2010). Aberrations of cellular content of cyclin D were also reported in leukemic lymphocytes. There are three known subtypes of this cyclin – D1, D2 and D3. Cyclin D3 is definitely overexpressed in CLL cells, what is confirmed by detection of its mRNA (Paul et al., 2005), as well as by the detection of its protein (Wołowiec et al., 2001). Studies concerning cyclin D2 are more discordant, with observations confirming the overexpression of the protein's mRNA (Delmer et al., 1995) and denying it (Paul et al., 2005), while intracellular content of cyclin D2 is elevated comparing to normal B lymphocytes (Wołowiec et al., 2001). Even cyclin E, appearing later in G_1 phase than cyclin D, is detectable in a significant subset of leukemic cells derived from peripheral blood (Decker et al., 2004) and from lymphatic nodes (Obermann et al., 2007). Expression of minichromosome maintenance protein 2 (Mcm-2) is a novel marker of cycling cells since this protein is

detectable from the beginning of G_1 phase, earlier than Ki-67 expression. A significant subpopulation of CLL lymphocytes are Mcm-2 positive and Ki-67 negative, what brings additional evidence for their arrest rather in early G_1, than G_0 cell cycle phase (Obermann et al., 2007). Protein p27[Kip1] is an inhibitor of the majority of known cdk – cyclin complexes, thus regarded as an important antiproliferative factor. CLL cells were demonstrated to express it in higher quantity than normal B lymphocytes and some studies suggested the relationship between higher p27[Kip1] expression and impaired in vitro apoptosis of leukemic cells, although mechanism of this protein antiapoptotic activity in these cells remained unknown (Vrhovac et al., 1998). Other observations carried out on early and intermediate stage patients did not confirm this connection, nevertheless they revealed negative prognostic significance of high p27[Kip1] expression in CLL, contrary to the majority of non-hematological malignances (Wołowiec et al., 2009).

3.3 Telomeres length and DNA synthesis in vivo

Investigations concerning telomeres brought another rationale for proliferation activity of CLL cells. Physiologically DNA composes long, repetitive sequences at the end of every chromosome: these structures are named telomeres. Their function is to protect cells from loss of information-coding segments of DNA during replication, when erosion of a genetic material on chromosomes ends takes place. After replication, an enzyme called telomerase restores lost fragments of telomeres, but only partially, what leads to gradual shortening of telomeres as a part of physiological aging. Therefore telomerase activation and shortening in telomeres length calculated proportionally to age are helpful markers of a cell proliferation (O'Sullivan & Karlseder, 2010). CLL lymphocytes are characterized by shorter telomeres and higher telomerase activity than normal B lymphocytes, what indicates on a greater number of their divisions in the past (Damle et al., 2004). Additionally, shorter telomeres are associated with genetic aberrations of defavourable prognostic signification, mainly unmutated status of the immunoglobulin heavy chain variable gene (Roos et al., 2008), and correlate with shorter progression-free and overall survival of CLL patients (Sellmann et al., 2011). These observations lead to a possible conclusion, that shorter lymphocyte doubling time – well known marker of the aggressive course of the disease – results from higher proliferation rate of neoplastic cells.

Recently designed technique measuring incorporation of deuterium (^{2}H) from heavy water (^{2}H$_2$O), or deuterated glucose into deoxyribose molecules allows to calculate DNA synthesis and proliferation rate of dividing cells in vivo with much higher sensitivity than classic methods like Ki-67 expression or 3H-thymidine incorporation (Busch et al., 2007). Used in CLL, this technique also revealed that malignant cells have measurable birth rates (Messmer et al., 2005), and that among whole population of CLL lymphocytes, those expressing CD38 have significantly higher proliferation rate comparing to CD38-negative cells (Calissano et al., 2009).

4. Summary and therapeutic implications

Although more and more is known about numerous anomalies of CLL cells apoptosis and proliferation, our knowledge still remains incomplete. Decades of research proved the crucial role of these disturbances in the appearance and clinical course of the disease, raising

hope, that their pharmaceutical corrections may evoke normal apoptosis of malignant cells, thus restraining CLL progression. Indeed, a lot of molecules, which influence signalling pathways regulating programmed cell death, are currently investigated towards their usefulness in a treatment of the disease (Robak, 2010). Nevertheless, a tremendous heterogeneity of CLL clinical course suggests significant differences of apoptosis and proliferation anomalies among individual patients, so probably no universal drug, efficient in every case, should be expected.

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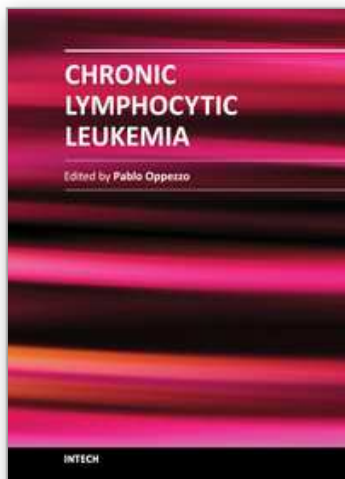
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Chronic Lymphocytic Leukemia

Edited by Dr. Pablo Oppezzo

B-cell chronic lymphocytic leukemia (CLL) is considered a single disease with extremely variable course, and survival rates ranging from months to decades. It is clear that clinical heterogeneity reflects biologic diversity with at least two major subtypes in terms of cellular proliferation, clinical aggressiveness and prognosis. As CLL progresses, abnormal hematopoiesis results in pancitopenia and decreased immunoglobulin production, followed by nonspecific symptoms such as fatigue or malaise. A cure is usually not possible, and delayed treatment (until symptoms develop) is aimed at lengthening life and decreasing symptoms. Researchers are playing a lead role in investigating CLL's cause and the role of genetics in the pathogenesis of this disorder. Research programs are dedicated towards understanding the basic mechanisms underlying CLL with the hope of improving treatment options.

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