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PROGRAMMED CELL DEATH PROTEINS AND CHRONIC LEUKEMIA

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Abstract - Apoptosis or programmed cell death is a genetically regulated process of cellular suicide. Apoptosis has been implicated in a wide range of pathological conditions, and mutations in apoptotic genes play important roles in the process of malignant transformation. Chronic leukemia represents a neoplastic disorder caused primarily by defective programmed cell death, as opposed to increased cell proliferation. This paper presents the main results of our ten-year research on the apoptosis of leukemia cells. The research included the morphological aspects of the process, the effect of antineoplastic agents on the induction of apoptosis in leukemia cells and expression analysis of the proteins involved in programmed cell death. Special attention was paid to the expression and interaction of the Bcl-2 family of proteins in leukemia cells. The ultimate aim of the study of apoptosis of leukemic cells is the discovery of new biological agents that might be used in the treatment of chronic leukemia.

Key words: B-Cell, myeloid cell, chronic, leukemia, apoptosis, mitochondria, Bcl-2 protein family, TP53, caspase, antineoplastic agents

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INTRODUCTION

Leukemia is a heterogeneous disease, characterized by the abnormal proliferation of blood precursor cells of myeloid or lymphoid origin, and it is generally categorized into fast-growing and aggressive acute leukemia, and slower progressive chronic leukemia. The World Health Organization (WHO) Neoplasms recognizes as distinct entities defined by a combination of morphology and cytogenetic abnormalities (Jaffe et al., 1999). Traditional approaches to the treatment of leukemia involve chemotherapy, radiation, and bone marrow transplantation. In recent years, specific targeted therapies have been developed for the treatment of leukemia (Ikeda et al., 2006).

Chronic leukemia

Chronic lymphocytic leukemia (CLL) represents the most common type of adult leukemia in Western countries, accounting for about 30% of the total cases (Chiorazzi et al. 2005). It is a neoplastic disease characterized by the accumulation of morphologically mature but immunologically dysfunctional monoclonal CD5+, CD19+, CD20+ and CD23+ B-cells in the blood, bone marrow and peripheral lymphatic organs (Matutes and Polliack, 2000). The accumulation of neoplastically transformed B-lymphocytes (CLL cells) is primarily the consequence of blocked apoptosis in these cells (Reed 1998; Brajušković et al. 2002a). CLL cases display recurrent genetic aberrations including trisomy 12 and monoallelic or biallelic deletion/inactivation of chromosomal regions 17p, 11q and 13q14 (Klein and Dalla-Favera, 2010).

Chronic myelogenous leukemia (CML) is a malignant myeloproliferative disease developing out of pluripotent hematopoietic stem cells that contain the

fusion Bcr-Abl gene, a product of the Philadelphia (Ph +) chromosome, named after the city in which it was discovered (Nowell and Hungerford 1960; Barnes and Melo 1995). The Ph chromosome is formed by a reciprocal translocation that fuses 5' sequences of the Bcr gene with sequences upstream of exon 2 of the c-Abl proto-oncogene on chromosome 22. The Bcr-Abl gene fusion product is a protein (210 kDa) in which the tyrosine kinase is constitutively active (Di Bacco et al. 2000). Approximately 90% of patients with CML have an acquired genetic abnormality, the Ph+ chromosome (Lugo et al. 1990). Based on clinical symptoms and laboratory findings, CML is classified into three clinical phases, often starting with a chronic phase, progressing to an accelerated phase and ultimately ending in a terminal phase called blast crisis (blast transformation). Blast crisis is the terminal phase of CML in which the patient has all the symptoms of acute leukemia (Chen et al. 2010). The translocation t(9;22), designated the Ph chromosome, is the genetic hallmark of CML (Sabattini et al., 2010). The mechanisms that lead to these changes at the molecular level are still unknown as well as mechanisms that increase the proliferative capacity of these cells (Chen et al. 2010). The main characteristics of this disease include adhesion independence, growth factor independence, and resistance to apoptosis (Di Bacco 2000; Fernandez-Luna 2000). It has been demonstrated that Ph+ cells can be uniquely resistant to the apoptosis induced by different type of stimuli (Keeshan et al. 2002).

Apoptosis – programmed cell death

Apoptosis is the specific process of programmed cell death regulated by numerous extracellular and intracellular mechanisms. The name apoptosis was given by Kerr, Wyllie and Currie in 1972 to the morphologic change culminating in cell death by a process clearly distinct from necrosis (Kerr et al., 1972). Morphologically, apoptosis is characterized by the nuclear and cytoplasmic condensation of single cells (shrinkage) followed by the loss of the nuclear membrane, fragmentation of chromatin, and subsequent formation of multiple fragments of nuclear material and cytoplasm - apoptotic bodies (Buja et al., 1993). It is thought that these changes reflect underlying biochemical processes, including DNA fragmentations, destruction of the cytoskeleton, phosphatidylserine externalization, proteolytic cleavage of a number of intracellular substrates and water loss (Gerschenson and Rotello 1992, Zimmermann et al., 2001).

Two major apoptosis-signaling pathways have been described: the 'extrinsic' pathway that is initiated by ligand-mediated activation of membrane death receptors, and the 'intrinsic' pathway that is controlled by members of the Bcl-2 family and mitochondria-derived proteins (Schmidt et al., 2004). Most apoptosis-signaling pathways finally result in the activation of caspases, a family of cysteine proteases organized in a branched proteolytic cascade. More than 14 caspases have been identified. Some of them (e.g. caspase 8 and 10) are involved in the initiation of apoptosis, others (e.g. caspase 6 and 7) execute the death order by destroying essential proteins in the cell. Caspase 3 is speculated to have a crucial role in apoptosis and is responsible for the cleavage of many critical cellular substrates, leading to characteristic morphological changes in apoptosis such as chromatin condensation, nucleosomal DNA fragmentation and the formation of apoptotic bodies (Hengartner 2000; Oliver and Vallette, 2005).

The Bcl-2 protein family, which includes 20 or more members in mammalian cells, are well-known modulators of this process. The founding member of this protein family is Bcl-2 (B-cell leukemia and lymphoma 2) protein. The bcl-2 gene was identified at the chromosomal breakpoint of t(14;18) bearing B cell lymphomas (Tsujimoto et al., 1984). The first proapoptotic homologue, the Bax (Bcl-2-associated x) protein, was identified by co-immunoprecipitation with Bcl-2 protein (Oltvai et al., 1993). Some members (such as Bcl-2, and Bcl-X_l) are antiapoptotic, while others (such as Bad or Bax) are proapoptotic. All contain at least one of the four conserved regions called the Bcl-2 homologous (BH) domains, with which family members dimerize (Schimmer et al. 2003). Antiapoptotic Bcl-2 proteins, Bcl-2, Bcl-X_l, Bcl-W, Mcl-1 (myeloid cell leukemia sequence 1) and A1/BFL 1, contain four BH domains (BH1–4). The

proapoptotic members of the family can be subdivided into two functionally and structurally distinct classes. The BH3-only proteins share only the BH3 domain. This class includes: Bim (Bcl-2-like protein 11), Puma (p53-upregulated modulator of apoptosis), Bid (BH3-interacting domain death agonist), Bad (Bcl-2-associated agonist of cell death), Bik (Bcl-2-interacting killer), Bmf, (Bcl-2 modifying factor), Harakiri (Hrk) and Noxa (Latin for *damage*). The BH1-3 proapoptotic proteins include Bax, Bak (Bcl-2-antagonist/killer) and Bok (Bcl-2-related ovarian killer), and contain multiple BH domains (BH1, BH2 and BH3) and are required downstream of BH3-only proteins to induce apoptosis (Kelekar and Thompson 1998; van Delft and Huang, 2006; Chipuk et al., 2010).

Korsmayer and his associates (1999) showed that Bcl-2 and Bax proteins can make homo- and heterodimers. The antiapoptotic effect of the Bcl-2 protein is based on its ability to form heterodimers with the Bax protein, and thus block the forming of Bax/Bax proapoptotic homodimers. The ratio of Bcl-2/Bax represents the cell autonomous rheostat, which determines the type of cell reaction to an apoptotic stimulus. Other BH3-only proteins, such as Bad, function predominantly by binding to the antiapoptotic repertoire proteins and not by directly activating Bak or Bax. The direct activation model of apoptosis suggests that activator BH3-only proteins are sequestered by binding to antiapoptotic proteins including Bcl-2. Increases in cellular levels of sensitizer proteins, including Puma and Noxa, result in the displacement of activator proteins, including Bim. The displaced Bim then interacts with Bax or Bak, which then change their conformation and insert into the outer mitochondrial membrane (Streele et al., 2009).

Mitochondria play a critical role in the control of apoptosis and represent the primary site of action of the Bcl-2 protein family (Desagher and Martinou 2000; van Loo 2002). The BH1-3 proapoptotic proteins undergo conformational activation leading to oligomerization and insertion in the outer mitochondrial membrane. This process is presumed to result in permeabilization of the outer mitochondrial membrane and egress of apoptogenic factors such as cytochrome c (Lomonosova and Chinnadurai, 2008). Bax translocation from cytosol to mitochondria is believed to be a crucial step for triggering cytochrome c release from mitochondria (Jia et al., 2001). Cytochrome c, a component of the mitochondrial electron transfer chain, initiates caspase activation when released from mitochondria during apoptosis. Cytosolic cytochrome c forms an essential part of the "apoptosome", which is composed of cytochrome c, Apaf -1, and procaspase 9. Only the caspase 9 bound to the apoptosome is able to efficiently cleave and activate downstream executioner caspases, such as caspase 3 (Wang 2001).

The p53 tumor suppressor gene plays a critical role in the regulation of cell proliferation, mainly through induction of growth arrest or apoptosis. It has been shown that the p53 protein as a regulatory protein of transcription has different effects: a) as a transcription suppressor gene whose protein products are needed for the normal propagation of the cell through the cell cycle; b) as a transcription activator gene whose protein products are not only involved in stopping the cell cycle in the G1 phase (eg. WAF1/CIP1), but also are involved in the apoptotic process (Prives and Hall, 1999). A number of proapoptotic Bcl 2 family genes are direct transcriptional targets for p53, e.g. Bax, PUMA and Bid (Zinkel et al., 2006). Under certain conditions, p53 induces apoptosis in the absence of transcription or protein synthesis (transcription-independent apoptotic mechanisms). A novel nontranscriptional mechanism identified in CLL cells involves the direct binding of p53 to antiapoptotic proteins, including Bcl-2, at the mitochondrial surface (Streele et al., 2008). The transcription-independent apoptotic activities of p53 have been demonstrated in transformed cells rather than in normal cells (Haupt et al., 2003). Mutant p53 cannot arrest cells at G1 and can deregulate apoptosis resulting in malignant transformation and proliferation (Lane et al. 1992). Around half of all human tumors carry p53 mutation (Škaro-Milić et al., 1997). The frequency of p53 mutations in hematological malignancies is relatively low compared to

other tumors (Peller and Rotter 2003). From a clinical standpoint, mutations in p53 are usually a poor prognostic indication in a variety of tumor types including gastrointestinal, hematopoietic, breast, and genitourinary cancers (McGill and Fisher, 1997). In general, p53 mutations are more frequent in aggressive diseases and are associated with poor survival (Bykov and Wiman 2003).

Apoptosis as a prognostic parameter in the treatment of chronic leukemia

Experimental studies, both *in vitro* and *in vivo*, have shown the capability of antineoplastic agents to induce the process of apoptosis (Cotter et al. 1992, Begleiter et al., 1994; Thompson, 1995). At the same time, resistance to antitumor treatment is also considered from the viewpoint of the loss of the capability of a certain antineoplastic agent to induce the apoptosis of malignant cells (Desoize 1994). Studies conducted on various model systems (acute lymphoblastic leukemia, lymphomas, etc.) have shown that the ability of antineoplastic agents to induce apoptosis of neoplastic transformed cells represents a positive prognostic parameter in the treatment of malignancy (Lynch 1993). Chlorambucil is able to induce typical features of apoptosis in B-CLL cells. There are no morphologically clear differences between the cells dying in spontaneous or therapy-induced apoptosis. The reduction of the nuclear volume is accompanied by the reduction of the cytoplasmatic volume, while many of organelles remain intact (Brajušković et al. 2003). Bax and Bak, two proapoptotic members of the Bcl-2 family, undergo conformational changes in CLL cells in response to drug-induced apoptosis (Bellosillo et al. 2002).

The first attempts at monitoring *in vivo* effects of chemotherapy showed that the level of spontaneous apoptosis, the maximal apoptotic response and the time of maximal apoptotic response are significant prognostic parameters that correlate with the therapeutic response of patients with malignant diseases. The level of spontaneous apoptosis represents the percentage of malignant cells that die by apoptosis without the influence of antineoplastic agents. Maximal apoptotic response is taken as the moment when the greatest percentage of cells is included in apoptosis during the treatment of patients with antineoplastic agents. The time needed to establish maximal response by apoptosis is the period in which the maximal apoptotic rate is achieved (Li et al. 1994). The results of the first Serbian study have shown that the established levels of spontaneous and therapy-induced apoptosis are in correlation with the clinical response of patients to the applied therapy and that the measurable apoptotic parameters can represent prognostic parameters in the treatment of hematologic neoplasms (Marjanović et al. 1999; Brajušković et al. 2002b).

The molecular basis of apoptosis in chronic leukemia

The disease outcome in chronic leukemia cannot yet be predicted. The molecular mechanisms that regulate apoptosis in CLL are complex but the importance of the Bcl-2 family of apoptosis-regulating proteins in CLL has been established for over a decade. A number of independent research groups have found that over-expression of the anti-apoptotic protein Bcl-2 is a hallmark of CLL (Brajušković et al. 2004a; Buggins and Pepper 2010). The mechanisms responsible for the high amounts of Bcl-2 remain enigmatic, but only rarely do they involve rearrangements of the Bcl-2 gene as a result of chromosomal translocations, unlike the follicular B-cell non-Hodgkin's lymphomas, and may entail Bcl-2 gene hypomethylation in its promoter region (Kitada et al., 1998). Aberrant expression of Bcl-2 is associated with a poor response to chemotherapy and decreased overall survival (Schimmer et al., 2003).

Our expression study of the Bcl-2 family proteins included the analysis of peripheral blood specimens from patients with CLL and specimens of healthy persons who represented the control group. Using Western blotting analysis, we quantitatively examined the protein expression of Bcl-2, Bax, Bad, and Bcl-X_I proteins. The level of Bcl-2 ($p = 3.68 \times 10^{-10}$), Bax ($p = 0.019$), and Bad ($p = 0.073$)

protein expression was significantly increased in all the analyzed peripheral blood samples of the patients, while the level of Bcl-X_l protein ($p = 0.75$) did not significantly differ compared to the control. The results of this study showed that the increased level of expression of Bcl-2, Bax, and Bad proteins represented the most striking feature of CLL cells. Moreover, the variations in the expression of only one protein of the Bcl-2 family have not been particularly helpful in predicting an outcome for patients with this disorder (Brajušković et al. 2004b).

Bcl-2 and Bax interaction, rather than the absolute level of Bcl-2 expression, is a more important determinant of CLL cell apoptosis (Bannerji and Byrd, 2000). The results of *in vitro* research have shown that Bcl-2/Bax ratios correlate with progressive disease and resistance to chlorambucil and fludarabine *in vitro* (Molica et al., 1998). We analyzed the Bcl-2 and Bax protein interaction in the B-lymphocytes of peripheral blood in patients with CLL. Specimens were precipitated with the anti-Bcl-2 monoclonal antibody, and immunoblotted with the anti-Bax polyclonal antibody (IP:Bcl-2/WB:Bax). Simultaneously, specimens were precipitated with the anti-Bax polyclonal antibody, and immunoblotted with the anti Bcl-2 monoclonal antibody (IP:Bax/WB:Bcl-2). The intensity of Bcl-2 and Bax proteins binding compared to the control samples of peripheral blood from healthy persons, was increased in the CLL cells (Brajušković et al. 2005). IP:Bax/WB:Bcl-2 showed a high level of "free" Bcl-2 protein which was not bound in the heterodimer form to the Bax protein. IP:Bcl-2/WB:Bax showed that a higher quantity of Bax protein was bound in the heterodimer form to the Bcl-2 protein as opposed to the quantity of pro-apoptotic Bax protein potentially bound in the homodimer form. Further studies involving larger groups of patients are necessary to explore the potential significance of the Bcl-2/Bax protein ratio as a prognostic parameter in CLL treatment. Simultaneously, decreased Bcl-2/Bax ratios are associated with increased sensitivity to cytotoxic drugs *in vitro* and improved the responses to chemotherapy in patients (Packham and Stevenson, 2005).

The complete release of cytochrome c is the result of the combined action of proapoptotic Bcl-2 family members and of changes in the complex morphology and ultrastructure of the organelle, controlled by the balance between fusion and fission processes (Soriano and Scorrano, 2010). Ultrastructural analyses of chlorambucil-treated CLL cells revealed mitochondrial changes (Brajušković et al. 2004c). The results of our study showed numerous damaged mitochondria in CLL cells during the apoptotic process as well. The most frequent mitochondrial abnormalities in apoptotic CLL cells were a reduction of size with a hyperdensity of their matrix (mitochondrial pyknosis), and markedly swollen with peripherally placed, disorientated and disintegrate cristae. In some apoptotic cells, we also detected a close association of mitochondria with loops of rough endoplasmatic reticulum. Unlike apoptosis induced by chlorambucil, the mitochondria of flavopiridol-treated CLL cells tended to be swollen with signs of autolysis (Hussain et al., 2008). The correlation between the ultrastructural damage and functional activity of the mitochondria in apoptotic B-CLL cells is still not clear and requires further investigation.

We analyzed the expression of caspase 3 protein using Western blotting analysis. The results showed an increased level of expression of caspase 3 in the peripheral blood of B-CLL patients treated by chlorambucil (Brajušković et al. 2004d). These results approved the proposed capability of chlorambucil to induced B-CLL cells apoptosis via activation of caspase 3. According to our investigation we concluded that caspase 3 may represent a logical molecular target for new approaches to overcoming drug resistance. Extensive studies have identified caspase 3 as the primary effector caspase in most mammalian cells including leukemia cells (Lu and Chen, 2011).

Using immunohistochemistry with the alkaline phosphatase/anti-alkaline phosphatase (APAAP) method we analyzed the expression of p53, Bcl-2 and Bax proteins in mononuclear bone marrow cells of patients with CML (Strnad et al., 2008). High expression of Bax protein was detected in all analyzed patients, but no significant differences were noticed

among them. An increased level of Bax protein expression is an essential characteristic of CML cells but it is not related with the clinical stage of disease.

Polymerase chain reaction single strand conformation polymorphism (PCR-SSCP) was used to analyze the presence of mut p53 gene in mononuclear peripheral blood cells and no mut p53 gene was detected in any of the analyzed samples (Strnad et al., 2008). Previous data suggested that changes in the p53 gene are commonly associated with the blast crisis of CML, but rarely with the chronic phase, and they are represented by rearrangements, deletions and point mutations (Lanza and Bi, 1995). The loss or mutation of the tumor suppressor gene, p53, is one of the most frequent secondary mutations in a CML blast crisis (Di Bacco et al. 2000). The lack of mutant p53 product in the peripheral blood and bone marrow cells in patients with CML suggests that this gene plays no important role in the disease pathology (Strnad et al., 2008). However, recent research has shown that wild-type p53 stabilization induces apoptosis in chronic myeloid leukemia blast crisis cells with or without Bcr-Abl mutation (Peterson et al., 2011).

In vitro studies have shown that bcl-2 and c-myc oncogenes cooperate in the development of malignant transformation (Škaro-Milić et al., 1997). The proliferative rate, c-myc and bcl-2 expression in CML change during the course of the disease. It is possible that the change in c-myc expression plays a causative role in the evolution of the blast phase from the chronic phase. In other words, an increase in c-myc expression may play an important role in disease progression (Handa et al., 1997). By a differential PCR method, we followed the presence of an amplified c-myc gene in the mononuclear peripheral blood cells in patients with CML (Strnad et al., 2006). The level of the expression of Bcl-2 protein was considerably higher in the bone marrow samples of the patients undergoing blast transformation of the disease. The amplification of the c-myc gene was detected in 30% of the patients in blast transformation of the disease. The results of our study confirmed that the expression of Bcl-2 protein and the amplification of the c-myc gene in CML cells are in correlation with the disease progression. The results of our study are correlated with results of another Serbian research group. Vidović and her associates showed that the Bcl-2 protein increased significantly with the progression of CML (Vidović et al., 2008).

The results of our research confirmed, once more, the role of the apoptotic process in pathogenesis in CLL and CML. The aim of future research based on up-to-date findings will be to discover new biological agents which would selectively induce leukemia cell apoptosis and which would be used for chronic leukemia treatment. Micro RNAs (miRNAs) are small endogenously expressed translational-repressor RNAs that regulate cellular pathways and are often aberrantly expressed in hematological malignancies (Caporaso et al., 2007). Because the miRNA expression levels in CLL patients differ from those of normal patients, they may be a novel biomarker of this disease (Ward et al., 2011). Some targets of miRNAs are antiapoptotic genes such as Bcl-2, and those miRNAs which are natural antagonists of these genes may be applied in leukemia therapy (Dong et al., 2009). MiRNA-expression profiling of human CLLs has identified signatures associated with diagnosis, staging, progression, prognosis, and response to treatment (Calin and Croce, 2009). The development and validation of miRNA biomarkers should have a significant impact in improving early cancer detection, stratification of the disease, in enhancing therapeutic successes, and increasing the life expectancy of patients (Moussay et al., 2011). Finally, we conclude that the time for a "miRNA revolution" in leukemia is here.

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