



Apoptosis and Necrosis Levels in Chemotherapy Treated Chronic Lymphocytic Leukemia Patients

*Abdul Hussein Moyet AL-Faisal , Manal Lafta Abdul Hassan

Institute of Genetic Engineering and Biotechnology for Postgraduate Studies, Baghdad University

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Abstract: The results showed that CLL patients chemotherapy treatment increase the expression of Bcl2 gene (via measuring the phosphatidylserine -PS level) which increased apoptosis and necrosis percentage. It is also showed that the Necrotic cells (%) were found to be significantly higher in patients samples (1.07 ± 0.03) than in healthy (0.17 ± 0.02) with no significance in viable cells (%) (82.88% in patients and 86.8% in healthy). The apoptotic cells % were found to be significantly higher in patients (15.04 ± 1.03) than healthy (4.40 ± 0.5). On the other hand, late apoptosis % showed no significant results (0.639 % in patients and 0.803 % in healthy).

Key words: CLL, Apoptosis, Bcl2, Mutations, PCR

*Corresponding author: should be addressed (Email: alfais2000@yahoo.com)

مستوى الاستماتة و التنخر عند مرضى ابيضاض الدم المزمن المقاومين للعلاج الكيميائي

عبد الحسين موييت الفيصل ، منال لفته عبد الحسن

معهد الهندسة الوراثية و التقنيات الاحيائية للدراسات العليا ، جامعة بغداد

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الخلاصة: بينت النتائج أن المعالجة الكيميائية لمرضى ابيضاض الدم المزمن أدت الى زيادة تعبير المورث و ذلك من خلال قياس مستوى الفوسفونايديل سيرين و الذي أدى الى زيادة نسبة الاستماتة و التنخر . بينت النتائج أيضا أن نسبة التنخر كانت عالية معنويا عند مجموعة المرضى (1.07 ± 0.03) مقارنة مع الاصحاء (0.17 ± 0.02) وعدم وجود فرق معنوي بينهما في حيوية الخلايا (86.8% و 82.88% على التوالي) . كما أن نسبة الخلايا المستماتة كانت عالية معنويا ايضا عند المرضى مقارنة (15.04 ± 1.03) مع الاصحاء (4.40 ± 0.5) بينما كانت نسبة الاستماتة المتأخرة غير معنوية بينهما (0.803 % و 0.639 % على التوالي) .

Introduction

Chronic lymphocytic leukemia, often referred to as CLL, is a type of cancer of the blood. It is one of a group of diseases that affects a type of white blood cells called a lymphocytes. Chronic lymphocytic leukemia, abnormally high numbers of ineffective lymphocytes are found in the blood and/or bone marrow (the spongy area in the middle of large bones where blood cells are formed) (1). The abnormal cells cannot fight infection as normal lymphocytes do, but instead collect in lymph nodes and other areas, such as the liver and spleen. The accumulation of ineffective lymphocytes can interfere with the production of other blood cells, such as red cells and platelets. CLL is thought to have its origins in the adaptive component of the immune system which consists of a highly specific detection and removal of foreign invaders (2). Adaptive immunity is characterized by an ability to recognize and remember these foreign antigens (2).

Apoptosis has been recognized as a typical and important mode of "programmed" cell death, which involves the genetically determined elimination of cells (3). Apoptosis is a multi-step, multi-pathway cell-death program that is inherent in every cell of the body. Many genes have been identified to have a role in apoptosis.

Bcl2 (B cell lymphoma/leukemia-2) gene is one of these genes. It is an oncogene that in follicular lymphoma is frequently linked to an immunoglobulin locus by the chromosome translocation t (14;18), it was the first example of an oncogene that inhibits cell death rather than promoting proliferation (4). The

site of action for the Bcl-2 is mostly on the outer mitochondrial membrane. Within the mitochondria are apoptogenic factors (cytochrome c, Smac/Diablo homolog, Omi) that if released activate the executioners of apoptosis, the caspases (5). Depending on their function, once activated, Bcl-2 proteins either promote the release of these factors, or keep them sequestered in the mitochondria. Whereas the activated pro-apoptotic Bak and or Bax would form mitochondrial apoptosis induced channel (MAC) and mediate the release of cytochrome c, the anti-apoptotic Bcl-2 would block it, possibly through inhibition of Bax and or Bak (6). The Bcl-2 family has a general structure that consists of a hydrophobic helix surrounded by amphipathic helices. Many members of the family have transmembrane domains.

Annexin V is a cellular protein in the annexin group. Annexin V has been proposed to play a role in the inhibition of blood coagulation by competing for phosphatidylserine binding sites with prothrombin and also to inhibit the activity of phospholipase A1. These properties have been found by in vitro experiments. Annexin V is used as a probe to detect cells that have expressed phosphatidylserine (PS) on the cell surface, an event found in apoptosis, necrosis as well as other forms of cell death.

In cancer, the apoptosis cell-division ratio is altered. Cancer treatment by chemotherapy and irradiation kills target cells primarily by inducing apoptosis. The current study aimed to measure the apoptosis and necrosis in CLL patients treated with chemotherapy (4,5,6).

Materials and Methods

Blood sampling

Three milliliters of blood was collected by vein puncture from 30 cases (CLL) who were admitted to the Center of Hematology /AlYarmouk Hospital from September 2012 till January 2013 .They clinically diagnosed by consultant medical staff at the center. Each collected blood sample was dispensed into EDTA tubes. 25 apparently healthy (blood donors) were also included as control.

Lymphocytes isolation (7):

1. Two millilitres of blood sample were added to an anticoagulant tube with potassium EDTA and diluted 1:1 ratio with phosphate buffer saline (PBS, pH 7.2) to wash and re-suspended the separated lymphocyte for different analytical procedures.
2. Four millilitres of the diluted blood sample were carefully layered (not mixed) on the top of four millilitres of lymphocyte separation media (Ficoll), which was dispersed in 15 millilitres siliconized glass conical centrifuge tube.
3. The tube was centrifuged at 2700 rpm at room temperature for 30 minutes. After centrifugation, the lymphocytes had formed a creamy-web like layer at the interface of the blood plasma and medium.
4. The lymphocytes layer was aspirated (not disturbed) by Pasteur pipette and transferred into another siliconized centrifuge tube.
5. The aspirated lymphocytes were washed by lysis buffer first time, and centrifuged for 15 minutes at 2700 rpm until a pellet was formed. The supernatant was discarded.

6. The pellet was washed by PBS (2ml), centrifuged for 15 minutes at 2700 rpm until a pellet was formed. The supernatant was discarded. PBS washing was repeated for more two times.
7. Finally, the lymphocyte pellet was ready to be used in the planned experiment.

Flow cytometry detection of apoptosis in lymphocytes

Lymphocytes were applied for flow cytometry according to Dillon *et al*,(8). Annexin V-FITC employed in cytometry was performed according to the human Annexin V-FITC Kit-ExBio Praha. This kit includes binding buffer, Annexin V-FITC, and Propidium iodide.

Procedure

1. Lymphocytes pellet isolated from previous steps were re-suspended with a 180 microlitres of diluted binding buffer (10X) in deionized water prior use(1x).
2. Ten microlitres of Annexin V-FITC and 10 microlitres of Propidium iodide were added to lymphocytes mixture and mixed gently.
3. The stained cells were incubated for 15 mins at dark at room temperature.
4. The cells were then centrifuged at 2700 rpm for 5 mins, the supernatant discarded and the pellet re-suspended with 180 µl of binding buffer.
5. The stained cells were analyzed by flow cytometer (Affymetrix eBioscience Company-USA).

Results and Discussion

The results showed that the Necrotic cells (%) was found to be significantly higher in patients samples (1.07 ± 0.03) than in healthy (0.17 ± 0.02) with no significance in viable cells (%) (82.88%

in patients and 86.8% in healthy). The apoptotic cells % were found to be significantly higher in patients (15.04 ± 1.03) than healthy (4.40 ± 0.5). On the other hand, late apoptosis % showed no significant results (0.639 % in patients and 0.803 % in healthy) (Table 1 and Figure 1).

The results showed that chemotherapy treatment increase the expression of Bcl2 gene (via measuring the PS level) which increased apoptosis and necrosis percentage. This finding agree with conclusion of Jitkaew *et al.*(9) who conclude that cancer treatment by chemotherapy and irradiation kills target cells primarily by inducing apoptosis. Increased PS exposure can be the result of membrane damage inflicted on cells. Sak (10) has been

demonstrated that PS exposure on the cell surface can occur before commitment to apoptotic death in some cell systems and may be reversible on withdrawal of the apoptotic stimulus. Although activation of caspases, as well as of other proteases, has been implicated in PS externalization in several studies of apoptotic cell lines (11). Translocation of PS to the cell surface has also been shown to occur independently of the apoptotic process. PS expression on the cell surface has been shown to have non apoptotic functions in viable lymphocytes, facilitating B-cell selection during maturation (8) and modulating the activities of membrane proteins in T lymphocytes (12,13).

Table 1: Flow cytometer parameters of healthy and CLL patients

Parameters	Mean \pm SE		Range	T-test
	Patients	Healthy		
Necrotic cells (%)	1.07 ± 0.03	0.17 ± 0.02	0 - 2.7	0.074 *
Late apoptosis cells (%)	0.639 ± 0.07	0.803 ± 0.04	0 - 1.6	0.344 NS
Viable cells (%)	82.88 ± 4.73	86.80 ± 3.79	58.5 - 97.2	7.844 NS
Apoptotic cell (%)	15.04 ± 1.03	4.40 ± 0.5	1.1 - 41.2	4.261 *
• (P<0.05) , NS: Non-significant				

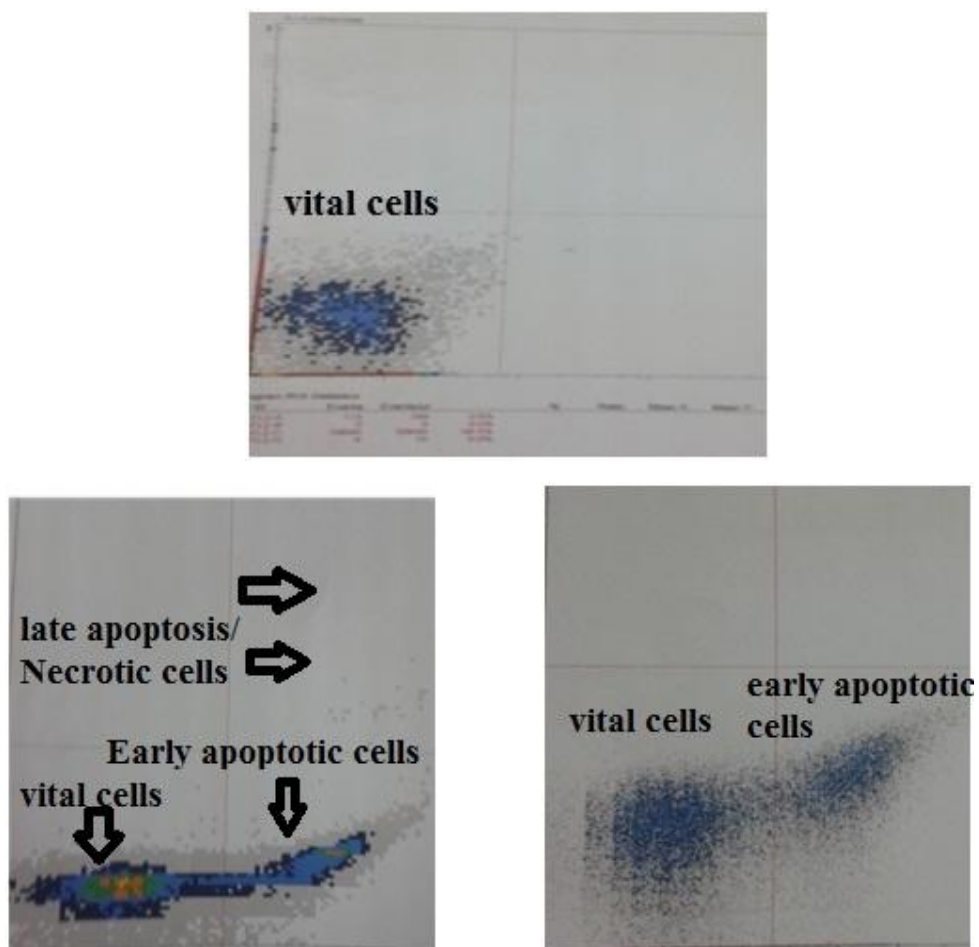


Figure 1 : Flow cytometry charts showing Annexin V expression in healthy (up) and CLL patients (below)

Increased levels of bcl-2 gene family expression were also detected by Pepper *et al.*(14) in a series of B-CLL patients in whom the expression of bax, a homologue of bcl-2 which promotes cell death, was also analyzed. Additional studies found that the CD5+ is the most common B-cell chronic leukemia, less than 10% are CD5-, in fact, the CD5- phenotype is associated with more advanced disease and shorter survival (14,15,16). However, the higher amount of bcl-2 levels of CD5+ B-cell leukemias might contribute to the expansion of the neoplastic cell population by prolonging cell survival.

In contrast, the relatively low bcl-2 expression observed in CD5- B-cells might increase tumor mass by accelerating the rate of cell division.

Apoptotic cells exclude all those dyes that are in use for cell viability assays, such as PI, while necrotic cells do not. However, late-stage apoptotic cells also undergo cell membrane damage in vitro and so these cells will also stain positive for PI. The number of apoptotic and necrotic cells increased with increasing drug concentration (17).

Although B-CLL cells showed a decrease ability to proliferate compared with normal B-cells, they have a longer

lifespan *in vivo*, which serves to maintain the tumor cell population. In contrast, the cells die by apoptosis (18). Incubation with cytotoxic drugs, such as chlorambucil, accelerates this process (19). The mechanism for the induction of apoptosis by these agents is yet to be determined but it seems likely that Bcl-2 and Bax proteins play important roles in determining whether cells undergo apoptosis (20,21). Although Bcl-2 gene rearrangements are unusual in B-CLL, high levels of Bcl-2 proteins expression have been consistently reported (22,23). Previous work has indicated that B-CLL cells undergo apoptosis more readily in patients with untreated, early-stage disease (24). Also Thomas *et al.*(24) evaluated B-CLL cells, using a semi-quantitative method, for their apoptotic response to drug treatment *in vitro* and found that cells with a high bcl-2 /bax ratio were more drug resistant than cells with a low bcl-2/bax ratio. The current data showed a trend towards an apoptosis-response phenotype with treatment, but it remains unclear as to whether this is due to selection of a response population or induction of drug response.

Drug resistance is the major obstacle to the successful management of B-CLL. Although the causes of drug resistance in B-CLL are probably complex, it

seems likely that dysregulation of the apoptotic pathway(s) may play a central role (25). The first-line therapies of choice in B-CLL, fludarabine and chlorambucil, are thought to induce their effects predominantly through a common apoptotic pathway that is reliant on p53 upregulation in response to DNA damage. No association was found between annexin-V expression and the age and sex of patients. This different results of Annexin-V expression (apoptotic cells) is due to various response to chemotherapy. The difference in the response to various chemical substances shown by normal and cancer cell could be explained by the fact that each cell type has its own characteristic metabolic behavior and that each cell line expresses its special and distinct receptors on cell surface which governs the rules and selectivity for binding (26).

In addition, DNA of tumor cells was found in a relaxed shape and the DNA molecule was found in unstable figure because of the longer distance between the H-bonds which connect the two strands of the DNA molecule compared to normal cells and this will subsequently make the DNA molecule of tumor cells more susceptible to the action of DNA interacting compounds (27,28).

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