



# *In vivo* monitoring of therapy-induced apoptotic process in patients with chronic lymphocytic leukemia and acute non-lymphoblastic leukemia

Goran BRAJUŠKOVIĆ<sup>1</sup>  
Slobodan MARJANOVIĆ<sup>2</sup>  
Anđelija ŠKARO-MILIĆ<sup>1</sup>

**BACKGROUND:** *The level of spontaneous apoptosis, the maximal response by apoptosis and the time when maximal apoptotic response is established are significant prognostic parameters that correlate with the therapeutic response of patients with malignant diseases.*

**METHODS:** *Our study included 12 patients with B-CLL (I group of 7 patients) and ANLL (II group of 5 patients). Detection of apoptotic parameters on semifine section of peripheral blood was performed. Bcl 2 and mutated p53 expression were analyzed using the monoclonal antibodies by APAAP techniques.*

**RESULTS:** *In our work, the established levels of spontaneous and therapy induced apoptosis correlate with clinical response of patients. All patients were bcl 2 strongly positive. Expression of p53 protein was not detected in these patients. The ultra structural analysis of the malignant cells has shown that a great number of cells react to antineoplastic agents' effect either by activating the cell response to stress or by apoptosis.*

**CONCLUSION:** *The results of our pilot study have shown that the established levels of spontaneous and therapy - induced apoptosis are in correlation with the clinical response of the patients to the applied therapy and that the measurable apoptotic parameters can represent prognostic parameters in treatment of hematologic neoplasms.*

**KEY WORDS:** *Apoptosis; Antineoplastic Agents; Leukemia; Gene Expression; Genes, bcl-2; Genes, p53*

*Archive of Oncology 2002, 10(2):55-60©2002, Institute of Oncology Sremska Kamenica, Yugoslavia*

<sup>1</sup>INSTITUTE OF PATHOLOGY, MILITARY MEDICAL ACADEMY,  
BELGRADE, YUGOSLAVIA  
<sup>2</sup>CLINIC OF HEMATOLOGY, MILITARY MEDICAL ACADEMY,  
BELGRADE, YUGOSLAVIA

## INTRODUCTION

**P**rogrammed cell death (PCD) - apoptosis is a special type of cell death essentially different from necrosis in nature and biological significance. Apoptosis is an active process of genetically regulated cell autodestruct ion and in most cases has a homeostatic function (1). Studies of the correlation of programmed cell death with proliferation and the multistage carcinogenesis process are in the focus of modern research. A great number of genes are known today, whose protein products take part in regulation of the PCD process (2). Mutations and deletions of apoptotic genes play important roles in carcinogenesis, tumor

growth, and tumor regression. Genes that have received the most attention in this regard are bcl 2 and p53 (3). It is known that the protein product of the bcl 2 gene plays role in the promotion of cell survival and in the inhibition of apoptosis (4). The recognition of a family of bcl 2 related proteins is also emerging that includes bax, a conserved homologue of bcl 2. It has been established that bcl 2 and bax proteins play important opposing roles in the regulation of apoptosis in many cell systems (5). Opposite to bcl 2, the wt p53 gene encodes a tumor suppressor protein, which plays a crucial role in cell cycle regulation and induction of apoptosis (6,7). Experimental studies, both *in vitro* and *in vivo*, have shown the capability of antineoplastic agents to induce the process of apoptosis (8-10). Studies conducted up to now on various models systems (acute lymphoblastic leukemia, lymphomas, Mycosis fungoideus, breast cancer, lung cancer, etc.) have shown that the ability of antineoplastic agents to induce apoptosis of neoplastic transformed cells represent a positive prognostic parameter in the treatment of malignancy (11). The first attempts at following *in*

Address correspondence to:

Dr. Goran Brajušković, Institute of Pathology, Military Medical Academy, 11002  
Belgrade, Crnotravska 17, Yugoslavia

The manuscript was received: 17. 06. 2002.

Provisionally accepted: 05. 07. 2002.

Accepted for publication: 09. 07. 2002.

*vivo* the effects of chemotherapy by a number of authors showed that the level of spontaneous apoptosis, the maximal response by apoptosis and the time when maximal apoptotic response are significant prognostic parameters that correlate with the therapeutic response of patients with malignant diseases (12).

In our study, the level of spontaneous apoptosis represents the percentage of malignant cells that die by apoptosis without the influence of antineoplastic agents. Maximal response by apoptosis 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. Histopathological diagnosis, previous treatment and response, duration of response, performance status of patient, immunophenotype of tumor, cytogenetics of tumor (when possible) represent clinically relevant data for analyzing PCD (11). 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 apoptosis of malignant cell (13,14).

## **MATERIALS AND METHODS**

Our study includes 12 patients with newly diagnosed B - chronic lymphocytic leukemia - B-CLL (I group of 7 patients) and acute non -lymphoblastic leukemia - ANLL - M4 (II group of 5 patients). The patients with B-CLL were treated by high dose - chrolambucil (HD-CLB), while the patients with ANLL-M4 were treated by intensive chemotherapy (Adryamicin, CytoAra and VP16). Patients included in this study were treated on the Clinic of Hematology, Military Medical Academy.

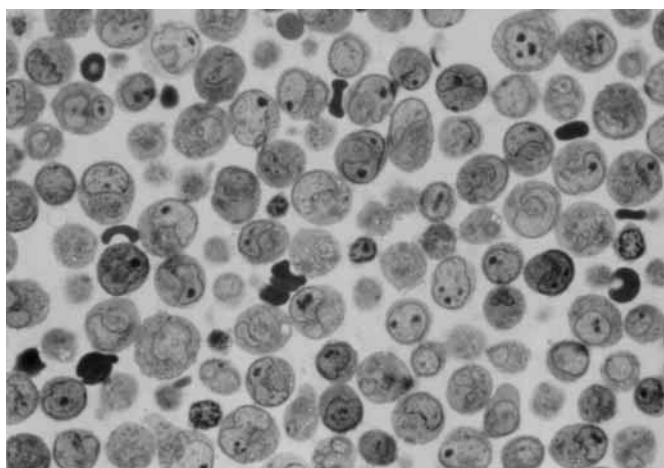
*Technique for preparation of peripheral blood buffy coat cells for electron microscopy.* The effect of the applied therapy on the induction of apoptosis was monitored by successive taking of peripheral blood of the patient prior to the administration of the therapy (to determine the level of spontaneous apoptosis) and during the first 5 days of the therapy. Detection of apoptotic parameters on semifine sections of peripheral blood was performed. The buffy coat cells in the peripheral blood of patients were enriched by centrifugation (1200 rpm, 15 min) (15). The specimens were fixed in 4% glutar-aldehyde buffered in 0.1 cacodylate buffer (pH 7.4) and postfixated in 1% osmium tetroxide in the same buffer. The specimens were dehydrated in a graded series of alcohol and embedded in EPON 812. The semifine and ultrathin sections were cut on the LKB ultramicrotome III. The semifine sections were routinely stained by 1% toluidine blue in borax (16). Samples were assessed under an optical microscope at a magnification of 400x in immersion. At least 1000 cells in randomly chosen fields were examined for each sample. The numbers of

apoptotic cells were presented as a percentage of the cell count. The ultrathin sections stained with uranyl acetate and lead citrate (17). Ultrastructural analysis of sections was performed on Philips electron microscope 201C.

*Immunohistochemistry.* Mononuclear cells from these peripheral blood specimens were separated by centrifugation (1500 rpm, 30') on Lymphocyte Separation Medium (Cat. No. 16-922-49, ICN Flow) and washed in phosphate-buffered saline (PBS). These specimens were used to prepare smears on microscopic slides. All smears were air-dried at room temperature for at least 2 hours. The cell smears were fixed in acetone for 10 minutes or methanol for 4 minutes at 4°C, before immunohistochemical staining. The antibodies used were monoclonal anti - bcl 2 antibodies, diluted 1:50 (DAKO A/S Denmark, Cat. No. M0887) and monoclonal anti - p53 DO-7 antibodies, diluted 1:25 (DAKO A/S Denmark, Cat. No. M7001). Slides were incubated for 24 hours with a dilution of the primary mouse antihuman monoclonal antibodies. Slides were washed twice with Tris buffered saline - TBS (this solution was prepared by mixing one part of Tris - HCL buffer, 0.5M, pH 7.6 with nine parts of 0.15 M saline) and incubated with a 1:25 dilution of Mouse Immunoglobulins (DAKO A/S Cat. No. Z 0259) for 30 minutes at room temperature. After rinsing in TBS, the slides were incubated with a 1:25 dilution of APAAP Mouse Monoclonal (DAKO A/S Cat. No. D0651) for 30 minutes at room temperature. The slides were rinsed again in TBS and incubated with chromogenic substrate (Tris buffer, 0.2M, pH 7.6, 50 ml; Naphthol AS phosphate, free acid - dissolved in 0.5 ml N, N-dimethylformamide, 15 mg; Fast red violet LB salt, 40 mg; 1 mM Levamisole, 12 mg) for 45 minutes at the room temperature. After chromogen development, the slides were washed in two changes of water and counterstained with Meyers's hemotoxylin for 4 minutes at room temperature. Finally, the slides were mounted with Glycergel (18,19). For immunohistochemical analysis, frozen sections of colonic carcinoma and normal tonsil tissue were used as positive controls. In our study, the TBS solution was used as a negative control. Samples were assessed under an optical microscope at a magnification of 400x. At least 500 cells in randomly chosen fields were examined for each sample. The data obtained were arbitrarily grouped in four classes of positivity: I. + (<20%), II. ++ (between 20 and 70%), III. +++ (between 70 and 90%), and IV. ++++ (>90%).

## **RESULTS**

*Quantitative analysis of measurable parameters of apoptosis.* The quantitative analysis of measurable parameters of apoptosis was conducted on semifine sections obtained from the patients' peripheral blood from both groups (Figure 1).



**Figure 1.** Semithin section of peripheral blood from patient with ANLL (day 5 of the therapy) (1.000x)

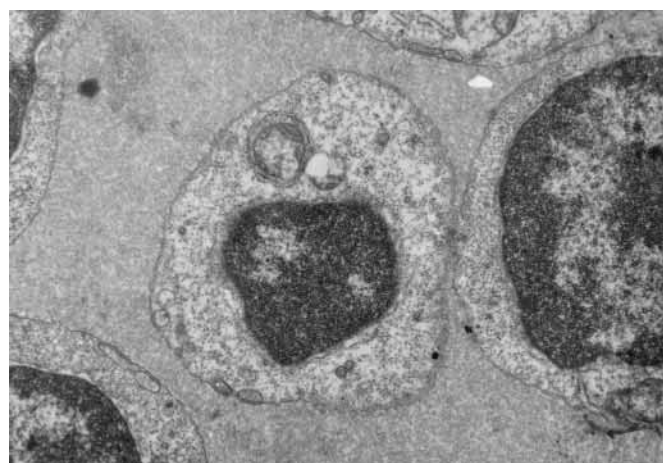
The level of spontaneous apoptosis was determined on semifine sections obtained from the peripheral blood of patients, taken prior to the introduction of chemotherapy. The percentage of the therapy-induced apoptotic cells was also determined on semifine sections obtained from the patient's peripheral blood that was successively taken during the first five days of the therapy. In the first group of patients with *de novo* discovered CLL, the level of spontaneous apoptosis ranged from 11.39% to 20.50%. In the group of ANLL-M4 patients it was significantly lower, and it ranged from 1.50% to 7.12%. The maximal apoptotic response was established with four patients with the CLL clinical diagnosis. The shortest maximal apoptotic response time was marked with a KM2 patient and it was 2 days. The maximal apoptotic response time in two patients was 3 days. One patient reached the maximal apoptotic response time of 4 days. A trend of growing number of malignant cells dying in the process of apoptosis was recorded in both analyzed groups of patients during the first five days of the chemotherapy (Table 1).

**Table 1.** The spontaneous and therapy-induced apoptosis in patients with CLL and ANLL

Initial.	Dg.	Rem.	Day 0	Day 1	Day 2	Day 3 (%)	Day 4	Day 5
S.M.	CLL	PR	11.39	12.44	13.05	13.62	17.17	21.28
Z. M.	CLL	PR	18.07	18.57	22.08	23.34	23.39	23.41
K. M.1	CLL	CR	16.72	19.82	21.65	25.63	26.12	26.42
F. Z.	CLL	PR	17.10	18.28	18.36	18.53	18.75	22.82
K. M.2	CLL	CR	20.50	21.05	23.42	23.50	23.18	24.12
C.R.	CLL	PR	18.05	19.95	15.40	21.49	21.12	20.11
I. M.	CLL	CR	19.81	21.12	21.49	24.39	26.36	26.43
K. R.	ANLL	without R	3.90	6.08	7.19	6.60	6.48	11.10
J. M.	ANLL	without R	7.12	7.33	8.09	8.40	8.86	
V. V.	ANLL	without R	6.91	7.60	8.15	8.57	9.00	10.20
D.M.	ANLL	without R	1.50	2.00	2.90	4.40	6.90	
S.A.	ANLL	without R	2.20	2.30	3.09	4.42	5.80	6.00

CR - complete remission  
PR - partial remission  
R - remission

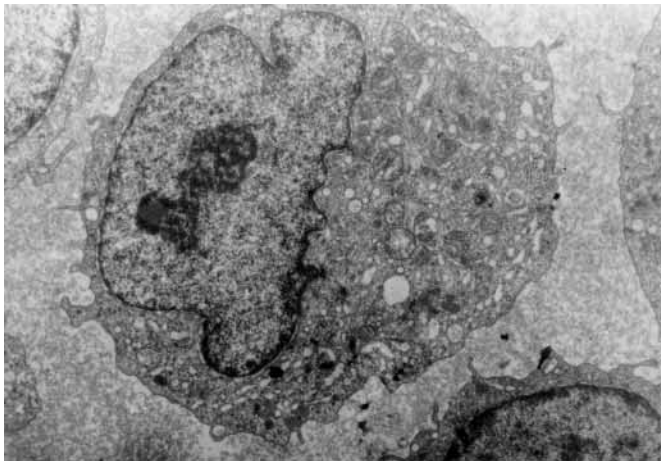
*The results of the electron microscopic analysis.* The results of the electron microscopic analysis of the buffy coat cells in the peripheral blood of patients (taken prior to the therapy administration and on the fifth day of the therapy). The malignant cells of the analyzed samples dying in the process of apoptosis showed typical morphological characteristics of this process, as described by many authors (20-22). It is possible to monitor a number of phases of the apoptotic process. The malignant cells of the analyzed samples dying in the process of apoptosis, initially showed decreased volume and marginated, highly osmiophilic chromatin. A total condensation of the nuclear chromatin and the reduction the nuclear volume occur at the later phases of the apoptotic process. The reduction of the nuclear volume is accompanied with the reduction of the cytoplasmic volume, while all organelles remain intact (Figure 2).



**Figure 2.** Electron microscopic appearance of apoptotic CLL cell (day 5 of the therapy) (16.000x)

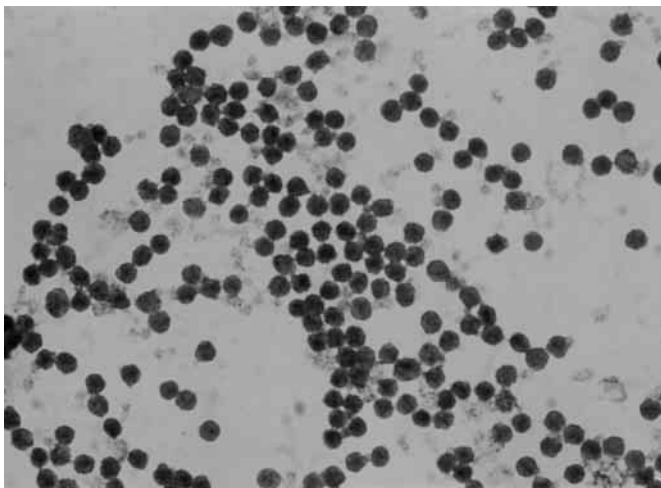
There are no morphologically clear differences between the cells dying in the spontaneous or therapy-induced apoptosis. The noted differences are more likely to be the differences in the morphologic characteristics of the cells that enter the apoptotic process, rather than the characteristics of the programmed cell death. Malignant cells, which do not die in the apoptotic process, on the fifth day of the therapy administration, show morphological characteristics of intensive synthetic activity. They have euchromatic nucleus and hypertrophic nucleolus. Dilated perinuclear spaces are a morphological sign of enhanced signal exchange between the nucleus and cytoplasm. The signal exchange is also made easier by the enlargement of the nucleus-cytoplasmic contact area, which is shown by the presence of pseudoinclusions (pseudoinclusions were not recorded with CLL cells, or with ANLL cells before the effect of the chemotherapy) (Figure 3).





**Figure 3.** Electron microscopic appearance of freshly isolated ANLL cell (day 5 of the therapy ) (11.200x)

The results of the immunohistochemical APAAP method for detection of protein products *mut. p53* and the *bcl 2* gene. Protein products of the *bcl 2* gene were detected in cell smears of isolated mononuclear cells of the peripheral blood of the both groups of patients (Figure 4). Simultaneously, the expression of mutated *p53* protein was not detected (Table 2).



**Figure 4.** Bcl 2 expression in CLL cells (1.000x)

**Table 2.** The results of the immunohistochemical APAAP method for detection of protein products *mut. p53* and the *bcl 2* gene

Initial	Dg.	mut. p53	bcl 2
C.R.	CLL	-	++++
S.M.	CLL	-	++++
I.M.	CLL	-	+++
K.M.1	CLL	-	+++
Z.M.	CLL	-	++++
K.M.2	CLL	-	++++
F.Z.	CLL	-	+++
J.M.	ANLL	-	++++
K.R.	ANLL	-	++++
V.V.	ANLL	-	++++
D.M.	ANLL	-	++++
S.A.	ANLL	-	+++

## DISCUSSION

By correlating the obtained values of the measurable apoptotic parameters (the level of spontaneous apoptosis, maximal apoptotic response and the maximal apoptotic response time) to the clinical response of the patients to the applied therapy, it is possible to divide all the patients in three groups:

- Patients with the CLL clinical diagnosis, who achieved a complete remission (CR) after the chemotherapy, and who had the highest percentage of dying cells due to therapy-induced apoptosis;
- Intermediary values of measurable apoptotic parameters were recorded in patients with the CLL clinical diagnosis, who are in the condition of a partial remission (PR);
- The lowest values, both in spontaneous and therapy-induced apoptosis, were recorded in patients with the ANLL clinical diagnosis, whose treatment had a lethal outcome.

The results of our pilot study have shown that the established levels of spontaneous and therapy-induced apoptosis are in correlation with the clinical response of the patients to the applied therapy and that the measurable apoptotic parameters can represent prognostic parameters in treatment of hematologic neoplasms.

A comparison of malignant cells from the patients' peripheral blood taken prior the chemotherapy and on the fifth day of the therapy, showed some morphological differences that can be attributed to the effect of cytostatic drugs. This was particularly noted with the analyzed peripheral blood samples of ANLL clinically diagnosed patients. We think that the described ultrastructural finding of the intensive metabolic activity of malignant cells, corresponds best with the morphological picture of cells with activated cell response to stress. It should be also pointed out that there is no significant morphological difference between the cells dying in the spontaneous apoptosis from those dying in the therapy-induced apoptotic process.

A high expression of the protein product *bcl 2* gene was detected immunohistochemically in all analyzed patients. The protein expression of the *bcl 2* gene blocks the apoptotic process in patients with the ANLL clinical diagnosis and it represents a negative prognostic parameter in treatment of such patients. A chlorambucil induced apoptotic process appeared in analyzed patients with the CLL clinical diagnosis in spite of a high level of immunohistochemically detected protein product *bcl 2* gene. Our results confirmed the numerous experimental studies of many authors that the high and constant expression of *bcl 2* gene in CLL cells excludes the pathogenic role of *bcl 2* oncogene in the development of clones of neoplastic cells (23-25). *Bcl 2* and *bax* interaction, rather than the absolute level of *bcl 2* expression, is more important determinant of CLL cell apoptosis (26). Simultaneously, a higher ratio of *bcl 2* to *bax* protein is associat-

ed with resistance to chemotherapy in CLL (27). The patients with CLL clinical diagnosis had a good clinical response to the applied therapy shown by a very short time in which they achieved a remission stadium, which was successfully maintained. Overexpression of bcl 2 blocks the apoptosis, and antineoplastic agents must act downstream of bcl 2 (28). At the same time, the absence of the protein product the mutated p53 gene in the peripheral blood of patients with the CLL clinical diagnosis is in correlation with their clinically good response to the therapy received. In the analyzed group of the ANLL clinically diagnosed patients, the expression of the protein product of the mutated p53 gene was not detected by the immunohistochemical method. The absence of the mutated p53 gene protein product does not necessarily mean that the functionally active protein product of the wt p53 gene is present. For example, cellular protein mdm 2 can inhibit the activity of the wt p53 gene (13,29,30). Functional inactivation of the protein product of the wt p53 gene, and not only the mutation of the p53 gene can be the reason for the low level of the spontaneous and therapy induced apoptosis in the ANLL clinically diagnosed patients. In 1996, Thomas et al., proved that the apoptotic process in the CLL cells was independent with the p53 gene. According to these authors, the main mediator of the apoptotic process in the CLL cells is the bax gene protein product. The discovery of new members of the bcl 2 gene family - bcl x, bad, bak, bag, bik and their influence on the programmed cell death makes this process even more complicated. Some of which are anti-apoptotic, whereas other members of the family, such as bax display pro-apoptotic function (28,31).

## CONCLUSION

Various experimental discoveries, as well as the results of our work, show that monitoring of the spontaneous and therapy induced apoptosis level, and of the prognostic parameters in the treatment of hematologic neoplasms, must be extended both to more members of the bcl 2 gene family and the p53 related genes.

### Acknowledgment

*We would like to thank Vitorović Miodrag for his technical assistance.*

## REFERENCES

- Schwarzman AR, Cidlowski JA. Apoptosis: The biochemistry and molecular biology of programmed cell death. *Endocrine Rev* 1993;14(2):133-51.
- Tang DG, Porter AT. Apoptosis: A Current Molecular Analysis. *Pathol Oncol Res* 1996; 2(3):117-31.
- Saikumar P, Dong Z, Mikhailov V, Denton M, Weinberg J, Venkatachalam M. Apoptosis: Definition, Mechanisms and Relevance to Disease. *Am J Med* 1999;107(5):489-506.
- Yang E, Korsmeyer SJ. Molecular Thanatopsis: A Discourse on the BCL 2 Family and Cell Death. *Blood* 1996;88(2):386-401.
- Pepper C, Thomas A, Hoy T, Bentley P. Chlorambucil resistance in B-cell chronic lymphocytic leukemia is mediated through failed bax induction and selection of high Bcl-2 expressing subclones. *Br J Haematol* 1999;104(3):581-8.
- Cox SL, Lane DP. Tumour suppressors, kinases and clamps: how p53 regulates the cell cycle in response to DNA damage. *BioEssays* 1995;17(6): 501-508.
- Velculescu VE, El-Deiry WS. Biological and clinical importance of the p53 tumor suppressor gene. *Clin Chem* 1996;42(6):858-68.
- Cotter TG, Glynn JM, Echeverri F, Green DR. The induction of apoptosis by chemotherapeutic agents occurs in all phases of the cell cycle. *Anticancer Res* 1992;12:773-80.
- Gruber J, Greil R. Apoptosis and therapy of malignant diseases of hematopoietic system. *Int Arch Allergy Immunol* 1994;105:368-79.
- Thompson CB. Apoptosis in the pathogenesis and treatment of disease. *Science* 1995;267:1456-62.
- Lynch JW. Programmed cell death. *Cancer Res Ther Control* 1993;3:213-9.
- Li X, Gong J, Feldman E, Seiter K, Traganos F, Darzynkiewicz Z. Apoptosis cell death during treatment of leukemias. *Leukemia and Lymphoma* 1994; 13(1):65-70.
- Fisher DE. Apoptosis in cancer therapy: Crossing the threshold. *Cell* 1994; 78:539-42.
- Desoize B. Anticancer drug resistance and inhibition of apoptosis. *Anticancer Research* 1994;14:2291-4.
- Mills AE, Emms M, Licata SG. A Simple Technique for Preparation of Bone Marrow or Peripheral Blood Buffy Coat Cells for Electron Microscopy. *Ultrastructural Pathology* 1990;14:173-6.
- Laschi R, Govoni E. Staining Methods for Semithin Sections. In: Johannessen JV, editor. *Electron Microscopy in Human Medicine. Vol 1 - Instruments and Techniques*. New York: McGraw-Hill International Book Company; 1978. p. 187-98.
- Seveus L, Johannessen JV. Embedding, Sectioning, and Staining. In: Johannessen JV, editor. *Electron Microscopy in Human Medicine. Vol 1 - Instruments and Techniques*. New York: McGraw-Hill International Book Company; 1978. p. 116-84.
- Cordell JL, Falini B, Erber WN, Gnosh AK, Abdulaziz Z, Macdonald S et al. Immunoenzymatic Labeling of Monoclonal Antibodies Using Immune Complexes of Alkaline Phosphatase and Monoclonal Anti-alkaline Phosphatase (APAAP Complexes). *J Histochem Cytochem* 1984;32(2):219-29.
- Chin-Yang L, Ziesmer SC, Yam LT, English MC, Janckila AJ. Practical Immunocytochemical Identification of Human Blood Cells. *AJCP* 1984;81(2): 204-12.
- Wyllie AH, Kerr JFR, Currie AR. Cell Death: The Significance of Apoptosis. *Int Rev Cytol* 1980;68:251-99.

21. Sen S. Programmed cell death : concept, mechanism and control. *Biol Rev* 1992 67:287-319.
22. Hockenbery D. Defining Apoptosis. *Am J Pathol* 1995;146(1):16-9.
23. Schena M, Larsson LG, Gottardi D, Gaidano G, Carlsson M, Nilsson K et al. Growth - and Differentiation - Associated Expression of bcl 2 in B - Chronic Lymphocytic Leukemia Cells. *Blood* 1992;79(11):2981-9.
24. Dyer MJS, Zani VJ, Lu WZ, O'Byrne A, Mould S, Chapman R et al. Bcl 2 translocations in Leukemias of Mature B Cells. *Blood* 1994;83(12):3682- 8.
25. Fuente M, Casanova B, Garcia Gila M, Silva A, Garcia Pardo A. Fibronectin interaction with  $\alpha 4\beta 1$  integrin prevents apoptosis in B cell chronic lymphocytic leukemia: correlation with bcl - 2 and bax. *Leukemia* 1999;13:266-74.
26. Bannerji R, Byrd J. Update on the biology of chronic lymphocytic leukemia. *Curr Opin Oncol* 2000;12(1):22-9.
27. Wierdan WG, Kipps TJ. Chronic lymphocytic leukemia. *Curr Opin Hematol* 1999;6(4):253-61.
28. Hannun YA. Apoptosis and the Dilemma of Cancer Chemotherapy. *Blood* 1997;89(6):1845-53.
29. Vogelstein B, Kinzler K. P53 function and dysfunction. *Cell* 1992;70:523-6.
30. Marks DI, Kurz BW, Link MP, Shuster JJ, Laver SJ, Brodsky I et al. High Incidence of Potential p53 Inactivation in Poor Outcome Childhood Acute Lymphoblastic Leukemia at Diagnosis. *Blood* 1996;87(3):1155-61.
31. Haunstetter A, Izumo S. Apoptosis-Basic Mechanisms and Implications for Cardiovascular Disease. *Circ Res* 1998;82:1111-29.