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PRELIMINARY STUDY, BY FLOW CYTOMETRIC PARAMETERS AND APOPTOTIC MARKERS, OF MITOCHONDRIAL DISFUNCTION IN LEUKEMIA CELLS

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ABSTRACT. The main purpose of the antineoplastic treatment is to induce apoptosis in the malignant cells. Apoptosis or programmed cell death is a process in which the deliberate degradation of the cell from the inside takes place, with the active selection of some genes and the failure of others, with the participation of the mitochondria for the propagation of the apoptotic signal, with changes in the phospholipid composition of the cell membrane, with the perturbation and alteration of the cell metabolism. The phases of apoptosis can be detected by flow cytometry. This way the changes in the phospholipid composition of the cell membrane can be determined, when phosphatidylserine (PS), usually on the inner side of the cell membrane, it is displayed on the surface. The aim of the present study is a preliminary analysis of the involvement of the mitochondria, as a major source of ROS in the cell, in the apoptotic process induced by the antineoplastic medication.

Keywords: apoptosis, leukemia, flow cytometer

INTRODUCTION

Mitochondria are subcellular organelles, whose function is to produce ATP through the oxidative phosphorilation system. Mitochondria are also essential in the processing like carbohydrates, amino acids and fatty acids. In addition, mitochondria are semi-autonomous organelles that perform an essential function in the regulation of cell death, signalling and free radical generation.(Czamecka A.M et al, 2007)

Alterations in respiratory activity appear to be a general feature of malignant cells.

Warburg pioneered the research on alterations of the mitochondrial respiratory chain in the context of cancer and proposed a mechanism to explain how they evolved during the carcinogenic process. In his series of landmark publications, he hipothesized that the key event in carcinogenesis could be the development of an injury to the respiratory machinery, resulting in compensatory increases in glicolytic ATP production.(Manash K.P. et al 2006)

Malignant cells produce their ATP through glycolytic mechanisms rather than through oxidative phosphorilation. In benign tumours the rate of aerobic glycolysis is only 1/3 that in malignant tumours. (Carew and Huang, 2002).

Mitochondria are the major source of ROS (reactive oxigen species) including super oxide anion production in cells. During transfer of electrons to molecular oxygen, an estimated 1 to 5% of electrons in the respiratory chain lose their way, most participating in formation of super oxide anion. Super oxides and lipid peroxidation are increased during apoptosis induced by myriad stimuli. However, generation of ROS may be a relatively late event, occurring after cells have embarked on a process of caspase activation. A decrease in the capacity of mitochondria to reduce NAD(P), together with a decline in the NAD(P)H/NAD(P) redox couple, permeabilizes the inner mitochondrial membrane. This favors the release of Ca2+ from the organelle and uncouples oxidative phosphorylation and these effects lead to depletion of ATP. (Pelicano H et al 2006).

The reduced ATP levels also lead to further impairment of other Ca2+ regulation system, in the plasma membrane and the endoplasmatic reticulum. In addition, the decrease in NAD(P)H compromises the activity of protective enzymes, which further increases the deleterious effects of ROS. (Sies H. et al, 1999 and Czamecka A.M et al, 2007)

ROS accumulation causes membrane permeability transition. These alterations in membrane organization may affect respiratory chain function.

Cancer cells exhibit unlimited proliferative potential, resistance to cell death stimuli and abnormal energy metabolism. (Czamecka A.M et al, 2007)

Diminished apoptotic capacity of cancer cells is assumed to the most essential condition responsible for the progeression of a tumor and mitochondria plays a vital role in the induction of apoptosis. (Schimmer A.D. et al 2001)

Apoptosis or programmed cell death is a process which involves the intentional degradation of the cell from the inside, the selective activation of certain genes and the inhibition of others, the participation of the mitochondria to propagate the apoptotic signal, the alteration of the phospholipid cell membrane composition, the perturbation and alteration of the cell metabolism. (Cruce M. et al 2004)

The mitochondrial mediated pathway for caspase activation is initiated by mitochondrial damage that leads to cytochrome c release. Cytochrome c is normally sequestered between the inner and outer

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membranes of the mitochondria. In response to a variety of proapoptotic stimuli, cytochrome c is released into the cytosol.

The exogenous cellular stresses, such as chemotherapy or increase level of reactive oxygen species (ROS) are activators to mitochondrial distruption. (Amuthan G. et al 2001)

Increase level of ROS in cells cause mitochondrial disfunction, which can induce apoptosis.

Apoptosis is characterized by a variety of morphological features. Changes in the plasma membrane are one of the earliest of these features. In apoptotic cells the membrane phospholipids, phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment (Gayon, 2002).

Annexin V is a 35-36 kDa Ca2+ dependent, phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes such as PE.

These formats retain their high affinity for PS and thus serve as sensitive probes for flow cytometric analysis of cells that are undergoing apoptosis.

Because externalization of PS occurs in the earlier stages of apoptosis, Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with Annexin V in conjunction with vital dyes such as 7-amino-actinomycin D (7AAD) allows the investigator to identify early apoptotic cells.(Annexin V positive, 7-AAD negative). (Apoptosis, instruction manual, 1998).

Flow cytometry offers the possibility of analyzing thousand of cells in a very short time (< 1 min) with precision and without the extensive preparation necessary.

MATERIALS AND METHODS

The antineoplastic drugs are inducing the apoptotic process in the sensitive cells. It has been studied leukemia cells. Using Annexin V-PE Apoptosis Detection Kit and flow cytometer, the amount of cells undergoing apoptosis, in various stages of the antineoplasic treatment, was detected. It was gathered blood, before treatment, after 12 hours of beginning the antineoplasic treatment, and then after 24 hours.

We were monitored, at the same time, the serum level of malondialdehyde, a standard method of assessing the oxidative stress.

Flow cytometric analysis

Flow cytometric analyses were peformed on a FACS Calibur cytometer, using Cell Quest Pro software for acquisition and analysis. Studied cells in suspension of isotonic PBS buffer were gated for the light scatter channels on linear gains, and the fluorescence channels were set on a logarithmic scale with a minimum of 10,000 cells analized in each conditions.

Determination the percentage of cells undergoing apoptosis

For determination of the percentage of cells undergoing apoptosis we used: *Annexin V-PE Apoptosis Detection Kit I***, catalog number 559763, from BD Bioscience, BD Pharmingen™**

Annexin V-PE is a sensitive probe for identifying apoptotic cells. It binds to negatively charged phospholipids surfaces (K_d of – 5x 10^{-2}) with a higher specificity for phosphatidilserine (PS) than most other phospholipids. Defined calcium and salt concentrations are required for Annexin V-PE binding as described in the Annexin V-PE Staining Protocol. Purified recombinant Annexin V was conjugated to PE under optimum conditions (Bakker-Schut et al, 1990).

Annexin V-PE is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early phase of apoptosis. In apoptotic cells, the membrane phospholipids phosphatidilserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing PS to the external environment.

Annexin V is a Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS, and is useful for identifying apoptotic cells with exposed PS. 7-Amino-actinomycin (7-AAD) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable to 7-AAD. Cells that stain positive for Annexin V-PE and negative for 7-AAD are undergoing apoptosis. Cells that stain positive for both Annexin V-PE and 7-AAD are either in the end stage of apoptosis are undergoing necrosis or are already dead. Cells that stain negative for both Annexin V-PE and 7-AAD are living cells (Copeland et al., 2002).

Reagents:

1. **Annexin V-PE.**
2. **7-AAD,** is a covenient, ready-to-use solution of the nucleic acid dye that can be used for the exclusion of nonviable cells in flow cytometric assays. 7-AAD fluorescence is detected in the far red range of the spectrum (650 nm long pass filter).
3. **10X Annexin V Binding Buffer.** The solution was 0.2 µm sterile filtered. For a working solution (1X), dilute 1 part binding buffer to 9 parts distilled water. This will yield a working solution of 10 mM Hepes/ NaOH (pH 7,4) 140 mM NaCl, 2,5 mM $CaCl_2$. Store both the 10X concentrate and working solution at 2-8 °C (Apoptosis, instruction manual, 1998).

It is the technique that allows the identification of cells of the beginning of apoptosis and undergoing an apoptotic process. Thus, the alteration of the phospholipid cell membrane composition can be determined, when during apoptosis, phosphatidylserine (PS), usually on the inner surface of the cell membrane, appears on the external surface (Bakker-Schut et al, 1990).

Malondialdehyde level determination

Its' determination represents a standard method of assessing the oxidative stress.

Malondialdehyde is one of the products of lipid peroxidation.

The dosage method is based on the reaction with thiobarbituric acid (TBA). The biological sample is heated with TBA, in acidic medium. As a result of the reaction, one molecule of MDA reacts with two molecules of TBA, with the production of a pink pigment, with a measured optical density at 530 nm using Pharmacia LKB Ultraspec III spectrophotometer. Normal values of the MDA serum levels are between 0.27- 1.02 nmol/ml.

Increased values of the MDA serum levels confirm the presence of the oxidative stress (Muresan et al, 2003).

RESULTS AND DISCUSSION

Flow cytometric analysis

The obtained results at flow cytometry FACS Calibur are graphically represented under dot-plot forms, in which every point of the bidimensional image represents an event, a cell that got in front of the laser waves. The dot-plots are divided into 4 quadrants, as it follows:

- UL - upper left quadrant;
- UR - upper right quadrant;
- LL - lower left quadrant;
- LR - lower right quadrant.

By the spreading of events, in different quadrants, the instrument calculates numeric and percentage the events in the quadrants, after which it can be, analyse and estimate the obtained results.

Cells that appear in the LL quadrant are alives. In the LR quadrant we are shown cells undergoing apoptosis, but in UR quadrants the cells are in apoptosis process or are already dead cells.

Figure 1 shows flow cytometric quadrant analyses of AnnexinV-PE/7-AAD double stained of untreated leukemia cells. These cells, were primarily Annexin V-PE and 7-AAD negative, indicating that they were viable and not undergoing apoptosis.

Figure 2 shows flow cytometric quadrant analyses of AnnexinV-PE/7-AAD double stained of leukemia cells after 12 hours of beginning the antineoplasic treatment. The majority of cells were undergoing apoptosis; they are Annexin V-PE positive, 7-AAD negative.

As shown in figure 3, after 24 hours of beginning the antineoplasic treatment, an increased proportion of the cells are in apoptosis process or are already dead, they are Annexin V-PE positive and 7-AAD positive.

The average percentages are shown in figure 4.

Before treatment the blood cells are alive in the percentage of 91.87%, the way as it can be observed examining the quadrants LL. After 24 hours the numbers of the alive cells reduces to 18.26%.

During the treatment, the initiation of the apoptotic process is present in the majority of cases, and after 24 hours, a percentage of 67.49%, from the cells are in apoptosis or are already dead cells, according to the events from the UR quadrant.

In the LR quadrant we are shown cells undergoing apoptosis. After 12 hours of beginning the treatment 51.43% of cells are undergoing apoptosis. It can be observed that the administrated antineoplasic drugs induce the apoptotic process of the cells, in a very short time from the beginning of the administration.

Serum level of malondialdehyde

We were monitored the serum level of malondialdehyde Normal values of the MDA serum levels are between 0.27- 1.02 nmol/ml. the average values of serum MDA level is shown in table 1.

Table 1

The average values of serum MDA level

	Serum level of MDA (nmol/ml)
Before treatment	**2,6**
After 12 hours	**3,0**
After 24 hours	**3,2**

High level of serum MDA, before and during the antineoplasic treatment was determined. The presence of the oxidative stress and the increase level of reactive oxygen species (ROS) were confirmed before the beginning of antineoplasic treatment and after.

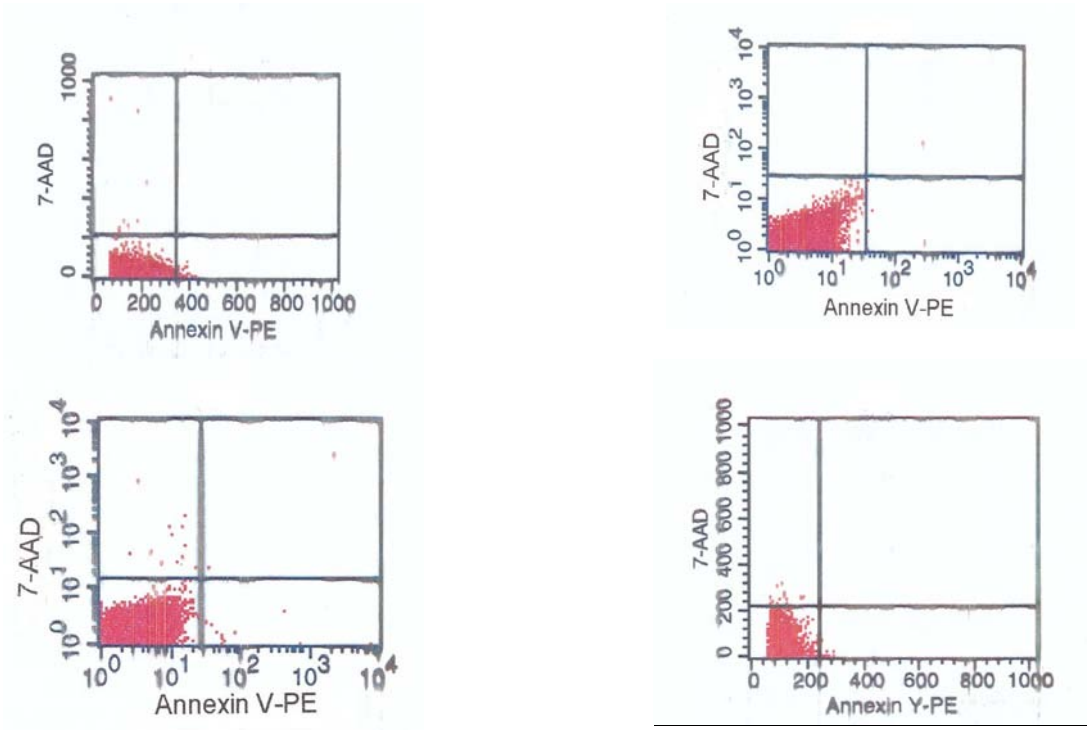

Fig. 1 Flow cytometric quadrant analysis of AnnexinV-PE/7-AAD double stained of untreated leukemia cells. Abscissae: log scale green fluorescence intensityof Annexin V-PE (FL-1). Ordinates: log scale red fluorescenceintensity of 7-Amino-actinomycin (FL 2). LL quadrant: viable cells, annexin V-Pe and 7-AAD negative, LR quadrant: apoptotic cells, annexin V-PE positive and 7-AAD negative cells; UR quadrant: dead cells, Annexin V-PE and 7-AAD positive cells.

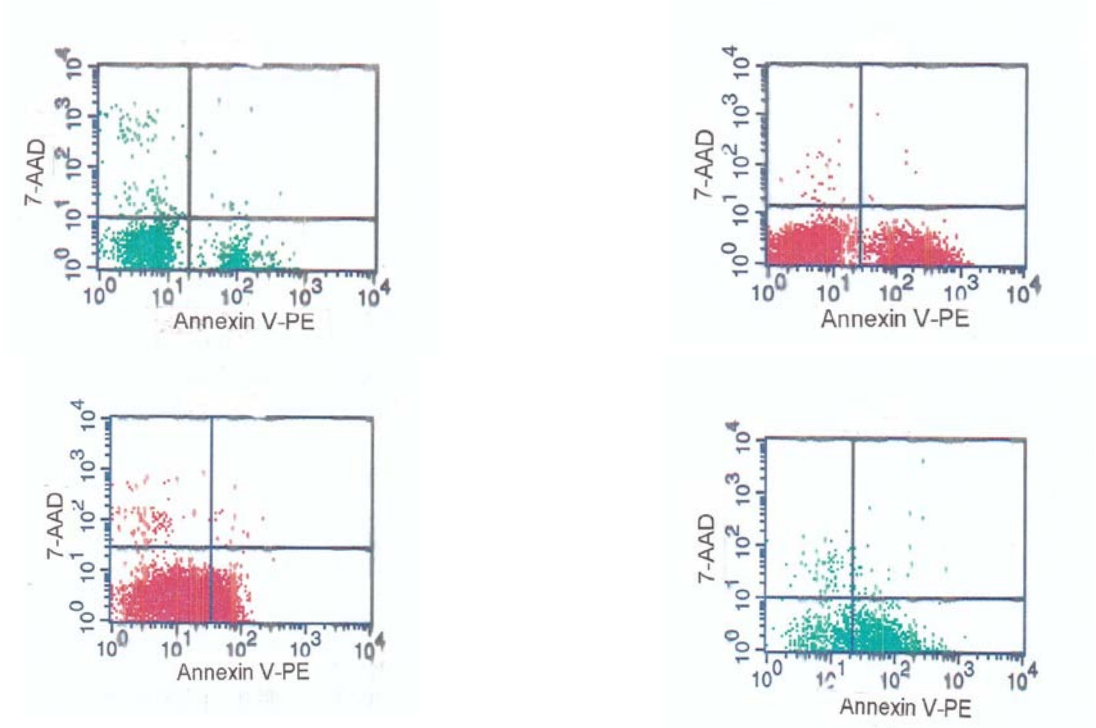

Fig. 2 Flow cytometric quadrant analysis of AnnexinV-PE/7-AAD double stained of leukemia cells after 12 hours of beginning the antineoplasic treatment. Abscissae: log scale green fluorescence intensityof Annexin V-PE (FL-1). Ordinates: log scale red fluorescenceintensity of 7-Amino-actinomycin (FL 2). LL quadrant: viable cells, annexin V-Pe and 7-AAD negative, LR quadrant: apoptotic cells, annexin V-PE positive and 7-AAD negative cells; UR quadrant: dead cells, Annexin V-PE and 7-AAD positive cells.

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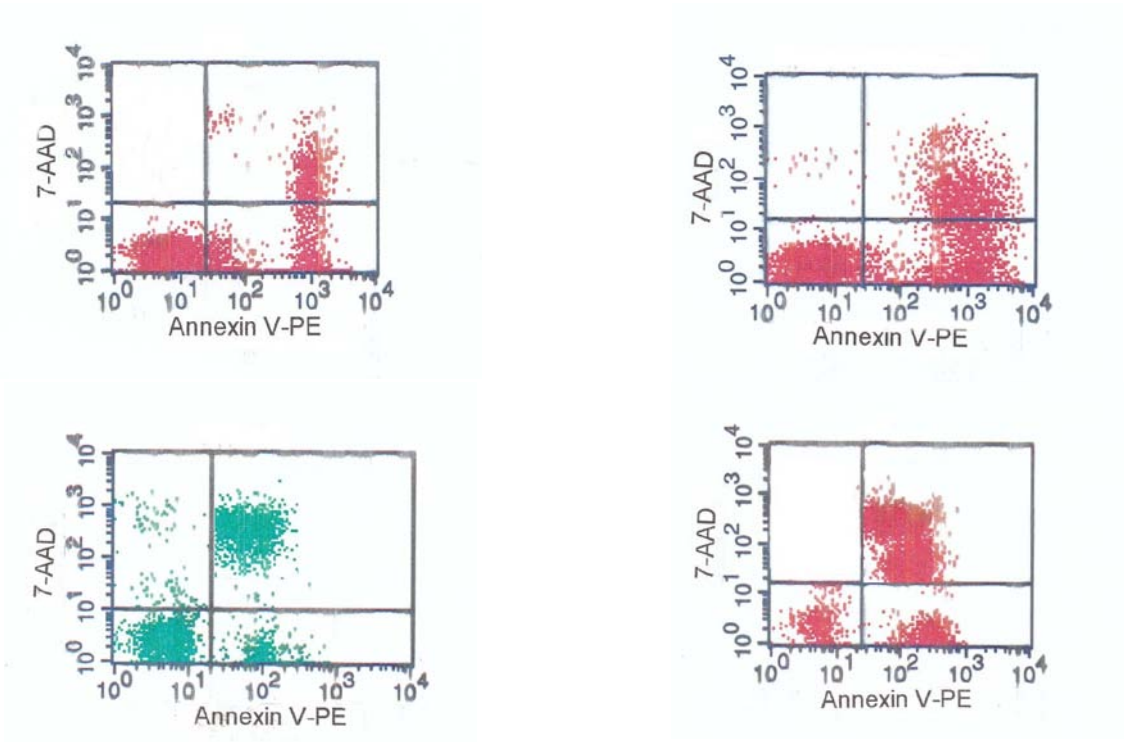

Fig. 3 Flow cytometric quadrant analysis of AnnexinV-PE/7-AAD double stained of leukemia cells after 24 hours of beginning the antineoplasic treatment. Abscissae: log scale green fluorescence intensityof Annexin V-PE (FL-1). Ordinates: log scale red fluorescenceintensity of 7-Amino-actinomycin (FL 2). LL quadrant: viable cells, annexin V-Pe and 7-AAD negative, LR quadrant: apoptotic cells, annexin V-PE positive and 7-AAD negative cells; UR quadrant: dead cells, Annexin V-PE and 7-AAD positive cells.

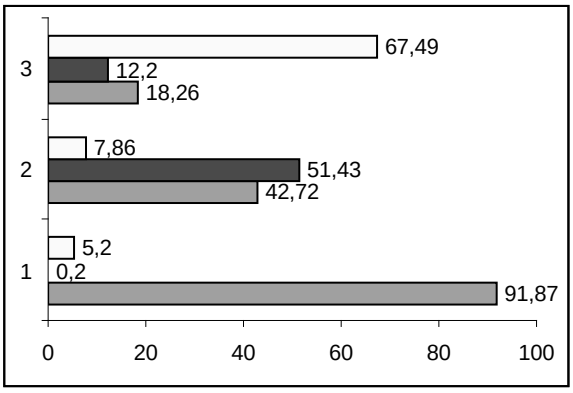

Fig. 4 The average percentages of flow cytometric quadrant analysis,
1 – before treatment: LL=91,87%; LR=0,20%; UR=5,20%;
2 – after 12 hours: LL=42,72%; LR=51,43%; UR=7,86%
3 – after 24 hours: LL=18,26%; LR=12,20%; UR=67,49%

CONCLUSIONS

Using flow cytometric assay, can be measured the percentage of cells undergoing apoptosis.

With comparative flow cytometric quadrant analysis of Annexin V-PE/7-AAD, can be measured the percentage of alive cells, the cells of the beginning of apoptosis and the apoptotic or necrotic cells in various stages of the antineoplasic treatment

It can be conclusioned that the administrated antineoplasic medicines induce the apoptosis for the target cells from a very short time after the administration.

The presence of the oxidative stress and the increase level of reactive oxygen species (ROS) was being confirmed.

Increase level of ROS (reactive oxygen species) in cells cause mitochondrial disfunction, the disfunction of the mitochondrial respiratory chain, which can induce an more efficient response to antileukemic therapy through beginning the apoptosis process (Bartnik et al, 2001).

The large quantity of ROS helps destroying the mitochondrial internal membrane helps to eliberation in the cytoplasma of the proapototic molecules, which determines the beginning of apoptosis.

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