MITOCHONDRIAL DISFUNCTION AND APOPTOSIS IN LEUKEMIA CELLS

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Abstract. Apoptosis or programmed cell death is a process which involves the intentional degradation of the cell from the inside, 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.

The antineoplastic drugs is inducing the apoptotic process in the sensitive cells.

It have been studied acute lymphoblastic 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. At the same time, were monitored, the serum level of malondialdehyde. The results obtained confirm the alteration of the mitochondrial metabolism. We can observed the mitochondrial dysfunction role in cell apoptosis.

Keywords: mitochondria, apoptosis, leukemia, cell metabolism, reactive oxigen species

INTRODUCTION

Acute leukemia is a neoplastic disease characterized by a rapid accumulation of primitive hematopoetic cells. Acute lymphocytic leukemia (ALL) is predominantly a disease of childhood with 75% of all cases occurring in patients younger than 15 years of age [18].

The majority of antineoplastic drugs act by genomic DNA degradation, thus inducing the apoptotic process in the sensitive cells [5].

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 [7].

Caspases are organized in a cascade with upstream (initiator) caspases responsible for activating the downstream (effector) caspases. Upstream caspases contain long prodomains that interact with their specific activators. The downstream caspases contain smaller prodomains and are activated by proteolytic cleavage by upstream caspases. Once activated, the downstream caspases cleave specific protein substrates, leading to the execution of apoptosis [4]. 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 membranes of the mitochondria. In response to a variety of proapoptotic stimuli, cytochrome c is released into the cytosol [18].

The exogenous cellular stresses, such as chemotherapy or increase level of reactive oxygen species (ROS) are activators to mitochondrial distruption [11].

Free radicals such as superoxide and hydroxyl radicals, can cause a damage in cellular components via peroxidation of proteins, nucleic acids, free amino acids, and lipoproteins. Free radicals first attack fatty acids, because the C=C double bonds in these molecules are sensitive to oxidative damage [9].

In a cell, where free oxygen radicals accumulate, the fatty acids, which would normally be subjected to beta-oxidation within the mitochondria, will be subjected to lipid peroxidation, production of toxic and reactive aldehyde metabolites such as malondialdehyde (MDA) which is commonly used as an index of lipid peroxidation [17].

Increase level of ROS in cells cause mitochondrial disfunction, which can induce apoptosis [14].

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 [10].

Annexin V is a 35-36 kDa Ca^{2+} 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).

The assay does not distinguish between cells that have already undergone an apoptotic death and those that have died as a result of a necrotic pathway because in either case the dead cells with stain with both Annexin V positive and 7-AAD positive.

Flow cytometry 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 [2].

MATERIALS AND METHODS

It has been determined the quantity of the cells entered in the apoptotic process in different stages of the antineoplasic treatment, between 2006-2008, at 8 children with ALL (acute limphoblastic leukemia). We were monitored, at the same time, the serum level of malondialdehyde, a standard method of assessing the oxidative stress.

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™ and flow cytometry ***FACS Calibur***.

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 [2].

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 alive [6].

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 was gathered blood, after 4 hours of beginning the antineoplasic treatment, and then after 24 hours.

At the same time, were monitored, the serum level of malondialdehyde, it's 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 [13].

RESULTS AND DISCUSSIONS

The obtained results at flow cytometry FACS Calibur are graphically represented under dot-plot forms, in which every point in 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. The results were present in **table 1.** and **2**.

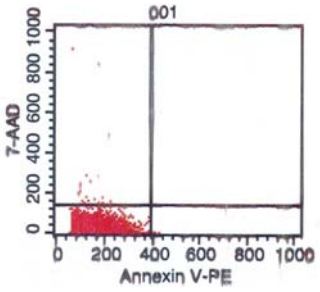
Table 1. Results after 4 hours at the beginning of treatment

Pacient no.	UL	UR	LL	LR
1.	1,15%	0,16%	77,39%	21,30%
2.	0,00%	0,01%	99,94%	0,05%
3.	0,31%	0,07%	63,72%	35,89%
4.	3,05%	0,73%	49,08%	47,14%
5.	1,63%	0,12%	80,60%	17,65%
6.	2,03%	0,10%	49,14%	48,73%
7.	0,63%	0,03%	54,32%	45,02%
8.	0,82%	0,18%	61,62%	37,38%

Table 2. Results after 24 hours at the beginning of treatment

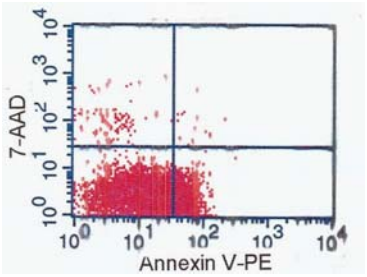
Pacient no.	UL	UR	LL	LR
1.	54,69%	0,22%	44,06%	1,03%
2.	74,12%	0,87%	23,42%	1,59%
3.	62,40%	1,93%	4,61%	31,05%
4.	75,06%	0,95%	22,39%	1,60%
5.	90,24%	1,63%	6,15%	1,98%
6.	18,03%	45,39%	36,23%	0,35%
7.	66,14%	8,06%	24,13%	1,67%
8.	70,32%	5,79%	17,35%	6,54%

Some significant dot-plots are shown in **figures 1, 2, and 3**.



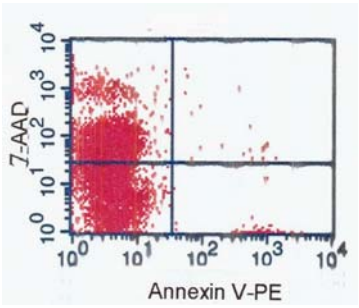
Quad	Events	%Gated	%Total	X Mean	X Geo Mean
UL	20	0.20	0.20	146.20	139.60
UR	0	0.00	0.00	***	***
LL	9976	99.76	99.76	148.16	137.98
LR	4	0.04	0.04	418.25	418.13

Figure 1. Dot-plot before the treatment. Subject no.1.



Quad	Events	%Gated	%Total	X Geo Mean
UL	106	1.15	1.15	3.79
UR	15	0.16	0.16	73.30
LL	7128	77.39	77.39	12.54
LR	1962	21.30	21.30	48.21

Figure 2. Dot-plot obtained after hours. Subject no.1.



Quad	Events	%Gated	%Total	X Geo Mean
UL	5006	54.69	54.69	3.77
UR	20	0.22	0.22	295.33
LL	4033	44.06	44.06	4.22
LR	94	1.03	1.03	655.31

Figure 3. Dot-plot obtained after 24 hours. Subject no.1.

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

The average percentages are shown in **figure 4**.

At the beginning of the treatment the blood cells are alive in the percentage of 66.97%, the way as it can be observed examining the quadrants LL. After 24 hours the numbers of the alive cells reduces to 22.29%.

During the treatment, the initiation of the apoptotic process is present in the majority of cases, and after 24 hours, a percentage of 63.84%, from the cells are in apoptosis or are already dead cells, according to the events from the UL quadrant.

A small percentage of 5.72 % shown through the events from the LR quadrant, are cells that are at the beginning of the apoptosis process at the beginning of the treatment, but after 24 hour there are a percentage of 31.64 %. Maybe the maximum level of the cells in apoptosis was touched between the two measures.

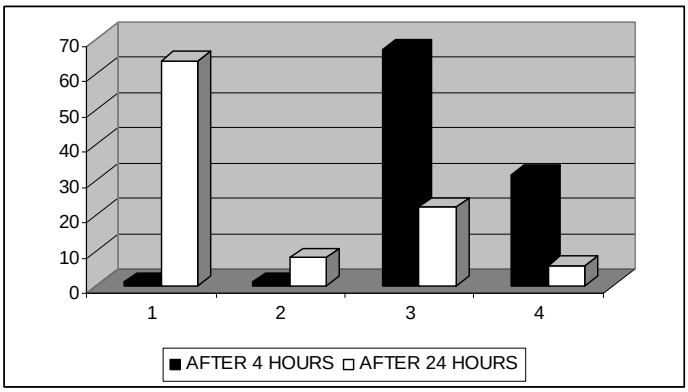


Figure 4. Average percentages: 1. quadrant UL; 2. quadrant UR; 3. quadrant LL; 4. quadrant LR

It can be observed that the administrated antineoplasic medicines induce the apoptotic process of the cells, in a very short time from the beginning of the administration.

Values of MDA serum levels are presented in **table 3**.

Table 3. Serum level of MDA

Subject no.	Serum MDA level (nmol/ml)
1.	2,60
2.	3,10
3.	2,70
4.	2,00
5.	1,90
6.	2,30
7.	2,50
8.	2,70

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

CONCLUSIONS

- The programmed cells death, the apoptosis is a genetic regulated process, power dependent, which eliminates the sick, injured cells, which became unuseful for the organism. The errors of the apoptotic programe, the way in the decreasing of the apoptotic inductive as in the working out of the excessive signals for surviving, favourises the tumourigenesis [15].
- Mitochondria are the powerhouse of the cell and play a vital role in the cellular metabolism and apoptosis. Mitochondrial dysfunction is closely related with a plethora of cancers [12].
- Cancer cells exhibit unlimited proliferative potential, resistance to cell death stimuli and abnormal energy metabolism. This metabolic alteration has been observed in many cancer types, including leukemia [8].
- It is now recognized that the Warburg effect (respiration chain deficient) represents a prominent metabolic characteristic of malignant cells.
- An efficient chemotherapy depends on the inductive of the apoptosis, or on the appearance of some modifications on the signal ways of the programmed death of cells [1].
- Before the treatment the cells are alive. During the treatment the beginning of the apoptotic process is present in the five cases after 4 hours and after 24 hours a great number of cells are dead at all 8 pacients.
- Due to the polichimiotherapy, the different ways of administration or of the different stages in which the patients are at the moment of gathering blood, there is a great difference in the percentage of apoptotic cells.
- It can be conclusioned that the administrated antineoplasic medicines induce the apoptosis for the target cells from a very short time after the administration. At the patients with high level of serum MDA, during the antineoplasic treatment, the apoptosis displays with a higher speed, a larger percentage of cells initiate the apoptotic process, after 24 hours of the beginning of the treatment.
- 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 [3].
- 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.
- We can observed the role of mitochondrial dysfunction in cell apoptosis and the potential therapeutic value in targeting mitochondria for antineoplasic drug development.

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