

Expression of cyclin A in human leukemia cell line HL-60 following treatment with doxorubicin and etoposide: The potential involvement of cyclin A in apoptosis

AGNIESZKA ZURYŃ¹, ALINA GRZANKA¹, ALEKSANDRA STEPIEN¹,
DARIUSZ GRZANKA², ROBERT DEBSKI³ and DARIUSZ SMOLINSKI⁴

¹Department of Histology and Embryology, Nicolaus Copernicus University, Collegium Medicum, Karłowicza 24, 85-092 Bydgoszcz; ²Department of Clinical Pathomorphology, ³Department and Clinic of Pediatric, Hematology and Oncology, Nicolaus Copernicus University, Collegium Medicum, Skłodowskiej-Curie 9, 85-094 Bydgoszcz; ⁴Department of Cell Biology, Institute of General and Molecular Biology, Nicolaus Copernicus University, Gagarina 9, 87-100 Torun, Poland

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Abstract. We investigated expression of cyclin A in HL-60 cells after induction of apoptosis with doxorubicin and etoposide. Following apoptotic trigger, both cells arrested in G2/M phase of the cell cycle and changes in morphology were noticed. Moreover, compared to control, the number of cells with cyclin A expression was changed and translocation of this protein from the nucleus to the cytoplasm was observed. The decrease in the number of cells with cyclin A expression, followed by the increase, and cyclin A distribution throughout the cell, appeared to be dose-dependent. Cells treated with lower doses of doxorubicin and etoposide as well as the untreated cells were found to have cyclin A scattered mainly throughout the nucleus. However, immunogold labeling of cyclin A in both cell lines treated with 5- and 10- μ M doses of doxorubicin, and 20 and 200 μ M of etoposide was observed more often in the cytoplasm than in the nucleus. Cells with features of apoptosis with bodies resembling micro-nuclei labeled with gold particles for cyclin A were recognized. However, the small amount of giant cells was also seen. These results suggest that cyclin A expression is linked to cell death pathways.

Introduction

Cyclin A is one of the major cell cycle regulatory proteins (1). The association with its catalytic partner, kinase (Cdk, cyclin-dependent kinase), leads to the partial activation of the

kinase complex cyclin A/Cdk, followed by the phosphorylation events resulting in its full activation (1,2). During the cell cycle, cyclin A is associated with Cdk2 or Cdc2 (cell division cycle 2), also known as Cdk1 (2). The cyclin A/Cdk2 complex activity is required for the regulation of S phase progression (2,3) whereas cyclin A/Cdk1 regulates G2/M transition (2). It has been widely accepted that cyclin A plays a very important role during the cell cycle. Cyclin A is present, not associated with Cdks, in the complex bound to chromatin as well as, complexed with Cdk2 or Cdk1 in S and G2 phase, respectively (4). It has been suggested that cyclin A/Cdk2 controls DNA replication by regulating activity of some replication machinery components (3-5). Moreover, activation of the cyclin A/Cdk2 complex has been found to be required for centrosome duplication (6-8) and centriole separation (9).

Some studies revealed the existence of a relationship between the level of cyclin A (10) or cyclin-dependent kinase (11) expression and apoptosis, thus between the cell cycle and programmed cell death (PCD). It has been also implicated that there is a link between cyclin A subcellular localization and its cell functions (11,12). It is well known that apoptosis characterized by membrane blebbing, nuclear fragmentation, depolymerization of the cytoskeleton and chromatin condensation, usually begins in the cytoplasm while DNA replication and mitosis are nuclear processes (13,14). Hiromura *et al* indicated that Cdk2 translocation from the nucleus to the cytoplasm is connected with its role in apoptosis and hypothesized that cyclin A/Cdk2 mediates both growth and death pathways (12). Furthermore, another report suggested that cyclin A may cause mitotic catastrophe (15), the process considered as the mode of non-apoptotic cell death (16).

The chemotherapeutic agents used in the present study, doxorubicin (DOX) and etoposide (VP-16), function as topoisomerase II inhibitors, thereby inhibiting DNA synthesis (17). The human promyelocytic leukemia cell line (HL-60) has been proven to be particularly susceptible to cytotoxic drugs (18). The reasons behind this are c-Myc overexpression which is known to promote tumorigenesis and apoptosis (19-21) as well as a deficiency of the tumor suppressor protein

Correspondence to: Dr Alina Grzanka, Department of Histology and Embryology, Nicolaus Copernicus University, Collegium Medicum, 24 Karłowicza St, 85-092 Bydgoszcz, Poland
E-mail: agrzanka@cm.umk.pl

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p53 (22-24). HL-60 cells express also oncoprotein Bcl-2, which is an important anti-apoptotic agent (22,25).

In this report we show the relationship between cell death pathways and the cell cycle protein - cyclin A alternations in the HL-60 cell line.

Materials and methods

Cell culture. The human promyelocytic leukemia cell line HL-60 (ATCC CCL 240) used in this study, was routinely cultured in RPMI-1640 medium containing 10% fetal calf serum and 20 μ l gentamycin in fully humidified atmosphere of 5% CO₂ at 37°C. Cells were incubated with 3 different concentrations of doxorubicin (Sigma, St. Louis, MO, USA) (0.5, 5 and 10 μ M) and 4 concentrations of etoposide (Sigma) (0.2, 2, 20 and 200 μ M) for 72 h. Control cells were grown in identical conditions. Cell viability was assessed by the trypan blue dye exclusion method. The experiment was repeated 10 times.

Immunocytochemistry. Cells fixed in 4% paraformaldehyde were centrifuged at a low speed and transferred to microscope slides. Cyclin A was detected by the streptavidin-biotin-peroxidase technique. Sections were treated with monoclonal antibody against cyclin A (Sigma) diluted 1:100 in phosphate-buffered saline for 1 h. Then, they were incubated for 40 min with biotin-labeled secondary antibody and Dako LSAB Kit peroxidase. Subsequently, the sections were developed with diaminobenzidine for 5 min and counterstained with Meyer's hematoxylin. Control specimens were incubated with non-immune antiserum (normal mouse serum, Dako, Glostrup, Denmark).

Immunofluorescence assay. Cells were fixed with 4% paraformaldehyde (pH 7.4) for 15 min, at 4°C, washed twice with PBS and collected directly onto microscopical slides using a cytocentrifuge (Megafuge1, OR, Heraeus, Sepatech). Cells on slides were incubated in permeabilization solution (0.1 Triton X-100 in PBS) and blocked with 1% BSA (Gibco). After permeabilization, cells were incubated with anti-cyclin A monoclonal antibody (Sigma) for 45 min at room temperature, washed three times with PBS and incubated with anti-mouse IgG TRITC conjugated antibody (Sigma) for 45 min, at room temperature. After incubation, cells were washed with PBS and mounted in gelvatol (Monsanto, St. Louis, USA). Nuclear staining was performed with DAPI (Sigma). Both cyclin A and DNA staining was examined with an Eclipse E600 microscope (Nikon, Tokyo, Japan) and with a confocal microscope (inverted microscope, Nikon, Eclipse TE 300).

Electron microscopy. To visualize Cyclin A at the ultrastructural level, a post-embedding streptavidin-gold method was used. Cells were fixed in 4% paraformaldehyde in PBS for 1 h at 4°C, washed overnight with PBS at 4°C, dehydrated in series of ethanol and embedded in LR White. Thin sections were collected on nickel grids. The grids were floated on a drop of non-immune rabbit serum (Dako) for 20 min and then transferred onto drops of mouse anti-human cyclin A antibody (Sigma) diluted 1:100. After incubation with primary antibody for 30 min, thin sections on grids were rinsed in PBS

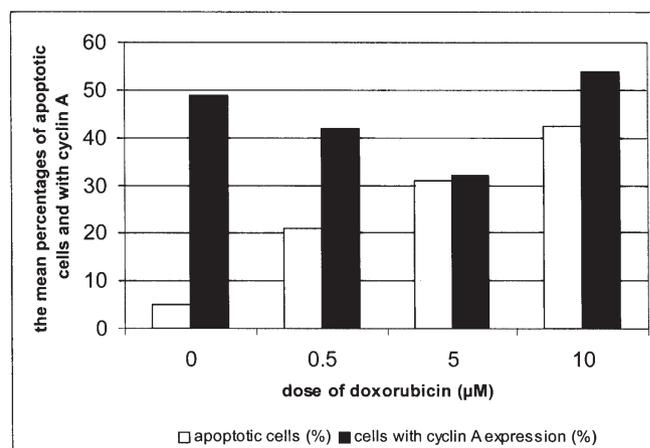


Figure 1. The percentages of apoptotic cells and with cyclin A expression in HL-60 cell line after treatment with doxorubicin.

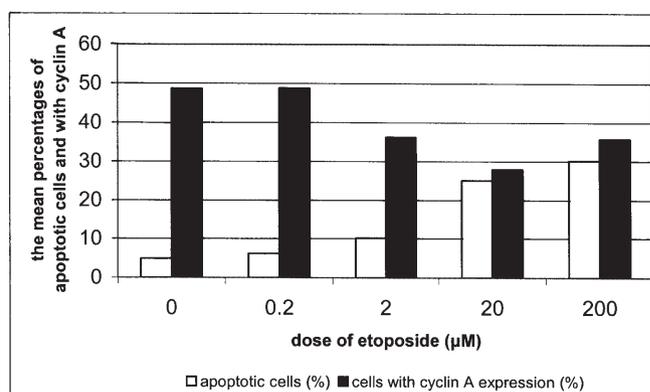


Figure 2. The percentages of apoptotic cells and with cyclin A expression in HL-60 cell line after treatment with etoposide.

and incubated with biotinylated rabbit anti-mouse antibody (Dako) diluted 1:100. Grids were then rinsed in PBS and transferred onto drops of solution containing 10 nm gold particles conjugated to streptavidin (Sigma), 1:20 dilution, and incubated for 30 min. After this final incubation grids were washed with PBS and dried. All incubation steps were performed at room temperature. Control specimens were incubated with non-immune antiserum (normal mouse serum, Dako). The preparations were examined using a transmission electron microscope JEM 100 CX (JEOL, Tokyo, Japan) at 80 kV.

Flow cytometric assessment of cell cycle and apoptosis. For the cell cycle analysis cells were stained with hypotonic propidium iodide solution (20 μ g/ml, DNA - Prep Kit) and 20000 events were analyzed with an Epis XL flow cytometer. Cell cycle phases were calculated by multicycle software (Phoenix Flow Systems, San Diego). Percentage of the cells in G1/0, S and G2/M phase was expressed as mean \pm standard deviation.

Apoptosis was analyzed, using the Annexin V-FITC apoptosis detection kit (BD Pharmingen, San Diego, CA). This assay was performed according to the manufacturer's instructions.

Dose (μM)	HL-60	
	Mean (%)	Standard variation
0.0	94.0	2.14
0.2	90.2	3.09
2.0	79.8	6.79
20.0	76.1	7.77
200.0	57.5	8.84

Statistical analysis. The Shapiro-Wilk test was used to test data for normal distribution. Statistical significance of the data was determined by one-way analysis of variance (ANOVA) and Tukey's *post hoc* test. Results were considered significant at $P < 0.05$.

Results

To determine the number of HL-60 cells with cyclin A expression as well as the amount of apoptotic cells after treatment with DOX and VP-16, light (LM), electron (EM) and fluorescence confocal microscopy (FCM) as well as flow cytometry were used.

The changes in the number of the cells with cyclin A expression and the number of the apoptotic cells were found to be dependent on doxorubicin (DOX) and etoposide (VP-16) concentration. The effects of DOX and VP-16 on HL-60 cells resulting in the changes in both the percentage of the cells with cyclin A expression and with morphological features of apoptosis are shown in Figs. 1 and 2. We observed a clear decrease in expression of cyclin A in treated with VP-16 at doses of 2 and 20 μM (Fig. 2) and DOX at 0.5 and 5 μM (Fig. 1), and an increase in its expression at 200 μM of VP-16

Table I. The effects of doxorubicin doses on cell viability.

Dose (μM)	HL-60	
	Mean (%)	Standard variation
0.0	94.0	2.14
0.5	86.1	4.31
5.0	79.9	6.78
10.0	64.0	5.70

(Fig. 2) and DOX at 10 μM (Fig. 1). It is important to note that at the doses of 200 μM of VP-16 and 10 μM of DOX the number of cells with cyclin A expression increased significantly and was parallel to the increase in the number of apoptotic cells (Figs. 1 and 2).

There was a statistically significant difference in the mean number of apoptotic cells after treatment with different doxorubicin and etoposide concentrations ($P < 0.01$) except for the difference between 0.2 μM VP-16 and control (0.0 μM). There was also a significant difference in the mean number of the cells with cyclin A expression after treatment with different doses of both anticancer compounds, except for the difference between 0.5 and 10.0 μM of DOX and control (0.0 μM) as well as between 2.0 μM and 200 μM of VP-16, 0.2 μM of VP-16 and control (0.0 μM).

Tables I and II show the effects of DOX and VP-16 on cell viability. The inhibition of the cell viability that had been also demonstrated by flow cytometric analysis (Tables III and IV) was evident. Moreover, Tables III and IV show results confirming the above-mentioned observation of the positive correlation between cytostatic doses and the number of dead cells. Also, the morphological changes in the cells were found to be dependent on the dose of DOX and VP-16. A number of the cells treated with the highest DOX and VP-16

Table III. Flow cytometric analysis of viable, apoptotic and necrotic HL-60 cells after treatment with etoposide using Annexin V and propidium iodide staining.

	Viable cells		Necrotic cells		Early apoptosis		Late apoptosis		Early and late apoptosis	
	(%)	SD	(%)	SD	(%)	SD	(%)	SD	(%)	SD
Control	93.53	0.82	4.46	1.24	1.96	0.29	0.08	0.09	2.04	0.35
Etoposide (0.2 μM)	94.23	1.18	3.55	0.67	2.13	0.79	0.10	0.09	2.23	0.73
Etoposide (2 μM)	67.50	2.16	20.67	1.63	11.02	1.13	0.84	0.29	11.86	0.97
Etoposide (20 μM)	30.33	5.97	11.94	2.89	55.43	6.48	2.24	1.89	57.68	7.93
Etoposide (200 μM)	15.77	3.72	7.07	2.35	70.77	2.72	6.39	1.13	77.15	1.92

Table IV. Flow cytometric analysis of viable, apoptotic and necrotic HL-60 cells after treatment with doxorubicin using Annexin V and propidium iodide staining.

	Viable cells		Necrotic cells		Early apoptosis		Late apoptosis		Early and late apoptosis	
	(%)	SD	(%)	SD	(%)	SD	(%)	SD	(%)	SD
Control	93.33	0.82	1.91	1.24	3.97	0.29	0.75	0.09	4.72	0.35
Doxorubicin (0.5 μ M)	81.40	1.18	3.20	0.67	12.93	0.79	2.42	0.09	15.35	0.73
Doxorubicin (5 μ M)	68.93	2.16	15.28	1.63	13.80	1.13	2.00	0.29	15.80	0.97
Doxorubicin (10 μ M)	49.10	5.97	21.06	2.89	25.73	6.48	4.06	1.89	29.76	7.93

concentrations, especially those at 10 μ M of DOX, were bigger in size (Fig. 3b) compared with non-treated cells (Fig. 3a). Our immunocytochemical labeling of cyclin A showed its presence predominantly in the nucleus of non-treated cells (Fig. 3a) whereas in the cells treated with 10 μ M of DOX (Fig. 3b), and 20 and 200 μ M of VP-16 (Fig. 3c) cyclin A was present mainly in the cytoplasm. Confocal laser microscopy images showed a similar pattern (Fig. 3d). Cyclin A distribution, at the ultrastructural level using immunogold labeling, was examined in the cells treated with anticancer compounds as well as in the control cells. In the cells treated with lower doses of DOX and VP-16 and non-treated, cyclin A was scattered predominantly throughout the nucleus (Fig. 4a). Immunogold labeling of cyclin A in the cells treated with 5- and 10- μ M doses of DOX, and 20 and 200 μ M of VP-16 was observed more often in the cytoplasm than in the nucleus (Fig. 4b). There were also cells with characteristic features of apoptosis with bodies resembling micro-nuclei labeled with gold particles for cyclin A (Fig. 4c). Cyclin A labeling was not found in the control cells incubated with non-immune serum (Fig. 4d).

The cell cycle analysis using flow cytometry demonstrated that DOX and VP-16 treatment induced the cell cycle arrest at G2/M in a dose-dependent manner (Figs. 5 and 6). The highest percentage (19.4%) of HL-60 cells at G2/M was observed at the dose of 200 μ M of VP-16 (Fig. 6). There was also an increase in the number of HL-60 cells arrested at G2/M phase after treatment with DOX at doses of 0.5 and 5 μ M (Fig. 5). A dose of 10 μ M of DOX caused a decrease in the number of HL-60 cells at G2/M phase.

Discussion

It has been shown that some of the cell cycle regulators are able to affect both division and death of the cell. These are, among others, Cdks, cyclins, c-Myc, p53 and Bcl-2 (21). Cyclin A is mainly known as the cell cycle protein (1,3,26). There are several reports however, demonstrating its pro-apoptotic role (10,11,27). Furthermore, there are reports considering this protein as a prognostic marker in cancer and

a predictive factor for chemotherapy response (28-34). A high level of cyclin A expression has been proven to be associated with good prognosis in AML (acute myeloid leukemia) patients (31). On the contrary, patients with cyclin A-positive non-small-cell lung carcinomas (NSCLCs) had a worse outcome than those with cyclin A-negative carcinomas (34). Similarly, overexpression of cyclin A was found to be associated with reduced overall survival in colorectal cancer patients (28). Another study has suggested the role for cyclin A/Cdk2 in the induction of apoptosis in SK-HEP-1 (human hepatoma cell line) after treatment with G-Rh2 (ginsenoside Rh2) (35). Other evidence indicates that overexpression of the wild-type form of cyclin A or Cdc2, Cdk2, and Cdk3 may circumvent the anti-apoptotic activity of the Bcl-2 in human HeLa cells (36). The ability of cyclin A to induce programmed cell death was also determined in rat fibroblasts exposed to low serum (10).

All the above-mentioned studies provide clear evidence that there is a link between cyclin A expression and apoptosis. Our previous report demonstrated this relationship as well. We found that in K-562 the mean percentage of the cells expressing cyclin A increased together with the percentage of cells with morphological features of apoptosis (37). In the present study we used the HL-60 cell line and two anticancer compounds, doxorubicin (DOX) and etoposide (VP-16), to investigate whether the previously observed relationship would be confirmed. The results obtained from the present study demonstrate that the used chemotherapeutics induce changes in the level of cyclin A expression in HL-60 cells in a dose-dependent manner. The number of apoptotic cells increased together with the increase of DOX and VP-16 concentrations. Conversely, under the same conditions, there was a decrease in the number of cells with cyclin A expression, except for the treatment using the highest concentrations of the drugs (200 μ M of VP-16, 10 μ M of DOX), where the number of cells with cyclin A labeling increased. The concentration-dependent decrease and increase in the level of cyclin A expression is intriguing. We suggest that the observed changes represent a kind of 'threshold' in which cyclin A changes its function from proliferating into proapoptotic.

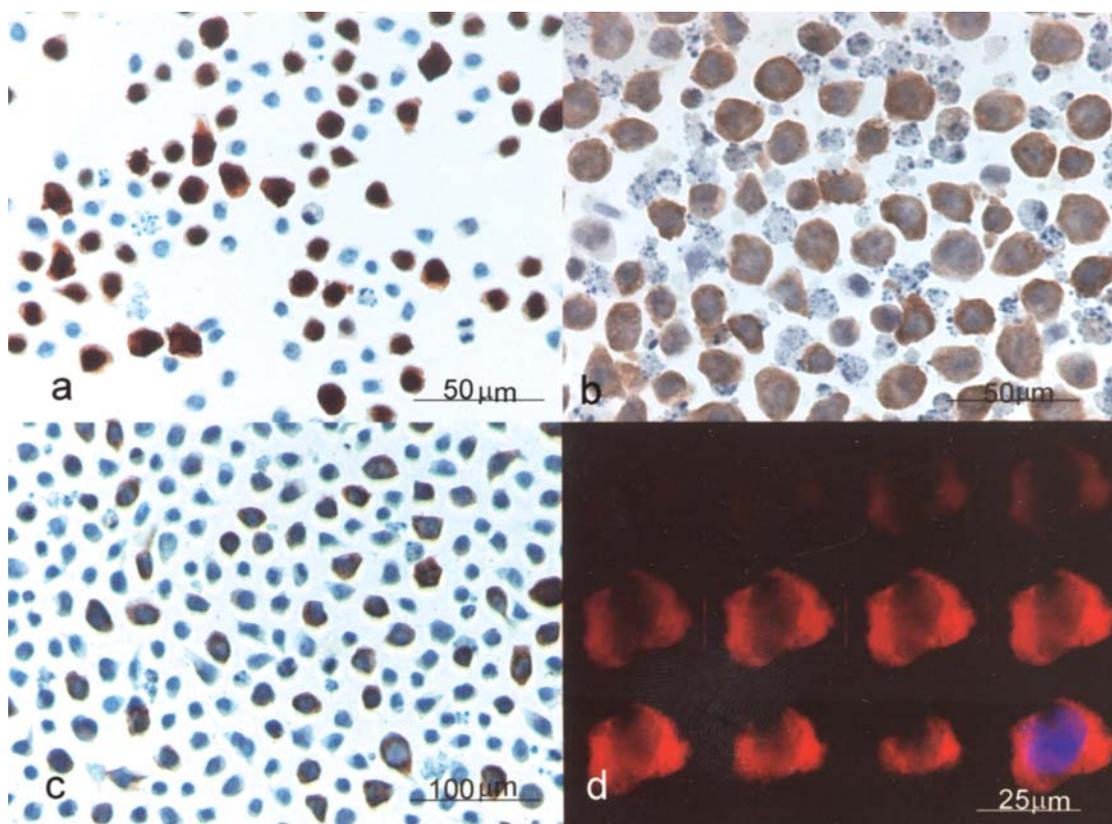


Figure 3. Non-treated HL-60 cells labeled with cyclin A (a). Cells treated with 10 μ M of DOX for 72 h. Cyclin A labeling was observed throughout the cytoplasm. Apoptotic cells were observed (b). HL-60 cells treated with 200 μ M of VP-16 for 72 h. Cells with cyclin A expression in the cytoplasm were recognized (c). Z-line construction through the HL-60 cell treated with 10 μ M of DOX for 72 h. Strong cyclin A labeling throughout the cytoplasm was evident. Cyclin A labeling was not observed in the nucleus (d).

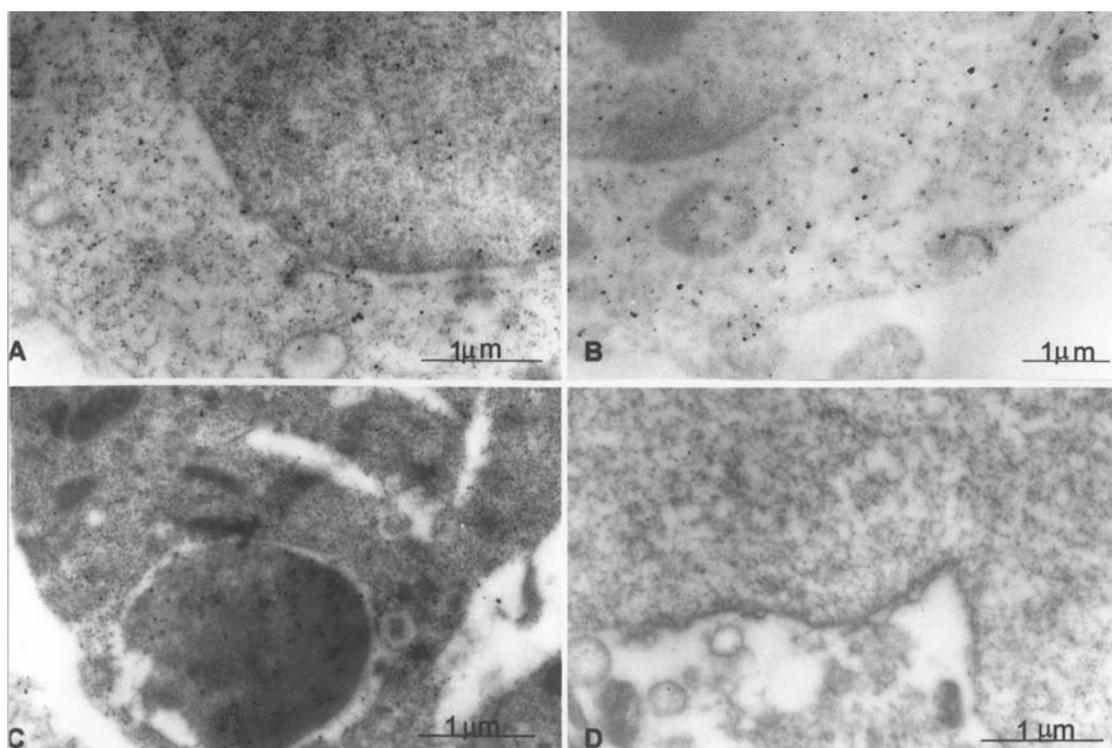


Figure 4. Electron microphotographs of HL-60 cells labeled with gold method. Non-treated control cells. The gold labeling for cyclin A was observed predominantly in the nucleus (a). Cells treated with 10 μ M of DOX for 72 h. Immunogold labeling for cyclin A was present in the cytoplasm (b). The body labeled with gold particles for cyclin A in HL-60 cells treated with 200 μ M of VP-16 (c). Control HL-60 cells incubated with non-immune serum. Immunogold of cyclin A was not observed (d).

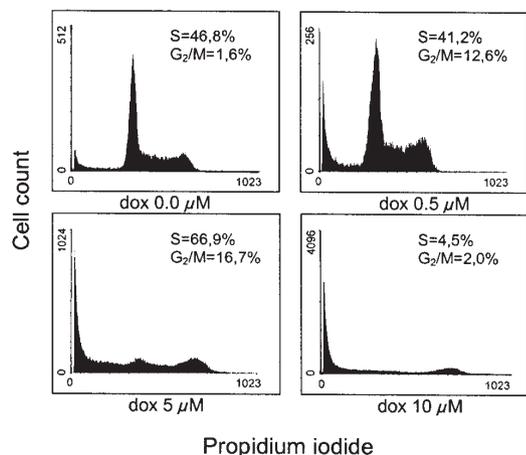


Figure 5. Cell cycle analysis of HL-60 cells after doxorubicin treatment.

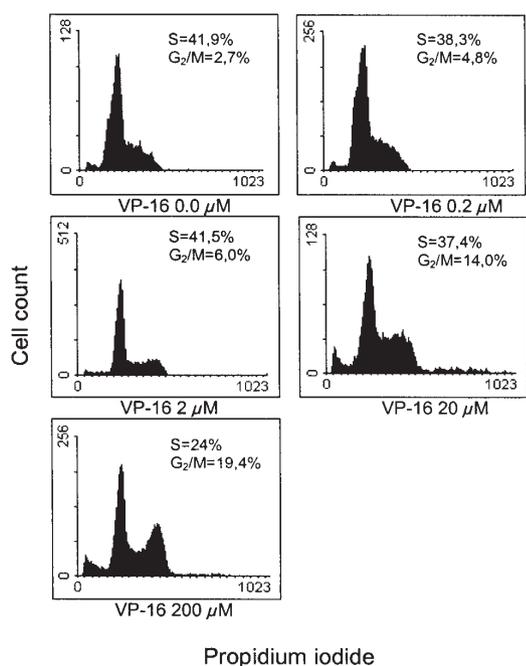


Figure 6. Cell cycle analysis of HL-60 cells after etoposide treatment.

It has been suggested that cyclin A is able to translocate from the nucleus to the cytoplasm, and thus interact with both nuclear and cytoplasmic substrates (38). Furthermore, there is growing evidence that subcellular localization of Cdks or cyclins may be connected with cell death pathways (12,39). Cytoplasmic cyclin A localization was also found to have an impact on cell transformation (39). We noticed that in HL-60 cells after exposure to doses of 5 and 10 μ M of DOX and 20 and 200 μ M of VP-16, cyclin A translocated from the nucleus to the cytoplasm. A similar relationship was observed by Hiromura *et al* (12) who studied the role of subcellular localization of Cdk2 during apoptosis induced by UV irradiation in mouse mesangial cells. They found that cyclin A/Cdk2 complexes were present predominantly throughout the cytoplasm following UV irradiation whereas, under these conditions, nuclear cyclin A/Cdk2 activity decreased significantly. The association of cyclin A/Cdks

with programmed cell death has been also found by Ekberg *et al*, who demonstrated that although the subcellular localization of cyclin A was nuclear and cytoplasmic, both in the normal hematopoietic cells and leukemic blasts from AML patients, the latter characterized the higher level of cyclin A expression. A decline in the level of nuclear cyclin A following ATRA (all-trans retinoic acid) treatment was also observed (40).

Another noteworthy observation in our study was that a number of cells arrested in G₂/M phase of the cell cycle in response to an apoptotic trigger. There are other studies demonstrating similar results (32,41-43). The arrest of the cell cycle in G₂/M was observed in the K-562 leukemic cell line after exposure to etoposide. This etoposide-induced event was found to impart resistance to programmed cell death and promote cell survival in K-562 cells (42). Similarly, in lymphoblastoid cell lines CEM and MOLT-4, cell cycle arrest at G₂/M following genotoxic agent etoposide was observed. This data showed that cells accumulated at G₂/M underwent cell death or survived by re-entering mitosis (43). Furthermore, in HL-60, inhibition of cell proliferation and accumulation of the cells in G₂/M followed by cell death after treatment with quercetin (QU) or QU combined with doxorubicin were also demonstrated (44). Moreover, other reports show the positive correlation between the amount of cyclin A RNA and the cumulative percentage of cells in G₂/M phase and, therefore, support the possibility to use cyclin A as a novel index to clinical oncology (32).

Based on evidence for the translocation of cyclin A from the nucleus to the cytoplasm occurring almost simultaneously with its expression increase after exposure to an apoptotic trigger, we conclude that cyclin A is involved in apoptotic cell death pathways in a dose-dependent manner. We are not able however to explain this phenomenon and link cyclin A into molecular pathway of cell death at this stage of our study.

Acknowledgements

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