

The effect of doxorubicin on the expression of cyclin A in K-562 leukemia cell line

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Two opposite processes such as apoptosis and cell division cycle play an important role in cancer biology. During apoptosis cells die by degrading proteins and genome, whereas in the cell cycle cells divide and the genome is duplicated. There are very few studies on the role of cell cycle proteins in apoptosis although their role is distinctly different from that in the cell cycle. The significance of expression of cyclin A and other cyclin cell proteins (eg. Cdk2) in apoptosis remains to be investigated. The aim of this study was to characterize the distribution pattern of cyclin A by using the streptavidin – biotin – peroxidase technique in K-562 cells treated with doxorubicin. The analysis of cell cycle phases using cytophotometric methods to estimate the cellular response to doxorubicin was also used. Studied cells were treated with doxorubicin in the range 0.5; 5.0 and 10 μ M. Expression of cyclin A in K-562 was 32.2; 41.8; 69.9%, respectively, according to doses of doxorubicin. The number of apoptotic cells was increasing together with the increase of doxorubicin doses as well as positive labeling for cyclin A. After doxorubicin treatment decrease of G1/G0 phase and the growth of cell percentage with dose dependent manner at G2/M phase, compared to control was observed. The results allow to suggest that expression of cyclin A may have pro-apoptotic role however more studies are required to clarify whether and what role cyclin A plays in apoptosis.

Key words: cyclin A, K-562 cells, apoptosis, cell cycle, doxorubicin

Cyclin A is one of the regulatory subunits of the cyclin dependent kinases (Cdk's) which control transition at different specific phases of the cell cycle. It is synthesized in the S phase and is degraded prior to mitosis. In the late part of S phase cyclin A forms a complex with Cdk2 [9]. The activity of this complex appears to be required for the initiation and progression of DNA replication [24]. Cyclin A associates with both the p34 cdc2 and p33 Cdk2 kinases and is involved in two major check-points (G1-S and G2-M) of the cell cycle. In G2/M phase transition of cyclin A signifies actively dividing cells [15]. Mutation or disruption of normal cyclin A expression causes cells arrest in G2 phase [25]. It has been identified in multimeric protein complexes that incorporate the E2F transcription factor, the p33 Cdk2 kinase, and p 107, which is related to the retinoblastoma protein [22]. In hematological malignancies, cyclin A expression correlates with the proliferation rate of these disorders [17, 20, 23].

NAKAMAKI et al observed that high levels of cyclin A1 and A2 are associated with good prognosis in acute myeloid leukemia patients [20]. There are some reports on the impact of cyclin A accumulation as a marker of proliferative cell fraction in other human cancers [2, 4, 11, 32]. However VOLM et al showed that negativity in cyclin A expression predicted outcome in non-small cell lung cancer patients [27, 28]. All these studies did not examine cellular distribution of cyclin A. The aim of this study was to characterize the distribution pattern of cyclin A at the light microscopy level in K-562 cell line treated with doxorubicin. Doxorubicin, like other topoisomerase II inhibitors also known as topoisomerase II poison, causes stabilization of the intermediate reaction between topoisomerase II and DNA [6, 18]. Topoisomerases have been suggested to play a role in DNA replication [21], gene expression [29] and cell division [14, 26]. From other studies it is known that topoisomerase II activity and content

increase in proliferating cells [7, 30]. However BORGNE and GOLSTEYN in their review found examples on proapoptotic and anti-apoptotic role of cyclins and cyclin dependent kinases in apoptosis and cell cycle [3]. Here we also tried to define the relationship between expression of cyclin A and cytotoxicity of doxorubicin throughout the apoptosis.

Material and methods

The human erythroleukemic cell line K-562 (ATCC, CCL 243) was used in this study. The cells were routinely cultivated in RPMI 1640 medium containing 10% fetal calf serum and 20 μ l gentamycin in fully humidified atmosphere of 5% CO₂ at 37C. Cells were incubated with 3 different concentrations of doxorubicin (Sigma, St. Louis, USA) 0.5, 5, 10 μ M/ml for 72 hours. Control flasks without doxorubicin were cultivated identically. The cell viability was determined by trypan blue. For study at the light microscope level cells were fixed in 4% paraformaldehyde, centrifuged at low speed and transferred onto slides. Cyclin A at the light microscope level was detected by the streptavidin-biotin-peroxidase technique. Slides were treated with monoclonal antibody against cyclin A (Sigma) diluted 1:100 in phosphate-buffered saline for 1 hour. Then incubated for 40 minutes with biotin labeled second layer antibody and DAKO LSA Kit peroxidase. After that developed with diaminobenzidine for 5 minutes and counterstained with Meyer's hematoxylin. Controls specimens were incubated with nonimmune anti-serum (normal mouse serum, DAKO, Glostrup, Denmark).

For the cell cycle analysis cells were stained with hypotonic propidium iodide solution (20 μ g/ml, DNA-Prep Kit) and 20,000 events were analyzed with an Epics XL flow cytometer. Cell cycle phases were calculated by multicycle software (Phoenix Flow Systems, San Diego). Percentage of cells in G1/0, S and G2/M phase was expressed as mean \pm standard deviation.

Results

The expression of cyclin A in leukemia K-562 cell line treated with doxorubicin was estimated. Changes in morphology and expression of cyclin A were dependent on doxorubicin concentration. The inhibition of cell growth in a dose dependent manner was observed (Tab. 1). The cellular response to damage by doxorubicin involved a decrease of

G1/G0 phase in K-562 cell line, the arrest at S phase and the growth of percentage of cells with a dose dependent manner at G2/M phase compared to control (Fig. 1). The effect of doxorubicin on percentage of cyclin A positive immunostained K-562 cells and mean percentage of cells with morphological apoptosis features are shown in diagram (Fig. 2). Morphological changes of the cells were also dependent on doxorubicin. Control cells and treated with 0.5 μ M of doxorubicin were much smaller in size compared to cells with higher doses especially with 10 μ M (Fig. 3–5). Among cells treated with 5 μ M and especially 10 μ M of doxorubicin

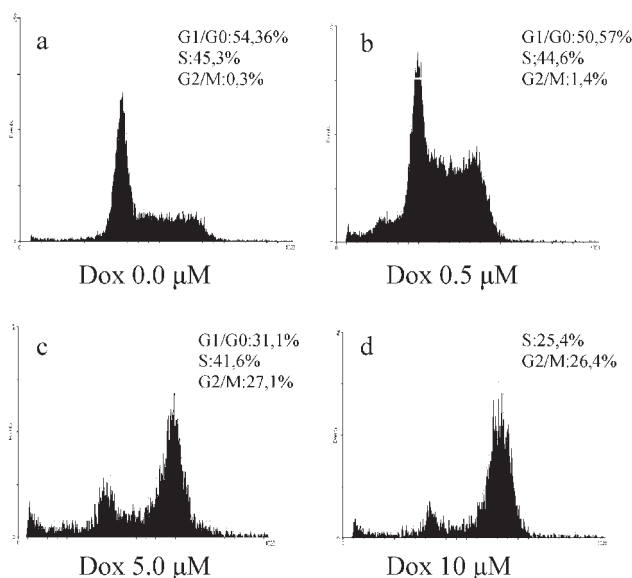


Figure 1. Effect of doxorubicin on cell cycle.

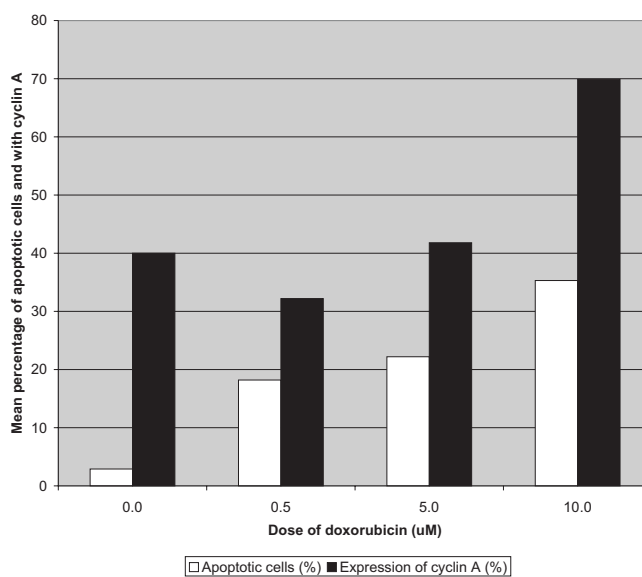


Figure 2. Expression of cyclin A and apoptotic cells in K-562 cell line.

Table 1. Effect of doxorubicin doses on cell growth

Dose (μ M)	K-562	
	Mean (%)	Standard variation
0.0	95.9	2.29
0.5	92.0	3.57
5.0	88.0	4.23
10.0	76.1	3.98

there were apoptotic cells without labeling of cyclin A and large cells with labeling in cytoplasm were observed (Fig. 6).

Discussion

In this study we have shown that doxorubicin in a dose-dependent manner induced the expression of the cyclin A in K-562 cell line. There are no earlier studies concerning the influence of doxorubicin on the expression of cyclin A in leukemic K-562 cell line. Previously, we studied the expression of cyclin A in HL-60 and K-562 cell lines not treated with cytostatics on the level of light and electron microscope [10]. Here, we tried to define our results with overexpression of cyclin A in relationship with increase of apoptotic cells. It is known that two important opposite cell processes, cell division cycle and apoptosis, are key aspect of cancer biology. In apoptosis cells die by selectively degrading proteins and their

genome, while in the cell cycle the genome is duplicated and two new cells are produced. Cancer is a disease in which deregulated cell proliferation and suppressed cell death are involved [8]. An analysis of the literature on apoptosis by BORGNE and GOLSTEYN showed examples in which cyclins A, B, D,E and also Cdk1 and Cdk2 have a pro-apoptotic role [3]. We are the opinion that expression of cyclin A in K-562 cell line treated with high dose of doxorubicin might have an influence on of the number of apoptotic cells. Here, we found among numerous large cyclin A labeled cells also a lot of apoptotic ones. The cells with cyclin A expression were most probably in the G2/M phase of the cell cycle, which was shown by cytophotometric method and was manifested by their large size. These results suggest that overexpression of cyclin A in cells of G2/M phase might participate in apoptosis and drive these cells on apoptotic death pathway. Although we could not yet explain it and place this protein into molecu-

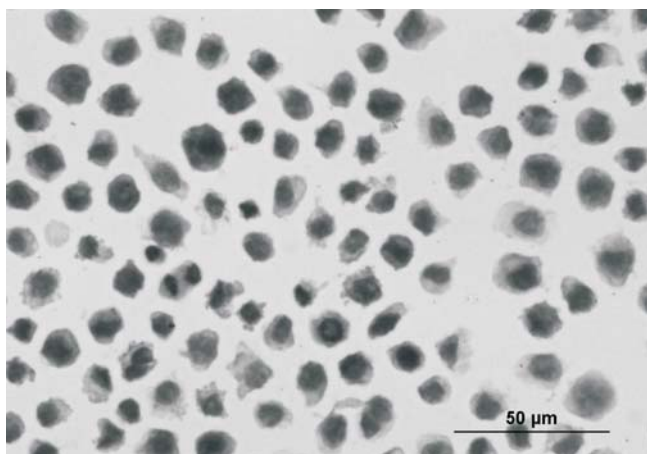


Figure 3. K-562 control cells.

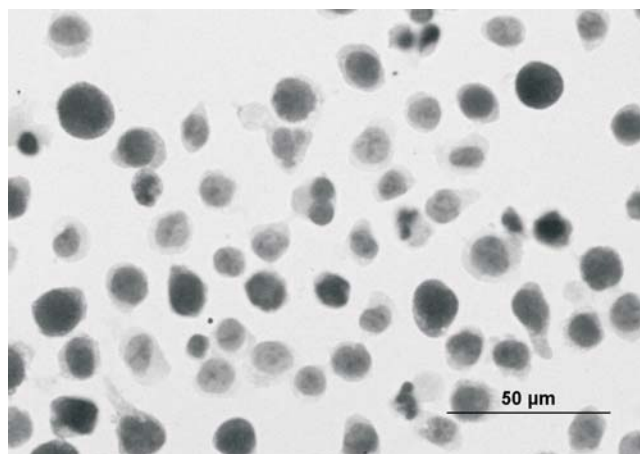


Figure 5. K-562 cells treated with doxorubicin 0.5 μM, cyclin A labeling present mostly in nucleus.

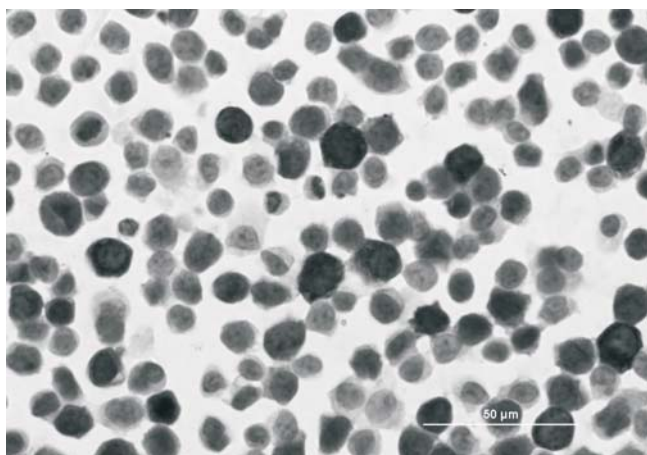


Figure 4. K-562 cells non-treated with doxorubicin.

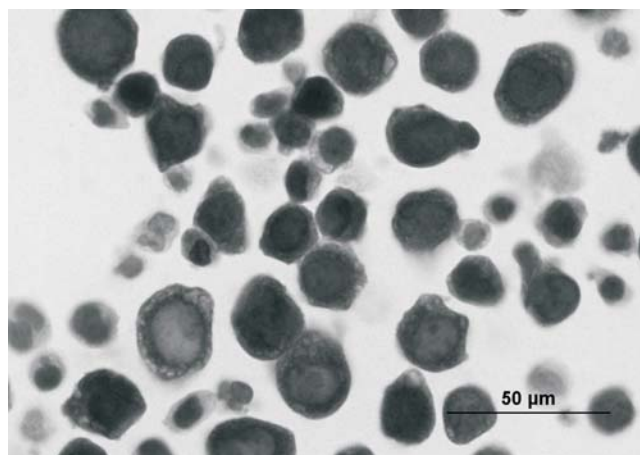


Figure 6. K-562 cells treated with doxorubicin 10 μM. Strong cyclin A labeling observed also in cytoplasm. Apoptotic cells are already to be seen.

lar pathway of apoptosis, we think this our supposition should be taken under consideration. Our studies are in agreement with others. JEONG et al found cyclin A expression at G2-arrest in K-562 cells treated with radiation and genistein [16]. CHEN et al also observed an increase of cyclin A after induction of apoptosis with aloe-emodin in HL-60 cells. Immunoblot analysis showed that aloe-emodin inhibited cell proliferation, induced G2/M arrest, apoptosis and increased cyclin A level in studied cells [5]. However YANG et al in studies with glycolic acid also showed increased percentage of cells in G2/M phase but decreased of the cyclin A expression [31]. ANDERSON et al. studied cell cycle division in *Xenopus* embryos and showed that cyclin A-Cdk2 complex must be involved in apoptosis of somatic cells. They found that high doses of gamma-irradiation (gamma-IR) before the midblastula transition induced apoptotic cell death and decreased levels of cyclin A1 and cyclin A1-Cdk2 complex activity. The addition of recombinant cyclin A1-Cdk2 induced the formation of apoptotic nuclei in *Xenopus* egg extracts, which suggested a role for cyclin A1-Cdk2 complex in apoptosis. Internucleosomal DNA fragmentation, pyknotic, condensed nuclei and loss of intercellular attachments, which are features of apoptosis, were present in embryos exposed to gamma-IR before the midblastula transition. Apoptotic cells accumulated in the blastocoel, are suggesting that before the midblastula transition *Xenopus* embryos use apoptosis to eliminate cells containing damaged DNA [1]. Other studies also showed that overexpression of cyclin A may predispose leukemic cells to undergo apoptosis through deregulated progression to the S or M phase of the cell cycle. Activation of cyclin A-Cdk complex and/or increased expression of cyclin A can induce apoptosis of tumor cells [13, 19]. HIROMURA et al studied the role of cyclin subcellular localization in apoptosis induced by UV irradiation in mouse mesangial cells [12]. They found that in UV induced cells complexes of cyclin A/Cdk2 were predominantly present in the cytoplasm. In our studies we also observed a decrease of cyclin A expression in the nucleus after treatment with doxorubicin. Others techniques are still needed to help us to find the role not only for cyclin A in apoptosis but also whether there is different role dependent on its localization. Studies on cyclin A by using electron and confocal microscope, biochemical methods and also other cell lines are now in progress.

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