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Cytoskeletal reorganization during process of apoptosis induced by cytostatic drugs in K-562 and HL-60 leukemia cell lines

A. Grzanka[a,b,*], D. Grzanka[b], M. Orlikowska[b]

[a]*Institute of Biology and Environment Protection, Bydgoszcz University of Kazimierz Wielki, Chodkiewicza 51, 85-667 Bydgoszcz, Poland*
[b]*Department of Clinical Pathomorphology, The Ludwik Rydygier Medical University in Bydgoszcz, 85-094 Bydgoszcz, Poland*

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Abstract

The aim of the present study was to investigate the reorganization of F-actin, vimentin and tubulin in K-562 and HL-60 cell lines during apoptosis induced by etoposide, doxorubicin and taxol. The distribution of cytoskeletal proteins was analyzed by fluorescence microscopy. Actin was also studied by confocal microscopy and at the ultrastructural level. Changes in the distribution of cytoskeletal proteins were found to be dose-dependent and appeared to be more intense in HL-60 cells. Etoposide- and doxorubicin-treated cells showed similar changes in the distribution of F-actin, vimentin and tubulin. The reorganization of cytoskeletal proteins seemed to be consistent with features of apoptosis. An increase in bright staining of F-actin, vimentin and tubulin at the site of apoptotic bodies formation was observed. Immunogold labeling of actin in HL-60 cells was associated with features typical for apoptosis, i.e. compaction and margination of nuclear chromatin. K-562 cells showed cytoplasmic actin-positivity in the cytoplasm. Significant changes in morphology of HL-60 cells were found in the following concentrations: etoposide 20, 200 μM; doxorubicin 5, 10 μM and taxol 2–10 μM. The investigated proteins seemed to be involved in the above-reported apoptotic changes. Bright staining of F-actin, vimentin and tubulin, concentrated at the site of apoptotic bodies formation might suggested importance of these proteins for this process. Moreover, the increase in actin labeling in areas of chromatin compaction and margination of nuclear chromatin especially in HL-60 cells, which are more susceptible to apoptosis might implicate that actin might be involved in the chromatin remodeling during apoptosis.

Keywords: Fluorescence; F-actin; Vimentin; Tubulin; Immunogold labeling; Apoptosis

1. Introduction

Actin microfilaments, intermediate filaments and microtubules are the major constituents of the cytoskeleton in eukaryotic cells. The functions of these cytoskeletal proteins include maintenance of morphology, cell motility, phagocytosis, intracellular transport, localization of cytoplasmic organelles and formation of the mitotic spindle during cell division [1]. Cytoskeleton also plays an important role in the processes of growth and differentiation and may be involved in signal transduction modulation. Furthermore, changes in the interaction between different cytoskeleton proteins occur during maturation [2]. Actin, one of the major constituents of the cytoskeleton in eukaryotic cells, is involved in cell locomotion, changes in cell shape, membrane ruffling and formation of lamellipodia [3]. The cytoskeletal components, particularly microtubules, are involved in the organization and distribution of the endoplasmic reticulum and other organelles, within the cell [4]. Vimentin filaments and microtubules are closely associated and disturbance of microtubules may also affect the organization of intermediate filaments [5–9]. Changes in the expression and alteration in distribution of vimentin have been described in leukemic cells [10]. Antitumor drugs used in our study show a variety of mechanisms in their cytotoxic action. Doxorubicin binds to DNA and also induces lipid peroxidation and has other membrane-damaging effects [11]. Etoposide (VP-16) is an inhibitor of DNA topoisomerase II. It causes single and double strand breaks in cellular DNA [12,13]. Taxol acts as a mitotic inhibitor blocking cells in the G_2/M phase of the cell cycle. It enhances tubulin polymerization and prevents microtubule depolymerization [14,15]. These drugs are used in the treatment of various types of neoplasms, such

* Corresponding author. Tel.: +48-525-85-4048; fax: +48-525-85-4048.
E-mail address: grzanka@ab-byd.edu.pl (A. Grzanka).
Abbreviations: DAPI, 4,6-diamidino-2-phenyindol.

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as ovarian, breast and lung cancer, malignant melanoma as well as leukemias [16–20].

In the last decade there has been a significant increase in research on cytoskeleton and its role in important biological cell processes. However, it still requires years of debates to give answers to the question we are still facing nowadays. This study describes alterations in the distribution of F-actin, vimentin and tubulin in K-562 and HL-60 cells in relation to apoptotic changes induced by cytostatic drugs in these leukemic cell lines.

2. Fluorescence microscopy studies

The presented experiment has maintained our assumptions based on the results of our earlier studies, which showed that treatment of human leukemia cell lines with DNA topoisomerase II inhibitors induced cytoskeletal changes [21,22]. Cytostatic drugs are strong poisons that damage many important intracellular processes, for example, synthesis of the cell macromolecules such as DNA, RNA, cell proteins or mitosis. These changes can induce programmed cell death (apoptosis) in which cytoskeletal proteins are involved. Cytoskeleton is involved in other processes such as cellular proliferation and differentiation. Induction of leukemia cell differentiation has been reported to be accompanied by changes in the cytoskeleton by Bernal and Stahel [23]. Leung *et al.* showed that microtubules may be involved in the transduction of signals during the initiation of HL-60 cell differentiation [2]. Other authors reported changes in microtubules and in vimentin of HL-60 cells induced to differentiate along the granulocytic or monocytic pathways [24,25]. Microtubules have been shown to be responsible for determining the polarity of cells and the intermediate filaments appear to be involved in physically organizing the interior of the cell [26]. Cotter *et al.* concluded that actin polymerization is a necessary step in the process of apoptotic body formation [27]. Etoposide and doxorubicin kill cells by inhibiting DNA topoisomerase II (stabilization of the DNA–topoisomerase cleavage complex) as a major intercellular target. Etoposide causes single and double strand breaks in cellular DNA and this DNA damage is considered to be the event leading to apoptosis in human tumor cells [28]. Doxorubicin also causes DNA breaks which are thought to be a critical event in cell killing. There are also other mechanisms proposed to explain doxorubicin cytotoxicity, for example, interaction with plasma membrane phospholipids, promotion of lipid peroxidation, free radical formation and deterioration of mitochondrial structural and functional integrity [11,29–31]. Taxol binds directly to polymerized tubulin, promoting microtubules assembly and inhibiting their disassembly [32,33]. Microtubule dysfunction results from the loss of ability of the microtubules to undergo the normal processes of tubulin subunit exchange at their ends [34]. It is now well established that cytoskeletal systems are interconnected and it is worth further investigation whether microtubular poison have influence on distribution of other filamentous network. Cytostatic drugs used in our studies caused significant changes in morphology and distribution of the three studied cytoskeletal proteins especially in HL-60 cells. In this study we used the following concentrations of the cytotoxic drugs: etoposide 20, 200 μM; doxorubicin 5, 10 μM and taxol 2–10 μM. Tubulin in etoposide- and doxorubicin-treated cells tended to translocate to the surface. Treatment of cells with taxol caused not only tubulin labeling at the apoptotic body formations but also intense labeling in

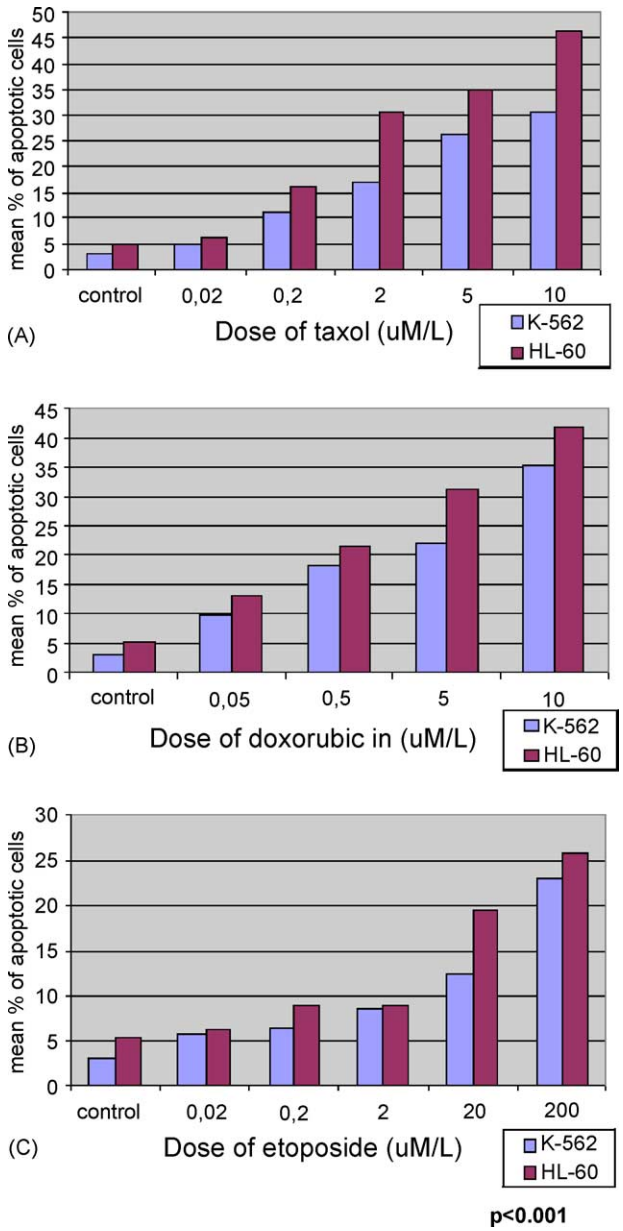

Fig. 1. (A–C) Effect of taxol (A); doxorubicin (B); etoposide (C) on mean percentage of apoptotic cells in K-562 and HL-60 cell lines. The cells were stained by Mayer hematoxylin. The criteria used to define apoptotic cells were nucleus fragmentation, cell shrinkage with the formation of membrane blebs and buds.

the center of the cells. Cells with bundled microtubules and multiple MTOC centers were also seen.

The antiproliferative activity of taxol was associated with marked reorganization of vimentin and actin as well. The increase of vimentin fluorescence was seen in cells treated with all cytostatic drugs used in this study. However, cells treated with microtubule stabilizing agent such as taxol demonstrate perinuclear collapse of the vimentin network. In contrast to this, F-actin microfilaments were less affected by taxol and there were cells with microfilaments distributed rather regularly in their cytoplasm, whereas etoposide- and doxorubicin-treated cells showed diffuse distribution of F-actin throughout the cytoplasm. It stands in agreement with the results of our previous investigations, which indicated that in fact, taxol causes reorganization of vimentin filaments while microtubules and actin microfilaments were less affected [35]. Taken together, our data suggest that the reorganization of actin, vimentin and tubulin leads to changes in cell morphology and seems to be connected with characteristic features of apoptosis (Fig. 1A–C). There were 22% (range, 19.5–25.9%) of HL-60 cells with blebs and buds treated with higher doses of etoposide and 36.6% (range, 31.5–41.8%) and 38.5% (range, 30.7–46.4%) with doxorubicin and taxol, respectively, while 18.7% (range, 12.5–23%) of K-562 cells treated with etoposide and 28.7% (range, 22.2–35.3%) and 23% (range, 16.9–30.5%) with doxorubicin and taxol. The studied cytoskeletal proteins seem to be involved in the formation of apoptotic bodies, especially in HL-60 cell line where cells with blebs and buds labeled for F-actin, vimentin and tubulin were seen more often (Fig. 2). In sections from confocal microscopy, rich labeling of F-actin at the site of the formation of apoptotic bodies was observed (Fig. 3). Levee *et al.* also showed, that apoptotic cells contained a network of fine microfilaments with bright staining concentrated at the site of apoptotic body formations [36]. Levee *et al.* suggest that reorganization of the microfilament network early in the process of apoptosis in combination with actin depolymerization may be important elements necessary for apoptotic body formations and release [36]. Gangemi *et al.* in HL-60 cells treated with taxol also observed appearance of morphological features consistent with the process of apoptosis [37]. Other reports also showed correlation between reorganization of

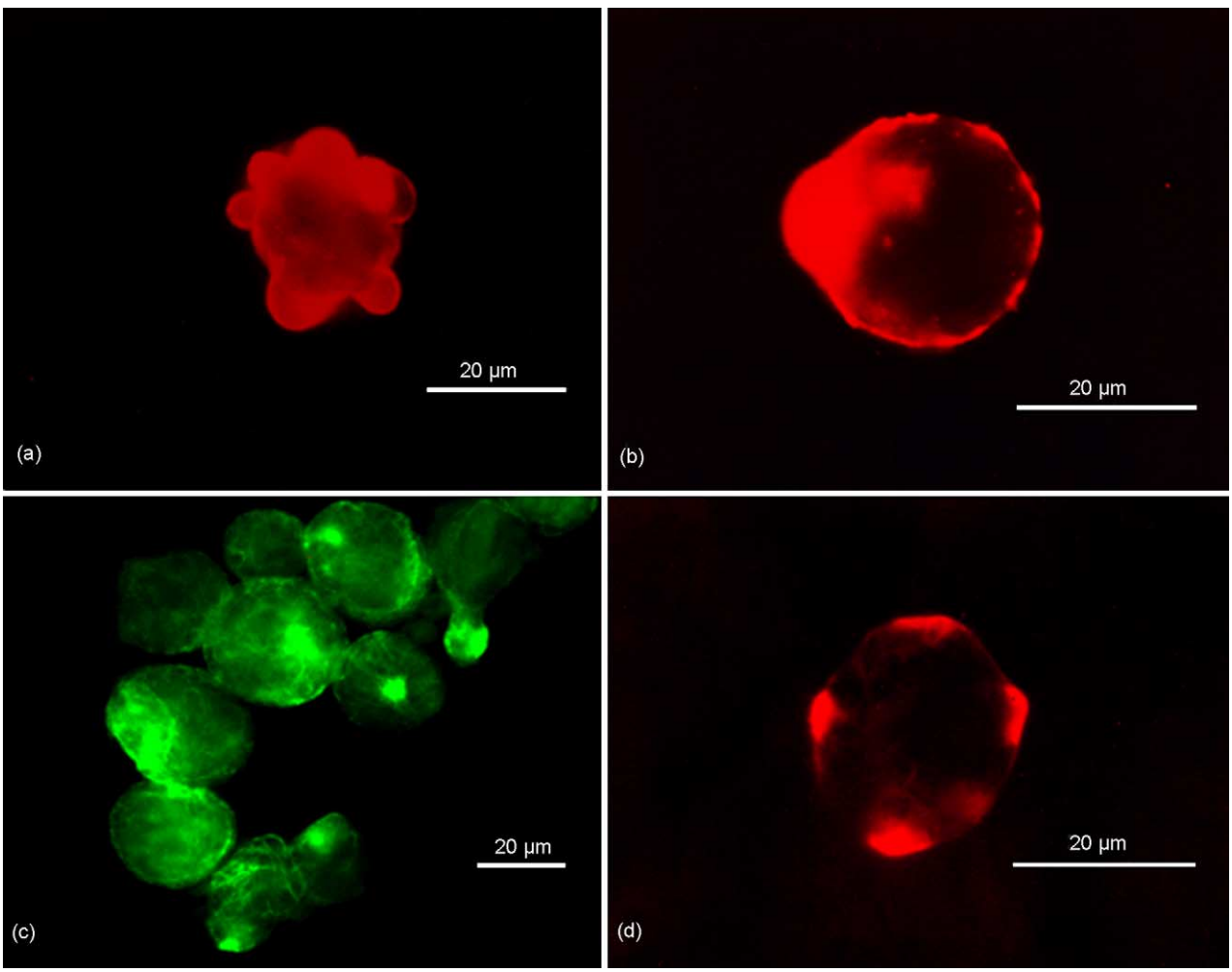

Fig. 2. (a–d) HL-60 cells treated with 10 μM doxorubicin for 72 hr. Cells with buds and blebs labeled for F-actin (a and b); vimentin (c) and tubulin (d).

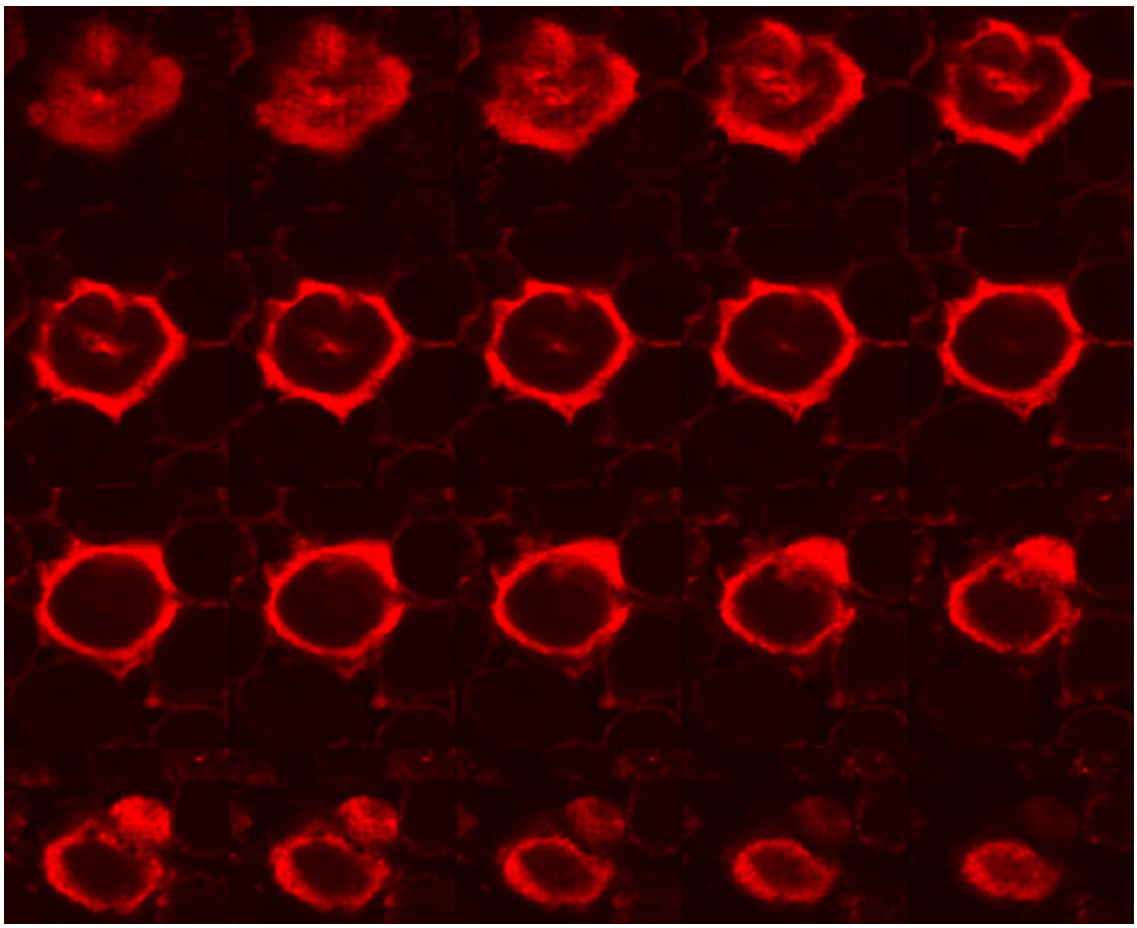

Fig. 3. Z-line construction through the HL-60 cell treated with 2 μM taxol for 72 hr with TRITC-phalloidin labeling for F-actin. There are in some sections rich labeling for F-actin at the site of budding structure is seen.

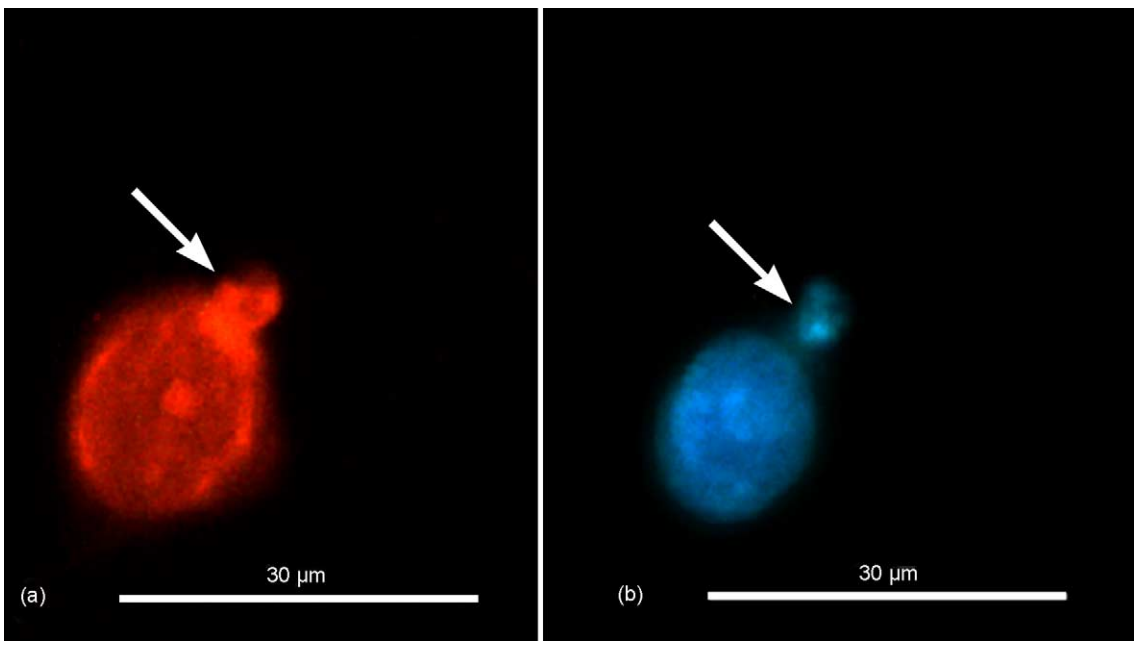

Fig. 4. (a and b) HL-60 cells treated with 10 μM doxorubicin for 72 hr. Shrinking cells with apoptotic bodies (arrows) stained for F-actin (a) and with DAPI (b) are seen.

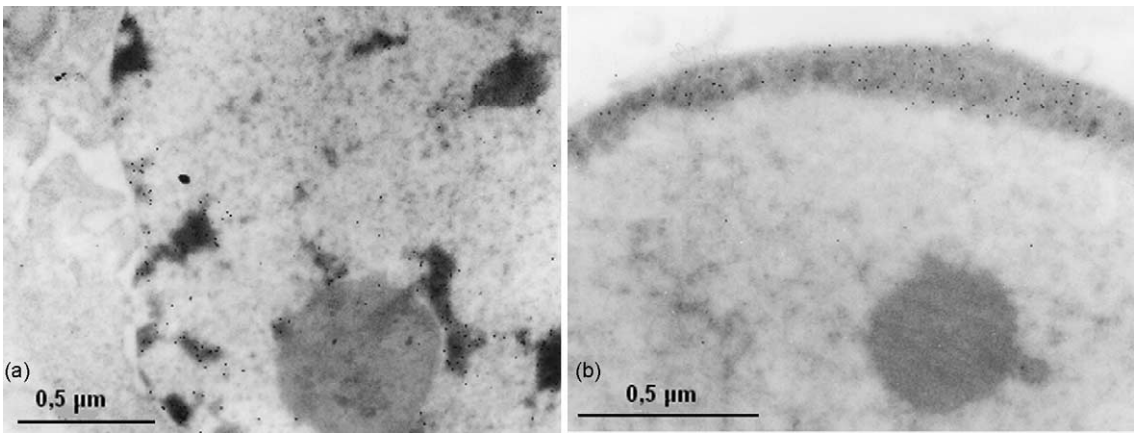

Fig. 5. (a and b) HL-60 cells treated with 10 µM doxorubicin for 72 hr. Gold particles are localized at the site of compaction of the chromatin (a); the fragment of nucleus of apoptotic cells from the culture with 200 µM etoposide. The gold particle labeling actin at the site of chromatin margination are seen (b).

cytoskeletal proteins and apoptosis [38–41]. However, Olins *et al.* concluded that cytoskeletal reorganization is independent of apoptosis [42]. Here, it was found that a lot of cells had blebs and buds marked with 4,6-diamidino-2-phenyindol (DAPI) (Fig. 4) but there were also cells with buds and blebs without DAPI staining. It may suggest that there are also other processes involved in membrane blebbing. Godman *et al.* showed that cytochalasin treatment itself has been associated with cell surface protuberances or blebs [43]. There are other reports denying the association between apoptosis and membrane blebbing or budding of the cells [44–47].

3. Immunoelectron microscopy studies

In order to study the presence of actin at the ultrastructural level, we carried out immunogold labeling on thin sections from low viscosity resin (LRW) embedded cells. Actin labeling in K-562 cells treated with cytostatic drugs was observed in the nucleus and cytoplasm, where gold particles were numerous and diffusely distributed. In our study, actin reorganization has been seen associated with compaction and margination of chromatin in the nucleus of HL-60 cells treated with higher doses of cytostatic drugs (Fig. 5a and b). This appears to be quite interesting and

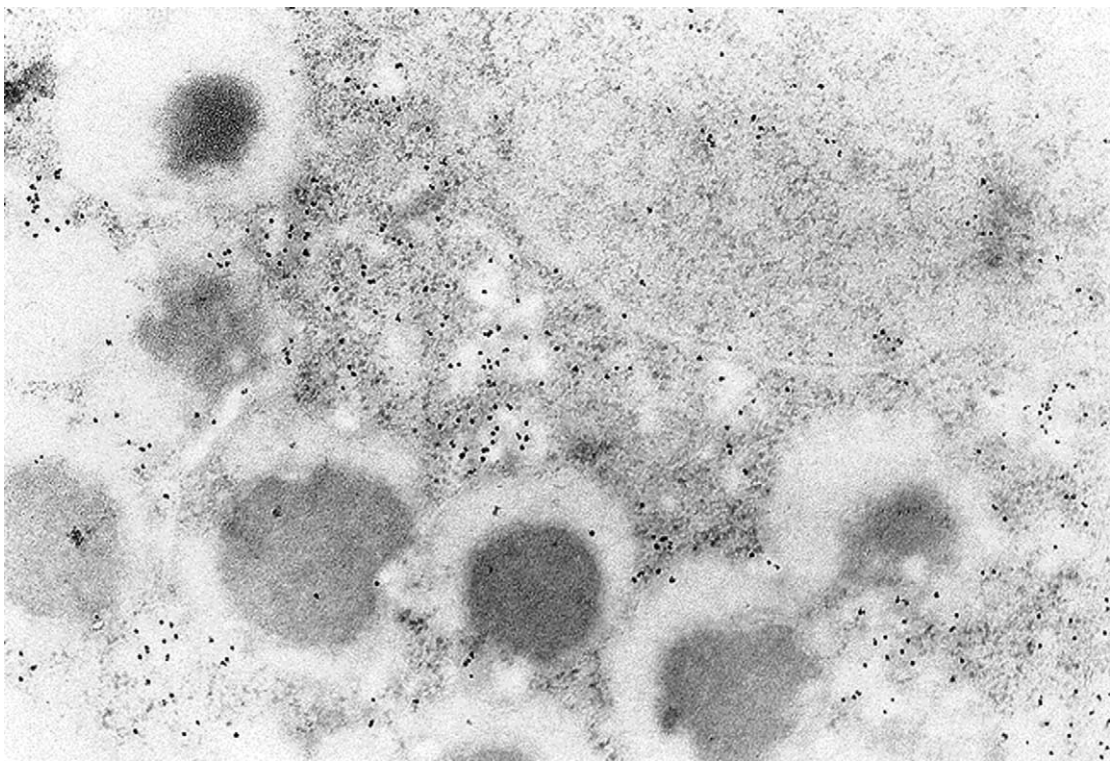

Fig. 6. K-562 cell treated with 5 µM doxorubicin for 72 hr. The gold particles are localized first of all in the cytoplasm.

might suggest that actin is involved in the chromatin reorganization during the process of apoptosis [21]. Moreover, the increase in actin labeling in the areas of chromatin compaction might correspond to actin translocation from cytoplasm to the nucleus in relation to the reorganization of chromatin. This finding could additionally be an explanation of the quite intense immunogold labeling in the cytoplasm of non-apoptotic K-562 cells, which are not as sensitive to apoptotic process as HL-60 cells (Fig. 6). We believe that our observations are worth consideration and further molecular and microscopic investigations on other cellular lines, employing different cytostatic agents. They might help to find the answer on question what role actin plays in nucleus and whether actin plays a role in chromatin remodeling during apoptosis. Continuation of our studies as well as other researches might be helpful in finding the answer to the questions about the role of actin in the nucleus and its involvement in chromatin remodeling during the process of apoptosis. This question requires further investigation. Wada *et al.* suggest that actin at least partially stays in the nucleus. They showed that actin contains two nuclear export signal sequences and defined a novel molecular mechanism controlling intracellular transport of actin. Moreover, they concluded that the presence of actin in the nucleus under normal conditions is harmful to the cell and this mechanism may be utilized to ensure its cytoplasmic localization. Another possibility in their opinion is that the shuttling of actin between the nucleus and the cytoplasm may have some physiological relevance [48]. Other works also showed reversible presence of actin in the nucleus [30,31]. Sanger *et al.* indicated that treatment of PtK2 and WI-38 cells with 10% dimethyl sulfoxide caused disappearance of stress fibers from the cytoplasm and appearance of actin in the nucleus [49]. The review by Rando *et al.* discusses the evidence for the presence of actin in the nucleus and summarizes recent works, which suggest the involvement of actin and actin-related proteins in the regulation of nuclear processes such as chromatin remodeling [50].

4. Summary

Taken together, our results show that reorganization of the studied cytoskeletal proteins in K-562 and HL-60 leukemia cells treated with cytostatic drugs is necessary for the formation of apoptotic bodies. The observations at the ultrastructural level suggest an actin involvement in chromatin reorganization during the process of apoptosis. Moreover, it seems to be related to the translocation of actin from the cytoplasm to the nucleus.

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