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Immunocytochemical Study on Microtubule Reorganization in HL-60 Leukemia Cells Undergoing Apoptosis

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ABSTRACT

Background: Microtubules (MT) are important components of cell cytoskeleton and play key roles in cell motility mitosis and meiosis. They are also the targets of several anticancer agents which indicating their importance in maintaining cell viability. Microtubular reorganization contributing to apoptotic morphology occurs in normal and neoplastic cells undergoing apoptosis induced by cytotoxic drugs. The aim of this study was to correlate the changes in the MT with behavior of the γ-tubulin in apoptotic cell, and to see if apoptitic MT showed biochemical characteristics of stable MT. **Methods:** Apoptosis was induced in the human leukemia cells (HL-60) by treatment with 1 μM of all-trans retinoic acid over a 5-day period. The time course of changes was assessed using flow cytometry, DNA fragmentation and immunocytochemistry in cells labeled for α-tubulins, acetylated α-tubulin and γ-tubulin. **Results:** The results indicated that γ-tubulin content is increased after cells have gone through the apoptosis with a diffuse cytoplasmic pattern. α-tubulin did not reveal any specific pattern of polymerization in apoptotic cells and acetylated α-tubulin content was also decreased in comparison with non-apoptotic cells. **Conclusion:** Our results support the idea that microtubule reorganization is an important factor of the mammalian cells response to apoptosis, and the altered properties of the MT did not reflect changes in function as apoptosis progresses. *Iran. Biomed. J. 11 (4): 209-214, 2007*

Keywords: Apoptosis, Microtubule, γ-tubulin, Immunocytochemistry

INTRODUCTION

Apoptosis is a term used to describe the terminal morphological and biochemical events seen in programmed cell death [1]. Apoptosis has recently been the subject of great interest because it has been clearly demonstrated to mediate cell death, not only during development but also in neoplasia in response to cancer chemotherapy and radiation [2, 3]. Studies on leukemia and lymphoma cells have been significant in determining the importance of apoptosis in the cancer process. Considerable morphological change occurs in apoptosis and it has been proposed that the cytoskeleton is involved. Some studies have directly implicated actin in the apoptotic process, particularly in the production of apoptotic bodies [4, 5]. Our studies have focused on microtubules (MT), which we have shown is reorganized, during apoptosis.

MT perform devices functions in mammalian cells, including transport of chromosomes and membrane enclosed organelles. This functional diversity is controlled at the transcriptional level, by several post-translational modifications, and also by differential binding of microtubule-associated proteins (MAP) [6-8]. MT exist in a state of dynamic instability *in vivo* [9-11] and *in vitro* [12]. In mammalian cells the MT nucleate from the microtubule organizing center (MTOC) or centrosome which stabilizes the minus end. Most cytoplasmic MT have rapid cycles of polymerization-depolymerization but there is a small proportion of relatively stable, slow turn over MT [13, 14]. Stable MT are biochemically distinct from newly formed MT. Certain post-translational modifications of α-tubulin, namely acetylation of Lys 40 and detyrosination of the c-terminal tyrosyl residue, are frequently found associated with stable MT [15-18]. Evidence suggests that such post-translational modifications occur preferentially in the microtubule and in a time-dependent manner [14]. Acetylation of α-tubulin is also associated with

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MT resistant to depolymerization by anti-mitotic drugs and cold shock [15, 17], and is regarded as marker of stable MT.

In the present work, we have used immuno-cytochemical staining and flow cytometry to examine changes in microtubule network and cell content of acetylated and non-acetylated α-tubulin in HL-60 cells undergoing all-trans retinoic acid (ATRA) induced apoptosis.

MATERIALS AND METHODS

Cell culture and induction of apoptosis. An established HL-60 human myeloid leukemia cell line was used for experiments (Iranian National Cell Bank, Pasteur Institute of Iran, Tehran, NCBI code C427). Cells were maintained in suspension culture at 37°C under 5% CO_2/air in RPMI 1640 (Gibco Inc. UK) supplemented with 10% FCS and antibiotics: 100 U/ml of penicillin and 100 µg/ml of streptomycin. The Cells were sub-cultured three times weekly. ATRA (Sigma Chemical Co., St. Louis, MO, USA), was used as an inductor of apoptosis. Immediately after inoculation, a solution of ATRA was added at a final concentration of 10^{-6} M. An equivalent volume of ethanol was added to control cells during the apoptosis process; the medium was removed at 48-h intervals and replaced with a fresh one containing ATRA. Simultaneously, fresh medium free of ATRA was also changed in the control flasks. Cell membrane integrity was confirmed using the trypan blue staining method.

Immunocytochemistry. Immunofluorescence microscopy was performed according to the reference [19] with some modifications. Cells were first centrifuged on poly-l-lysine coated coverslips, then the coverslips bearing cells were immersed in -20°C methanol for 5 minutes. Coverslips were then rehydrated with PBS, and were washed three times with Ab buffer (PBS, 3% BSA, 0.1% Triton X-100, 0.02% azide). The medium was supplemented with DAPI fluorescent dye (Sigma-Aldrich, Inc. USA) at a concentration of 1 µg/ml to stain DNA. Primary antibodies anti α-tubulin (Cat no. T-9026), α-acetylated tubulin (Cat. no. T-6793) and γ-tubulin (Cat. no. T-3559), all from Sigma-Aldrich Inc. (USA) chemicals were diluted in Ab buffer and added to the coverslips and incubated for 30 minutes in the room temperature. The samples were washed three times with PBS and incubated in secondary fluorescent conjugated antibody for 30 minutes. The coverslips were mounted on slides using glycerol, observed in a Leitz fluorescence microscope and fluorescence micrographs were recorded using CCD camera (Hitachi, kp-D20BP, Japan). The apoptosis process was detected in individual cells on the basics of characteristic changes in nuclear morphology (chromatin collapse and nucleus breakdown). In the apoptosis cells, MT and γ-tubulin distribution were assessed and compared with the cytoskeleton of interphase and mitotic cells.

Hoechst assay. The cells were washed with PBS, and then were fixed for 5 minutes in ice cold 3% paraphormaldehyde solution in PBS. Cells were centrifuged and washed with PBS, and then were resuspended in 50 µl Hoechst 333492 solution as described before [20]. The cells were mounted on a glass slide using glycerol. Cells were visualized with a DAPI filter (Ex = 330 A, Emission = 480) under an immunofluorescence microscope.

Immunoblotting. Western-blot analysis was performed as described before [20]. Briefly, equal numbers of cells were lysed using a 30-s sonication in the lysis buffer [DTT 1 mM, EDTA 1 M, PMSF (10 µM) and the protein concentration in the lysate was measured using Bradford assay. Equal amounts of proteins (10 µg) were loaded into the mini gel (10% Lamelli acrylamide gel) electrophoresed at constant voltage of 100 V for 3 hours, and then transferred overnight to nitrocellulose membranes. Membranes were incubated for 2 hours in the blocking solution containing 5% skim milk, then incubated in primary antibodies against α, α-acetylated and γ-tubulin. The Membranes were washed in washing buffer and were further developed in the secondary antibodies for 1 h (ALK-P conjugated for γ-tubulin HRP-conjugated and for α- and α-acetylated detection) and finally was incubated in the corresponding substrate for secondary antibodies after a three-time washing in the wash buffer until the bands were developed.

DNA laddering. DNA fragmentation was performed according to Porter and Lee [21] with some modifications. Cells were lysed with digestion buffer (Tris-HCl, 10 mM; NaCl, 100 mM; EDTA, 25 mM; SDS 1%, proteinase k, 0.2 mg/m) and were incubated at 50ºC for 2 hours. The lysate was extracted with phenol/chloroform/isoamyl alcohol and twice with phenol/chloroform/isoamyl alcohol (25/24/1) and once with chloroform/isoamyl alcohol (24/1). DNA was precipitated by adding ammonium

acetate to final concentration of 2.5 M, mixed and 2 volumes of pure ethanol was added, and then incubated at -20ºC overnight. DNA was pelleted by centrifugation at 12,000 ×g for 30 minutes, washed with 70% ethanol, air dried and dissolved finally in the Tris-EDTA buffer (Tris-HCl (pH = 8), 10 mM; EDTA, 1 mM) and visualized using ethidium bromide containing agarose gels.

Annexin-5 assay. Annexin-5 assay was performed as described before [21] using commercial kit (from IQ Products Company, The Netherlands). Briefly, cells were washed twice in calcium buffer, incubated at 4°C for 20 minutes at dark in Annexin-5 FITC washed with calcium buffer and incubated for 10 minutes in propidium iodide and analyzed using an FAC scan (Becton Dickinson) flow cytometer.

RESULTS

Induction of apoptosis by ATRA. The human HL-60 leukemia cell line was treated with 1 µM ATRA for various periods of time, and cells were harvested for morphological, flow cytometric and DNA analysis. The morphological changes typical of apoptosis were visible 4-5 days after treatment. DNA fragmentation was observed at five days post-treatment (Fig. 1). Flow cytometric analysis of ATRA-treated cells by Annexin V is shown in Figure 2. The percentage of cells positive for Annexin 5 staining was 10.87% four days after treatment and significantly increased (24.43%) five days after treatment. For non-treatment cells, the percentage of positive cells was h.54% (data not shown).

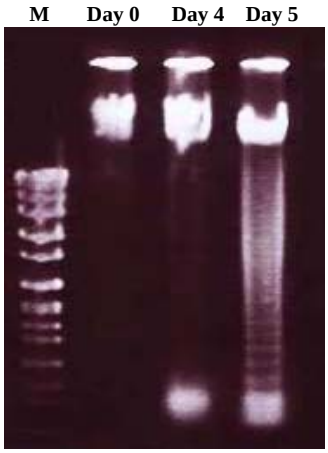

Fig. 1. Detection of apoptosis by DNA laddering. Apoptosis was documented after 5 days of ATRA treatment.

Relative content of γ, α and α-acetylated tubulin. The content of γ-tubulin was significantly increased following apoptosis. Acetylated-α tubulin and α-tubulin were decreased following apoptosis induction in comparison with non-apoptotic control cells as identified by Western-blotting (Fig. 3).

Changes in dynamic of MT following apoptosis. A dual staining both for the nucleus and microtubule network allowed identifying individual cells by the morphology of nucleus. Apoptosis was characterized by fragmented nuclei as identified by Hoechst assay, so those cells were at the latest stage of apoptosis. Examination of α-tubulin-stained cytocentrifuge preparations showed that all cells both treated and untreated showed some tubulin positivities. In apoptotic cells, two patterns of MT were observed: lack of staining and faint, disperse pattern of staining. No obvious MTOC was observe (Fig. 4 a and b). In normal cells, half displayed polymerized tubulin structures in cytoplasm and a

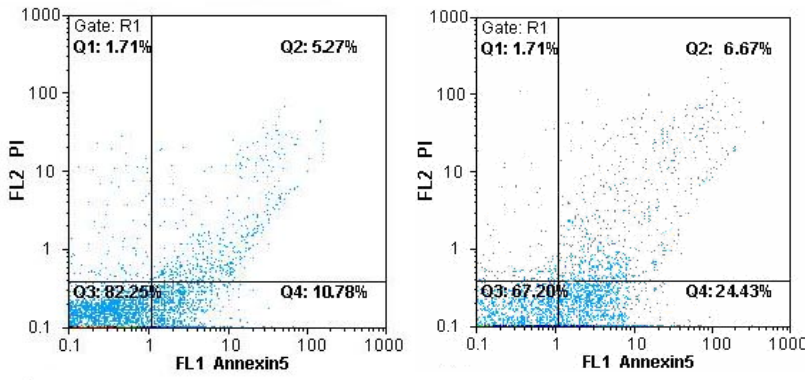

Fig. 2. Indications of apoptosis as analyzed by Annexin-5 assay. Percentage of cells positive both for propidium iodide and annexin-5 staining was significantly increased after 5 days of ATRA treatment **(right)** in comparison to day 0 treatment **(left)**.

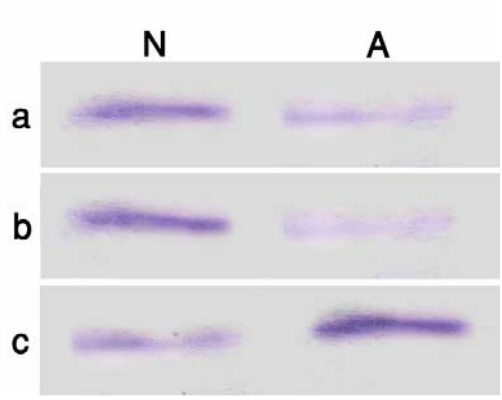

Fig. 3. Immunoblot analysis of α-tubulin **(a)**, acetylated-α-tubulin **(b)** and γ-tubulin **(c)** in normal non-apoptosis and apoptotic HL-60. N, non-apoptotic; A, apoptotic.

Faint microtubular network were seen on or around the nucleus (Fig. 4 c and d). Immunofluorescence staining for the α-acetylated MT showed changes comparable to that of α-tubulin, but there was no consistent staining of α-acetylated cell in apoptotic cells (Fig. 5 a and b). In non-apoptotic cells, a fine rim of acetylated α-tubulin was seen around the nucleus (Fig. 5 c and d).

γ-tubulin distribution in different stages of apoptosis. In normal non-apoptotic cells, the staining was mostly confined to the centrosomal areas and a faint, localized and very weak staining for γ-tubulin was also observed in cytoplasm (Fig. 6 a and b). In the apoptotic cells, there was no centrosomal staining but areas of cytoplasmic staining were more intense than non-apoptotic cells (Fig. 6 c and d).

DISCUSSION

Pattern of MT in a typical tissue culture cell is usually in the form of MT distributed to their minus ends clustered together and their plus ends extending out into the cytoplasm. The canonical model for how such arrays are generated is that MT, nucleated at centrosomes, grow through the cytoplasm. Dynamic instability describes the stochastic behavior of MT in which individual MT transit between phases of growth and fast shrinkage. Upon contact with capture sites, they would be stabilized, forming patterns.

Tubulin self-assembles *in vitro* when a critical concentration is exceeded, indicating that MTOC are not absolutely required for MT nucleation. However, even in the normal cytoplasm of living cells, concentration of tubulin is not high enough to account for a free nucleation of MT, independent of an MTOC [22]. On the other hand, over-expressed γ-tubulin is shown to be associated with ectopic microtubule nucleation outside the centrosome in mammalian cell cytoplasms. This can be considered as an explanation of our observed increase in γ-tubulin content after apoptosis. It is possible that γ-tubulin might be increased in response to a decreased tubulin content for the formation of multiple MTOC that might form in apoptosis as suggested by other investigators [23], possibly as a defense mechanism against progression of apoptosis. Increased cytoplasmic staining of γ-tubulin observed in our study is also supportive for this hypothesis. Induction of apoptosis by cytostatic drug taxol in HL-60 cells [5] and VP-16 in CCRF-CEM cells also

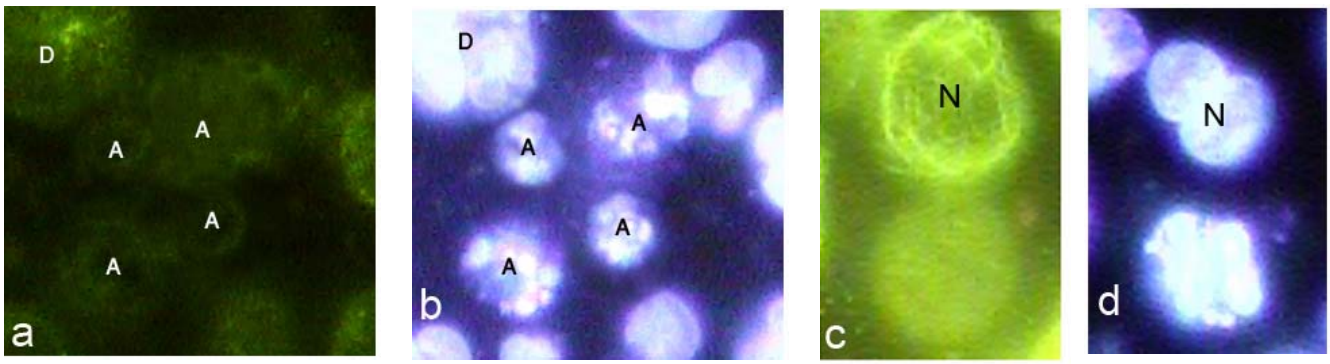

Fig. 4. Immunofluorescent staining of α-tubulin in (**a** and **b**) apoptotic and (**c** and **d**) non-apoptotic HL-60 cells. Cells were fixed and stained with α-tubulin monoclonal antibody and fluorescein-conjugated secondary antibodies, as described in material and methods. Nuclear morphology in the same subset which signifies the stage of apoptosis is clarified using Hoechst staining on the same cells (c and d). A, apoptotic; N, non-apoptotic; D, differentiated.

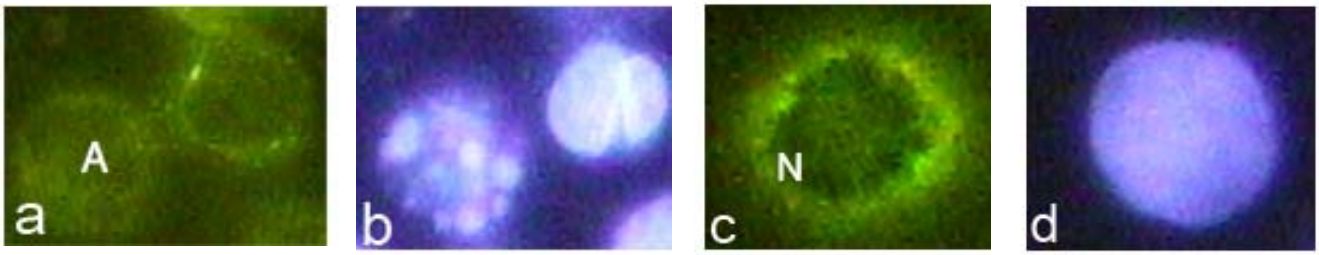

Fig. 5. Immunofluorescent staining of acetylated α-tubulin in **(a)** apoptotic and **(c)** non-apoptotic HL-60 cells which shows a fine labeling of acetylated α-tubulin around the nucleus. Nuclear morphology in the same subset is clarified using Hoechst staining on the same cells (**b** and **d**). A, apoptotic; N, non-apoptotic.

induces multiple MTOC [24]. On the other hand, the increased γ-tubulin content might only reflect that its high level in differentiated cells have not returned to normal yet. ATRA-induced apoptosis in HL-60 cells is claimed to be a process independent of differentiation [25, 26] which argues against this assumption, although a recent proteomics analysis [27] have suggested that differentiation is a pre-requisite of apoptosis induced by ATRA.

Dynamic MT rapidly grows using free unmodified tubulin, and frequently shrinks, never existing long enough to be significantly (detectably) modified. Stable MT, being older, simultaneously accumulate significant concentrations of post-translationally modified tubulin forms (acetylated and tyrosinated) [13]. Selective stabilization of MT has been discussed as a general model for morphogenesis and acetylation and tyrosination of tubulin is shown to be a major event in differentiated related phenomena [28]. In this model, a cell in the absence of an outside morphogenetic signal randomly polymerizes MT in all directions with the rapid turn-over predicted by the property of dynamic instability *in vitro* as well as the behavior observed in living cells.

According to our observations, apoptotic cells would have less abundant α and α-acetylated tubulin and will have disperse cytoplasmic γ-tubulin, α and α-acetylated tubulin without having and organized structure of any of these components. This is comparable to the previous report showing microtubule disruption in ATRA-induced apoptosis of HL-60 cells [29]. We believe that these findings about the role of γ-tubulin and microtubule cytoskeleton in APL (Acute promyelocytic leukemia) apoptosis would be enough justification for performing further studies on the function of γ-tubulin in ATRA resistant subsets of leukemia cases.

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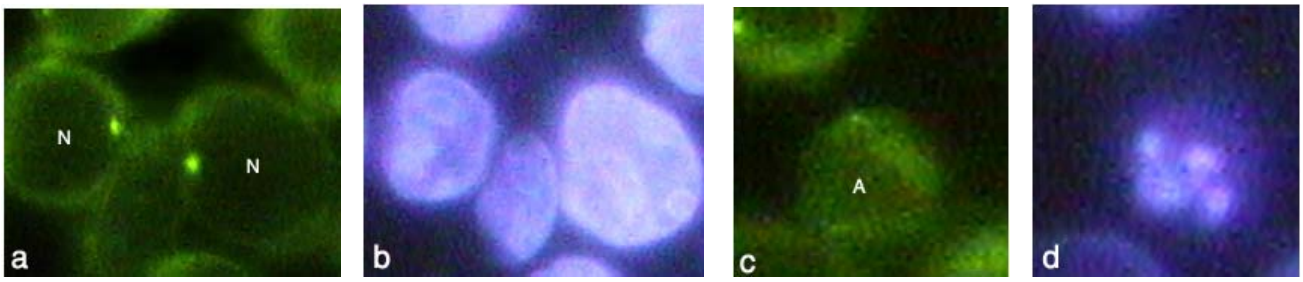

Fig. 6. Immunofluorescent staining of γ-tubulin in **(a)** non-apoptotic and **(c)** apoptotic HL-60 cells. In non-apoptotic cells, staining is mostly confined to the centrosomal area. In apoptotic cells, there is no centrosomal staining, but the cytoplasmic staining is more intense than non-apoptotic cells. Nuclear morphology in the same subset is clarified using Hoechst staining on the same cells **(b and d)**. N, non-apoptotic; A, apoptotic.

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