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A Study of Correlation between NPM-Translocation and Apoptosis in Cells Induced by Daunomycin

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ABSTRACT. Human leukemia K562 and HeLa cells were treated with daunomycin (DA) for 1–4 hr. With the indirect immunofluorescence technique, we observed that the nucleolar protein nucleophosmin/B23 (NPM) shifted its location from the nucleolus to the nucleoplasm (NPM-translocation). The degree of NPM-translocation was determined by the relative immunofluorescent intensity in the nucleoli vs the nucleoplasm (defined as localization index, LI). We found that NPM-translocation, as determined by the decrease of LI, correlates with cytotoxicity. The degrees of NPM-translocation, chromatin condensation, and DNA fragmentation in HeLa cells were determined after treatment with 0.1, 0.5 and 1 μg/mL DA for 1 hr. We found that NPM-translocation (LI < 2.5) was observed in cells during the treatment with 0.5 and 1 but not with 0.1 μg/mL DA. Also, cells treated with 1 μg/mL remained in an NPM-translocated state for a longer time (5–6 hr) than those cells treated with 0.5 μg/mL (1–2 hr). Cells treated with 0.5 and 1 μg/mL DA showed increased levels of chromatin condensation beginning at 5 hr after the drug treatment. The number of cells with condensed chromatin increased with both time and drug concentration. No cells with condensed chromatin were observed in samples treated with 0.1 μg/mL DA, which also showed no significant NPM-translocation. Similar results were observed for induction of DNA fragmentation. We found that the drug concentration required for induction of DNA fragmentation and chromatin condensation coincided with the drug concentration required for NPM-translocation. Taken together, these results indicate that NPM-translocation correlates with apoptosis induced by daunomycin. BIOCHEM PHARMACOL **57**;11:1265–1273, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. nucleophosmin; NPM-translocation; daunomycin; apoptosis; tumor cells

A striking feature of cancer cells is their enlarged, pleomorphic nucleoli, which are the sites of ribosome synthesis [1]. Intricate mechanisms that mobilize hundreds of proteins, RNAs, and other elements in the nucleolus are orchestrated in a concerted manner to make ribosomes [2, 3]. In addition, the nucleolus also is involved in other cellular activities, including mRNA processing [4] and hosting viral proteins HIV Rev and tat [5, 6]. The fact that many anticancer drugs (Adriamycin^R, actinomycin D, mitoxantrone, and camptothecin) localize in nucleoli or have effects on nucleoli [1, 7–10] indicates that the nucleolus is one of the target areas of the drugs. These anticancer drugs alter nucleolar structure (segregation of the fibrillar and the granular components) and inhibit its function [1].

NPM† (nucleophosmin/B23, NO38, Numatrin) [11–13] is a major nucleolar phosphoprotein that is 20 times more abundant in cancer cells than in normal cells [11]. The abundance of NPM is directly proportional to cell proliferation. The human *NPM* gene was cloned and characterized recently [14]. It was found that the *NPM* gene is located on chromosome 5, and the gene is at the breakpoints of the t(2:5), t(3:5), and t(5:17) chromosome translocations of certain lymphomas [15–17]. The putative function of NPM is ribosome assembly and transport. The basic and acidic domains in the NPM molecule may minimize the nonspecific interactions between ribosomal proteins and rRNA during assembly [18]. NPM is also a mobile nucleolar protein that shuttles between nucleoli and cytoplasm [19]. Recent studies suggest that NPM may recruit protein factors (yy1, p120, Gu, Rev) into nucleoli for rDNA transcription or other functions [5, 20–22]. We found that the cellular localization of NPM is affected by the levels of GTP and ATP. NPM requires GTP to localize in the nucleolus [23, 24] and ATP for dissociation to nucleoplasm [25, 26].

In the past, we found that NPM shifts from nucleoli to the nucleoplasm when cells are exposed to certain anticancer drugs (actinomycin D, doxorubicin, DA, tiazofurin, or camptothecin) [23, 27–32]. We have studied this drug-induced phenomenon (NPM-translocation) and found it to be useful in determining the effectiveness of drugs. The shifted cellular location of NPM is detected by visual observation of the immunofluorescent cells. The degree of NPM-translocation therefore was difficult to assess. To bypass this obstacle, this study employed an imaging

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† *Abbreviations:* DA, daunomycin; LI, localization index; NPM, nucleophosmin/B23; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; and DRB, dichlororibobenzimidazole.

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method to determine NPM-translocation. With this method, we reported a direct relationship between NPM-translocation, cytotoxicity, and apoptosis in K562 and HeLa cells induced by DA. These results also agree with the notion that NPM-translocation is a part of the apoptosis pathway that involves damage to the nucleolus.

MATERIALS AND METHODS

Cell Cultures and Drug Treatments

HeLa cells were grown as a monolayer in Minimum Essential Medium supplemented with 10% newborn bovine serum in a 5% CO_2 humidified incubator at 37°. K562 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, glutamine, and antibiotics. K562 cells were fed 2 hr before drug treatment. HeLa cells were fed overnight before the drug treatment. After cells were treated with drugs for 1 or 4 hr, they were cultured in drug-free medium for an additional 24 hr. Cells were removed from cultures at different times for analysis of NPM-translocation and apoptosis (chromatin condensation and DNA fragmentation).

Immunofluorescence Assay

About 5–10 $\times$ 10^4 K562 cells were cytocentrifuged (HeLa cells were grown for 2 days) onto a clean glass slide, fixed with 2% formaldehyde (20 min) and permeabilized with cold (−20°) acetone (5 min). The slides were washed with 4 M urea (10 min) and then with PBS. Cells were incubated first with a mouse anti-NPM antibody (2 hr at room temperature), washed, and then incubated with FITC-conjugated goat anti-mouse IgG antibody (1 hr at room temperature).

Determination of NPM-Translocation

The immunofluorescence images of cells were examined with a fluorescence microscope (Zeiss, model Axiovert 100). Fluorescent images were captured by a CCD camera (COHU, model #4915), which was equipped with a frame store (Colorado Video, model 440) and an LG-3 frame grabber (Scion Corp.). Images were analyzed using the software NIH Image (written by Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov). The full gray-scale is 256 units (from complete darkness at 0 to full luminescence at 255). The nucleoli and the nucleoplasm of an individual cell were identified by the phase-contrast and fluorescent images. Their fluorescence intensities were measured. The ratio of nucleolar over nucleoplasm fluorescence intensities (defined as LI) then was calculated. About 100–200 cells from each sample were measured. To reduce variability and to establish the same basis for fluorescence measurement, pictures were taken using the same conditions for each experiment. These conditions included (a) time of exposure to the mercury light source, (b) the gain setting in the camera, (c) the numbers of picture frames collected, and (d) internal positive and negative controls. To reduce bleaching of fluorescence by the mercury light source, 2% *n*-propyl gallate [33] was incorporated in the glycerol mounting solution.

Determination of Cytotoxicity with the MTT Assay

The MTT assay was used to measure the cytotoxicity induced by DA [34, 35]. Cells were grown in a 96-well microtiter plate for 1 day before drug treatments. After the drug treatment (4 hr for K562 cells and 1 hr for HeLa cells), fresh medium without the drug then was added, and the cells were allowed to grow for an additional 20 hr. A MTT solution (5 mg/mL in culture medium without serum and antibiotics) was added to the cell culture (final concentration 0.5 mg/mL). Incubation at 37° was continued for 1 hr. Cells and crystals that formed were collected by centrifugation, and the supernatant was aspirated from the wells. The formazan (product of the reduction of tetrazolium by viable cells) in the pellet was dissolved in 100 μL isopropanol containing 0.04 N HCl, and the absorbance at 595 nm was measured. The percentage of cytotoxicity is calculated as [1 − (absorbance of experimental wells/absorbance of control wells)] $\times$ 100.

Determination of Chromatin Condensation

HeLa cells were grown on glass slides before the drug treatment. At various times after the drug treatment, cells were fixed with 2% paraformaldehyde before staining with 20 μg/mL Hoechst 33258 in PBS for 5 min. Apoptotic cells were identified with a fluorescence microscope (Fig. 5), and the percentage of apoptotic cells was tallied. Over 5000 cells per sample were counted.

DNA Fragmentation

The method described by Skladanowski and Konopa [36] was used. Following the treatment with various concentrations of DA (1 hr), HeLa cells were washed twice with fresh medium without the drug and allowed to grow for an additional 20 hr. Cells were collected by scraping and washed once with ice-cold PBS. Cells (10^6/20 μL) were lysed in buffer (10 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl, 0.5% SDS, pH 7.4, and 0.5 mg/mL proteinase K) at 50° for 1 hr. RNase A (DNase-free) then was added, and incubation was continued for 2 more hours. Following incubation, the NaCl concentration was raised to 1 M. The mixture was shaken and centrifuged (30 min, 500 *g*). The supernatant was collected, and DNA was precipitated with 2.5 vol. of ethanol overnight at −20°. The DNA dissolved in TE buffer was separated by electrophoresis in 1.8% agarose gel (3 V/cm). DNA was visualized with ethidium bromide.

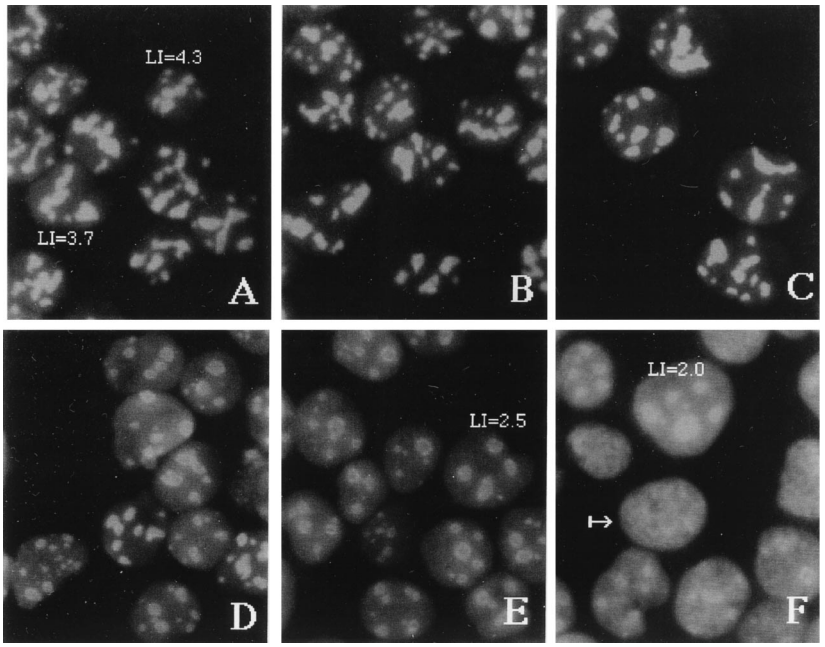

FIG. 1. Immunofluorescence of NPM in K562 cells after exposure to DA for 4 hr. A: control; B: 0.01 μg/mL; C: 0.1 μg/mL; D: 0.5 μg/mL; E: 1 μg/mL; F: 5 μg/mL. Immunofluorescent pictures were captured by a CCD camera. The NPM-localization index (LI) also is depicted.

RESULTS

Human leukemia K562 cells were treated with different amounts of DA (0.01 to 5 μg/mL) for 4 hr, after which NPM-translocation was determined immediately. Figure 1 shows the immunofluorescence images of treated cells. Nucleoli of the K562 cells are shaped irregularly. We observed bright nucleolar fluorescence in control cells or cells treated with 0.01 to 0.1 μg/mL DA. The nucleoplasm fluorescence was relatively weak (Fig. 1, panels A–C). When cells were exposed to a higher drug concentration (0.5 to 5 μg/mL DA), an increase of nucleoplasm fluorescence was observed (Fig. 1, panels D–F). The ratio of the fluorescence intensity in nucleoli to the nucleoplasm (defined as LI) for each individual cell was determined. Figure 2 shows histograms of LI of cells treated with DA. The LI values of control cells and cells with low drug concentrations ($\leq$ 0.1 μg/mL) were in the range between 3 and 5.5 with an average value of 4. This result indicates that the majority of NPM was localized in nucleoli even though the distribution of NPM in the nucleoplasm of these cells was quite heterogeneous. We conclude that with concentrations $\leq$ 0.1 μg/mL DA, there was no NPM-translocation. However, NPM-translocation was observed (with decreased LI) when cells were treated with a higher drug concentration ($\geq$ 0.5 μg/mL). With 5 μg/mL DA, the average LI in cells was approximately 2.0. We considered that cells with LI $<$ 2.5 were NPM-translocated cells.

To study the correlation between NPM-translocation and cytotoxicity, MTT formation after the drug treatment was analyzed [34, 35]. Figure 3A and B show the concentration-response curves of NPM-translocation and the inhibition of MTT formation, respectively. We found that with DA concentrations $\leq$ 0.1 μg/mL, there was no significant NPM-translocation or inhibition of MTT formation. However, both NPM-translocation (indicated by a decrease of LI) and cytotoxicity (indicated by a decrease of MTT formation) were observed with a DA concentration higher than 0.1 μg/mL. The LI values for cells treated with $>$ 5 μg/mL DA were not determined because at this concentration, the brightness of the nucleoplasm fluorescence approached that of the nucleolus (arrow in Fig. 1F), which made it difficult to identify the nucleolus for the calculation of LI. The upper limit of the LI measurement is approximately 1.5.

We also used HeLa cells to study the correlation between NPM-translocation and cytotoxicity. HeLa cells were treated with 0.01 to 2 μg/mL DA for 1 hr. Figure 4 shows the correlation between LI and the inhibition of MTT formation. Similar to those observed in K562 cells, a good correlation between NPM-translocation and cytotoxicity was observed.

Apoptosis in cells is characterized by the appearance of chromatin condensation and DNA fragmentation. To study the relationship between NPM-translocation and apoptosis, HeLa cells were treated with 0.1, 0.5, and 1 μg/mL DA for 1 hr. NPM-translocation, chromatin condensation, and DNA fragmentation then were determined at various times afterward.

Figure 5 shows the cellular localization of NPM and chromatin condensation in HeLa cells before (controls, Fig. 5A) and during the drug treatment (Fig. 5B). NPM-translocation was observed during the drug treatment. However, while the majority of cells had reverted and showed bright nucleolar fluorescence at 24 hr after the drug treatment, we found that identical cells showed chromatin condensation and NPM-translocation as highlighted by the arrows in Fig. 5C.

Figure 6 shows the histograms of LI of these cells at 0, 1, 3, 5, 7, and 24 hr after the drug treatment. No significant NPM-translocation was observed with 0.1 μg/mL DA at all

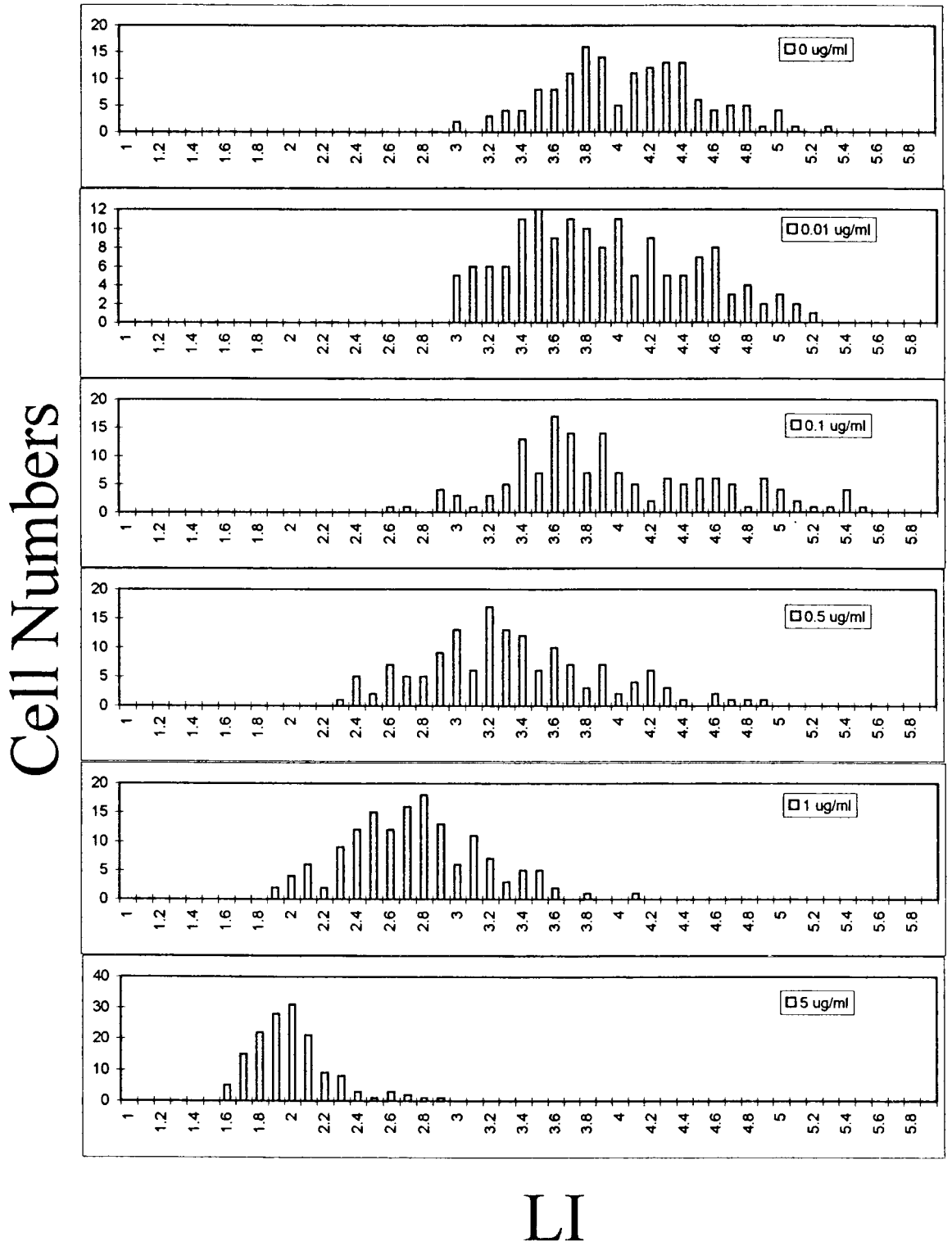

FIG. 2. Histograms of LI of K562 cells after treatments with various concentrations of DA (inserts). The data were collected from three experiments in which over 150 cells were calculated. Abscissa: LI values; ordinate: number of cells with the specific LI.

time points (Fig. 6A). With DA at 0.5 and 1 μg/mL, NPM-translocation was observed during and after the drug treatment and then gradually reverted to its normal state (Fig. 6B and C). The duration of NPM-translocation was dependent on the drug concentration. Cells treated with 1 μg/mL remained in the translocation state (LI $<$ 2.5) for about 5–6 hr (Fig. 6C), whereas in cells treated with 0.5 μg/mL, translocation lasted only 1–2 hr. Because no NPM-translocation was observed in cells treated with 0.1 μg/mL, the result also indicated that a minimum of 0.5 μg/mL DA was required for NPM-translocation.

Chromatin condensation (a characteristic of cells under-

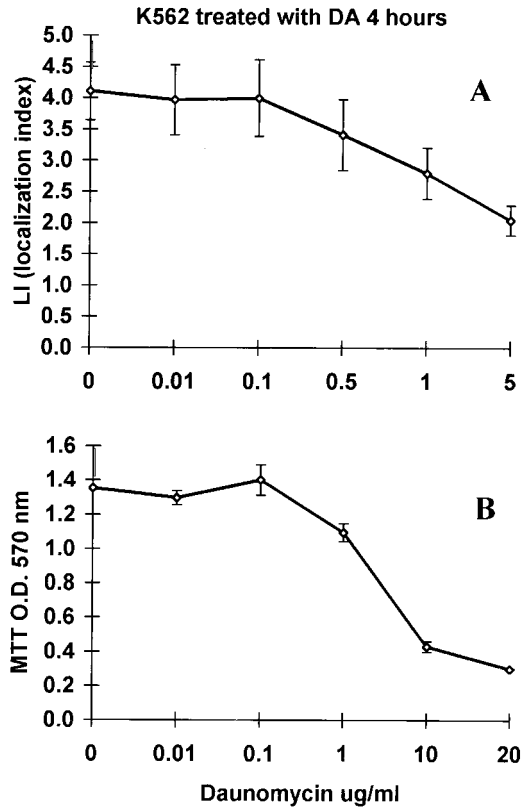

FIG. 3. Concentration–response curves of (A) localization index (LI) and (B) MTT formation in K562 cells treated with DA (4 hr). The data in A represent the average value of three experiments. Error bars are standard deviations (SD) with N = 150. The data in B represent the average of two experiments (total sample size N = 20). Error bars stand for the range of the mean.

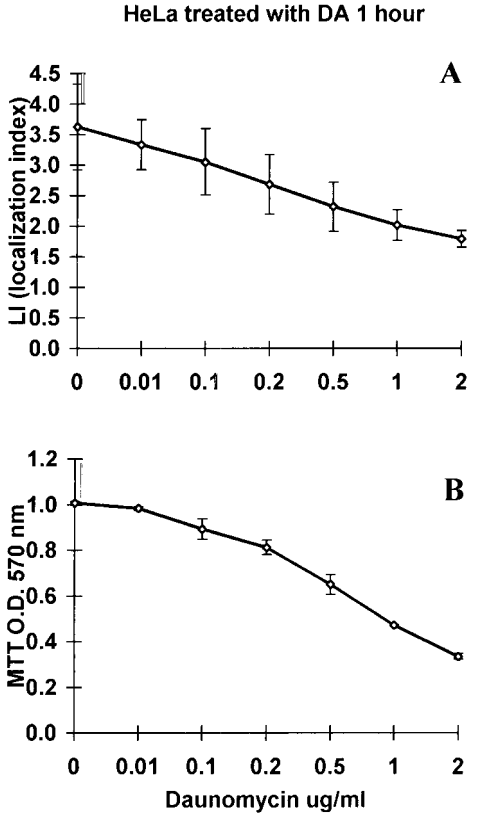

FIG. 4. Concentration–response curves of (A) localization index (LI) and (B) MTT formation in HeLa cells treated with DA (1 hr). The data in A represent the average values of three experiments. Error bars are SD with N = 150. The data in B represent the average of two experiments (total sample size N = 20). Error bars stand for the range of the mean.

going apoptosis) in cells after drug treatment was identified with the Hoechst-33258 stain (arrows in Fig. 5C). Normally, we found that about 0.1 to 0.2% of cells were apoptotic cells. However, we found a significant increase of cells with chromatin condensation beginning at 5 hr after drug treatment (Fig. 7). The increase was concentration- and time-dependent. The increase was observed with 0.5 and 1 μg/mL DA but not with 0.1 μg/mL DA. We found that the drug concentration for producing apoptotic cells coincided with the drug concentration for induction of NPM-translocation with LI < 2.5 (Fig. 6).

DNA fragmentation in drug-treated cells was analyzed at 20 hr after the drug treatment (Fig. 8) with 1.8% agarose gel electrophoresis. DNA fragmentation was not observed in control cells or cells treated with 0.1 μg/mL DA. Prominent DNA fragmentation was observed with 0.5 and 1 μg/mL DA. The drug concentrations for inducing DNA fragmentation coincided with the drug concentrations required for NPM-translocation. Based on these results and those of the MTT assay, we concluded that NPM-translocation (with LI < 2.5) correlated with the degree of apoptosis in cells induced by DA. These results suggest that NPM-translocation may represent a cytotoxic drug-induced apoptosis pathway that involves damage to the nucleolus.

DISCUSSION

Current theory suggests that apoptosis may result from many different induction pathways. In general, stresses imposed on cells, such as radiation or exposure to cytotoxic agents, are among the major causes of apoptosis [37]. Our results show that damage of nucleolar structures and functions, which imposes stresses on cells, may cause apoptosis. NPM is a major nucleolar protein in cells. There are 6×10^6 NPM molecules in a HeLa cell [38]. Dissociation of NPM from nucleoli is the initial step of nucleolar segregation [1]. This process renders the nucleolar DNA vulnerable to attack by nucleases. As a result, it may trigger the DNA repair mechanism and activate the *p53* gene and apoptosis [39]. In addition to ribosome synthesis, nucleoli are also involved in other cellular functions. It was reported that other nucleolar proteins, including topoisomerase I (DNA unwinding enzyme), Ki67 (associated with cell proliferation) and Gu (a RNA/DNA helicase) [22, 40–42] also are dissociated from nucleoli upon treatments with NPM-translocating drugs (camptothecin, actinomycin D, and DRB). Dissociation of these proteins from nucleoli affects nucleolar functions, which may indirectly induce apoptosis. For example, inhibition of topoisomerase I or II activities may

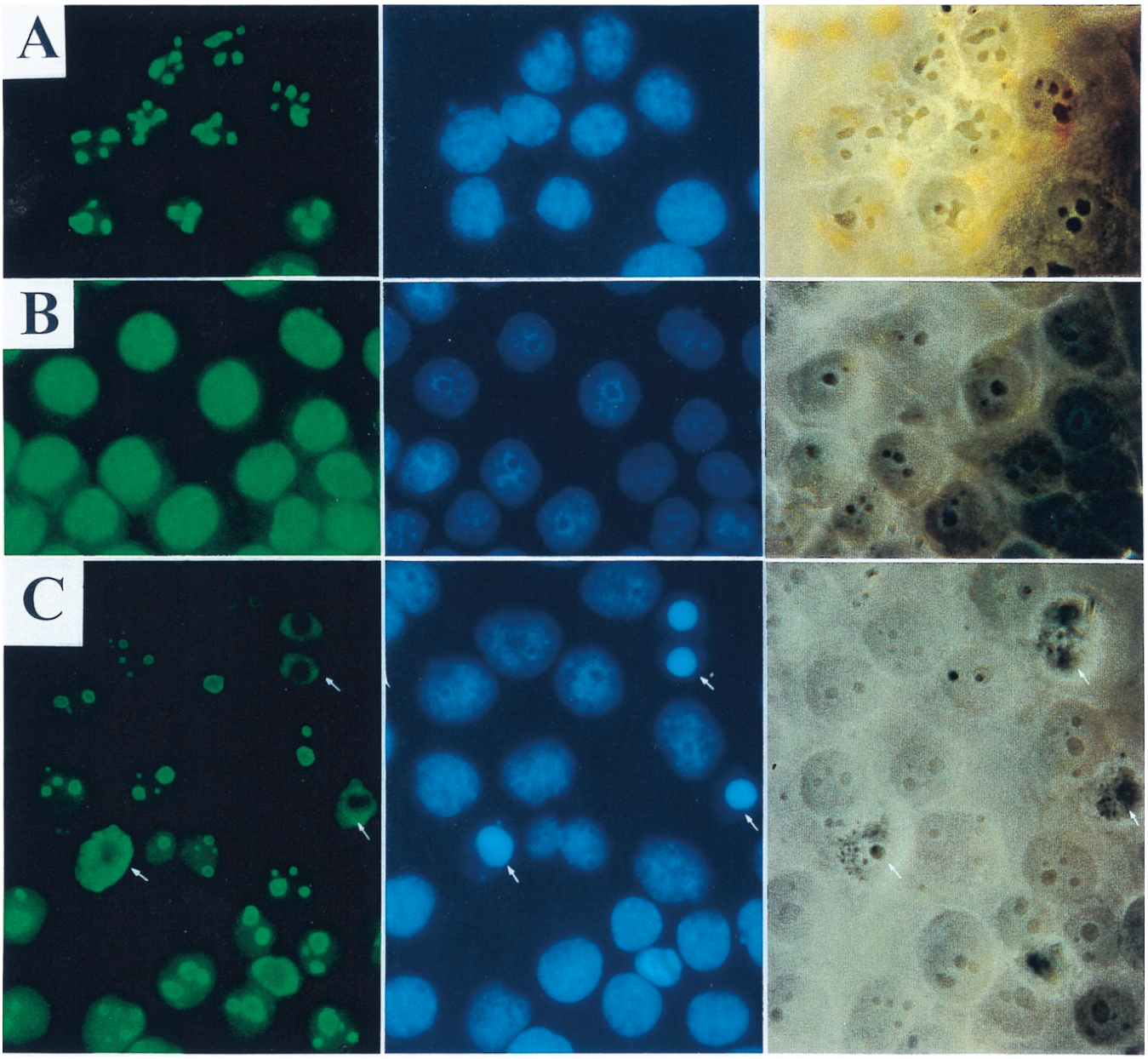

FIG. 5. NPM localization and chromatin condensation in HeLa cells treated with DA (1 μg/mL). (A) Before drug treatment; (B) during the drug treatment (1 hr); (C) 24 hr after removal of the drug. Pictures from left to right are of the same field. The picture on the left is immunofluorescence; center: Hoechst 33258-stain; right: phase contrast. Arrows in C point to apoptotic cells.

alter Bcl2 expression or change the balance of Bcl2/Bax complex formation [43] during NPM-translocation.

Our results support the idea that during NPM-translocation, an "unknown event" triggers apoptosis. Firstly, NPM-translocation occurs prior to other apoptotic events such as chromatin condensation and DNA fragmentation. Secondly, we observed a good correlation between NPM-translocation and apoptosis. We found that the degree and the duration of NPM-translocation correlated with the magnitude of apoptosis. A certain degree of NPM-translocation (LI < 2.5) was necessary for the induction of apoptosis. Given cells with NPM-translocation, the number of apoptotic cells and the magnitude of DNA fragmentation were proportional to the duration of NPM-translocation. Cells with 5 hr of NPM-translocation showed more apoptotic cells and a higher degree of DNA fragmentation than those with a brief duration of NPM-translocation (Fig. 6). The cause-effect relationship between NPM-translocation and apoptosis remains to be investigated. Nonetheless, the current results indicate that destruction of nucleolar structure and function was associated with apoptosis.

Our studies reaffirm that NPM-translocation is a cell-based assay that is potentially useful in measuring the effectiveness of DA in cancer cells. It can be used, for example, in detecting DA-resistant cells [28, 29]. Leukemia cells may be removed from leukemia patients and tested *ex vivo* for drug effectiveness [32]. The whole assay can be completed within 1 day. Since NPM-translocation is also

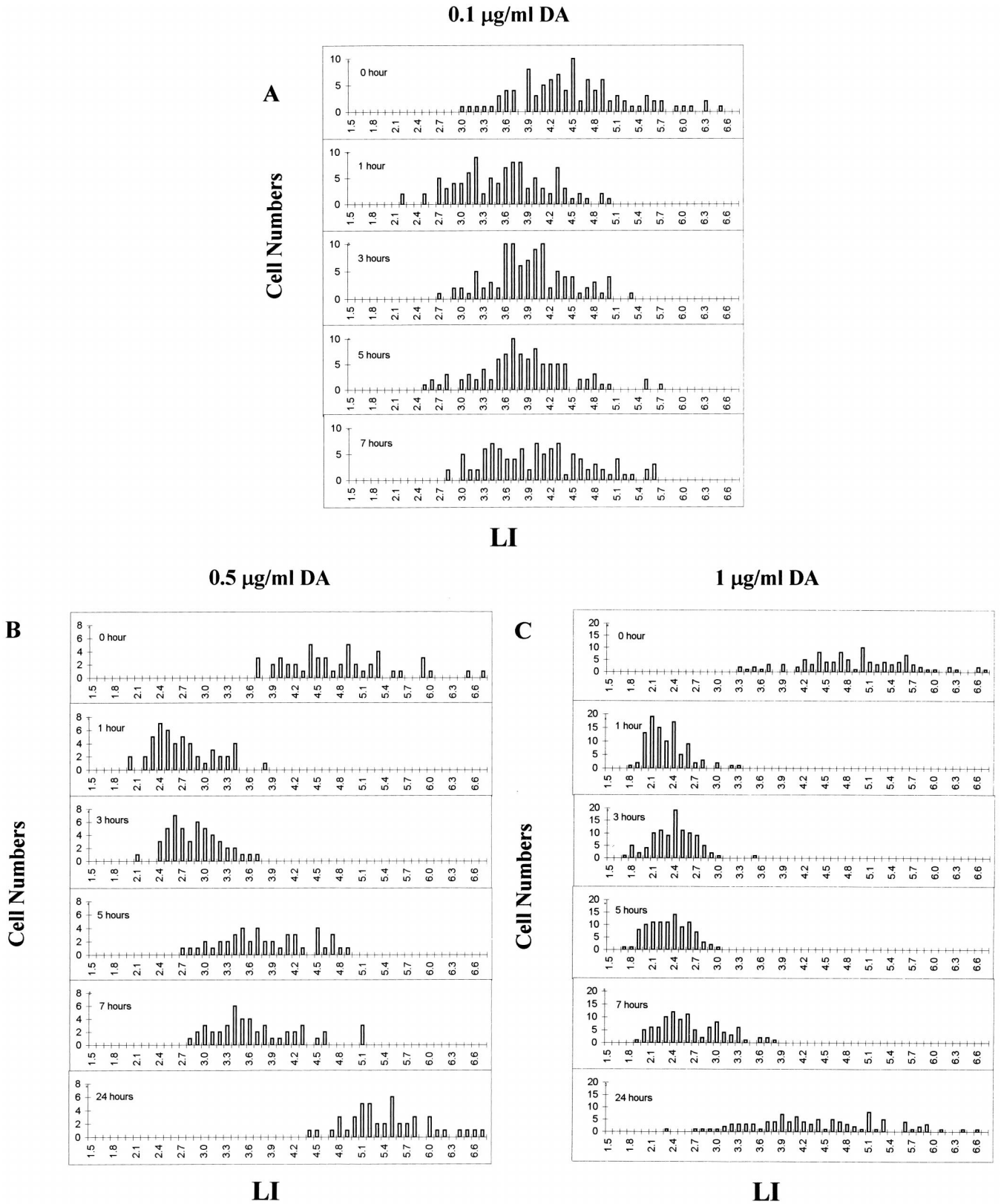

FIG. 6. Distribution of LI of HeLa cells at 0, 1, 3, 5, 7, and 24 hr after the drug treatment (1 hr) with (A) 0.1; (B) 0.5 and (C) 1 μg/mL DA. The data represent the results of a typical experiment in which about 50 cells were measured at each time point. Abscissa: LI values; ordinate: number of cells with the specific LI.

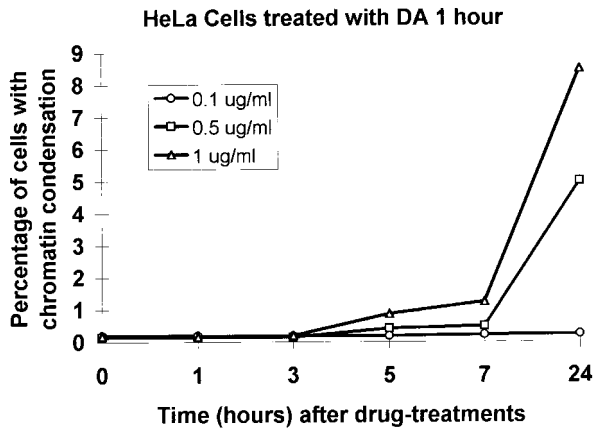

FIG. 7. Chromatin condensation in HeLa cells after drug treatment. Ordinate: percentage of cells with chromatin condensation; abscissa: time (hr) after treatment with DA. The data represent the average of three experiments in which 3000–5000 apoptotic and non-apoptotic cells were counted for each time point.

induced by other anticancer drugs including camptothecin, actinomycin D, and tiazofurin, it may also be useful in the selection of these effective drugs for an individual patient.

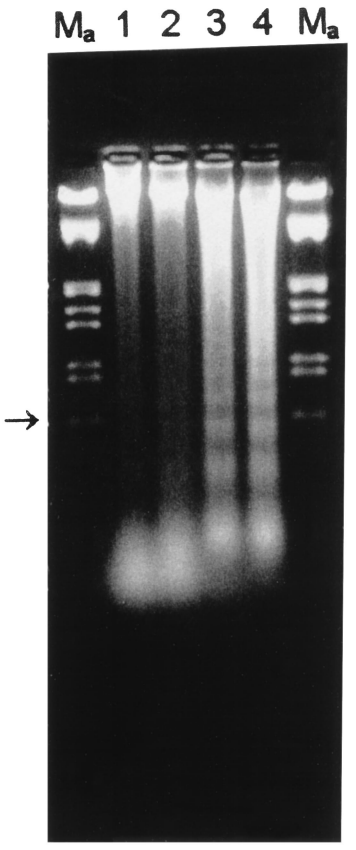

FIG. 8. DNA fragmentation in HeLa cells treated with DA. Lane 1: control; lane 2: 0.1 μg/mL; lane 3: 0.5 μg/mL; lane 4: 1 μg/mL. M_a: DNA markers (Lambda DNA/*Eco*RI + *Hin*dIII cut). The arrow indicates 564 bp.

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