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Nucleolar Protein B23 Translocation after Doxorubicin Treatment in Murine Tumor Cells[1]

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ABSTRACT

Rats bearing Novikoff hepatoma ascites cells were given i.p. injections of actinomycin D, doxorubicin, or daunorubicin. Four hours after injection, tumor cells were removed from the ascites fluid and analyzed for protein B23 translocation using an immunofluorescence technique. Bright nucleolar fluorescence was observed in untreated cells. Treatment with actinomycin D (1.25 mg/kg), doxorubicin (25 mg/kg), or daunorubicin (12.5 mg/kg) produced a uniform nucleoplasmic fluorescence. This change in immunofluorescence distribution indicated that protein B23 translocated from the nucleolus to the nucleoplasm after drug treatment. These results are an extension of previous studies with HeLa cells (Yung *et al.*, Cancer Res. *46:* 922–925, 1986).

Doxorubicin-resistant and -sensitive mouse leukemia cells (P388) were cultured in medium containing various doses of doxorubicin for 4 h, and the responsive levels of the cells to doxorubicin were compared. At 50 μg/ml doxorubicin, 86% of the doxorubicin-sensitive cells showed uniform nucleoplasmic fluorescence, and less than 2% of the cells retained nucleolar fluorescence. At this same dose, only 9% of the resistant cells showed nucleoplasmic fluorescence, and 75% of the cells retained nucleolar fluorescence. At 100 μg/ml, about 26% of the resistant cells showed translocation, in contrast to 100% of the sensitive cells that showed B23 translocation. About 57% of the resistant cells showed an intermediate effect, and about 17% of the resistant cells maintained bright nucleolar fluorescence at this dose. The resistant cells also showed less responsiveness to actinomycin D. These results suggest that identification of "B23 translocation" may be used to detect drug-resistant cells and to study the efficacy of certain antitumor agents.

INTRODUCTION

Protein B23 is a nucleolar phosphoprotein which is more abundant in tumor and growing cells than in normal resting cells. This protein is associated with preribosomal particles and is localized in the granular region of the nucleolus (1–4). Recent studies indicated that protein B23 translocates from nucleoli to the nucleoplasm during serum deprivation (5) or during actinomycin D (6) or toyocamycin treatment (7). "Protein B23 translocation" correlates with the inhibition of rRNA synthesis and processing. Inhibition of protein synthesis has no effect on B23 translocation (5, 7). Experiments using luzopeptin analogues (BBM 928 A, B, C, and D) (8, 9) with different antitumor activities (10) have shown that "B23 translocation" and inhibition of RNA synthesis correlate well with the antitumor activities of luzopeptins (11). These studies indicated that the detection of B23 translocation may be used in a rapid and simple screening method for the selection of antitumor agents which inhibit ribosomal RNA synthesis.

All of the previous studies used HeLa cells that were grown in culture medium. To extend these studies to various cell types and culture conditions, this study employed Novikoff hepatoma cells that were grown in rat ascites fluid and mouse leukemia cells (P388) that were grown in suspension.

MATERIALS AND METHODS

Chemicals. Actinomycin D, Adriamycin (doxorubicin), and daunorubicin were purchased from Sigma Chemical Co., St. Louis, MO. EM-grade *p*-formaldehyde was purchased from Polysciences, Inc., Warrington, PA. Other chemicals were of reagent grade.

Cells. (A) HeLa S3 cells were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum, glutamine, and antibiotics (100 units/ml penicillin, 100 μg/ml of streptomycin) in a 5% CO_2-humidified incubator at 37°C. For immunofluorescence studies, cells were grown on slides in a Petri dish.

(B) Novikoff hepatoma ascites cells were implanted i.p. in adult male albino Holtzman rats (200 g) and were left to grow for 6 days. Actinomycin D (50 μg/ml in 50% ethanol), daunorubicin (20 mg/ml in 0.9% NaCl), or doxorubicin (20 mg/ml in 0.9% NaCl) was injected i.p. into the hepatoma cell-bearing rats and left for 4 h. Hepatoma cells were then removed from the ascites fluid and analyzed.

(C) Mouse leukemia cells (P388 D_1, ATCC CCL-46; American Type Culture Collection) were grown in suspension in Fischer's medium for leukemia cells of mice (GIBCO Laboratories 430-1100) supplemented with 10% horse serum and 2 mM glutamine. Doxorubicin-resistant P388 cells were obtained from Dr. B. Long at the Bristol-Baylor Laboratory and were grown in suspension in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum, 2 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin, and 25 μM 2-mercaptoethanol. Both cell lines were grown at a cell density of approximately 6 × 10,000 cells/ml and were split twice weekly. Cells were split (diluted with fresh medium) the day before the drugs were added. Doxorubicin or actinomycin D was suspended in deionized water or 50% ethanol, respectively. Various amounts of drugs were added to the cell culture, and the cells were incubated at 37°C for an additional 4 h.

Slide Preparation. HeLa cells (grown on slides) were fixed immediately after incubation with the drugs. Novikoff cells and mouse leukemia cells were cytocentrifuged onto clean slides. The slides were fixed first in 2% *p*-formaldehyde in PBS[4] (8.45 mM Na_2HPO_4, 1.6 mM NaH_2PO_4, 145 mM NaCl) for 20 min at room temperature and then were washed 3 times in PBS with gentle shaking for 5 min/wash. The slides were fixed in acetone at −20°C for 3 min, rinsed briefly 4 times in PBS, and blown dry with cool air.

Immunofluorescence Assay. The first antibody (a monoclonal IgG that recognizes only protein B23 in total HeLa cell extracts) was diluted 1:16 in PBS (to a concentration of 3.4 mg/ml) and applied to the slides. For an experimental control, NS-1 supernatant (Cappel 0101-2407) or PBS alone was added to some slides to determine whether there would be any background fluorescence. The slides were incubated in a moist chamber overnight at 4°C. The slides were washed 4 times, for 15 min/wash, in PBS with gentle shaking. The slides were blown dry with cool air. The second antibody (Cappel 1611-3151, 1 mg/ml fluorescein-conjugated goat anti-mouse IgG, heavy- and light-chains specific, affinity purified) was diluted 1/20 in PBS and applied to the slides. The slides were incubated in a moist chamber for 1 h at room temperature. The slides were washed 4 times with PBS, blown dry, and mounted in 50% glycerol in PBS (pH 9). The slides were viewed under a fluorescence microscope.

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[4] The abbreviations used are: PBS, phosphate-buffered saline; RNP, ribonucleoprotein particles.

Enzyme-linked Immunosorbent Assay. The enzyme linked immunosorbent assay was carried out as described by Yung *et al.* (7). Monoclonal antibody to protein B23 was diluted 1:20,000 in PBS before use.

[^{3}H]Uridine Incorporation Determination. Inhibition of RNA synthesis in cells after drug treatment was determined following the method described previously (11).

RESULTS

Table 1 shows the results of the immunofluorescence studies of Novikoff hepatoma cells stained with protein B23 antibody. Control cells with no drug treatment showed bright nucleolar fluorescence with little or no nuclear fluorescence. Cells with this type of fluorescence pattern were classified as A. After actinomycin D (1.25 mg/kg), doxorubicin (25 mg/kg), or daunorubicin (12.5 mg/kg) treatment for 4 h, uniform nucleoplasmic fluorescence, instead of nucleolar fluorescence, was observed. Cells with this fluorescence pattern were classified as C (see Materials and Methods). Fig. 1 shows the immunofluorescence and phase-contrast photomicrographs of Novikoff hepatoma cells after treatment with actinomycin D. Phase-contrast nucleoli were observed in the control and the drug-treated cells. When a reduced amount of drug or a shorter incubation time was used, weak nuclear and nucleolar fluorescence were observed. Cells with this fluorescence pattern were classified as B. Table 1 also shows the resulting protein B23 translocation in Novikoff hepatoma cells after injection of various doses of drugs. A dose-dependent B23 translocation was observed. The minimum doses of drugs that are needed to produce protein B23 translocation are 1.25 mg/kg of actinomycin D, 25 mg/kg of doxorubicin, and 12.5 mg/kg of daunorubicin.

Table 2 shows the resulting immunofluorescence patterns and inhibition of RNA synthesis of HeLa cells after treatment with daunorubicin. At a dose of 0.5 μg/ml, both nuclear and nucleolar fluorescence were observed (B pattern). At doses of 1 μg/ml or higher, RNA synthesis inhibition of more than 60% and a uniform nucleoplasmic fluorescence were observed. These results agree with previous studies using other antitumor agents (6, 7, 11).

Fig. 2 shows the immunofluorescence study on mouse leukemia cells (P388). Both doxorubicin-sensitive and -resistant cells were used in these studies. Bright nucleolar fluorescence was observed in both doxorubicin-sensitive and -resistant cells before drug treatment (Fig. 2, *A* and *E*). A gradual shifting of nucleolar to nuclear fluorescence in both sensitive and resistant cells was observed with increasing doses of doxorubicin (Fig. 2). The resistant cells required a higher dose of drug to produce B23 translocation. Table 3 summarizes the quantitative analysis of these studies. At 10 μg/ml of doxorubicin, 87% of the sensitive cells began to show B23 translocation with both nuclear and nucleolar fluorescence (B pattern). A majority of the resistant cells (87%) retained bright nucleolar fluorescence (A pattern) at this dose. When the doxorubicin-sensitive cells were treated with 50 μg/ml of doxorubicin, complete translocation (C pattern) was observed in 86% of the cells (Fig. 2*C*), and only 2% of the cells showed nucleolar fluorescence (Table 3). When a similar dose of doxorubicin was applied to the resistant cells, only 9% of the cells showed complete translocation while 75%

Table 1 *"B23 translocation" on Novikoff hepatoma ascites cells*

Novikoff hepatoma cells were grown in rat ascites fluid. Rats were given i.p. injections of antitumor drugs and were left for 4 h. Tumor cells were removed, fixed, and immunostained with anti-B23 antibody.

Drug	Dose (mg/kg)	% of cells in each translocation pattern[a]		
		A	B	C
Actinomycin D	0.000	100	0	0
	0.300	99	1	0
	0.625	91	9	0
	1.250	0	79	21
	2.500	0	16	84
Doxorubicin	0.000	100	0	0
	25.000[b]	0	0	100
Daunomycin	0.000	100	0	0
	2.500	84	16	0
	7.500	22	66	12
	12.500	3	9	88
	20.000	0	0	100

[a] A, cells showed bright nucleolar fluorescence with little or no nuclear fluorescence; B, both nuclear and nucleolar fluorescence were observed in cells; C, cells showed homogeneous nuclear fluorescence with no distinct nucleolar fluorescence.

[b] It was noted that at this dose of drug a weak greenish autofluorescence (due to the drug itself) was observed. However, this background fluorescence did not affect the detection of the bright yellowish-green fluorescein isothiocyanate immunofluorescence conjugated to the second antibody.

Table 2 *"B23 translocation" in HeLa cells treated with daunorubicin*

HeLa cells were cultured on slides. Doses of daunorubicin were added to the culture medium. The cellular incorporation of [^{3}H]uridine and the localization of protein B23 were then determined after 4 h of incubation. Viability of the cells as determined by trypan blue dye exclusion was over 95% under these conditions.

Dose (μg/ml)	RNA synthesis inhibition (%)[a]	% of cells in each translocation pattern[b]		
		A	B	C
0.01	ND[c]	100	0	0
0.05	ND	97	2	1
0.50	42.1	0	95	5
1.00	61.6	0	0	100
2.00	73.2	0	0	100
10.00	91.8	0	0	100

[a] Percentage of RNA synthesis inhibition was calculated as

$$100 \times \left(1 - \frac{[^3\text{H}]\text{uridine uptake in treated cells}}{[^3\text{H}]\text{uridine uptake in control cells}}\right)$$

[b] Classification of immunofluorescence pattern is defined in the footnotes of Table 1.

[c] ND, not determined.

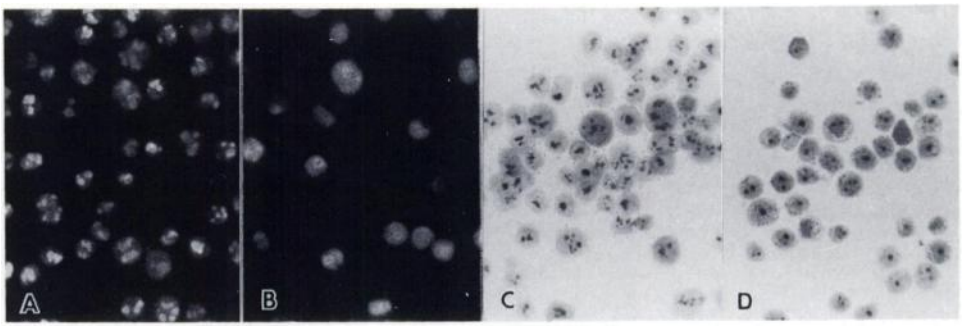

Fig. 1. Phase-contrast and immunofluorescence photomicrographs of Novikoff hepatoma cells after treatment with actinomycin D. *A* and *C*, control cells without drug treatment. *B* and *D*, cells treated with actinomycin D (1.25 mg/kg) for 4 h. *A* and *B*, immunostained with antibody. *C* and *D*, phase-contrast pictures of cell stained with azure C. Bright nucleolar fluorescence was observed in control cells (*A*); only nuclear fluorescence was observed in drug-treated cells (*B*). Phase-contrast nucleoli are visible in both the control (*C*) and drug-treated cells (*D*).

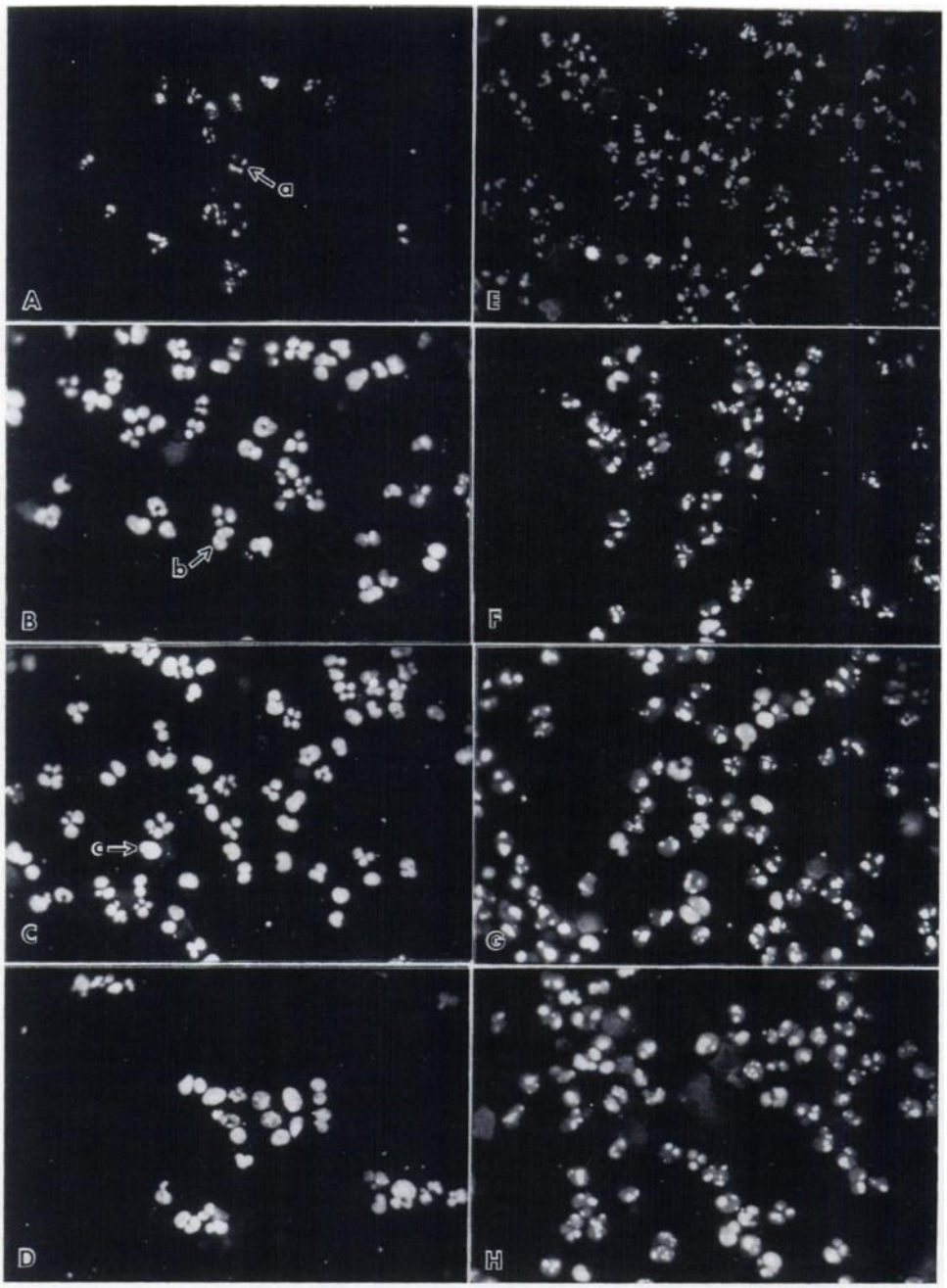

Fig. 2. Effects of doxorubicin on doxorubicin-sensitive and -resistant mouse leukemia (P388) cells. Doxorubicin-sensitive and -resistant cells were grown in suspension in medium containing various doses of doxorubicin (as described in "Materials and Methods") for 4 h. Cells were cytocentrifuged onto glass slides, fixed, and immunostained with anti-B23 antibody. *A* to *D*, doxorubicin-sensitive cells; *E* to *H*, doxorubicin-resistant cells; *A* and *E*, control cells without drug treatment; *B* and *F*, 20 μg/ml; *C* and *G*, 50 μg/ml; *D* and *H*, 100 μg/ml. *Arrow a*, bright nucleolar fluorescence; *arrow b*, both nuclear and nucleolar fluorescence; *arrow c*, uniform nucleoplasmic fluorescence.

Table 3 *Comparison of B23 translocation in doxorubicin-sensitive and -resistant mouse leukemia (P388) cells*

Mouse leukemia cells (P388) were grown in suspension in medium containing various doses of doxorubicin or actinomycin D for 4 h. Cells were cytocentrifuged onto slides, fixed, and immunostained with B23 antibody.

Drug	Dose (μg/ml)	% of cells in each translocation pattern[a]					
		Sensitive cells			Resistant cells		
		A	B	C	A	B	C
Doxorubicin	0.0	100	0	0	100	0	0
	1.0	100	0	0	93	7	0
	5.0	99	1	0	94	7	0
	10.0	13	87	0	87	13	0
	20.0	1	73	26	78	21	0
	50.0	2	12	86	75	16	9
	100.0	0	0	100	17	57	26
Actinomycin D	0.0	100	0	0	100	0	0
	0.01	1	74	25	98	2	0
	0.1	0	31	69	0	87	13
	1.0	0	3	97	0	6	94
	10.0	0	0	100	0	1	99

[a] About 500 cells were counted. Classification of immunofluorescence is defined in the footnotes of Table 1.

of cells retained bright nucleolar fluorescence (Table 3). At 100 μg/ml of doxorubicin, all of the sensitive cells showed completely nuclear fluorescence while only 26% of the resistant cells showed complete translocation. Of the resistant cells 17% maintained bright nucleolar fluorescence (Fig. 2). These results indicate that resistant cells are less responsive to doxorubicin than sensitive cells.

Table 3 also shows the immunofluorescence of the sensitive and resistant cells after incubation with actinomycin D. The resistant cells were also less responsive to actinomycin D. At 0.1 μg/ml, 69% of the sensitive cells (Table 3) showed translocation, but only 13% of the resistant cells responded to actinomycin D treatment.

DISCUSSION

All of our previous studies (5–7, 11) on B23 translocation were done on HeLa cells that were grown on glass slides. The present studies indicate that protein B23 translocation is also

observed in Novikoff hepatoma cells grown in the ascites fluid of rats after treatments with actinomycin D, doxorubicin, and daunorubicin. Doxorubicin and daunorubicin are about 10 times less potent than actinomycin D in producing B23 translocation (Table 1).

There is a significant difference in dose response of B23 translocation between doxorubicin-resistant and -sensitive cells. The immunofluorescence pattern of the sensitive cells at 20 μg/ml of doxorubicin is similar to the pattern of the resistant cells at 100 μg/ml, thus indicating that the resistant cells are at least 5 times more doxorubicin resistant. While the majority of the doxorubicin-sensitive cells responded to the drug uniformly, one fifth of the resistant cells (17%) retained bright nucleolar fluorescence (Fig. 2*H*), even at high doses of doxorubicin (100 μg/ml). These results indicate that there is heterogeneity among the resistant cells in responding to doxorubicin.

The cause-effect relationship of B23 translocation and antitumor activity is not known. Our previous studies (7, 11) indicated that protein B23 binds to certain elements in the nucleolus (pre-rRNA, proteins, or matrix structure) and plays an essential role in ribosome synthesis. One hypothesis is that, when cells are treated with antibiotics (actinomycin D, toyocamycin, and leuzopeptin), RNA synthesis and RNP processing are inhibited, and protein B23 loses its binding target in the nucleolus and diffuses into the nucleoplasm. Studies by Fields *et al.* (12) indicated that protein B23 is associated with the internal nuclear matrix of rat liver cells. Our previous studies (7) found that protein B23 is associated with both the 55S and 80S RNPs. The 55S RNP is a precursor of the large ribosomal subunit. It is possible that protein B23 is involved with the transport of large ribosomal subunits from the nucleolus to the nucleoplasm through the internal nuclear matrix network.

In conclusion, B23 translocation, as observed by immunofluorescence, may be used for studying the drug resistance of tumor cells and the efficacy of certain antitumor agents.

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