Enhanced Sensitivity of Nucleoli in Human Proliferating Cells to Inhibition of Protein Synthesis with Anisomycin

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We describe the reaction of nuclei in cultured human cells from different tissues to inhibition of total protein synthesis with anisomycin – ribotoxin, which is now considered as a potential antitumor drug. It was shown that nucleoli in sensitive cells demonstrate typical reaction: under the action of the inhibitor, labile nucleolar protein, a component of RNA polymerase I transcription complex (A3 antigen), rapidly migrates from the nucleolus to numerous discrete foci in the nucleoplasm. These changes are specific for translation suppression and are not induced by other influences on the cells. Migration of A3 antigen into the nucleoplasm manifests primarily in cells at the stage of DNA replication and is absent in resting cells. These results suggest that localization of A3 antigen can be a marker of artificial suppression of translation in proliferating human cells *in vitro*.

Key Words: *anisomycin; nucleoli; A3 antigen; replication; cultured human cells*

The nucleolus is the main structural domain of the cell nucleus responsible for transcription of ribosomal genes (rDNA), processing of pre-rRNA into mature rRNA (18S, 5.8S, and 28S), and assembly of ribosomal particles. Nucleoli of mammalian cells are characterized by high plasticity manifesting in variation of their size, number, morphology, and functional activity under the effect of various factors. The state of the nucleoli changes in different stages of the cell cycle, during cell activation and proliferation, or, on the contrary, under conditions of exhaustion of growth factors in the medium and exposure to stress factors leading to cell death [6]. These factors include inhibition of transcription, inhibition of kinases phosphorylating the major nucleolar proteins [13], UV radiation [9], and exposure to heavy metals salts [4]. The nucleolus responds specifically to most of these exposures. Thus, inhibition of transcription leads to spatial separation of its main structural components, segregation [13]. Inhibition of the main protein kinases (*e.g.* kasein kinase 2) is associated with loosening of nucleolar packing up to appearance of single transcribed genes [13]. The effect of mercury chloride leads to typical migration of fibrillarin, the main protein of early pre-rRNA processing, from the nucleolus into the cytoplasm [4].

Inhibition of summary protein synthesis with antibiotic anisomycin currently used in many studies of long-term memory mechanisms and a potential antitumor drug and is an effective inductor of cell death [10,11]. Anisomycin inhibits translation by binding to a site of 28S rRNA located in peptidyltransferase center of the ribosome. It should be noted that toxic effects of anisomycin appear only upon its contact with functionally active ribosomes and is accompanied by activation of stress kinases, which differs it from other translation inhibitors cycloheximide, puromycin, and emetine [7]. However, at the cytological level, the reaction of cells with different activity of ribosomes to anisomycin remains unclear. The reaction of the nucleolus, the only place of 28S rRNA synthesis, is also poorly studied [6]. We previously showed that

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anisomycin changes the localization of A3 antigen, a nucleolar protein entering the composition of RNA polymerase I transcription complex, in HeLa cells [1]. However, the question whether the nucleolar response to anisomycin is universal for all types of cells was never studied.

Here we studied the reaction of nucleoli in human cells with different proliferative activity and from different tissues to incubation with anisomycin.

MATERIALS AND METHODS

Human cells of different strains (Table 1) were cultured in DMEM medium (PanEko) containing 10% embryonic cattle serum (ECS, HyClone) and penicillin and streptomycin in standard concentrations (100 U/ml) at 37°C and 5% CO_2. Monolayer cultures were grown on coveslips and were used on day 2 after seeding, *i.e.* during the exponential phase of growth. Skin fibroblasts were synchronized in G_0-period by 72-h incubation in a medium containing 0.1% ECS. B-lymphoblastoid cells (Ramos strain) were grown in a suspension in a mixture of DMEM and F12 (1:1, PanEko) containing 10% ECS and standard antibiotics. Human peripheral blood lymphocytes were isolated and activated for proliferation with phytohemagglutinin for 48 h as described elsewhere [5]. Ramos cells and resting and activated lymphocytes were placed on poly-L-lysine-precoated coverslips. Anisomycin (Sigma) was added to the culture medium in a final concentration of 100 μM for 2 h. We previously showed that changes in the localization of A3 antigen in HeLa cells were most pronounced under these conditions [1]. The cells were fixed in 2% paraformaldehyde in phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.2-7.4) for 20 min at room temperature, treated with 0.1% Triton X-100 for 10 min, and stained with A3 antibodies [1] and then with FITC-conjugated antibodies to murine immunoglobulins (Sigma).

S-phase cells were detected using bromodeoxyuridine (BDU; Sigma) as described previously [2]. BDU in a final concentration of 20 μM was added to the culture medium for 30 min before cell fixation with paraformaldehyde. DNA was hydrolyzed with 1 n HCl for 10 min at room temperature. After thorough washout in phosphate buffered saline, the cells were incubated with mouse monoclonal antibodies to BDU (Sigma) conjugated with Texas red. For simultaneous detection of A3 antigen and BDU, the cells after hydrolysis were incubated with A3 antibodies and then with FITC-conjugated antibodies to mouse immunoglobulins, fixed in 2% paraformaldehyde for 10 min, and incubated with antibodies to BDU (Sigma) and then with antibodies to mouse immunoglobulin conjugated with Texas red. Additional fixation with paraformaldehyde stabilized with antibody–antigen complex formed in the first immunolabeling reaction and excluded the interaction between Texas red-conjugated antibodies with A3 antigen. The preparations were embedded in Moviol (Calbiochem) and examined under an Axiovert 200 epifuorescent microscope equipped with 100x Plan-Neofluar/1.3 Ph objective and the corresponding filter sets. The images were grabbed with a CoolSnap$_{cf}$ 13-bit monochrome camera (RoperScientific) and processed using AdobePhotoshop CS3 10.0 software. At least 500 cells per point were analyzed. The significance of differences between the means was evaluated using Student *t* test; the differences were significant at $p \leq 0.05$.

RESULTS

All cultures except skin fibroblasts synchronized in G_0-period by serum depletion in the medium and peripheral blood lymphocytes from healthy donors (cells in the natural state of proliferative rest [5]) contained proliferating cells (Table 1).

A typical variant of HEp-2 cell staining with antibodies to A3 antigen and the general appearance of cells in phase contrast regimen are presented in Fig. 1, *a, b*). A3 antigen forms grain clusters of different size located exclusively above the nucleolar area. No signals were found in the nucleoplasm and cytoplasm. Similar pattern of A3 antigen distribution was observed in other cell types except resting lymphocytes, whose nucleoli contained only one large granule corresponding to the location of RNA polymerase I and argentophilic proteins (data not shown [1]). Resting fibroblasts contained lower number of granules in the nuclei (<30) compared to HEp-2 and HeLa cells (up to 100).

Incubation of HEp-2 cells with anisomycin led to appreciable changes in the localization of A3 antigen in many cell nuclei (Fig. 1, *c, d*). In these nuclei, A3 antigen was detected not only in the nucleolus, but also in the nucleoplasm in the form of numerous discrete foci. In some atypical nuclei, predominant localization of A3 antigen above the nucleoli was retained, while in others in was evenly distributed over the nucleus (Fig. 1, *c*). It can be hypothesized that these patterns of A3 antigen distribution reflect successive stages of its dislocation from the nucleolus into the nucleus. Apart from nuclei containing A3 antigen in the nucleoplasm, cells not differing from the control by the staining pattern were seen; A3 antigen was seen in them only above the nucleoli (Fig. 1, *a*, *b*). It should be noted that changes in A3 antigen localization were not accompanied by appreciable changes in cell and nucleus morphology or nucleolus disintegration (Fig. 1, *d*).

Changes in the localization of A3 antigen induced by anisomycin were previously described for HeLa

TABLE 1. Cultures of Human Cells Used in Experiments (*M*±*m*)

Cells	S-phase cells in the control, %	Cells with abnormal localization of A3 antigen after incubation with 100 μM anisomycin, %
HEp-2 (laryngeal carcinoma)	40±5	32±9
HeLa (cervical carcinoma)	43±11	36±10
Ramos (B-lymphoblastoid strain)	32±10	17±9
Normal skin fibroblasts, asynchronous culture	25±5	12±5
Normal skin fibroblasts synchronized in G_0-period	0	0
Inactive lymphocytes (G_0-period)	0	0
Peripheral blood lymphocytes 48 h after activation with phytohemagglutinin	25±8	13±7

Note. We analyzed 500 cells in 3 independent experiments for each experimental point. The mean percent of S-phase cells and cells with abnormal localization of A3 antigen in different cell cultures did not differ significantly ($p>0.05$).

cells [1] belonging similarly to HEp-2 cells to tumor cells. To evaluate the response of A3 antigen to anisomycin in other cell types, including non-tumor cells, we used skin fibroblasts and lymphocytes isolated from healthy donors. Analysis of the obtained findings showed that incubation with anisomycin leads to the appearance of abnormal cells in all proliferating cultures (HEp-2, HeLa, Ramos, asynchronous skin fibroblasts, and lymphocytes activated for proliferation). In resting cells (fibroblasts and lymphocytes at the state of proliferative rest), no changes in the localization of A3 antigen were observed after incubation with actinomycin (Table 1). These observations drove us to the following conclusions. First, migration of A3 antigen from the nucleolus into the nucleus induced by anisomycin was not characteristic of tumor cells only (HEp-2, HeLa, Ramos), but was also seen in the nucleoli of normal cells (skin fibroblasts and lymphocytes). Second, anisomycin induces changes in the localization of A3 antigen only in cultures containing proliferating cells. The reaction of nuclei was different in different cells (Fig. 1, *c, d*).

Heterogeneity of the nucleolar response in proliferating cultures suggests that the nucleoli of S-phase cells are most sensitive to translation inhibition. To test this hypothesis, HeLa cells were incubated with anisomycin for 1.5 h with anisomycin and then for 30 min with anisomycin and DNA synthesis precursor BDU. A3 antigen and incorporated precursor were detected using A3 antibodies and anti-BDU antibodies (Fig. 1, *e, f*). Comparison of these pictures showed that the nuclei containing A3 in the nucleoplasm were labeled with BDU, but the precursor did not incorporate into nuclei with normal localization of A3 antigen. The reverse regularity was also true: in nuclei incorporating the precursor, the A3 antigen was present in the nucleoplasm, while in unlabeled nuclei it was located only above the nucleolus. These observations showed that nucleoli of cells at the stage of DNA replication were most sensitive to translation inhibition. Most HeLa cells with A3 antigen in the nucleoplasm (about 90%) were labeled with BDU, *i.e.* were in the S-period of the cell cycle (Table 2). In most cells beyond the S-phase (about 90%), A3 antigen was located only in the nucleoli. Similar conclusion can be made from the data that the relative content of cells with active DNA replication in HeLa and HEp-2 cultures was 43 and 40%, respectively, while the relative content of

TABLE 2. Increased sensitivity of nucleoli in S-phase HeLa cells to translation inhibition with anisomycin (100 μM, 2 h; *M*±*m*)

Cell type	Relative content of cells, %	
	not labeled with BDU	labeled with BDU
Cells with unchanged localization of A3 antigen	9±2	91±10*
Cells containing A3 antigen only in nucleoli	89±8	11±2*

Note. Total number of cells with changed and normal localization of A3 antigen was taken as 100%. The experiment was repeated twice; at least 500 cells were analyzed in each case. *$p<0.001$ compared to cells not labeled with BDU.

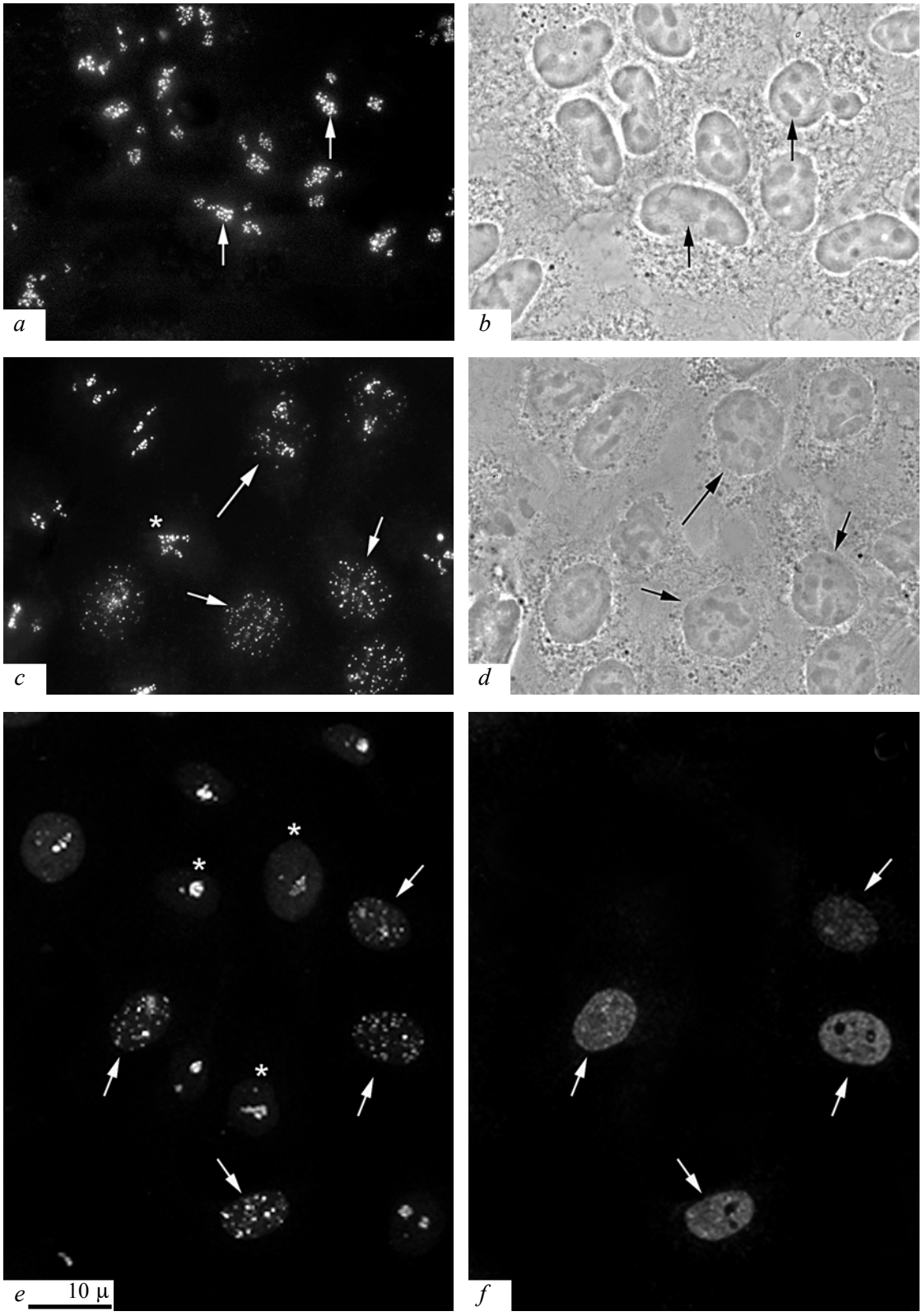

Fig. 1. Immunocytochemical detection of A3 antigen in HEp-2 (*a-d*) and HeLa cells (*e, f*) in the control (*a, b*) and after treatment with 100 μM anisomycin (*c-f*); *e* and *f*: co-localization of A3 antigen with DNA synthesis precursor BDU. For *a*, *b*: arrows show nucleoli. For *c, d*: asterics show nuclei with A3 antigen grain clusters only above the nucleoli; short arrows show nuclei with A3 antigen grains above the nucleoli and in the nucleoplasm; long arrows show nuclei with even distribution of the antigen. For *e, f*: arrows show nuclei with A3 antigen in the nucleoplasm and positive BDU labeling; asterics show nuclei with A3 antigen localization only above nucleoli and not incorporating BDU.

cells with abnormal localization of A3 antigen was 36 and 32%, respectively (Table 1). This incomplete coincidence between the number of abnormal cells and S-phase cells is most likely due to partial inhibition of replication, which is a side effect of anisomycin-induced suppression of protein synthesis [7, 9,11]. Moreover, it can result from difficulties in interpretation of A3 antigen location in individual nuclei, because single granules located outside the nucleolar clusters (Fig. 1, *e*) can correspond to either small nucleoli, or initial stages of A3 antigen migration from the nucleolus to the nuclei.

The mechanisms underlying enhanced sensitivity of the nucleoli in S-phase cells to inhibition of total protein synthesis leading to changes in the localization of A3 antigen remain unknown. The closest analog of the observed phenomenon is migration of UBF protein, a specific RNA polymerase I co-factor, observed during infection of HeLa cells with adenovirus [8] or herpesvirus [12]. In both cases, UBF migrated from the nucleolus into discrete nuclear loci coinciding with viral DNA replication sites. Migration of UBF was observed also during exposure of HeLa cells with translation inhibitor ementine [3], but the correlation between the replication and atypical localization of UBF was not described. Taking into account the fact the A3 antigen is a component of RNA polymerase I complex, an enzyme participating in rRNA synthesis, we can hypothesize that under conditions of translation inhibition it starts to perform a new function related to DNA replication.

As was mentioned above, the nucleolus due to its high plasticity often rapidly and specifically responds to external influences. However, only suppression of translation induces migration of components of RNA polymerase I complex to discrete complexes in the nucleoplasm. This suggests that the pattern of A3 antigen distribution allows detection of protein inhibition signs at the cellular level.

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REFERENCES

1. A. A. Grigoryev, T. I. Bulycheva, E. V. Sheval', *et al., Thitologiya*, **50**, 338-346 (2008).
2. V. V. Gurchenkov, M. A. Polzikov, K. Magoulas, *et al., Bioorgan. Khimiya*, **31**, 578-585 (2005).
3. O. Yu. Smirnova, A. V. Mishina, and O. V. Zatsepina, *Thitologiya*, **45**, 1179-1187 (2003).
4. M. Chen, A. von Mikecz, *Ann. N. Y. Acad. Sci*., **1051**, 382-389 (2005).
5. N. N. Dergunova, T. I. Bulycheva, E. G. Artemenko, *et al., Immunol Lett*., **83**, No. 1, 67-72 (2002).
6. S. Ferreira-Cerca and E. Hurt, *Nature*, **459**, 46-47 (2009).
7. M. S. Iordanov, D. Pribnow, J. L. Magun, *et al*., *Mol. Cell. Biol*., **17**, No. 6, 3373-3381 (1997).
8. F. J. Lawrence, B. McStay, and D. A. Matthews, *J. Cell Sci.*, **119**, Pt. 12, 2621-2631 (2006).
9. C. P. Rubbi and J. Milner, *EMBO J.*, **22**, No. 22, 6068-6077 (2003).
10. J. W. Rüdy, J. C. Biedenkapp, J. Moineau, and K. Bolding, *Learn. Mem*., **13**, No. 1, 1-3 (2006).
11. S. Ruller, C. Stahl, G. Kohler, *et al., Clin. Cancer Res*., **5**, No. 10, 2714-2725 (1999).
12. N. D. Stow, V. C. Evans, and D. A. Matthews, *J. Gen. Virol.,* **90**, Pt. 1, 69-73 (2009).
13. O. V. Zatsepina, R. Voit, I. Grummt, *et al., Chromosoma*, **102**, 599-282 (1993).