EXPERIMENTAL BIOLOGY

Changes in the Status of Nucleolus during Long-Term Culturing of Human HeLa Cells

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Translated from B*yulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 144, No. 9, pp. 321-324, September, 2007
Original article submitted February 12, 2007

Changes in the immunocytochemical status of the nucleoli during long-term (6-8 months) *in vitro* culturing of HeLa (carcinoma of the cervix uteri) cells were described using new A3 monoclonal antibodies selectively reacting with human cell nucleoli. The appearance of cells with abnormal location of A3 antigen was paralleled by a significant increase of culture sensitivity to some external factors (protein synthesis inhibition and oxidative stress). The data indicate that location of one of the nucleolar antigens is an indicator of the qualitative status of HeLa cells in the culture.

Key Words: *HeLa cells; nucleolus; immunocytochemistry; apoptosis*

Long-term culturing of cells can lead to changes in their initial characteristics, including decrease in growth rate (prolongation of population doubling time) and sensitivity to external factors, so-called "aging" of cell culture. Detection of other objective signs of "aging" culture is practically interesting and important for studies of the molecular mechanisms of cell variability. Status of the nucleolus (the main structural domain of the cell nucleus) is interesting in this respect. The nucleolus is one of the most plastic cell organelles; its status depends on various factors, including culturing conditions.

Here we studied changes in the location of a specific nucleolar antigen in HeLa cells (human carcinoma of the cervix uteri) during their long continuous culturing using new A3 monoclonal antibodies.

MATERIALS AND METHODS

Human HeLa cells were kindly provided by Laboratory of the Nucleus Dynamics and Plasticity, Curie Institute (Paris), in 2003. The cell status corresponded to International standards. One portion of cells was frozen in liquid nitrogen at -196°C, while another portion were used for culturing. The cells were cultured in plastic flasks (Greiner) or on coverslips placed into sterile plastic dishes (Medpolymer) in DMEM (PanEko) with 10% FCS (HyClone), 100 U/ml penicillin and streptomycin (PanEko) at 37°C and 5% CO_2. The cells were reinoculated twice a week using a mixture of 0.25% trypsin and 0.025% EDTA (1:1, PanEko). The cells were cultured under these conditions for at least 6 months. Then, aliquots of cells stored at -196°C were used for culturing. Mouse monoclonal A3 antibodies specifically reacting with nucleoli of human and primate cell were used in the study. The cells were fixed, incubated with antibodies, stained with DNA-DAPI (4',6-diamidino-2-phenylindole; 1 μg/ml, 10 min), embedded in Mowiol (Calbiochem), and examined under an Axiovert 200 epifluorescent microscope (Carl Zeiss) [2,6]. Total protein synthesis was suppressed by adding 100 μM anisomycin (Sigma) to the culture medium for 1-4 h [7,8]. Oxidative stress was induced by adding 100 μM H_2O_2 (Sigma) for a period of 30 min to 8 h [3]. Dead (apoptotic) cells

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were identified by chromatin condensation and nucleus fragmentation detected by DAPI staining. The percentage of cells with abnormal location of A3 antigen and the apoptotic index were estimated per 1000 randomly selected cells.

RESULTS

At the initial stages of HeLa cell culturing ("early" culture) A3 antibodies reacted with the antigen located exclusively in the nucleolar zone (Fig. 1). Sites of A3 antigen binding formed numerous discrete foci, their number reached several tens per nucleus. The location A3 antigen corresponds to the molecular components of RNA polymerase I transcription complex, such as subunits 1 (RPA190) and 2 (RPA116) and the RNA polymerase I specific cofactor UBF protein [12]. However, after 2-3-month culturing we noted the appearance of cells (3-5% of total population) with discrete A3-positive foci in the nucleoplasm (outside the nucleolar zone), in addition to characteristic location of A3 antigen in the nucleoli (Fig. 1, *c*). The percent of cells with abnormal localization of A3 antigen progressively increased and after 6-8 months these modified cells constituted about half of the entire population. Interestingly, the general morphology of cells and nuclei evaluated in the phase contrast mode or by DAPI staining was virtually the same in cells with normal and abnormal location of A3 antigen (Fig. 1, *b*, *d*).

The causes of these changes in the location of nucleolar A3 antigen during long-term culturing of HeLa cells are not studied. No phenomena of this kind were described in available literature, though it is well known that the nucleolus is one of the most plastic components of the cell and its morphology and immunoreactive characteristics change in response to various stress exposures. For example, under conditions of suppression of ribosome gene transcription with actinomycin D, rDNA transcription regulatory proteins accumulate on the surface of the nucleolus, while proteins essential for terminal stages of ribosomal particle assembly migrate from the nucleolus into the nucleoplasm [10]. Incubation of cells with 5-40 μM $HgCl_2$ causes characteristic changes in the location of fibrillarin, early rRNA processing factor, but has no effect on the location of other nucleolar proteins [5]. Suppression of transcription of structural genes with 5,6-dichloro-1-β-ribofuranosylbenzimidazole (casein kinase II inhibitor) leads to characteristic redistribution of the majority of the nucleolar proteins [2]. It seems that changes in the immunocytochemical status of the nucleolus, most close to changes de-

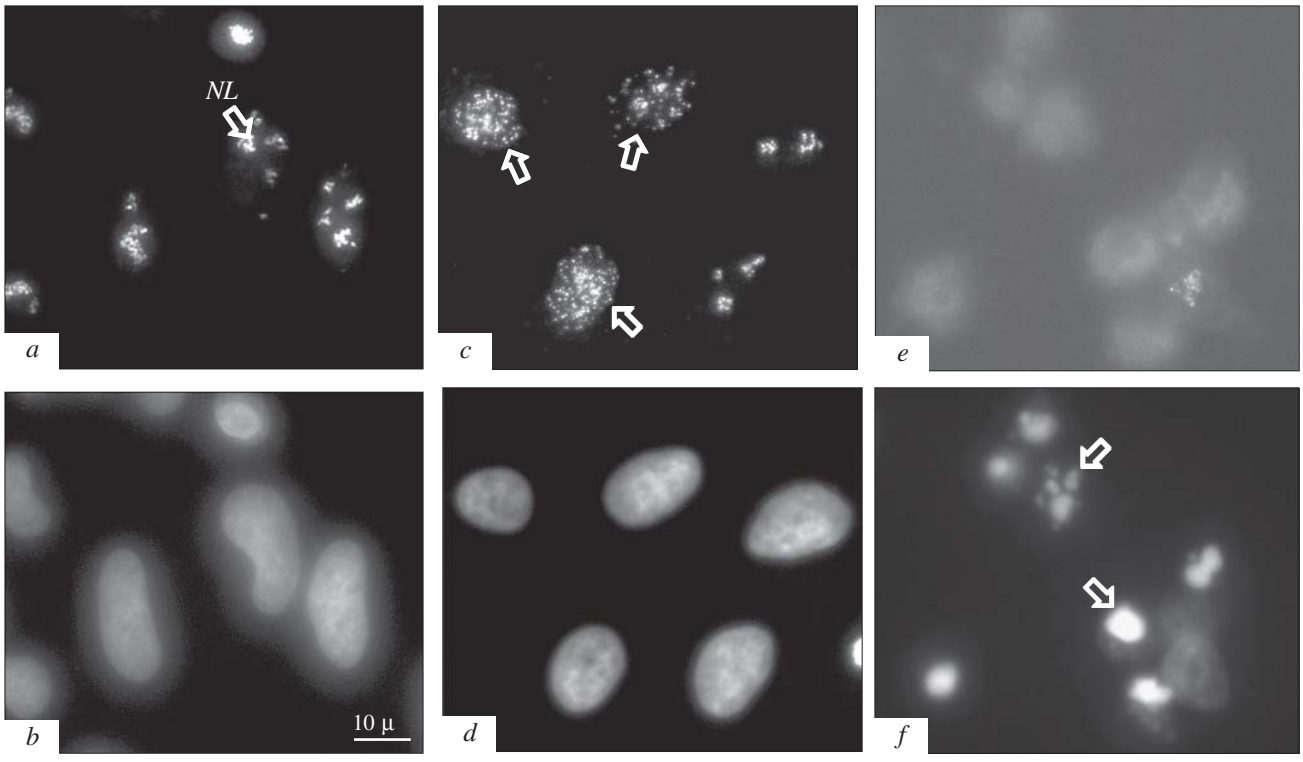

Fig. 1. Location of A3 antigen in the nucleoli of "early" (*a*) and "late" (*c*, *e*) HeLa culture in the control (*a*, *c*) and after 4-h treatment with anisomycin (100 μM) (*e*) and signs of apoptosis in DAPI staining of the nuclei (*b*, *d*, *f*). Arrows show cells with A3 antigen in the nucleolus and nucleus (*a*) and dying cells (*f*). *NL*: nucleolus.

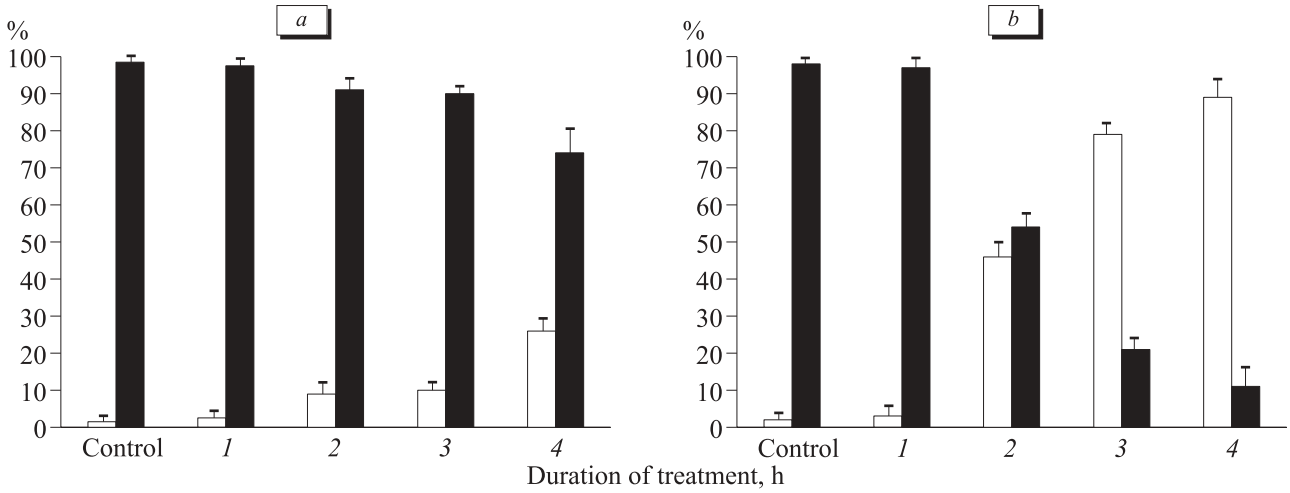

Fig. 3. Percent of viable (dark bars) and dying (light bars) cells in "early" (*a*) and "late" (*b*) HeLa cultures in the control and after anisomycin (100 μM) treatment.

scribed in this work, take place during treatment of HeLa cell with alkaloid emetine, an effective inhibitor of protein synthesis [4]. Presumably, the presence of A3 antigen not only in the nucleoli, but also in the nucleus is a result of inhibition of total protein synthesis observed in HeLa cells during their long-term and continuous culturing.

In order to clear out, whether changes in the location of A3 antigen reflect changes in the general status of the culture, we evaluated the reactions of the "early" (1-2 months) and "late" (6-8 months) HeLa cell cultures to two apoptosis inductors: protein synthesis inhibitor anisomycin and oxidative stress inductor H_2O_2. One hour after addition of anisomycin, the percent of dying cells in the "early" and "late" cultures just slightly surpassed the control (Fig. 2), but after 2 h the percent of dying cells in "early" and "late" cultures differed more than 4-fold. These differences became still more pronounced 3 and 4 h after the start of exposure: the count of dying cells in the "early" culture did not exceed 15-25%, while in "late" culture this parameter reached 80-90%. DAPI staining showed that cells died by apoptosis in both cases, which could be seen from chromatin condensation and nucleus fragmentation (Fig. 1, *e*, *f*) [1,6]. In dying cells, no A3 antigen was detected by immunocytochemical methods (Fig. 1, *e*), which attests to its probable degradation. By this sign A3 antigen corresponded to nucleolar Ag-NOR proteins, UBF (RNA polymerase I cofactor), and nucleolin (protein involved in the regulation of rDNA transcription and rRNA maturation) [9,11]. Hence, the appearance of cells with abnormal location of A3 antigen in HeLa culture was associated with increased sensitivity of the culture to suppression of total protein synthesis with anisomycin.

Similar results were obtained after treatment of "early" and "late" HeLa cultures with H_2O_2 in a concentration inducing oxidative stress and apoptotic cell death [3]. General morphology and pattern of DAPI staining of cells virtually did not change 8 h after treatment with 100 μM H_2O_2 in comparison with intact cells, while in the "late" culture up to half of cell nuclei contained condensed and fragmented chromatin during this period. It seems that high sensitivity of "late" HeLa culture is a general characteristic manifesting under conditions of stress exposure with different mechanisms of action.

Hence, the location of A3 antigen can be used as a new (immunocytochemical) criterion for evaluation of HeLa cell status in culture.

The study was supported by the Russian Foundation for Basic Research (grants No. 05-04-08131 and No. 06-04-49392).

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