BIOPHYSICS AND BIOCHEMISTRY

Early Expression of Nucleolar SURF-6 Protein in Mouse Spleen Lymphocytes Activated for Proliferation *In Vitro*

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Using specific antibodies we studied the content of nucleolar SURF-6 protein, which participates in rRNA processing, in mouser spleen lymphocytes activated for proliferation with concanavalin A and compared it with the content of nucleolar nucleophosmin/B23 protein and DNA replication factor PCNA, well-known markers of proliferating cells. Using immunocytochemistry and immunoblotting methods we demonstrate that the concentration of all these proteins increases simultaneously with increasing the proportion of proliferating cells. Unlike nucleophosmin/B23, SURF-6 protein was not revealed in quiescent lymphocyte nucleoli, while the increase of its level in activated lymphocytes preceded elevation of PCNA level. These observations suggest that nucleolar protein SURF-6 can act as a marker of early T lymphocyte activation for proliferation and that it could participate in cell cycle regulation in mammals.

Key Words: *mouse spleen lymphocytes; SUFR-6, nucleophosmin/B23; concanavalin A; proliferation*

Activation of mammalian cells for proliferation is followed by elevation of the content of various nuclear proteins, *e.g.* chromatin proteins whose expression increases abruptly at the stage of DNA replication, transcription factors (MYC, p53, retinoblastoma family proteins), replication factors (PCNA and Ki-67), cyclins, cyclin-dependent kinases [11], and proteins of nucleolus, the largest structural domain of the cell nucleus responsible for ribosome production [13]. Induction of proliferation modulates the expression of proteins participating in ribosomal RNA (rRNA) processing and ribosomal particle assembly, such as nucleophosmin/B23, nucleolin, and Ag-NOR proteins. The increase of the level of these proteins was described in rat hepatocytes [5] and in human peripheral blood lymphocytes activated for proliferation with phytohemagglutinin [4]. Elevated level of nucleophosmin/B23 and Ag-NOR proteins is also typical of tumor cells [3,10]. These observations led to the hypothesis that hyperplasia of the nucleolus and activation of ribosome synthesis can lead to impairment of cell cycle regulation, promote uncontrolled cell growth and malignant transformation [3,13]. Thus, identification of nucleolar proteins with expression varying during cell cycle is important for applied studies as well as for basic research.

In order to reveal nucleolar proteins (new potential markers of cell activation for proliferation) we evaluated the content of nucleolar protein SURF-6 in mouse spleen lymphocytes activated for

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proliferation with concanavalin A and compared it to the level of two other proteins, nucleophosmin/B23 and PCNA, whose level is elevated in proliferating cells [4,8]. SURF-6 protein belongs to the group of evolutionary conservative vital proteins, its homologues are present in other eukaryotes including humans [9,10]. In mammals, SURF-6 is most thoroughly studied in murine cells, which was substantially promoted by elaboration of high-affinity antibodies that bind to SURF-6 at the cellular level and in immunoblots [8]. Murine SURF-6, a protein consisting of 355 amino acid residues with pI 10 and electrophoretic mobility corresponding to a molecular weight of 43 kDa, is localized predominantly in the granular part of the nucleolus and is characterized by high affinity to rRNA *in vitro* and *in situ* [1,9]. These data indicate that SURF-6 in mammals can participate in rRNA processing and ribosomal particle assembly.

MATERIALS AND METHODS

The spleens were isolated from mature of (CBA×C57BL/6)F_1 mice according to a standard protocol [6]. The spleen was placed in a 6-cm Petri dish containing 5 ml DMEM (PanEko) under sterile conditions, cleansed from fat, and transferred to another dish with medium. Splenic capsule was cut in 2-3 sites and the content was squeezed out into the medium. After sedimentation of large particles, the medium was collected and centrifuged at 1000 rpm. The cell pellet was suspended in 1 ml medium and mixed in 2 ml 10% PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4, pH 7.2-7.4) for erythrocyte lysis. After 1-2 min, 5 ml DMEM containing 8% FCS was added to the suspension and it was centrifuged at 1000 rpm for 7 min. The precipitate was thoroughly suspended, lymphocytes were counted in Goryaev chamber. The mean yield was 10^7 cells from one spleen.

The isolated lymphocytes in a final concentration of 0.5-1×10^6 cells/ml were transferred to Petri dishes containing 5 ml α-MEM (PanEko), containing 15% ECS and 3 μg/ml concanavalin A (PanEko) and cultured at 37°C and 5% CO_2 for 16, 24, 48, and 72 h. Lymphocyte distribution over the cell cycle was analyzed by flow cytofluorometry. To this end, lymphocytes were precipitated by centrifugation at 700*g* for 10 min, twice washed in PBS, fixed in 70% ethanol at 4% for 15 min, and incubated in a buffer containing 50 μg/ml propidium iodide (Sigma) and 0.5 mg/ml RNAse A (Sigma) in PBS for 1 h at 37°C. Analysis was performed on an EPICS ELITE flow cytofluorometer (Coulter) using argon laser CYONICS (UNIPHASE). At least 10^4 cells were analyzed for each point. The data were processed using MULTIGRAPH program (Coulter).

Total lymphocyte and fibroblast lysates from NIH/3T3 mice were obtained as was described before [1]. Protein concentration in the lysates was estimated using Spectronic Genesis 10Bio spectrophotometer (Thermo Electronic Co) according to Lowry method modified by Peterson. For electrophoresis, 20 μg protein in 5-fold standard Laemmli buffer (250 mM TRIS-HCl pH 6.8, 50% glycerine, 10% dodecyl sulfate, 500 mM β-mercaptoethanol, 0.5% bromphenol blue) was applied to each row in 12% PAAG. Electrophoretic separation of proteins and transfer on Protran nitrocellulose membrane (pore diameter 0.22 μ, Schleicher and Schuell) was conducted according to a standard protocol [11]. The membrane was incubated in 5% delipidated dry milk in TBS-T buffer (20 mM tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.6) for 1 h, then with rabbit polyclonal antibodies to SURF-6 [9] (dilution 1:500 in TBS-T buffer containing 5% dry milk), mouse monoclonal antibodies to B23/nucleophosmin (diluted 1:500, Sigma) or with rabbit polyclonal anti-PCNA antibodies (1:500, Santa Cruz) for 1 h at room temperature. Then the membrane was incubated with antibodies to rabbit or mouse immunoglobulins conjugated with horseradish peroxidase (1:20,000, Sigma) for 40 min at room temperature. Antibodies were detected using ECL+Plus Detection Kit (AmershamPharmaciaBiotech) and HyperFilm ECL X-ray film (AmershamPharmaciaBiotech) according to manufacturer's instructions. Bound antibodies were washed from the membrane (when needed) in a buffer containing 100 mM β-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM tris-HCl pH 6.7 for 40 min at 40-50°C. After blotting, the gels were fixed in 10% trichloroacetic acid for 40 min and stained in colloid 0.08% Coomassie blue G-250 (Amresco) containing 20% ethanol, 1.6% orthophosphoric acid, 8% ammonium sulfate and 70% water overnight at vigorous agitation.

For immunocytochemical staining, the lymphocytes were precipitated on coverslips coated with 0.1% poly-L-lysine (Sigma) and fixed in absolute ethanol at -20°C for 10 min (optimal conditions of SURF-6 detection at the cellular level) [11]. The cells were stained with antibodies to SURF-6 (dilution 1:100), B23/nucleophosmin (dilution 1:100) or with their mixture for 1 h at room temperature. Staining with FITC-conjugated antibodies to rabbit immunoglobulins (dilution 1:50), Texas-red-conjugated antibodies to mouse immunoglobulins (dilution 1:400), or with a mixture of two secondary antibodies was performed at room temperature for

45 min. All antibodies were obtained from JacksonImmunoResearch Lab. Lymphocytes stained with secondary antibodies only were used as control. The cells were counterstained with chromatin-binding dye DAPI and studied under an Axiovert 200 inverted epifluorescence microscope (Carl Zeiss) using 100× Plan-Neofluar/1.3 Ph lens and appropriate sets of filters. Images were recorded using 13-bit monochrome Coolsnap$_{cf}$ camera (RoperScientific) and processed using graphic editor. In statistical analysis, no less than 500 cells per point were analyzed.

RESULTS

Changes in the number of proliferating lymphocytes at different time after activation with concanavalin A are shown on a histogram plotted on the basis of cytofluorimetric data obtained in 3 independent sets of experiments. The count of S-phase lymphocytes starts to increase after 16 hours and reaches its maximum 48 h after mitogen activation (Fig. 1). However, the number of proliferating lymphocytes after 48 and 72 h did not differ significantly. These results agree with the known data on the time course of change in the level of marker protein of proliferating cells PCNA [8] and the time course of changes observed in this study (Fig. 2). PCNA was not detected neither in total lysates of non-activated lymphocytes, nor in cell lysates 16 h after activation (Fig. 2). At later terms, the amount of PCNA progressively increased and after 72 h became comparable to the content of this protein in asynchronous NIH/3T3 mouse fibroblast culture containing about 30% S-phase cells [1].

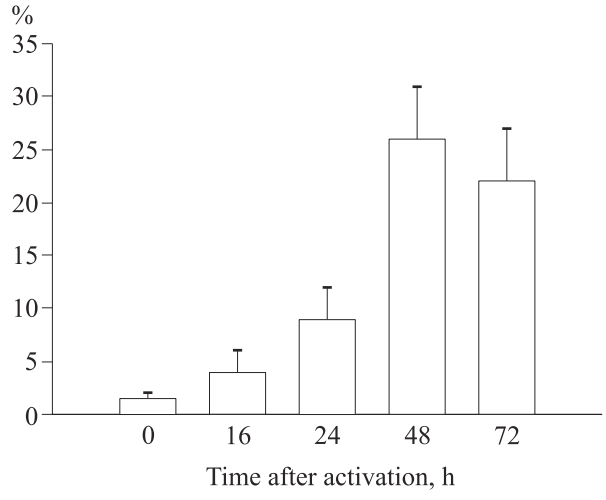

Fig. 1. Proportion of S-phase lymphocytes after activation for proliferation with concanavalin A. Ordinate: ratio of S-phase cells to total number of cells.

SURF-6 was not detected in non-activated lymphocytes (Fig. 2, row 1), but its amount progressively increased after activation (Fig. 2, rows 2-4). A slight reduction of SURF-6 amount is observed at 72 h (Fig. 2, row 5), which is probably related to lymphocyte death after long-term *in vitro* culturing and protein degradation. Published data [4,8] and the results of cytofluorometry also showed partial lymphocyte death 72 h after the start of culturing (data not presented).

The content of another nucleolar protein, B23/nucleophosmin, also increased with increasing the proportion of proliferating lymphocytes (Fig. 2). However, in contrast to SURF-6, B234/nucleophosmin protein was detected in non-activated lymphocytes and its level peaked 72 h after activation. The changes in B23/nucleophosmin level in mouse spleen proliferating lymphocytes are in line with the data on its content in peripheral blood lymphocytes from healthy donors and in lymphocytes activated for proliferation with phytohemagglutinin [4]. The difference in B23/nucleophosmin and SURF-6 content observed 72 h after lymphocyte activation can be explained by higher resistance of B23/nucleophosmin to proteolysis after cell death. For instance, it was demonstrated that degradation of B23/nucleophosmin does not occur after apoptotic death of HeLa cells induced by TNF-α [2].

In order to find out how the content of SURF-6 and B23/nucleophosmin changes at the cellular level, quiescent and activated lymphocytes were stained with antibodies in the reaction of indirect immunofluorescence: B23/nucleophosmin is present in non-activated lymphocytes (Fig. 3, *a*). It should be noted that fluorescence was predominantly observed along the nucleolar periphery, which agreed with previous reports [14]. In contrast, SURF-6 was not detected in the nuclei of the same lymphocytes (Fig. 3, *b, c*). Weak fluorescence of the

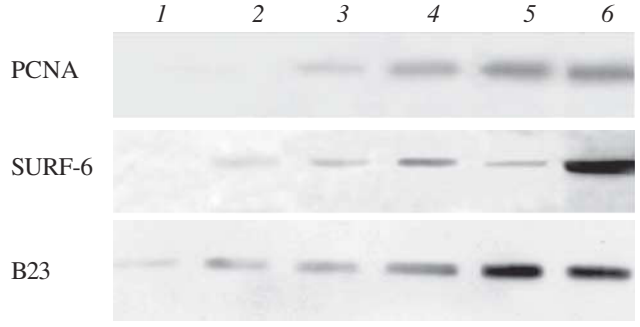

Fig. 2. PCNA, SURF-6 and B23/nucleophosmin content in non-activated lymphocytes and in lymphocytes at different terms after activation for proliferation with concanavalin A. *1*) non-activated lymphocytes; *2-5*) lymphocytes at 16, 24, 48, and 72h after activation for proliferation, respectively; 6) asynchronous cells from NIH/3T3 mice.

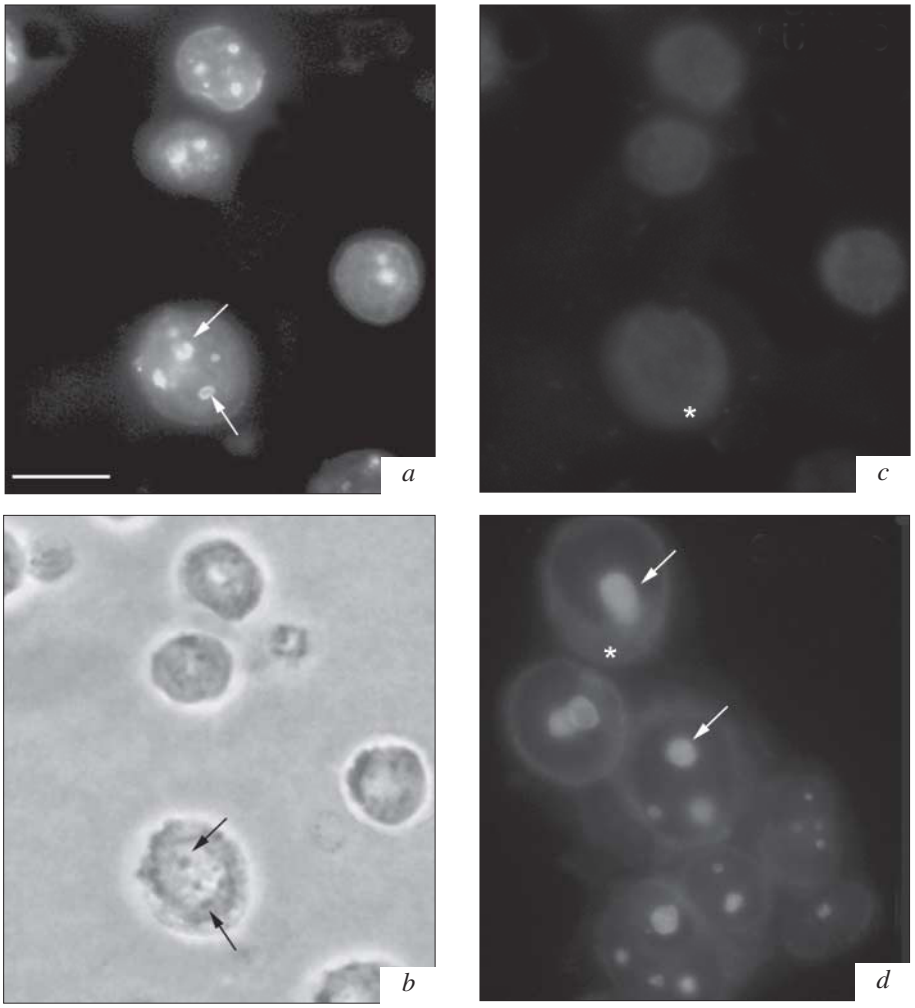

Fig. 3. B23/nucleophosmin (*a*) and SURF-6 (*b*, *c*) detection by immunocytochemistry in non-activated mouse spleen lymphocytes (*a-c*) and in lymphocytes 48 h after activation for proliferation with concanavalin A (*d*). *b*) phase contrast view of lymphocytes demonstrated on *a* and *c*. Arrows show nucleoli. Scale: 10 μ. Asterisk shows cell nucleus.

cytoplasm observed in all cells was most likely caused by non-specific binding of secondary antibodies, it was present in all control specimen (data not presented) as well as in lymphocytes at later stages of culturing (Fig. 3, *d*). SURF-6 appeared in lymphocyte nucleoli 16 h after activation, *i.e.* when PCNA was absent (Fig. 2). However, the proportion of SURF-6-positive lymphocytes among the total number of lymphocytes at this term did not exceed 30%. Twenty-four, 48, and 72 h after adding concanavalin A, SURF-6 was present in the nucleoli of all cells (Fig. 3, *d*). As anticipated, lymphocyte activation was followed by their significant enlargement (Fig. 3, *c, d*). B23/nucleofosmin was present in the nucleoli at all terms of activation (data not presented). Thus, the results of detection of nucleolar proteins SURF-6 and B23/nucleophosmin by immunoblotting and immunocytochemistry do not disagree: in non-activated spleen lymphocytes only B23 was detected, while SURF-6 was not revealed (Fig. 2, 3, *a-c*). This feature of SURF-6 is similar to PCNA and Ki-67, well-known activation markers in human cells. It was demonstrated that both proteins are absent in quiescent peripheral blood lymphocytes of healthy man, but they emerge in nuclei (PCNA) and nucleoli (Ki-67) of T lymphocytes during the S-phase [4,8].

Thus, it can be concluded that nucleolar protein SURF-6 is a new marker of T lymphocyte activation for proliferation. Moreover, in mouse splenic T lymphocytes activated for proliferation, SURF-6 protein can be detected at earlier stages than PCNA, which allows to regard this protein as early markers of lymphocytes activation *in vitro*. Nucleolar proteins with similar properties have not yet been described in literature.

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