Subcellular localization of S6K1 and S6K2 forms of ribosomal protein S6 kinase in primary monolayer culture of rat thyrocytes

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The main aim of this work was to determine subcellular localization of S6K1 and S6K2 forms of ribosomal protein S6 kinase in the primary monolayer culture of thyrocytes obtained from undamaged follicles. In the thyroid follicles S6K1, S6K2 have been detected predominantly in the cytoplasm of the cells, however, in the monolayer culture of thyrocytes in the course of follicles outspreading S6K1 and S6K2 have been observed in nuclei as well. Such redistribution of S6K was not directly related to the appearance of proliferating Ki-67 positive cells. At the same time there was a correlation between the appearance of S6K1, S6K2 positive nuclei in monolayer thyrocyte culture and decrease in thyroglobulin content in cultured cells. Thus, the obtained results indicated that the down regulation of thyrocyte functional activity caused by the loss of follicle organization was accompanied by subcellular redistribution of S6K1 and S6K2.

Key words: ribosomal protein S6 kinase, thyrocytes, primary culture.

Introduction. The principal regulators of thyroid growth and hormone synthesis are thyroid stimulating hormone (TSH) and insulin and dysfunction of thyroid is often connected to abnormalities of signal transduction into the cell [1, 2]. Recent investigations have shown that both of them exhibit their effect on target cells through PI3K signal transduction pathway [3–5]. The ribosomal protein S6 kinase (S6K) is an important member of this pathway. The S6K along with protein kinase C and B belong to the AGC family of Ser/Thr protein kinases. There are two forms of S6 kinase, S6K1 and S6K2, each one of them has cytoplasmic and nuclear variants derived from alternative splicing at N-terminus [6]. Initially ribosomal protein S6 (rpS6) was considered to be the only substrate for S6K. Phosphorylation, catalyzed by S6K, leads to activation of rpS6, which consequently takes part in regulation of cell size, protein synthesis, and glucose homeostasis. But recently several new substrates including eukaryotic translation initiation factor 4B (eIF4B), cAMP-responsive element modulator (CREM), 80 kDa subunit of the nuclear Capbinding complex (CBP80), and S6K1 Aly/REF-like target (SKAR) have been identified [7–9]. The inhibitory effect of S6K was demonstrated for insulin receptor substrates IRS1, IRS2, BAD, and eukaryotic elongation factor 2 kinases (eEF2K) [10].

Currently, there are a lot of evidences suggesting the involvement of S6K in the regulation of cell cycle [11–13]. These data indicate the possible link between subcellular localization of S6K and its role in the processes of cell proliferation and maintenance of the tissue functional activity.

Previous investigations of our laboratory on breast cancer model have shown that subcellular localization of S6K is not static. It was demonstrated that S6K2 appeared in the nuclei of malignant cells as opposed to normal breast tissue [14].

It is well established that endocrine activity of the thyroid gland depends on its follicular organization [15, 16]. The disturbances in follicular structure at various levels affect synthesis of thyroid hormones. Such consequences have been observed upon changes at tissue (follicle damage, hypo- and hyperplasia) [17] cellular, and molecular (derangements of desmosome distribution, change of quantitative and qualitative composition of extracellular matrix, etc.) [18] levels. Earlier it was shown that the loss of follicular structure *in vitro* leads to decrease in functioning of thyrocytes [19]. Currently, there are several culture models available for investigation of thyrocytes. Thyrocytes in primary culture can be studied as monolayer [20], as reorganized follicles in suspension [21], embedded in collagen gel [22], as floating aggregates of follicles [23], and as monolayer on filters set between two chambers [24]. Functional activity of cultured thyrocytes could be assayed using such markers as iodine uptake and transport [25], thyroglobulin synthesis [26], thyroperoxidase and deiodinase activities [27], T3 and T4 level [28]. In this study we have employed an approach, which allows monitoring the changes in thyrocytes in the course of their rearrangements from undamaged follicles to uniform monolayer. Primary monolayer culture utilized in this study was initiated from undamaged follicles that were obtained from initial rat thyroid tissue. Our previous studies, performed on the primary monolayer culture of thyrocytes, demonstrated multiple changes including decrease in thyroglobulin content, activation of cell migration, and proliferation [23]. The main goal of the present work was to study subcellular localization of S6K in rat thyrocytes in the course of follicles rearrangement under conditions of monolayer culture.

Materials and Methods. *Reagents*. RPMI-1640 medium, glutamine, amphotericin B, penicillin-streptomycin mixture, dispase, trypsin inhibitor, monoclonal anti-Pan cytokeratin (Pck) mouse antibodies were obtained from «Sigma» (USA). Collagenase CLS 2 was purchased from «Worthington Biochemical Corporation», USA. Foetal calf serum (FCS) was supplied by «Helena Bioscience» (USA). Monoclonal anti-thyroglobulin and anti-Ki-67 antibodies were obtained from «Dakopatts» (Denmark). Monoclonal antibodies to S6K1 and S6K2 were obtained in our laboratory using the method, described in [14, 29].

Cell culture. For each experiment samples of sterile thyroid glands excised from three Wistar rat males were placed into Petri dish with RPMI-1640 medium, supplemented with 5-fold concentration of penicillin/streptomycin and were freed from the fibrous capsule. The thyroid tissue was cut into small fragments (1.5–2.0 mm), rinsed in phosphate buffered saline, pH 7.2 (PBS), and incubated with a mixture of collagenase (0.5 mg/ml), dispase (1 mg/ml), trypsin inhibitor (1 mg/ml) in PBS for 20 min at 37 °C.

The enzyme mixture was changed once, and the fragments of thyroid tissue were incubated for another 30–40 min at 37 °C with agitation every 10 min. After that, 5 ml of growth medium (RPMI-1640 with 17 % FCS, 4 mM glutamine, 50 units/ml penicillin, 50 g/ml streptomycin, 1 g/ml amphotericin B) were added to the tissue sample. Disaggregated by pipetting material was then filtered through nylon mesh (d_{pore} = 180 m) and collected on nylon mesh (d_{pore} = 40 m). The isolated follicles have been washed with 10 ml of growth medium and collected by centrifugation (8 min, 1500 rpm). The pellet was then resuspended in growth medium and the follicles were transferred into 60 mm dishes, coated with 1 % gelatine layer. Cultivation was carried out at 37 °C under humidified 7 % CO_2-air.

Immunocytochemical and immunohistochemical analysis. S6K1/S6K2 localization, thyroglobulin, Ki-67 antigen, epithelial antigens (Pck) were determined by indirect immunoperoxidase method using paraffin sections of initial rat thyroid tissue and primary cell cultures. Representative sections obtained from paraffin blocks were deparaffinized and rehydrated. Endogenous peroxidase was quenched with 1 % H_2O_2 in PBS for 1 hour. After blocking of non-specific staining with 10 % FCS in PBS, sections were incubated for 2 hours at 37 °C with primary antibodies, and thereafter they were incubated with peroxidase-conjugated secondary antibodies for 1 hour at 37 °C. The reaction was developed with

3,3'-diaminobenzidine solution. Thyrocyte cultures were fixed with cooled methanol for 20 min at –20 °C. After fixation, the cells were permeabilized with 0.2 % Triton X-100 in PBS for 15 min. The following dilutions of antibodies were utilized: S6K1, 1:50; S6K2, 1:200; Tg, 1:100; Ki-67, 1:50; Pck, 1:100. All experiments were repeated three times. All microscopy studies were performed using Leica DM1000 light microscope (Germany).

Results and Discussion. *Detection of thyroglobulin content in cultured thyrocyte*. The approach, applied in our work, allows obtaining the suspension of undamaged follicles. The follicles were isolated from thyroid by double-filtration of enzymatically treated tissue. The epithelial origin of cultured cells was confirmed using monoclonal antibodies to epithelial antigens cytokeratins (Pck) (unpublished data). Thyroglobulin content was used as a marker of thyrocyte functional activity. Thyroglobulin was detected on paraffin sections of rat thyroid (Fig. 1, *a*, see plating) and in the culture of outspreading follicles on the 3rd, (Fig. 1, *b*), 6th (Fig. 1, *g*), and 10th (Fig. 1, *j*) day of cultivation using immunoperoxidase technique. Monolayer culture conditions employed for thyroid follicles resulted in complete loss of their follicular organization and in formation of uniform monolayer. Apparent difference in thyroglobulin content between thyrocytes on the 3rd and 10th days serves as a strong evidence of decrease of thyrocyte functional activity.

Subcellular localization of S6K1 and S6K2 in rat thyroid tissue and cultured thyrocytes. Subcellular localization of S6K1 and S6K2 was examined on paraffin sections of rat thyroid by immunohistochemical technique using monoclonal antibodies to S6K1 and S6K2. The data suggest that in rat thyroid tissue S6K1 and S6K2 are detected predominantly in cell cytoplasm (Fig. 1, *b*, *c*). Analysis of S6K1 and S6K2 distribution in thyrocytes on the 3rd day of cultivation, when attached follicles began to spread, also showed their cytoplasmic localization (Fig. 1, *e*, *f*). Later, on the 6th and 10th day of cultivation both forms of S6K were detected in the cytoplasm and in the nuclei of thyrocytes (Fig. 1, *h*, *i* and Fig. 1, *k*, *l*). It is noteworthy that cells with S6K1, S6K2-positive nuclei appeared at the leading edge of outspreading follicle, whereas in the centre of follicle the nuclei were still non-stained (Fig. 2 *a*, *b*, see plating).

Detection of Ki-67 positive thyrocytes in culture. Detection of proliferating cells was carried out in paraffin sections of thyroid and in thyrocyte cultures on the 3rd, 6th, and 10th days of cultivation by indirect immunoperoxidase method using anti-Ki-67 antibodies. Ki-67 positive nuclei were not observed on histological sections of thyroid and in thyrocytes on the 3rd day of cultivation. However, the thyrocytes positive to Ki-67 were detected on the 6th, and especially on the 10th day of cultivation (Fig. 3, see plating). Therefore, the data suggest that Ki-67 positive cells appeared when S6K1 and S6K2 had translocated into nuclei. Analysis of spatial arrangement of Ki-67 positive thyrocytes revealed that they are evenly distributed within outspreading follicles. Unlike S6K1, S6K2-positive cells, the appearence of Ki-67-positive thyrocytes was not selectively attributed to the edge of outspreading follicles (Fig. 3).

Follicle is an essential functional unit of the thyroid gland. It is composed of epithelium arranged as tightly-packed spherical unit. Each follicle is lined by a single layer of specialized thyroid epithelium, which rests on a basement membrane and encloses a lumen filled with thyroid colloid which is a homogeneous proteinaceous material rich in thyroglobulin. This structure is responsible for the synthesis of thyroid hormones, which regulate multitude of physiological processes, including regulation of oxygen uptake, thermogenesis, increase of striated and heart muscles contractility, activation of the metabolism of proteins, lipids, carbohydrates, mineral agents etc. [30]. The disturbance of follicle organization or merely flattening of thyroid epithelium may lead to abnormalities in functional properties of thyroid gland [17].

Upon selection of primary cell culture conditions it is very important to consider strong polarization of thyrocytes [31]. Due to specific follicular structure of thyroid gland *in vivo* it was suggested that receptors to hormones (TSH), growth factors (IGF), iodine transporters, etc., are located on the basal membrane of thyrocytes [32]. Since the follicular structure of thyroid tissue is tightly connected to hormone synthesis, the loss of this organization in monolayer culture and the absence of TSH (conditions used in our experiments)

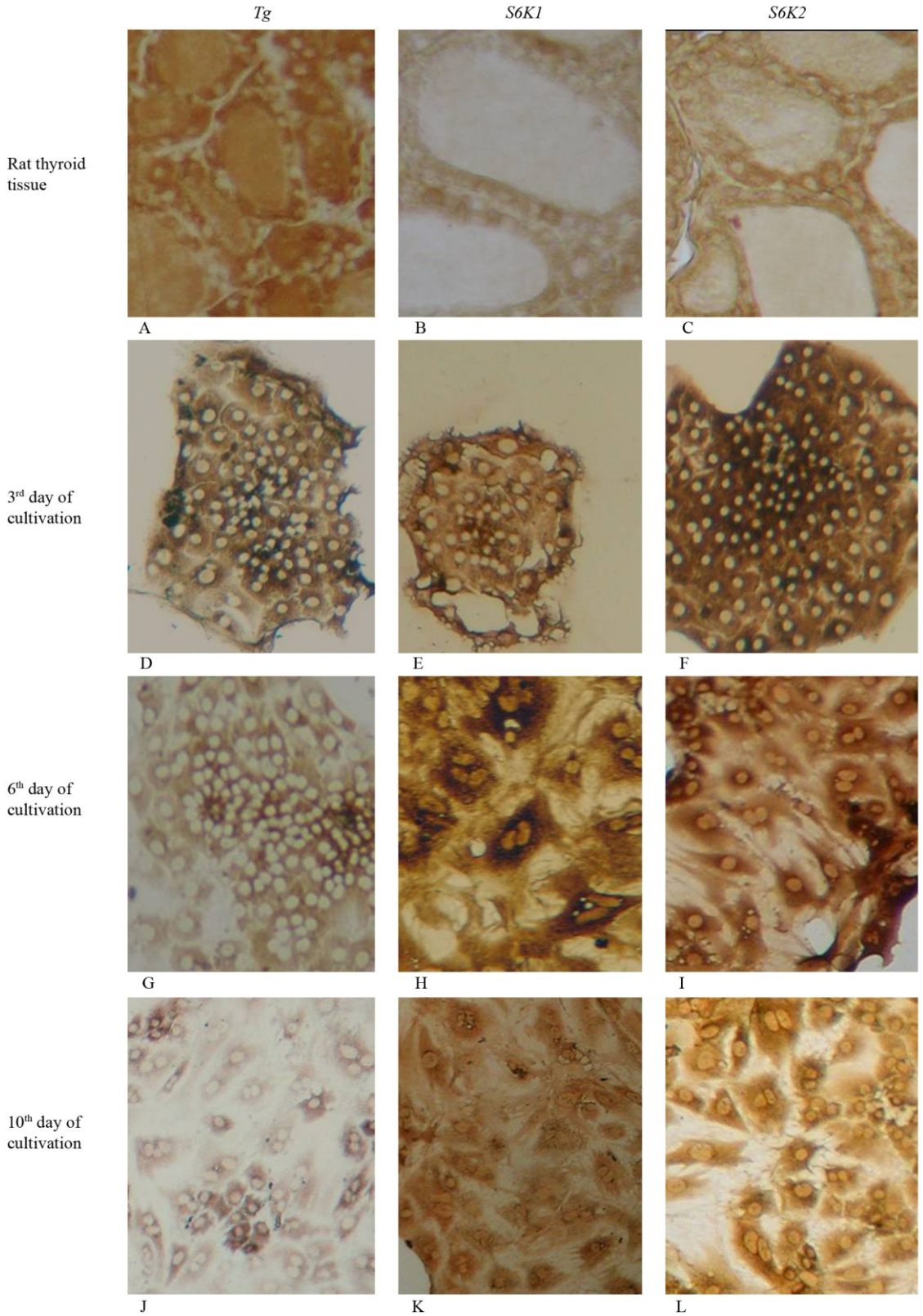

Fig. 1. Immunohistochemical detection of thyroglobulin, S6K1 and S6K2 in normal rat thyroid tissue (*A–C*), and in cultured thyrocytes at the 3rd (*D–F*), 6th (*G–I*) and 10th (*J–L*) days of cultivation. Objective · 20, ocular · 10

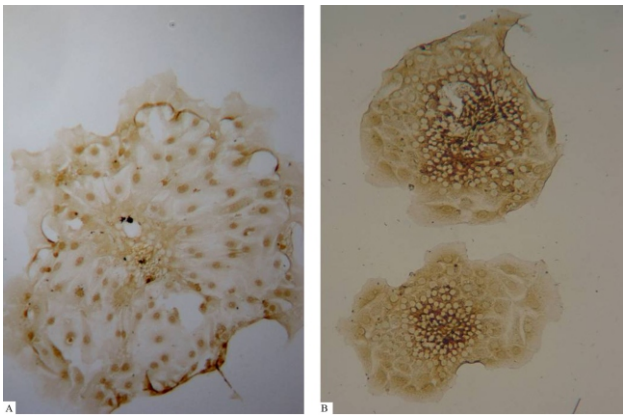

Fig.2 Immunocytochemical detection of S6K1 (A) and S6K2 (B) localization in outspreading follicles on the 4th day of cultivation. S6K1 and S6K2 negative nuclei are located in the central area while positive ones are visible at the outer edge. Objective x20, ocular x10.

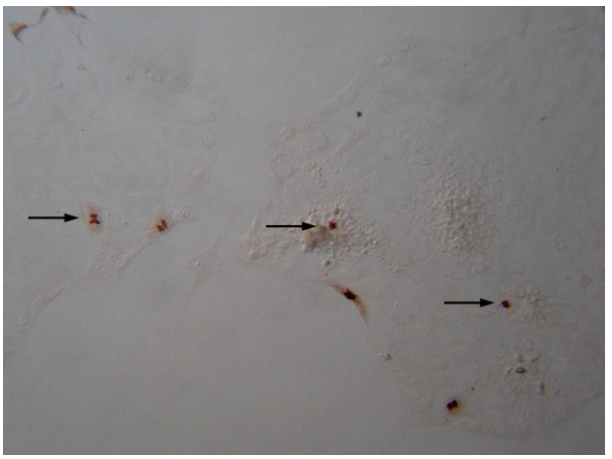

Fig.3 Detection of proliferating thyrocytes on 10th day of cultivation. Arrows show Ki-67 positive cells in the centre of outspreading follicles. Objective x40, ocular x10.

lead to decrease of thyrocyte functional activity and cell dedifferentiation. The level of thyrocytes differentiation could be detected using several methods, including measurement of iodine uptake, iodoperoxidase and deiodinase activity, thyroglobulin, T3 and T4 synthesis [25–28]. In the present study, the thyroglobulin content was detected at several time points of thyrocytes cultivation. The immunocytochemical reaction of cultured thyrocytes observed using light microscopy on the 3rd day of cultivation was significantly stronger as opposed to on the 6th and 10th days of cultivation. Therefore, these data suggest that in the course of follicle transformation to uniform monolayer the functional activity of thyrocytes was reduced. This culture model was applied for investigation of subcellular distribution of S6K1 and S6K2. Obtained data suggest that in the initial tissue both S6K1, and S6K2 are localized mainly in cytoplasm of rat thyrocytes. Likewise human thyroid gland [29], the immunohistochemical reaction detected on rat tissue was feebly marked. On the 3rd day of cultivation the positive reaction for S6K1, S6K2 was still observed predominantly in cytoplasm. Later, on the 6th and the 10th days of cultivation, both nucleic and cytoplasmic fractions of cultured thyrocytes were positive for S6K1 and S6K2. At least two kinases – PKC and CK2 – could be partially responsible for such distribution of S6K [6, 33].

It is very likely that the nuclear substrates of S6K play an important role in thyroid follicles rearrangements, first of all, in thyrocytes dedifferentiating determined by the loss of thyroglobulin synthesis. There are several nuclear substrates for S6K currently known. These include CREM , CBP80, SKAR, and protein from Aly/REF family of RNA binding protein [10].

Since the localization of Ki-67 positive cells did not correlate with nuclear translocation of S6K1, S6K2, the latter event probably has no connection to the activation of cell proliferation. Furthermore, in some experiments we noted the appearance of small quantity of S6K2 positive nuclei on the 4th and 5th days, while Ki-67 positive cells were registered mainly after 6 days of cultivation.

Our data demonstrate that S6K nuclei positive thyrocytes appeared at the leading edge of outspreading thyroid follicles, whereas the nuclei of cells located in the central area were still unstained. We can not exclude that activation of locomotor properties of cultured thyrocytes could be triggered by such redistribution of S6K. According to Zhou et al. [34] the invasive/migratory phenotype of ovarian cancer cells activated by HGF could be blocked by specific inhibitors of the phosphatidylinositol-3-kinase (PI3K) cascade and particularly by inhibitor of S6K1. A significant role of S6K1 in cell invasion is supported by the observation that expression of constitutively active

forms of S6K1 is sufficient to induce invasive and migratory phenotypes in ovarian cancer cells [34, 35].

Subcellular localization of S6K1 and S6K2 was earlier studied in our laboratory using smears of normal and cancer breast tissue. The data obtained suggested that the level of S6K1 and S6K2 expression in breast tumours is much higher, compared to that of the normal tissue. In addition the intensive cytoplasmic and nuclear staining of S6K2 was demonstrated in tumor samples but not in normal tissue. Collectively these data indicate that S6K is involved in the process of cell transformation accompanied by cell dedifferentiation and invasion [36].

In conclusion, our investigation suggests that decrease in thyrocyte functional activity, triggered by the loss of follicle organization *in vitro*, is accompanied by subcellular nuclear-cytoplasmic redistribution of ribosomal protein S6 kinases S6K1 and S6K2. Additional studies are necessary to understand fully the underlying mechanism of this phenomenon.

А. И. Хоруженко, О. В. Чередник, В. В. Филоненко

Субклеточная локализация S6K1 и S6K2 форм рибосомной протеинкиназы S6 в первичной монослойной культуре тиреоцитов крыс

Резюме

Иммунохимически определена субклеточная локализация S6K1, S6K2 в ткани и монослойной культуре клеток щитовидной железы крыс. В исходной ткани S6K1 и S6K2 локализовались преимущественно в цитоплазме. При распластывании фолликулов положительная реакция появлялась и в ядрах тиреоцитов. Такое перераспределение напрямую не связано с появлением пролиферирующих Ki-67 положительных клеток. При этом отмечалась корреляция между появлением S6K1, S6K2 положительных ядер и снижением содержания тиреоглобулина в культивируемых клетках. Полученные нами результаты свидетельствуют о том, что снижение функциональной активности тиреоцитов вследствие потери фолликулярной организации сопровождается изменением субклеточной локализации S6K1 и S6K2.

Ключевые слова: рибосомная протеинкиназа S6, тиреоциты, первичная культура.

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