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Subcellular localization of S6K1 and S6K2 forms of ribosomal protein S6 kinase in rat thyrocytes under conditions of two- and three-dimensional culture

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The aim of this work was to study the link between activation of processes of migration, proliferation, loss of thyrocyte follicle organization at different culture conditions and subcellular localization of S6K1/2. The subcellular redistribution of S6K1/2 takes place in the rat thyrocyte monolayer culture where S6K1/2 was detected not only in the cytoplasm but in the nuclei of cells as well|. In addition, the content of S6K1/2 in proliferating cells was increased. The subcellular| localization of S6K1/2 in thyrocytes cultivated as follicles was similar to that observed in normal thyroid tissue. Subcellular relocalization of S6K1/2 was detected only in certain cellular population, which for some reasons lost follicle organization and, consequently, functional activity. Thus, the changes in subcellular localization of S6K1/2 in cultivated thyrocytes are directly related to the level of differentiation, unlike proliferation and migration of these cells.

Keywords: ribosomal protein S6 kinase, thyrocytes, level of differentiation.

Introduction. The main function of thyroid, as an endocrine organ, consists in the production of thyroid hormones which play an important role in the metabolism of human and mammalians. (A) Basic structural and functional unit of thyroid is a follicle, which consists of closed prismatic or cuboidal epithelium monolayer with an apical part forming a lumen. The latter is filled with a colloid which contains a glycoprotein thyroglobulin. Follicle organization of thyrocytes ensures the multistep process of thyroid hormones synthesis. It was shown on cultivated thyrocytes that the loss of follicle organization and depolarization lead to dedifferentiation of these cells and to a decrease in thyroglobulin content [1, 2].

The functions of thyroid are regulated by a few factors with the main role of pituitary thyroid stimulating hormone (TSH). It was shown, that TSH influences the thyroid cells through the Phosphatidylinositol 3-Kinase (PI3K) signal transduction pathway [3]. The link between the receptor of TSH and regulatory p85 subunit of PI3K was demonstrated on cultivated thyrocytes. It was noted that TSH stimulates the interaction between the receptor of TSH and PI3K, which results in PI3K- and protein kinase A (PKA) dependent translocation of

phospholipid dependent kinase 1 (PDK1). TSH stimulates ribosomal protein kinase S6K through PI3K-, PDK1- and PKA dependent pathway and as a consequence affects *in vitro* proliferation and functional activity of follicular cells [4].

S6K belongs to (an) AGC family of serine/threonine protein kinases which includes protein kinase C, protein kinase B, SGKs, and 90 kDa ribosomal S6 protein kinase [5]. There are two forms of S6 kinase - S6K1 and S6K2 and each (of them) has cytoplasmic (S6K1II and S6K2 II) and nuclear (S6K1 I and S6K2 I) izoforms [6]. It was demonstrated that activity of S6K1/2 is regulated by phosphorylating/dephosphorylating in response to different extracellular stimuli, which include growth factors, cytokines and hormones. Nowadays no highly specific inhibitors of S6K are known. Numerous data demonstrate that PI3K/mTOR signalling pathway play(s) a key role in complete activating S6K.

Earlier an analysis of S6K expression was performed on the tumours of human thyroid, normal tissue and cell lines [7, 8, and 9]. Overexpression of these kinases was detected in papillary carcinoma in comparison to normal tissue. Studies carried out by Western-blot analysis and immunohistochemical technique revealed cytoplasmic localization of S6K1 in normal tissue and in papillary carcinoma of human thyroid.

We have shown that in rat thyroid gland S6K1 and S6K2 are localized mainly in the cytoplasm of thyrocytes [10]. At the cultivation of the isolated follicles of thyroid under the conditions of monolayer culture during spreading of follicles and loss of follicle organization of thyrocytes, a positive immunocytochemical reaction was detected not only in the cytoplasm but in the cell nuclei as well. Initially (the) S6K1/2 positive nuclei were detected on the edge of cell colonies, later a positive reaction was determined in the nuclei of all other cells [10]. These data led to an assumption about the link between activation of cell migration and subcellular redistribution of (the) indicated kinases. On the other side, it is known that S6K is involved in control of cell proliferation [11]. In the previous studies we didn't found a correlation between the expression of Ki-67 antigen and the appearance of S6K in the nuclei of thyrocytes [10]. That is why a possible role of S6K1/2 in regulation of thyrocyte proliferation has to be studied further. Besides, it was shown that after 10-days of the cultivation of follicles under conditions of two-dimensional culture the content of thyroglobulin in thyrocytes is substantially diminished [10]. It points out a possible connection between the decline of functional activity of thyrocytes *in vitro* and the changes in subcellular localization of S6K1 and S6K2. Consequently, the process of thyrocyte cultivation is accompanied by activating the migration processes, cell proliferation and changes in tissue structural organization as well as the functional activity. However, it concerns to a lesser degree the follicles cultivation at under three-dimensional conditions [2].

The aim of this work was to study the relation between these processes and the content and subcellular localization of S6K1/2 in (the) thyrocytes cultivated under different culture conditions. The research included generation of the monolayer cultures and three-dimensional cultures with the maintenance of follicle organization of thyrocytes. The cell migration was studied as well using polycarbonate filters [2, 12] with a subsequent immunocytochemical analysis. The detection of proliferating cells and thyroglobulin content was carried out by the immunoperoxidase and immunofluorescence methods.

Materials and methods. *Reagents.* Reagents for the cell culture and chemiluminiscent reagent were obtained from "Sigma" (USA). Antibodies to the thyroglobulin and Ki-67 were from "Dako" (Denmark); antibodies to mitogen-activated protein kinases (Phospho-p42-44 MAPK) were obtained from "BioLabs" (USA). Polyclonal and monoclonal antibodies to S6K1/2 were generated in our laboratory. Transwell with the pore diameter of polycarbonate membrane 8 m were obtained from "Costar" (USA).

Generation of thyrocyte cultures. The suspension of thyroid follicles from rats of Wistar line was obtained by the method described earlier [10]. Thyroid tissue was cut into small fragments, incubated with (the) mixture of collagenase/dispase with addition of trypsin inhibitor during 2 hours at 37^0C. Thereafter (the) growth medium (RPMI-1640, 17 % FCS, 4 mM

glutamine, 50 u/ml of penicillin, 50 g/ml streptomycin, 1 g/ml amphotericin B) was added. The disaggregated material was filtered through a nylon mesh with (the) pore diameter (of) 180 m and collected on a nylon filter with (the) pore diameter (of) 30 m. The isolated follicles were washed with (the) growth medium and centrifuged. To obtain the monolayer cultures the follicles were cultivated in Petry dishes. The cultures of aggregates of follicles were obtained on dishes, preliminary coated with 1 % agarose. Cultivation was carried out at 37 ^{0} C, in a humidified gas phase 7 % CO_2/air.

Migration of thyrocytes through Transwell. The suspension of follicles was transferred into the upper chamber of transwell, in RPMI-1640 supplemented with 1 % human serum albumin, 4 mM glutamine and (the) mixture of antibiotics. The lower chamber contained RPMI-1640 with 20 % FCS to stimulate the chemotaxis of thyrocytes. (The) Cultivation was carried out for 4 days; the medium in lower and upper chambers was changed daily. (The) Filters were fixed with methanol for 5 min, cells in the upper chamber were removed by a cotton tampon, retaining the cells that migrated to the opposite filter surface.

Immunocytochemical and immunohistochemical analysis. Detections of thyroglobulin (TG), S6K1 and S6K2 were performed by the indirect immunoperoxidase method on the paraffin sections of the cultivated aggregates of follicles, monolayer cultures and in the thyrocytes, cultivated in transwells. Histological sections were deparaffinised and rehydrated. Endogenous peroxidase was quenched with 3 % H_2O_2 for 30 min. After blocking non-specific binding by 10 % FCS, (the) sections were incubated with primary monoclonal antibodies (S6K1, 1:50; S6K2, 1:200; TG, 1:100) for 90 min at 37^{0}C. Incubation with the secondary antibodies, labelled with peroxidase, was carried out for 1 h at 37^{0}C. The reaction proceeded in 3, 3'-diaminobenzidine tetrachloride solution. For double immunochemical reaction the first antigen was detected by the immunoenzymatic method using corresponding first antibodies (anti-thyroglobulin 1: 100, anti-MARK 1: 50, anti- Ki-67 1: 50) and secondary antibodies labelled with horse- radishperoxidase. S6K1 and S6K2 were determined by the immunofluorescent method, using the secondary antibodies labelled with FITC. To decrease the autofluorescence background the preparations were incubated for 30 min in 10 mM $CuSO_4$ and 50 mM of CH_3COONH_4 at pH 5.0. Control preparations were incubated without primary antibodies.

All experiments were repeated no less than 3 times. Microscopic studies were carried out using Leica DM1000 microscope (Germany).

Results and discussion. To detect S6K1/2 in proliferating thyrocytes in monolayer cultures a double immunocytochemical staining was applied. This approach was initially tested using the MCF-7 cell line which is characterized by a high level of the ribosomal protein S6 kinases expression. The data obtained demonstrate that after three days of incubation of follicles suspension at the conditions of (the) monolayer culture an amount of proliferating cells positive for Ki-67 antigen was insufficient. Starting from (the) sixth day of cultivation the number of Ki-67 positive cells increased. In these thyrocytes the increased reaction on S6K1 and S6K2 was revealed as well (Fig. 1, b, d). In addition, double immunocytochemical staining of activated MAP kinases and S6K1 or S6K2 in thyrocytes was carried out. Like in the case of Ki-67 antigen in the cells positive on phosphorylated MAP kinases (Fig. 1, e, g), an increased reaction on S6K1/S6K2 (Fig. 1, f, h) was detected. Thus, on a primary culture of rat thyrocytes an increased content of S6K1/S6K2 in proliferating cells was demonstrated.

The obtained results correlate with the literature data on the involvement of S6K in regulation of cell proliferation. (An) Activation of S6K1 and MARK was revealed during stimulation of human endothelial cells proliferation by angiopoetin [13]. In addition, the activation of protein synthesis, mediated by S6K in the human vascular endothelium, was determined as a key stage in passage through the cell cycle [14]. In the inducible mice tumours producing TSH, the activation of S6K was detected at aberrant pituitary growth [15]. However, as we reported earlier (,) the alterations in subcellular localization of S6K1/2 kinases are not connected directly with proliferation of thyrocytes [10].

For the detection of S6K1 and S6K2 localization in migrating rat thyrocytes the transwells were used. Since the initiating of thyrocytes migration through a porous membrane requires follicles attachment to the plastic surface and their spreading out, the cultivation of follicles was carried out for 4 days. This term of cultivation was chosen experimentally.

In the control experiments to avoid the stimulation of chemotaxis, the contents of FCS in upper and lower chambers were identical. The cells were fixed on membranes with methanol with subsequent immunocytochemical determination of a subcellular localization of S6K1 and S6K2. On the opposite side of the membrane the colonies of migrating cells of the regular shape or lacking central part were found. This fact leads to the assumption that the boundary cells, unlike those located in a centre of the colony have higher mobility potential. In thyrocytes migrating to the opposite surface of the membrane, and also in those which remained on topside, the localization of S6K1 and S6K2 was predominantly cytoplasmic (Fig. 2).

There are no literature data concerning subcellular localization of S6K1/2 in migrating thyrocytes. However, there are some data relating to the participation of PI3K signal pathway and S6K in migration. Recently it was shown that suppression of mTOR by rapamycin leads to inactivation of this pathway that in turn inhibits (in dose-dependent manner) the migration of the NUGC4 line cells, which originate from the carcinoma of stomach [16]. The above-mentioned data are in accordance with the work of Zhou H.Y. performed on the cells of ovarian carcinoma. It was shown that the constitutively active form of S6K1 caused induction of invasive and migratory phenotype of cells. In addition, activating S6K1 predetermined an increase in the expression level and proteolytic activity of matrix metalloproteinases, in particular MMP-9 [17]. Our data do not exclude a possibility of activation of indicated kinases in a cytoplasm of thyrocytes, but point out that appearance of S6K1 and S6K2 in the nuclei does not relate to the cell migration.

Earlier we have shown that dedifferentiation of thyrocytes is accompanied by subcellular relocalization of S6K1 and S6K2 in the monolayer culture [10]. That is why (in this work) we studied subcellular localization of S6K1/2 in thyrocytes of the follicles cultivated on the agarose layer at conditions when the follicles form aggregates and keep tissue organization of the thyroid cells. However, because of partial damage during isolation the follicles collapsed which resulted in aggregate areas with lost follicle organization. The aggregates of follicles were fixed and then (the) localization of indicated kinases was determined immunohistochemically on 3^{d}, 6^{th} and 10^{th} days of cultivation (Fig. 3). It was revealed that in the areas with the follicle structure organization S6K1 and S6K2 were localized mainly in a cytoplasm of thyrocytes.

It should be noted that the maintenance or loss of follicle organization at cultivation under three-dimensional conditions correlated with the functional state of thyrocytes that was determined by thyroglobulin content in the cells (Fig. 4 a, c). It was detected that cells in follicles were positive on a thyroglobulin, however, the cells with lost primary tissue organization did not contain a thyroglobulin which indicated the decreased level of their functional activity. Double immunohistochemical detection of thyroglobulin and S6K1/S6K2 in the cells was performed. In the cells containing thyroglobulin S6K1 and S6K2 had mainly cytoplasm localization, as well as those in the initial rat thyroid tissue. After 10 days of cultivation in the population of cells which did not save the follicle organization and, accordingly, did not contain thyroglobulin the positive reaction on S6K1/2 was detected in thyrocyte nuclei as well as in the case of prolongated thyrocyte cultivation in the monolayer culture (Fig. 4). In addition, the immunostaining of S6K1/2 in the thyroglobulin negative cells was greater than in the cells containing thyroglobulin. These findings enable to suggest that ribosomal protein S6 kinases – S6K1 and S6K2 - are involved into the process of thyrocytes differentiation. At condition of artificially initiated dedifferentiation there is a relocalization of these kinases in comparison with the normal thyroid tissue. The role of S6K in differentiation was studied previously on neural cells, neutrophiles and other types of cells [18, 19]. It was shown that suppression of S6K1 delays the differentiation of neural cells. It should be noted that (earlier in our laboratory was demonstrated) the

presence of positive reaction on S6K2 in the nuclei of human breast carcinoma cells, which was not observed in normal tissue. Besides, it was revealed that the amount of S6K1/2 detected in nuclei of malignant cells increased considerably in the tumours of low differentiation grade [20].

Thus, our data demonstrate that the proliferation and migration of thyroid cells *in vitro* are not associated directly with the changes in S6K1 and S6K2 localization. Alternatively, the connection between cell proliferation and increase of S6 kinases cell content has been shown. As a result of the performed research, the dependence between subcellular localization of S6K1/2 and functional state of thyrocytes has been found that can be useful for an analysis of different compounds modulating the thyroid function at pathology.

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Субклітинна локалізація S6K1 і S6K2 форм кінази рибосомного білка S6 у тиреоцитах щурів за умов дво- та тривимірної культури

Резюме

За умов моношарової культури тиреоцитів щура відбувається внутрішньоклітинний перерозподіл S6K1/2, які поряд з цитоплазмою детектуються у ядрах клітин. Метою роботи було дослідити зв'язок між активацією процесів міграції, проліферації, втрати фолікулярної організації тиреоцитів за різних умов культивування та субклітинною локалізацією S6K1/2. Виявлено загальне зростання вмісту S6K1/2 у проліферуючих клітинах. У тиреоцитах, культивованих у вигляді фолікулів, внутрішньоклітинна локалізація S6K1/2 не змінюється відносно нормальної тканини. Субклітинну релокалізацію S6K1/2 відзначено лише для окремої популяції клітин, що за певних причин втратили фолікулярну організацію і відповідно функціональну активність. Отже, зміна субклітинної локалізації S6K1/2 у культивованих тиреоцитах безпосередньо пов'язана зі зміною рівня диференціації на відміну від проліферації та міграції цих клітин.

Ключові слова: кіназа рибосомного білка S6, тиреоцити, рівень диференціації.

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

Субклеточная локализация S6K1 и S6K2 форм киназы рибосомного белка S6 в тиреоцитах крыс в условиях двух- и трехмерной культуры

Резюме

В условиях монослойной культуры тиреоцитов крысы происходит внутриклеточное перераспределение S6K1/2, которые наряду с цитоплазмой детектируются в ядрах клеток. Цель работы состояла в изучении связи между активацией процессов миграции, пролиферации, потери фолликулярной организации тиреоцитов при разных условиях культивирования и субклеточной локализацией S6K1/2. Обнаружено общее повышение содержания S6K1/2 в пролиферирующих клетках. В тиреоцитах, культивируемых в виде фолликулов, внутриклеточная локализация S6K1/2 не изменялась по сравнению с нормальной тканью. Субклеточная релокализация S6K1/2 отмечена лишь для отдельной популяции клеток, которые по различным причинам теряли фолликулярную организацию и соответственно функциональную активность. Таким образом, изменение субклеточной локализации S6K1/2 в культивируемых тиреоцитах непосредственно связано с изменением уровня дифференциации в отличие от пролиферации и миграции этих клеток.

Ключевые слова: киназа рибосомного белка S6, тиреоциты, уровень дифференциации.

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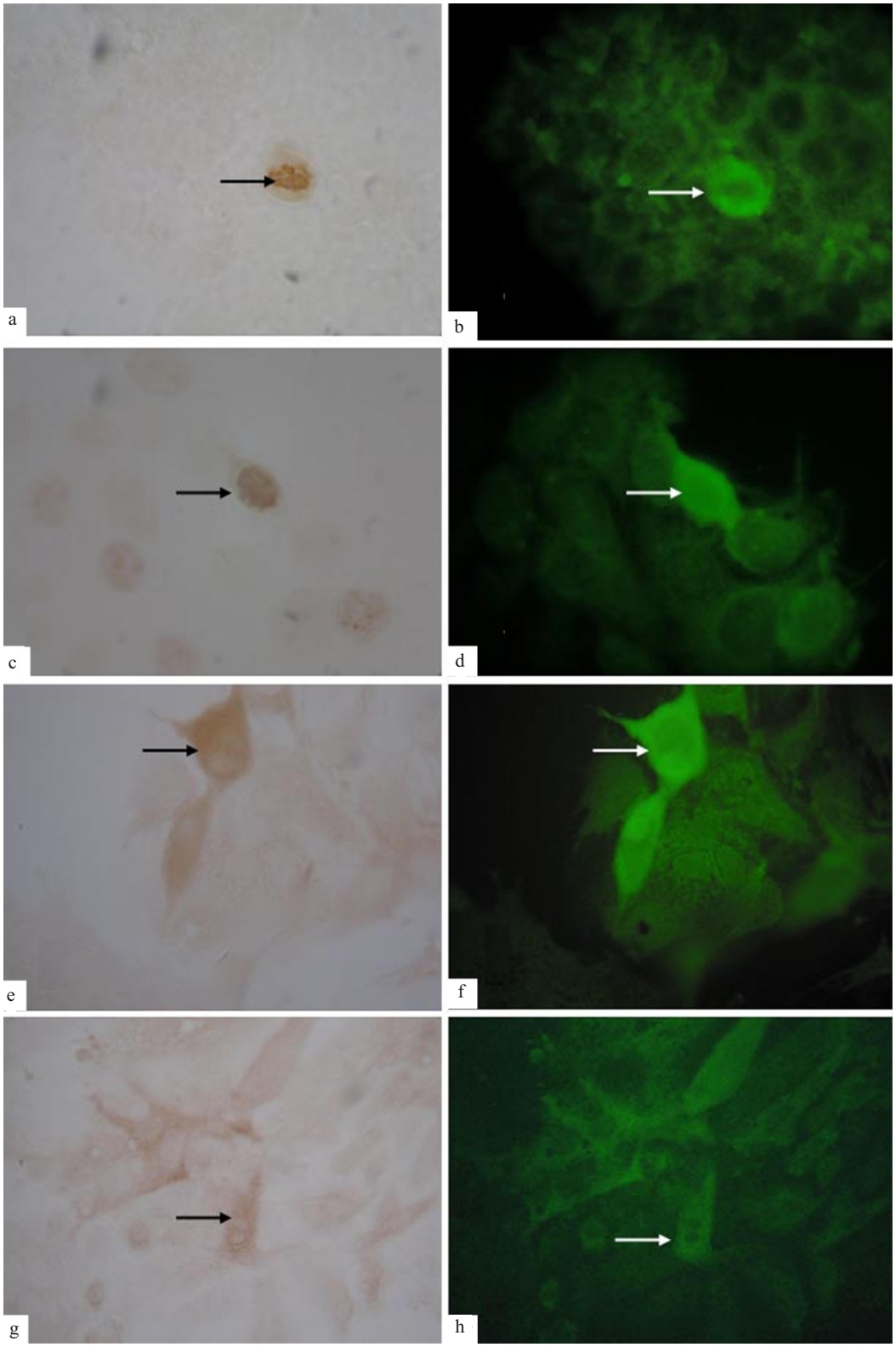

Fig. 1. Double immunochemical detection of Ki-67, MAPK, S6K1 and S6K2 in rat thyrocytes under conditions of monolayer culture. a, c, e, g – immunoperoxidase reaction; b, d, f , h – immunofluorescent reaction. a, c – detection of an antigen Ki-67, b, d - detection of S6K1 and S6K2 correspondingly in the same thyrocytes, both arrows show the same cell. e, g – detection of MAPK, Ocular x10, objective x40.

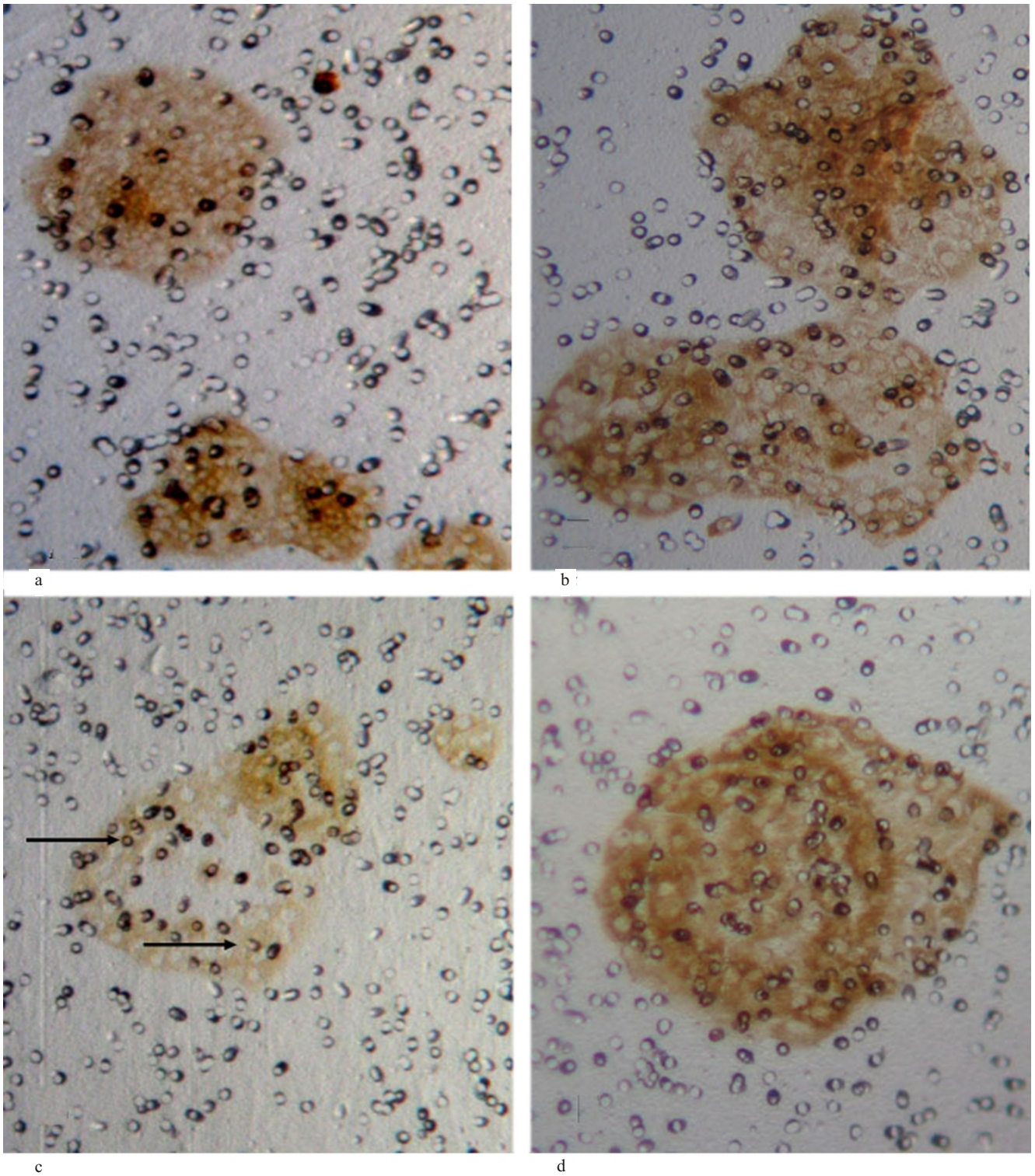

Fig. 2. Imunoperoxidase detection of localization of S6K1 (a, c) and S6K2 (b, d) in thyrocytes on the topside of transwell membrane (a, b) and on a lower surface after migration (c, d). There is cytoplasmic localization of S6K1/2 in all cases. During migration of thyrocytes from the spreaded follicle, the cells located at an edge of the colonies migrate faster than the cells of central area (Fig. 2B.) Ocular x10, objective x5.

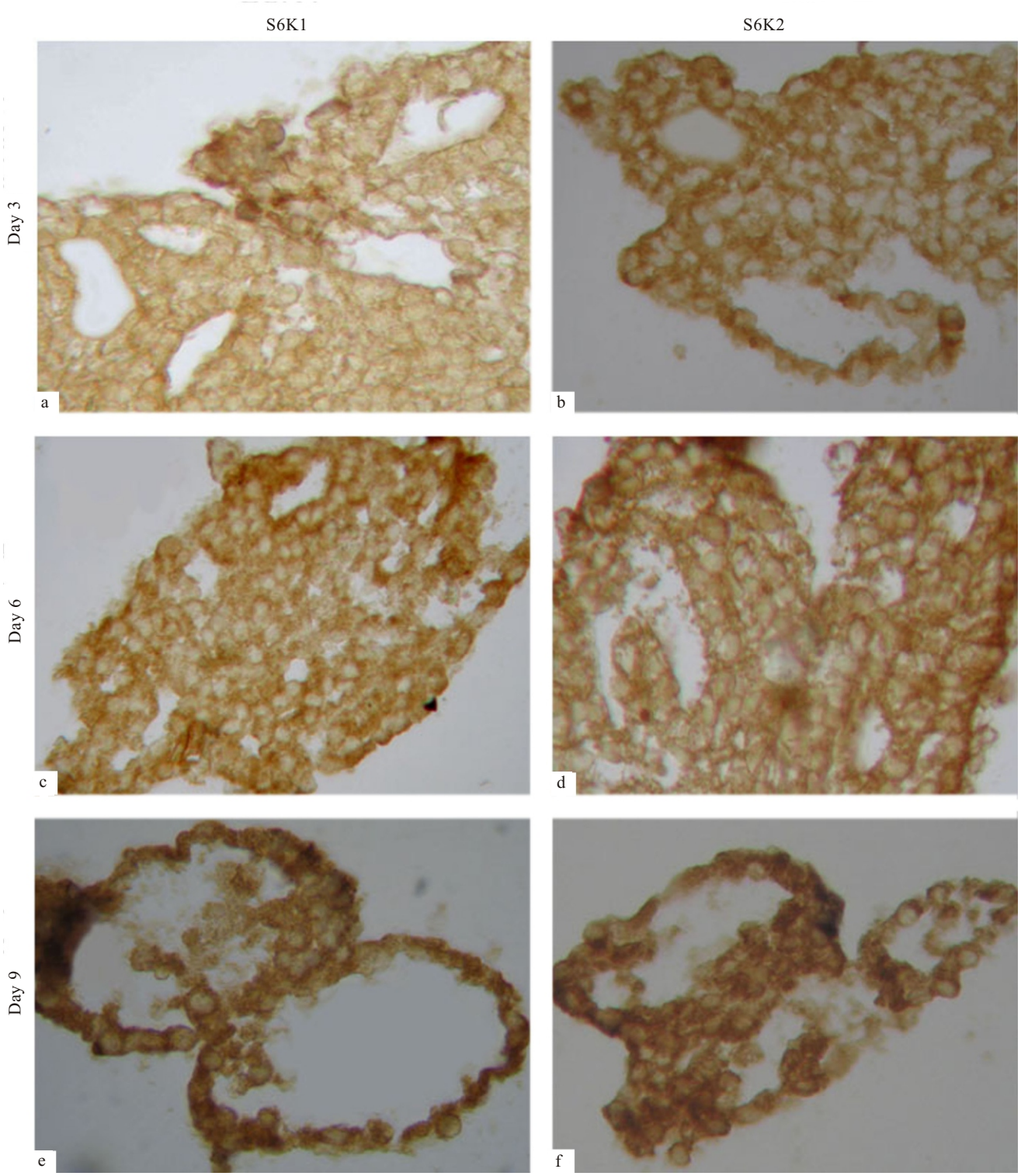

Fig. 3. Detection of subcellular localization of S6K1 (a, c, e) and S6K2 (b, d, f) in the cells of aggregates of follicles cultivated under three-dimensional conditions for 3 days (a, b), 6 days (c, d) and 10 days (e, f). There is mainly cytoplasmic localization of indicated kinases in thyrocytes. Ocular x10, objective x40.

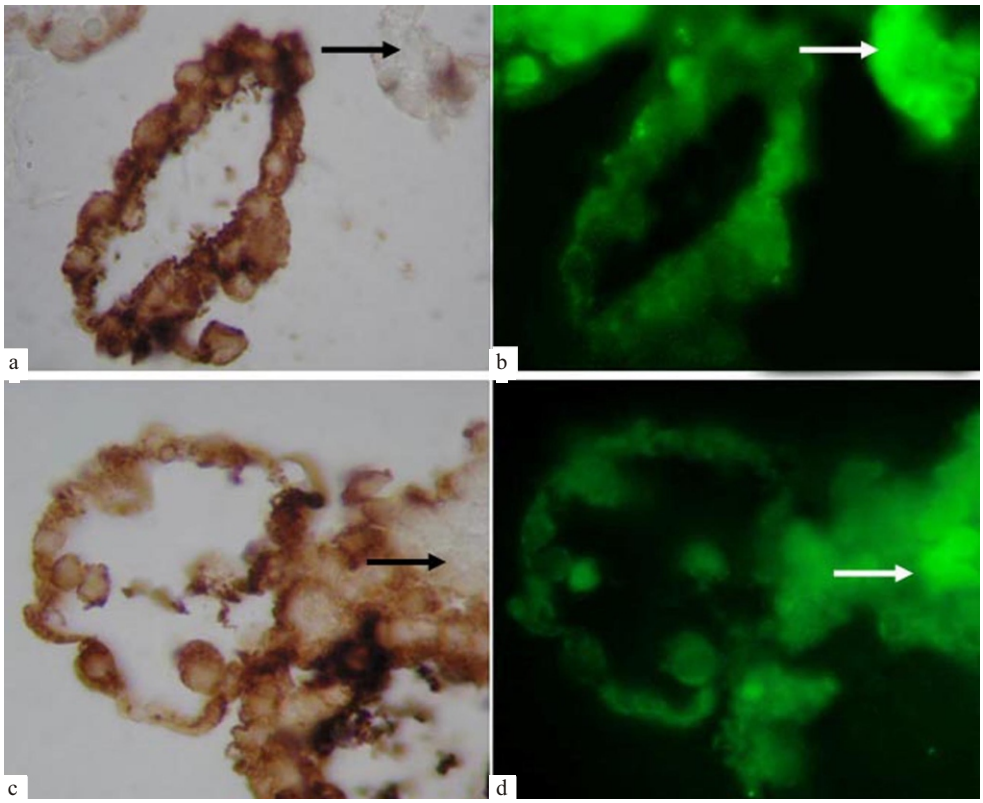

Fig. 4. Double immunocytochemical detection of colocalization of thyroglobulin (a, c – immunoperoxidase method) and S6K1/2 (accordingly, b, d – immunofluorescent method) in the aggregates of follicles on the 10th day of cultivation. Arrows show areas where a follicle structure and thyroglobulin are absent. In thyrocytes without thyroglobulin S6K1/2 positive nuclei are revealed. Ocular x 10, objective x 40.