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METHODS

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Optimization of tumor cell culture conditions in soft agar for subsequent immunohistochemical analysis

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Aim. *The aim of this work is to optimize conditions of malignant cells cultivation in soft agar for subsequent immunohistochemical analysis of formed three-dimensional colonies.* ***Methods***. *Cultivation of breast carcinoma cell line MCF-7 in soft agar, immunohistochemical and immunofluorescence detection of epithelial antigen and mTOR kinase in cultured cells.* ***Results***. *We describe a methodical approach to the culti- vation of cells in soft agar, which allows to carry out morphological, morphometric and immunochemical analysis of the studied cells.* ***Conclusions***. *The proposed method provides an additional characteriza- tion of cells growing in soft agar, which will be useful in basic research and in evaluation of the effectiveness of anticancer drugs.*

Keywords: soft agar, evaluation of colony formation, three-dimen- sional culture of malignant cells.

Introduction. The efficiency of colony formation by tumor cells in soft agar is rather widely used to characterize malignant cells in vitro [1, 2]. The method consists in determination of the number of cells which are capable of giving rise to new colonies. In some approximation this test is considered as the ability of cells for substrate-independent growth, which could indicate a higher metastatic potential of the investigated cells. For this purpose a suspension of separate cells is placed in semisolid agar, which prevents interaction between the cells and cell adhesion to the growth surface. Thus, the resulting colonies originate from single cells. The number of cells that give rise to new colonies characterizes the carcinogenic properties of cell population [3]. However, it should be noted that an issue of the origin of metastasis from a single cell or a group of cells remains controversial, which in turn open a questions about an adequacy of this method. However, numerous studies have demonstrated a very high correlation between the efficiency of colony formation by malignant cells in soft agar and the results obtained on laboratory animals [4]. At present, the evaluation of colony formation in soft agar is considered more appropriate method for studying tumor cells than conventional monolayer culture. The experimental technique is not very complicated and used quite extensively. Usually a Petri dish is covered with a layer of 1 % agarose, and then a cell suspension in 0.3 % agarose solution is layered. An external layer of 1 % agarose is often applied to simplify the procedure of culture medium replacement. After a certain period of cultivation the colonies of cells are analyzed under a light microscope [5, 6]. The colony formation efficiency, defined as the percentage ratio between the number of the colonies formed and the number of initial cells, is used as an index of the degree of cell malignancy. Additional hardware and software are often used to calculate the number of colonies [7, 8]. However, the morphological and immunohistochemical analysis of obtained colonies of malignant cells is of particular interest. It allows both

quantitative and qualitative (by the content of investigated antigens) characterization of the growth of malignized cells in conditions of three-dimensional cultures. This paper presents an approach to obtaining histological sections for immunohistochemical analysis of cell colonies isolated from soft agar.

Materials and Methods. C*ell culture*. Cell line MCF-7 derived from breast carcinoma were cultured in medium DMEM (Sigma, USA) supplemented with 10 % fetal bovine serum (FBS, Hy Clone, USA), 50 U / ml penicillin, 50 μg / ml streptomycin, 4 mM glutamine (PAA, USA), until they reached 80–90 % monolayer confluence. The cells were removed with the solution of 0.25 % trypsin-0.02 % EDTA (PAA) and calculated in hemocytometer (Goryaev's chamber). 0.6 % agarose solution was previously prepared (Serva, USA) using deionized water, and autoclaved. Also the two-fold concentrate of cultural medium was previously prepared for subsequent mixing with agarose. At a temperature up to 40 °C $6 \cdot 10^3$ cells were mixed with 1 ml of two-fold culture medium and 1 ml of 0.6 % agarose and added into 15 ml sterile plastic tube. In 10–15 min 2 ml of culture medium was layered and the cells were cultured for 10 days, with replacing 2 ml of culture medium every 4–5 days. The fixation was performed using formalin (final concentration of 5 %) for 30 min.

Immunohistochemical analysis. The suspension of cell colonies was filtered through nylon mesh filter with the pore diameter of 40 μm with subsequent five-fold washing with buffered saline, pH 7.2 (0.8 g NaCl, 0.02 g KCl, 0.02 g KH_2PO_4, 0.082 g Na_2HPO_4, 0.013 g NaH_2PO_4 per 100 ml of buffer). Histological sections were obtained according to [the] standard histological technique with slight modification. Then the sections were stained with hematoxylin and eosin. The modification consisted in decrease of time of microsample incubation in alcohols and xylenes. Taking into account small sample size and the rate of diffusion of fixatives, alcohols and xylenes, the incubation period was decreased to 30 min. The histological sections were boiled twice in the microwave oven for 5 min in 10 mM citrate buffer for the exposure of determinants. Epithelial antigens were defined using mouse monoclonal antibodies to cytokeratins (anti-Pan cytokeratin, Clone 11, Sigma) in dilution 1:100. Mouse monoclonal antibodies (F11) obtained in our laboratory were applied to determine subcellular localization of mTOR kinase in 1:100 dilution. The immunohistochemical analysis was performed using UltraVision LP Value Detection System (Thermo, USA). Goat antibodies to mouse IgG, conjugated with FITC were used for immunofluorescent analysis (Jackson Immunoresearch, USA).

The sections were analyzed using Leica DM 1000 microscope (Germany) and Zeiss LSM 700 confocal microscope (Germany).

Results and Discussion. As usual the determination of cell capability to colony-formation in soft agar is performed by the three-layer method. The layer of 1 % agarose is applied on the bottom of cultural dish for the prevention of cell adhesion to the growth surface. Then 0.3 % agarose containing a cell suspension is layered. The upper layer of 1 % agarose allows replacement of the nutrient medium without the removal of a part of cell suspension.

The approach, proposed by us, implies the use of disposable plastic tubes instead of cultural dishes. Since the surface of such tubes is non-adhesive, there is no need to cover them with a bottom layer of 1 % agarose. Cell suspension in soft agar is placed directly into a sterile tube. So, both the bottom layer of 1 % agarose and the upper one are not applied, because it is possible to change the nutrient medium in the tube without touching the upper layer of cell suspension in agarose.

While placing cultures into CO2-incubator it is necessary to provide the gas exchange between the incubator and the tube content. Nowadays a number of producers, including TPP, Ctlltreat and others, supply sterile plastic tubes with lids, containing a filter, ensuring the gas exchange in cultivation conditions, but preventing the contamination of cultures. The cell suspension in agarose was placed into the tubes and then into the refrigerator for 10–15 min at the temperature of 4 °C for agarose solidification. Then, in the conditions of laminar box, the growth medium was gently layered onto the surface of soft agar with cells. The cells were cultured for 10–14 days at 37^0 C, 5 % CO_2.

Thereafter three-dimensional cell cultures were fixed by the addition of either glutaraldehyde to the final concentration of 2.5 % or formalin to the concentration of 5 %. Fixation was performed for 30 min up to 2 h. The amount of PBS equal to the volume of soft agar with cells was added. Thus, the final concentration of

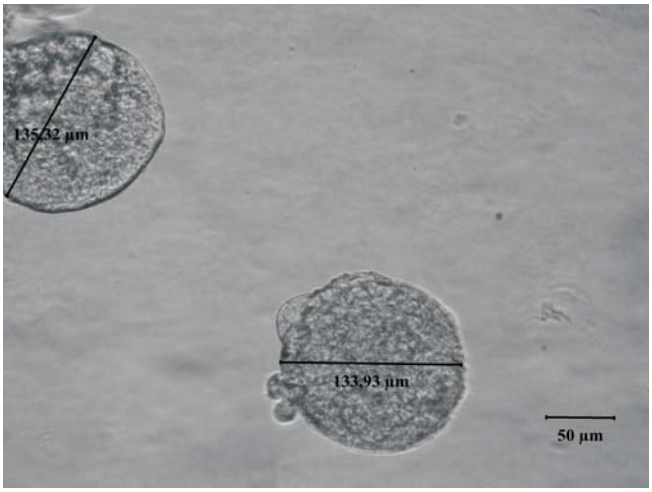

Fig. 1 Colonies of MCF-7 cells, cultivated in soft agar. Determination of the size of colonies

agarose was 0.15 %. After fixation, the suspension of colonies was placed into a water bath (60–75 °C) and incubated for 30 min with subsequent slow mixing by rotation until even suspension of colonies was reached. The suspension of cell colonies (20–100 µl) was transferred to the glass slide using the tip with a large outlet and covered by the glass sheet of 18 x 18 mm. The colony size is defined under a microscope (Fig.1) Under these conditions, all colonies of cells were arranged in one plane.

Colony growth in soft agar in tubes significantly simplified the procedure of histological sections obtaining. For this purpose the colonies were collected on the nylon filter with the pores diameter of 40–100 µm to remove the agarose. After washing off the residues of agarose, the cell cultures were dehydrated by alcohols of increasing concentration; the histological sections are obtained in accordance to the technique, described in Materials and Methods.

For example, (Fig.2) demonstrates a section of MCF-7 cell colony, grown in soft agar. The possibility of immunohistochemical techniques application for the analysis of these sections has been proved by determining the epithelial antigens, namely cytokeratins, (Fig. 3). Moreover, the subcellular localization of mTOR kinase was established in the MCF-7 cells cultured in tubes: mTOR kinase nucleolar localization (in addition to cytoplasmic) was revealed for the first time. It was further confirmed by histological sections

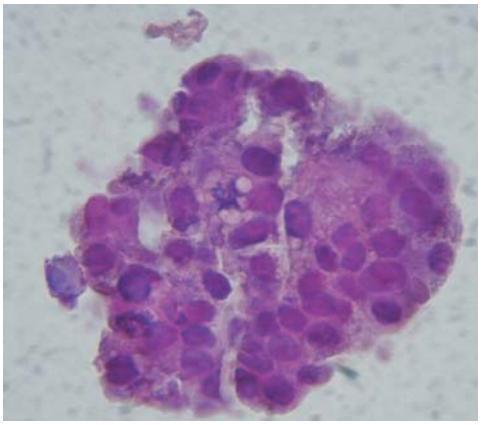

Fig. 2 Colonies of MCF-7 cells, cultivated in soft agar. Staining with hematoxylin-eosin. Ob. 10x, Oc.40 x.

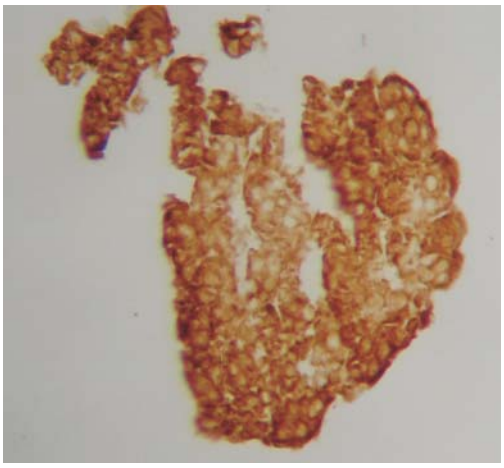

Fig. 3 Immunohistochemical detection of epithelial antigens (cytokeratins) in three-dimensional cultures of cell line MCF-7. Ob.10 x, Oc.40 x.

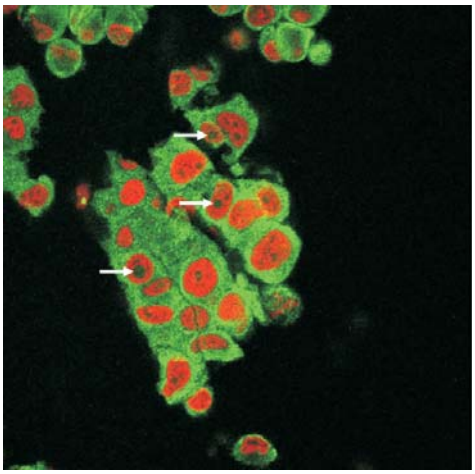

Fig. 4 Immunofluorescence analysis of subcellular localization of mTOR kinase in MCF-7 cells cultivated in soft agar. Nucleoli are indicated by arrows. Ob.10 x, Oc.63 x.

of malignant tumors of breast cancer and monolayer cultures of MCF-7 line cells (Fig.4).

Conclusions. The method proposed for the estimation of colonies, formed by malignant cells in soft agar, will allow rather fast and efficient analysis of investigated cells and considerable simplification of the procedure of their cultivation. Further immunohistochemical analysis of cells growing in three-dimensional conditions, will provide an additional information regarding the availability of specific antigens in the cells under study.

In the author's opinion, this method will be useful for basic researches of malignant properties of cells in vitro as well as for the analysis of anticancer drugs on cells of different origin.

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А. И. Хоруженко

Оптимизация условий культивирования опухголевых клеток в мягком агаре для их дальнейшео иммуногистохимического анализа

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Резюме

Цель. *Оптимизировать условия культивирования злокачественных клеток в мягком агаре для последующего иммуногистохимического анализа образованных трехмерных колоний клеток.* ***Методы***. *Культивирование клеток линии MCF-7 карциномы молочной железы в мягком агаре, иммуногистохимическое и иммунофлюоресцентное выявление эпителиальных антигенов и киназы mTOR в культивированных клетках.* ***Результаты***. *Описан методический подход к культивированию клеток в мягком агаре, позволяющий проводить морфологический, морфометрический и иммунохимический анализ исследуемых клеток.* ***Выводы***. *Предложенный метод предоставляет дополнительную характеристику клеток, растущих в мягком агаре, что может быть полезным как для базовых исследований, так при оценке эффективно- сти противоопухолевых препаратов.*

Ключевые слова: мягкий агар, оценка эффективности колониеобразования, трехмерные культуры злокачественных клеток.

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Оптимізація умов культивування пухлинних клітин у напіврідкому агарі для їхнього подальшого імуногістохімічного аналізу

Резюме

Мета. *Оптимізувати умови культивування злоякісних клітин у напіврідкому агарі для подальшого імуногістохімічного аналізу отриманих тривимірних колоній клітин.* ***Методи***. *Культивування клітин лінії MCF-7 карциноми молочної залози у напіврідкому агарі, імуногістохімічне та імунофлуоресцентне виявлення епітеліальних антигенів і кінази mTOR у культивованих клітинах.* ***Результати***. *Описано методичний підхід до культивування клітин у напіврідкому агарі, що дозволяє проводити морфологічний, морфометричний та імунохімічний аналіз досліджуваних клітин.* ***Висновки***. *Запропонований метод надає додаткову характеристику клітин, які ростуть у напіврідкому агарі, що може бути корисним як для базових досліджень, так і при оцінці ефективності протипухлинних препаратів.*

Ключові слова: напіврідкий агар, оцінка ефективності колоніє- утворення, тривимірні культури злоякісних клітин.

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