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In vitro model for study the interaction between tumor and stromal cells

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Aim. *To develop a model to study the interaction between tumor and stromal cells in three-dimensional culture.* ***Methods***. *Cultivation of HeLa cell lines and human dermal fibroblasts in monolayer and three-dimensional culture, immunofluorescent and immunohistochemical analysis.* ***Results***. *In this work we present an approach based on a direct interaction between the cells of multicellular tumor spheroids and spheroids of fibroblasts. Subsequent immunofluorescence analysis allows to determine an origin of cells in the area of their contact.* ***Conclusions***. *This model will be useful to study the basic mechanisms of carcinogenesis, and to find targets for anticancer therapy.*

Keywords: interaction of stromal and tumor cells, three-dimen- sional cultures of malignant cells, multicellular spheroids.

Introduction. The growth and development of a malignant tumor in organism occur in the context of complex homo- and heterotypical intercellular interactions that have significant effect on the survival and proliferation of tumor cells as well as on the processes of invasion, angiogenesis and metastasis [1]. Fibroblasts are the main cellular component of the malignant tumor stroma. They have been shown to secrete the components of extracellular matrix such as fibrillar collagens, fibronectin and tenascin C along with the production of a considerable amount of auto- and paracrine growth factors [2, 3]. Besides, fibroblasts have also been shown to participate in extracellular matrix remodeling via the secretion of several kinds of proteolytic enzymes, such as matrix metalloproteinases MMP2, MMP3 and MMP9 [1].

Cancer cells are known to modulate the properties of surrounding connective-tissue stroma and change its features, thus creating favorable microenvironment for further tumor progression. The process of malignant tumor development involves the activation of stromal fibroblasts, similar to that observed during wound healing and tissue fibrosis [3]. The process of fibroblast activation includes the change in a number of morpho-functional features as well as in the expression of some specific biochemical markers, such as smooth muscle actin, fibroblast-specific protein (FSP) and fibroblast-activating protein (FAP) etc. The activated fibroblasts stimulate further tumor progression through the secretion of a high number of growth factors, hormones and cytokines, including PDGF-α/β, TGFβ1, bFGF, IL-6, LPA and eicosanoids; they also affect the tumor development via establishment of direct intercellular contacts and remodeling the surrounding matrix [1, 4]. The tumor-associated fibroblasts do not only stimulate the proliferative activity of tumor cells, but also participate in the initiation of their epithelial-mesenchymal transition. In addition, they are capable to initiate the formation of cancer stem cells phenotype in the population of malignant cells [4] and play an important role in the formation of organ-specific metastatic niches, which are favorable for the extravasion of circulating cancer cells and the formation of secondary tumors [5].

In vivo and in vitro experiments have demonstrated that stromal elements affect the sensitivity of malignant cells to anti-cancer drugs and participate in the formation of tumor chemoresistance. At the same time there is convincing evidence of the fact that in some cases the normal cellular microenvironment is capable to affect the development of a malignant tumor, decreasing its proliferative activity and reducing the risk of invasion [6]. Therefore, heterotypical in vitro models employing stromal cells (e.g. fibroblasts) may become a convenient tool for investigation of molecular basis of reciprocal effect of stromal and transformed cells and their role in the initiation and inhibition of oncogenesis processes. Moreover, they will supply the information, required for the development of new approaches to prevention and therapy of oncological diseases [2, 4, 6].

There are a lot of methods which have been applied to study intercellular interactions in vitro, including indirect methods, based on the determination of the effect of a conditioned medium, obtained from fibroblast or other stroma cells, on the behavior of cancer cells in cell culture, different technologies for co-culturing cells of two and more types, etc. Both two- and three-dimensional systems, consisting of two and more cell types are used as well as other additional modifications [7, 8].

The main advantage of three-dimensional cultivation is the maintenance of the polarity and morphology as well as of the similarity of gene expression profile and activation of intracellular signaling cascades, specific for the initial tissue. It has been demonstrated that cultured cells in 2D and 3D conditions significantly differ in a variety of morphological and biochemical parameters, including the expression of receptors to the growth factors and a number of paracrine regulators, as well as in the level of proteolytic enzyme activity, migration ability and sensitivity to some therapeutic agents [8].

There are a number of approaches to organotypic three-dimensional cultures, the most often used is the formation of multicellular spheroids of one or more cell types.

To obtain three-dimensional cellular aggregates the cell suspension is applied to Matrigel or non-adhesive substrate like agarose or their analogues, which promotes the initiation of intercellular contacts and the formation of multicellular spheroids [7]. Recently, the increased application of 3D culture allowed the development of a great number of methods to obtain these aggregates, beginning from traditional techniques, like a hanging drop method, the roller flasks method and the sandwich method, and up to the creation of various bioengineering systems and microreactors for rapid isolation of multiple spheroids [8, 9].

The study of cancer and stromal cells behavior in three-dimensional conditions gives a better understanding of the heterotypical intercellular interactions in the structure of a malignant tumor and of the role of stroma components in the regulation of different stages of oncogenesis [9, 10]. The following methods are most frequently used to obtain heterotypical aggregates: the formation of spheroids of a mixed type from the cell suspension, the cultivation of spheroids of one type of cells with a monolayer of cells of another type, the addition of the cell suspension to formed spheroids of another type of cells [10, 11].

Here we present the method based on the interaction of two types of multicellular spheroids originated from human fibroblasts and HeLa cells.

Materials and Methods. Cell culture. HeLa cells originated from human cervical carcinoma were cultured in the DMEM medium (Sigma, USA) supplemented with 10% fetal calf serum (FBS, Hy Clone, USA), 50 units/ml penicillin, 50 μg/ml streptomycin, 4 mM glutamine (PAA, USA) until reaching 80–90 % monolayer confluence. Then the cells were detached using 0.25 % trypsin-0.02 % EDTA (PAA) solution and calculated in Goryaev's chamber. 1 % agarose gel was prepared ahead of time (#11400, Serva, USA) and autoclaved. 1 ml of hot agarose was layered in the wells of 24-well plate. After cooling it was washed with the serum-containing medium. Approximately $5 \cdot 10^3$ cells were added to each well and cultivated for 5 days to observe the formation of multicellular aggregates.

The primary culture of human dermal fibroblasts was kindly provided by K. A. Nizheradze (A. V. Dumansky Institute of Colloid and Water Chemistry, NAS of Ukraine). The fibroblasts were cultivated in F-12 medium with 10 % fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, 4 mM glutamine. As described above for HeLa cells, the fibroblasts were trypsinized by 0.25 % trypsin-0.02 % EDTA solution.

$5 \cdot 10^3$ cells per well were transferred into the 24-well plate, covered with agarose to allow the formation of multicellular aggregates. After five days of cultivation the aggregates, formed by HeLa cells and fibroblasts, were placed in one well and their fusion was observed. The aggregates were fixed in 10 % formalin, paraffin sections were obtained by the standard histological method.

The immunohistochemical and immunofluorescent analysis. The histological sections were boiled in 10 mM citrate buffer, pH 6, in the microwave oven (700 W) twice for 5 min with the 5 min break, for antigen retrieval procedure [12]. The autofluorescence of specimens was inhibited by the addition of 10 mM copper sulfate, 50 mM ammonium acetate buffer, pH 5, for 20 min at room temperature. The epithelial antigens were determined using mouse monoclonal antibodies to cytokeratins (anti-Pan cytokeratin, Clone 11, Sigma) in 1:100 ratio, and the fibroblasts – using antibodies to vimentin (Dako, Denmark) in 1:100 ratio. The visualization of antigens was conducted using secondary anti-mouse FITC-stained antibodies (Jacksonimmunoresearch, USA), and the immunoperoxidase reaction – using Ultra Vision LPValue Large Volume Detection system (Thermo Scientific, USA). The specimens were analyzed using Leica DM 1000 microscope (Germany).

Results and Discussion. The problem of interactions between a malignant tumor and its stroma is very complicated. Earlier it was demonstrated that during the initial stages of breast cancer formation the stromal compartment can significantly inhibit the tumor growth and spreading, but at the later stages it may stimulate the tumor progression [5]. The problem of bilateral interactions of tumor and stromal cells has been a subject for a great number of studies, thus many models have been elaborated to investigate these interactions. The current work presents a modified approach to the modeling ofstroma-tumor interaction. Classic models suggest the use of mixed cell suspension, placed in non-adhesive conditions. Our work presents the method, based on the interaction of already formed multicellular aggregates of fibroblasts (as main cellular stromal elements) and tumor cells. This approach enables studying the direct interactions between tumor cells and fibroblasts using morphological and immunohistochemical methods.

The initial culture of dermal fibroblasts was analyzed by the immunohistochemical method using antibodies to the surface antigen of human fibroblasts; no cells with negative reaction were observed (the data are not presented). The mentioned fibroblasts served as a source to obtain multicellular aggregates, spheroids.

HeLa cells formed spheroids on the second day of cultivation on the agar coated surface. The fusion of aggregates was observed during the co-cultivation of fibroblast spheroids and HeLa spheroids on the following day (Fig. 1, see the insert). In many cases the number of fibroblast spheroids was lower than the number of HeLa aggregates. In course of the interaction of aggregates, formed by HeLa cells with the fibroblast aggregates, the epithelial cells tended to locolize on top of the fibroblast spheroids, that corresponds to the general histogenesis mechanisms (Fig. 2, 3, see the insert). After the completion of cultivation term the combined three-dimensional cell cultures obtained were fixed with 10 % formalin and the paraffin sections were prepared according to the histological protocol. Immunofluorescent analysis was applied to determine fibroblast and epithelial cells in the formed aggregates. The nuclei of cells were stained with the propidium iodide. A strong immunofluorescent reaction with antibodies to the surface of human fibroblasts on monolayer culture was observed, but there was a considerably weak reaction on histological sections. Therefore, the antibodies to vimentin were used to distinguish epithelial cells and fibroblasts. Strong immunohistochemical reaction with anti-vimentin antibodies was observed in fibroblasts from the sections of interacting aggregates, but a weak reaction was present in HeLa cells as well, which corresponds to the literature data (Fig. 4, 5, see insert). Thus, the character of reaction in the cells was obviously different. The antibodies to epithelial antigens have demonstrated a positive reaction in HeLa cells, but there was no reaction in fibroblasts (Fig. 2, 3, see insert).

So, we managed to fix the area of the contact of tumor cells and fibroblasts at the histological sections of 3D cultures. This co-culture of stromal and malignant cells will be used in our further research on the intracellular signaling in tumor cells and fibroblasts.

The presented model to study the interaction between tumor and stroma can be useful for the investigation of both fundamental mechanisms of

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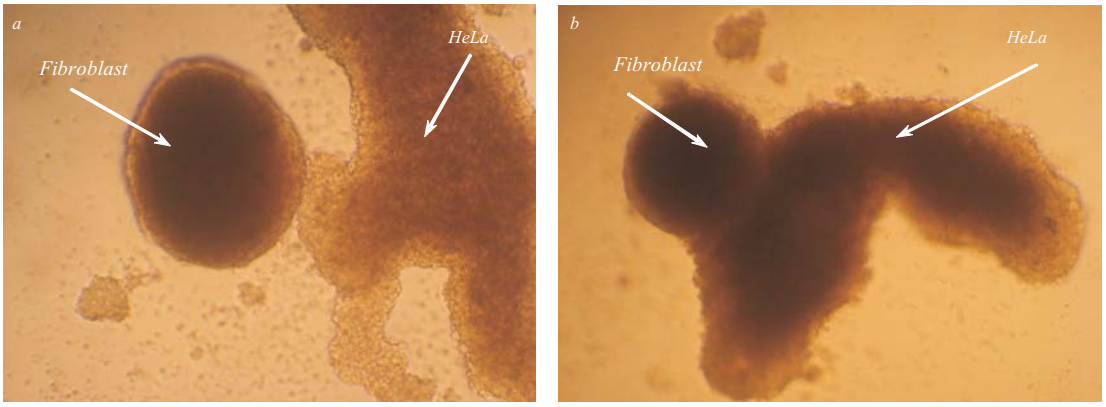

Fig.1. Co-culture of spheroids of human dermal fibroblasts and HeLa cells. *a* - 1 day of cultivation. Oc. 10x, Ob. 20x; *b* - 4 days of cultivation. Oc. 10x, Ob. 10x

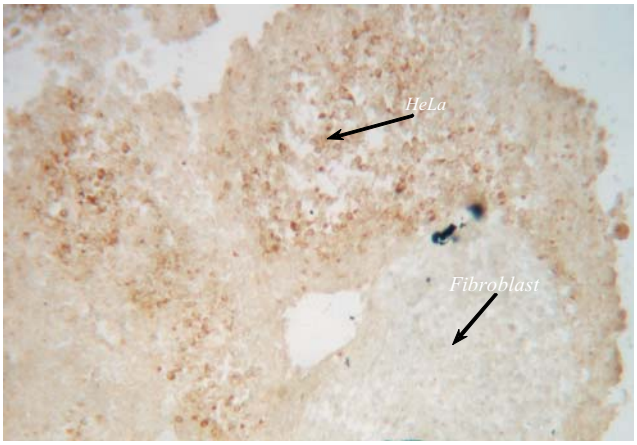

Fig.2. Multicellular aggregate obtained after fusion of spheroids of HeLa and fibroblast spheroid. Immunoperoxidase reaction (brown staining) revealed epithelial, cytokeratin positive cells surrounding fibroblast spheroid. Oc. 10x, Ob. 10x

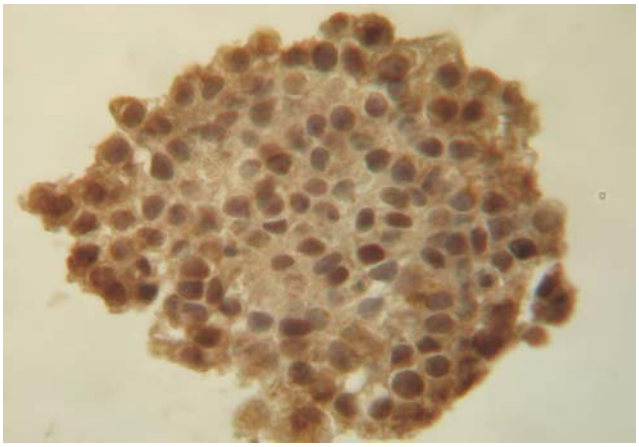

Fig.3. Immunohistochemical revealing of epithelial antigens (cytokeratins, intermediate filaments of epithelium) in HeLa cells forming spheroid. Oc. 10x, Ob. 40x

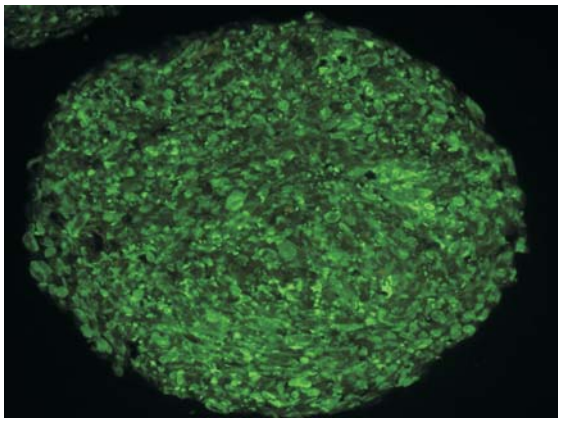

Fig.4. Immunofluorescent revealing of vimentin (fibroblast intermediate filaments) in spheroid formed fibroblasts. There is a green staining of cytoplasm. Oc. 10x, Ob. 40x

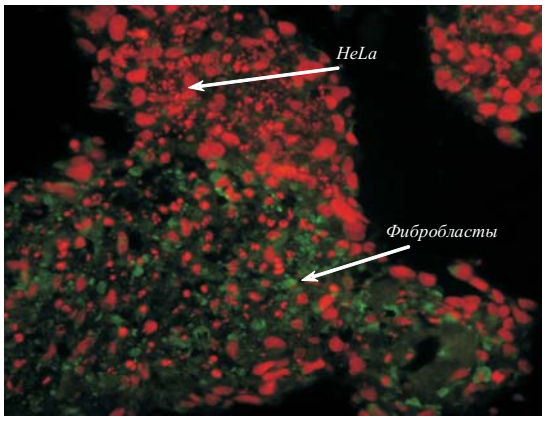

Fig. 5. Immunofluorescent detection of fibroblast by vimentin content (green) in aggregate in co-culture of HeLa and fibroblast spheroids. Cell nuclei are stained with PrI. Oc. 10x, Ob. 40x

carcinogenesis and the search for new anticancer therapy targets.

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Модель *in vitro* для изучения взаимодействия стромальных клеток и клеток опухолевого происхождения

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Summary

Цель. *Разработать модель для изучения взаимодействия опухолевых и стромальных клеток в условиях трехмерной культуры.* ***Методы***. *Культивирование клеток линии HeLa и дермальных фибробластов человека в монослойной и трехмерной культуре, иммунофлуоресцентный и иммуногистохимический анализ.* ***Результаты***. *Предложен подход, базирующийся на исследовании непосредственных взаимодейст- вий клеток многоклеточных сфероидов опухолевых клеток и сфероидов фибробластов. Последующий иммунофлуоресцентный анализ дает возможность определить происхождение клеток в зоне их контакта.* ***Выводы***. *Описанная модель будет полезна как для изучения базовых механизмов канцерогенеза, так и при поиске мишеней для противоопухолевой терапии.*

Ключевые слова: взаимодействие стромальных и опухолевых клеток, трехмерные культуры злокачественных клеток, многоклеточные сфероиды.

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Модель *in vitro* для дослідження взаємодії стромальних клітин і клітин пухлинного походження

Резюме

Мета. *Розробити модель для вивчення взаємодії пухлинних і стромальних клітин за умов тривимірної культури.* ***Методи***. *Культивування клітин лінії HeLa і дермальних фібробластів людини в моношаровий і тривимірній культурі, імунофлуоресцентний та імуногістохімічний аналіз.* ***Результати***. *Запропоновано підхід, що базується на дослідженні безпосередньої взаємодії клітин багатоклітинних сфероїдів пухлинних клітин і сфероїдів фібробластів. Подальший імунофлуоресцентний аналіз дає можливість визначити походження клітин у зоні їхнього контакту.* ***Висновки***. *Описана модель буде корисною як для вивчення базових механізмів канцерогенезу, так і за пошуку мішеней для протипухлинної терапії.*

Ключові слова: взаємодія стромальних і пухлинних клітин, тривимірні культури злоякісних клітин, багатоклітинні сфероїди.

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