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COMPARISON OF 2D AND 3D PRIMARY CELL CULTURES OBTAINED FROM EXPLANT OF HIGH-GRADE UROTHELIAL BLADDER CANCER

Studying the biological characteristics of bladder cancer in primary culture can be an effective way for diagnostic and prognostic purposes, as well as choosing a scheme for personalized therapy. **Aim.** To characterize and compare 2D and 3D primary cell cultures obtained from the same tumor sample resected from a patient with high-grade bladder cancer. **Materials and Methods.** 2D and 3D primary cell cultures were obtained from explants of resected bladder cancer. Glucose metabolism, lactate dehydrogenase (LDH) activity, and level of apoptosis were studied. **Results:** Multicellular tumor spheroids (3D) differ from planar culture (2D) by more pronounced consumption of glucose from the culture medium (1.7 times higher than 2D on Day 3 of culture), increased lactate dehydrogenase activity (2.5 times higher on Day 3 *vs.* Day 1 of cultivation, while in 2D culture LDH activity is constant), stronger acidification of the extracellular environment (pH dropped by 1 in 3D and by 0.5 in 2D). Spheroids demonstrate enhanced resistance to apoptosis (1.4 times higher). **Conclusion.** This methodological technique can be used both for tumor characterization and for selection of optimal postoperative chemotherapeutic schemes.

Keywords: bladder cancer, primary cell culture, spheroids, lactate dehydrogenase, apoptosis.

Conventional two-dimensional (2D) cell cultures are widely used in cancer research, especially for drug screening and assessing drug resistance. Nevertheless, there are several limitations of their application since in 2D cultures since the complex microenvironment of tumor tissue

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as well as tumor heterogeneity are lacking. To overcome these limitations, the technology of three-dimensional (3D) cultures has been developed [1]. The method of 3D spheroid culture of cells obtained directly from cancer tissue can be applied to various types of cancer including bladder cancer. The characteristics of cells in primary 3D cultures (spheroids) are more representative resembling those of cancer cells *in vivo.* [2]. This methodology can be especially advantageous for the selection of personalized cancer therapy [3].

The study of the characteristics of primary culture cells is important from the point of view of their maximum approximation to the state of cells in the body tissues. 2D and 3D models of cell cultures obtained from primary tumors can be instruments for therapeutic testing and studying cytopathophysiology of tumors. Therefore, the characterization of such cells is an important task.

The aim of this work was to characterize the primary cell culture obtained by the explant method from aggressive high-grade urinary bladder cancer and to compare their properties in 2D and 3D cultures.

Materials and Methods

The primary cell culture of bladder cancer was obtained by the explant method from the postoperative cancer tissue resected during the surgery of a 70-year-old male patient with a history of painless gross hematuria due to early relapsing high-grade bladder cancer. The patient underwent TURBT for the primary tumor and radical cystectomy for the relapse of cancer. The ethics committee of the Clinical Hospital "Feofaniya" approved this study. The patient provided his consent for the use of surgical samples for scientific research.

3 mm^3 fragments of cancerous tissue were excised and seeded on 6-well plates (4—7 explants per well). The explants were cultured under standard conditions in a RPMI-1640 medium with 10% FBS and 100 μl of antibiotic–antimycotic agent (Sigma, USA). After 7 days of culture, we isolated the multicellular spheroids (3D growth) and adhesive cells (2D growth). Then each of the fractions was planted onto 12-well plates, and the biochemical and morphological analyses were performed.

The glucose consumption from the incubation medium of primary bladder cancer cell culture was assessed at the end of Days 1, 2, and 3 of culture starting from cell isolation from the explants. The initial cell concentration was 1×10^5 cells/ml in the sample volume of 200 μl. Measurement of glucose level (mmol) was carried out using a standard set based on the glucose-oxidase reaction modified for the cell culture medium [4]. The intensity of glucose absorption was assessed by the decrease in its amount in the medium.

The lactate dehydrogenase (LDH) activity was assessed by the spectrophotometric method in the same time intervals by the rate of NADH oxidation during the reduction of pyruvic acid (pyruvate + NADH + $H^+ \leftrightarrow$ L-lactate + NAD^+) [5]. The samples were incubated with pyruvate and NADH, and then the decrease in NADH absorption at 340 nm was measured. The reduction in the extinction (ΔE) by 1 μmol min^{-1} was measured. The results were presented in nanokatals (1 U equals 16.67 nanokatals).

The pH in the culture medium was determined using a portable pH meter with a microsensor InLab Ultra-Micro-ISM (Mettler Toledo, Belgium).

The level of apoptosis in the cell culture on Day 3 of incubation was determined by the generally accepted flow cytometric method with an AnnexinV-FITC/PI (Beckman Coulter, USA) [6].

Morphological analysis of cultured cells and cell complexes was performed using an inverted microscope Axiovert 40C (Zeiss, Germany) with the AxioVision software.

Results and discussion

Postoperative material was obtained from the resected tumor representing histologically confirmed high-grade bladder cancer with invasion to muscles graded as pT2aN0M0 G3 urothelial bladder cancer, stage II.

Urothelial carcinoma is known to have a wide range of histological variants [9, 10]. The determination of its variants bears significant clinical significance for the diagnosis, treatment, and prognosis of the disease.

In our case, we observed an infiltrative lesion, in which the neoplastic cells invaded the bladder wall as nests or small clusters, irregular in size and shape. In the adjacent areas, an inflammatory cell infiltrate was noted. The neoplastic cells were enlarged and had modest amounts of pale to eosinophilic cytoplasm and nuclear atypia (Fig. 1). No lymphatic or vascular invasion was seen. Histologically, this tumor type could be graded as a nested variant of urothelial carcinoma [9].

The morphology of the explants (after separation of the upper cell mass on Day 7 of culture) is presented in Fig. 2. These structures retain a tissue-like composition and fairly abundant remnants of vascularization. It is possible that this organization contributes to the maintenance of active proliferation and the formation of new cell layers around the explant. The newly-formed cells migrate intensively and abundantly synthesize an extracellular matrix, which confirms their fibroblast-like properties (Fig. 2). Cells migrating from the explant attach to the substrate, and in the remote areas, the proliferation of cells and large aggregates of blood cell elements are observed.

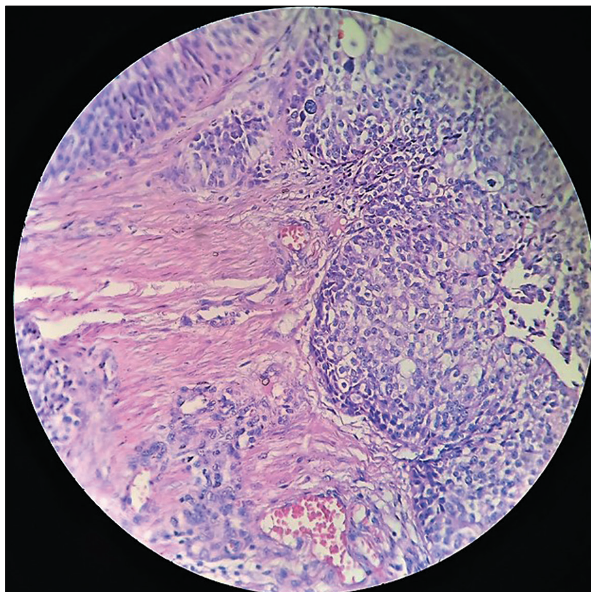

Fig. 1. Histology of high-grade invasive urothelial carcinoma. H&E, 400×

Among the migrating cells, one can detect attached large cells, which may belong to either fibroblast-like cells or endothelial cells. Also, one can see abundant red blood cells and platelets, persisted in the process of explant production as physiological buffers used do not cause lysis of red blood cells.

The cells of the primary culture obtained after 7 days of incubation of the initial explant of tumor tissue were isolated for further cloning on a 24-well plate. Subpopulations of cells with different growth characteristics in culture were obtained. After 3 days of incubation, in some wells, so-called spheroids were observed, which accounted for up to 80—90% of the cell population, and the remaining cells were attached to the substrate in the form of an adhesive monolayer cell subpopulation (Fig. 3).

The obtained 3D (spheroid) and 2D (planar) cultures demonstrate the absence of red blood cells and stroma cells. The cultures consisted of identical types of cells. However, in 2D culture, cells with fibroblast-like morphology were noted, and their number grew from 2—3% to 5—7% from Day 1 to Day 3. In 3D cultures, such cells were not detected.

Cells in 3D culture consumed glucose from the incubation medium more intensively compared to 2D culture (Table 1). The intensity of the consumption increased by 1.7 times on Day 2 and almost tripled on Day 3 compared to Day 1.

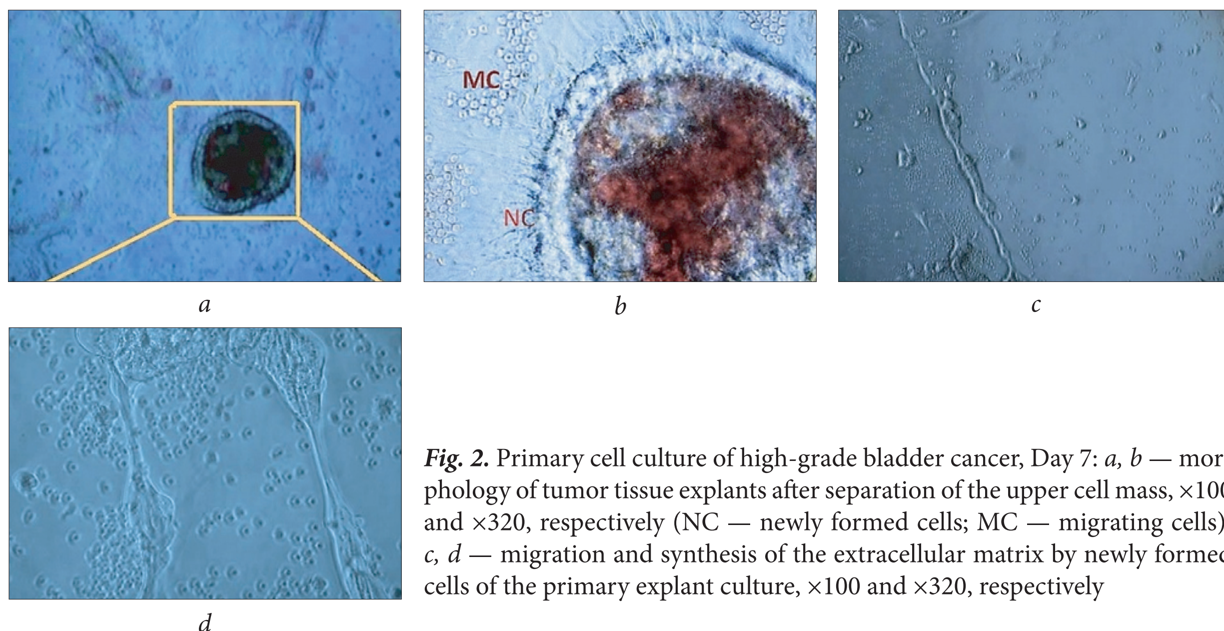

Fig. 2. Primary cell culture of high-grade bladder cancer, Day 7: *a, b* — morphology of tumor tissue explants after separation of the upper cell mass, ×100 and ×320, respectively (NC — newly formed cells; MC — migrating cells). *c, d* — migration and synthesis of the extracellular matrix by newly formed cells of the primary explant culture, ×100 and ×320, respectively

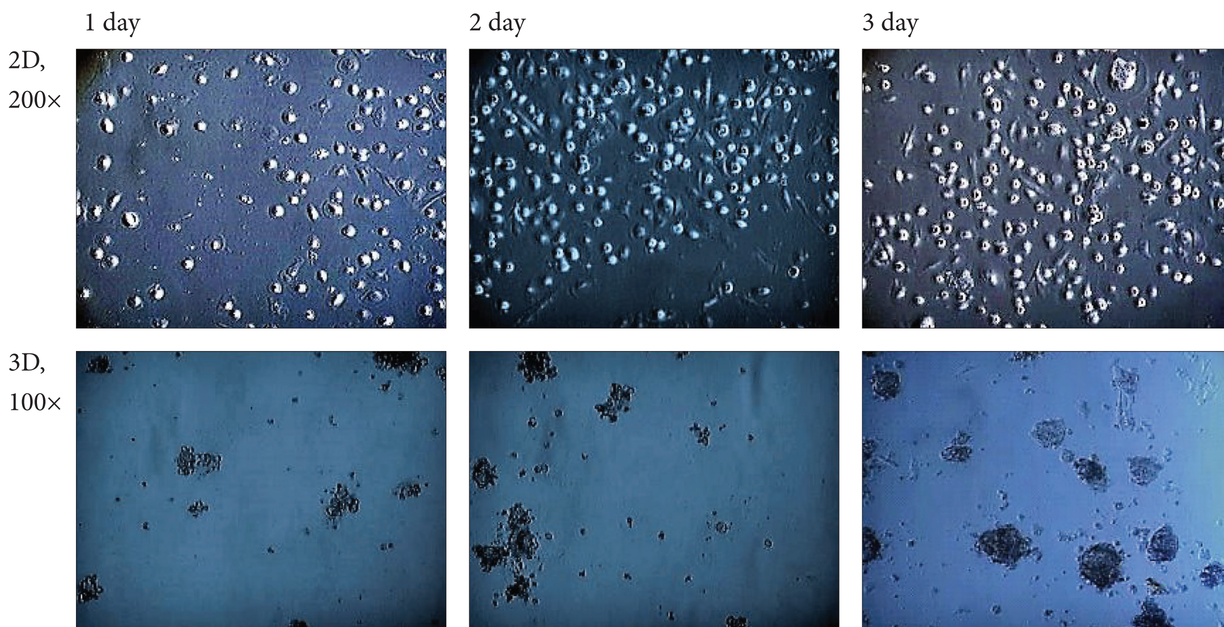

Fig. 3. Formation of 2D (adhesive subpopulation) and 3D (spheroidal complexes) cultures from cells in the primary explant culture after 7 days of pre-cultivation

At the same time, the monolayer cell culture demonstrated a less active increase in glucose consumption, only by 1.6 times on Day 3. Also, a higher increase in the total LDH activity in the microenvironment of the 3D culture was determined on Day 3 — 2.5 times higher ($p < 0.05$). The level of LDH in monolayer 2D culture did not change.

The data presented above highlight the fact that either planar (2D) or spheroid (3D) formation of the primary culture of explanted high-grade urothelial carcinoma cells occurs in the

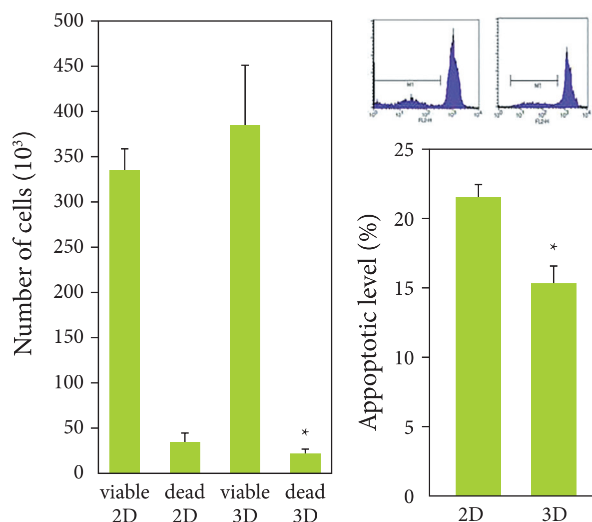

Fig. 4. Concentration of live and dead cells and the level of apoptotic cells on Day 3 of cultivation after culture fractionation for 2D and 3D growth. *$p < 0.05$ compared to 2D growth

wake of the metabolic reprogramming characteristics of cancer cells such as the predominance of glycolysis manifested by increased glucose uptake and lactic acid production, which is more pronounced in 3D cell culture (spheroids).

An increase in the activity of LDH in cancer cells and related accumulation of lactate are the reasons for the acidic shift in pH balance in these cells and in their extracellular environment. According to our data, the acidification was more intense in 3D culture.

Increased glucose uptake and lactate accumulation are common features of cancer cells. Lactate itself causes migration of cells and their complexes, and also induces the secretion of hyaluronic acid by tumor-associated fibroblasts, creating a favorable environment for migration [11]. Another important property of lactate is its ability to stimulate VEGF production by endothelial cells, leading to enhanced migration and resulting in lactate-induced angiogenesis independently of oxygenation [12].

The dysregulation of pH is a well-known hallmark of solid tumors. Cancer cells are characterized by a pH increase inside the cell and a decrease in the surrounding environment [13, 14]. Such shifts in acidity accompany both the metabolic reprogramming of cancer cells and their increased oncogenic properties such as proliferation survival, migration, and metastasis [14]. In our case, the decline in pH allows us to state that the 3D culture bears more pronounced tumorigenic properties.

Such tumorigenic properties of cells growing in spheroids are also confirmed by a comparative assessment of the apoptosis level and cell survival in different forms of growth. By Day 3, the number of apoptotic cells in 3D culture was 1.4-fold lower than in the 2D culture monolayer (15.4%

Table 1. **Parameters of glucose metabolism in monolayer primary cell culture (2D) and spheroid cell culture (3D) from high-grade urothelial carcinoma**

Parameters	Type of culture	Day of culture		
		1	2	3
Level of LDH-activity (mkat/l)	2D	0.056 ± 0.003	0.062 ± 0.004	0.057 ± 0.001
	3D	0.039 ± 0.007*	0.067 ± 0.002	0.096 ± 0.01*
Level of glucose in medium (mM)	2D	9.3 ± 0.6	8.9 ± 0.5	5.8 ± 0.9
	3D	9.3 ± 0.6	5.4 ± 0.3*	3.2 ± 0.3*
pH of culture medium	2D	7.3	7.1	6.8
	3D	7.2	6.5*	6.2*

Note: *$p < 0.05$, 3D vs. 2D.

vs. 21.6%), which means a higher cellular survival rate in 3D primary culture (Fig. 4). Moreover, the percentage of dead cells was lower in 3D culture.

In the primary culture of urothelial carcinoma, cells forming spheroids have more pronounced tumorigenic properties and can be considered as a more adequate model for studying the properties and behavior of aggressive invasive bladder cancer.

This methodological technique can be used for both tumor characterization and selection of optimal postoperative chemotherapeutical schemes.

Authors' contributions

All authors equally contributed to this work.

Competing interests

The authors have no competing interests in regard to the publication of this study.

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ПОРІВНЯННЯ 2D І 3D ПЕРВИННОЇ КУЛЬТУРИ КЛІТИН, ОТРИМАНОЇ З ЕКСПЛАНТАТА НИЗЬКОДИФЕРЕНЦІЙОВАНОГО УРОТЕЛІАЛЬНОГО РАКУ СЕЧОВОГО МІХУРА

Вивчення біологічних характеристик раку сечового міхура в первинній культурі є важливим для встановлення діагнозу, визначення прогнозу та вибору схеми персоналізованої терапії. **Мета.** Охарактеризувати та порівняти 2D і 3D первинну культуру, отриману з одного зразка пухлинної тканини хворого на низькодиференційований рак сечового міхура. **Матеріали та методи.** 2D і 3D первинну культуру отримували з експлантата клітин раку сечового міхура. Визначали метаболізм глюкози, активність лактатдегідрогенази та рівень апоптозу. **Результати.** Багатоклітинні сфероїди пухлинних клітин (3D) відрізняються від культури 2D більшим рівнем споживання глюкози із поживного середовища (в 1,7 рази на 3-ю добу культивування), підвищеною активністю лактатдегідрогенази (збільшується в 2,5 рази на 3-ю добу в 3D культурі та залишається незмінною на 3-ю добу в 2D культурі), більшим закисленням позаклітинного середовища (pH знижується на одиницю в 3D культурі і на 0,5 в 2D культурі). Сфероїди демонструють більшу резистентність до апоптозу (в 1,4 рази). **Висновки.** Описаний методологічний підхід може бути корисним для характеристики пухлини, а також для оптимізації схем післяопераційного лікування.

Ключові слова: рак сечового міхура, первинна культура клітин, сфероїди, лактатдегідрогеназа, апоптоз.