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ORIGINAL ARTICLE

Experience in creating primary cultures of endometrial cancer and studying cells carrying phenotype of cancer stem cells

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ABSTRACT

Purpose of the study. Was to investigate the possibility of applying the method of spheroid formation in culture for assessment of the endometrial cancer (EC) tumor stem cells (TSC) content in complex samples containing various tumor cells and microenvironment.

Materials and methods. Primary cultures were obtained from fragments of tumors removed during surgery as a first stage of treatment at the Department of Gynecological Oncology, the National Medical Research Center for Oncology. After enzymatic disaggregation of tissue, cell suspension was passaged in DMEM medium containing 10 % fetal bovine serum and 1 % gentamicin to obtain primary two-dimensional cultures. To study the ability of cells to form spheroids, the primary culture was removed from the culture plate and passaged with 2.0×10^4 cells per well of a six-well plate (n = 6) in DMEM medium containing 0.35 % agarose and growth factors EGF (20 ng/ml) and FGF (20 ng/ml). After two weeks of cultivation, the average size, number of formed spheroids, and frequency of spheroid formation were determined. For those cultures that had formed spheroids, immunofluorescent staining of the two-dimensional culture for the marker CD133 was performed, after which the frequency of CD133+ cells was determined.

Results. A total of nine primary cultures of EC were obtained, five of which formed spheroids within two weeks of cultivation under non-adhesive conditions. In these cultures, small polygonal CD133+ cells showed the strongest association with spheroid formation, which were associated with the largest spheroids (98–110 μm in diameter).

Conclusion. There is a large number of microenvironmental cells in mixed cultures of CSC, some of which may express CD133, including healthy stem cells that also form spheroids in soft agar. A more detailed study of CSC subpopulations compared to normal endometrium is required to establish a link between the observed diversity of cells in culture and their ability to form spheroids and other characteristics of tumor stem cells.

Keywords: endometrial cancer, primary cell cultures, cancer stem cells, cell spheroids

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Compliance with ethical standards: the work followed the ethical principles set forth by World Medical Association Declaration of Helsinki, 1964, ed. 2013. The study was approved by the Committee on Biomedical Ethics at the National Medical Research Centre for Oncology (extract from the minutes of the meeting No. 25 dated 09/08/2022). Informed consent has been obtained from all participants of the study

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3.1.6. Онкология, лучевая терапия
ОРИГИНАЛЬНАЯ СТАТЬЯ

Опыт создания первичных культур рака эндометрия и исследования в них клеток, обладающих фенотипом опухолевых стволовых клеток

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РЕЗЮМЕ

Цель исследования. Изучить возможность применения метода сфероидообразования в культуре для оценки содержания опухолевых стволовых клеток (ОСК) рака эндометрия (РЭ) в сложных образцах, содержащих различные клетки опухоли и микроокружения.

Материалы и методы. Первичные культуры получали из фрагментов опухолей, удаленных в ходе оперативного вмешательства, проводимого в качестве первого этапа по лечению РЭ в отделении онкогинекологии ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации. После ферментативной дезагрегации ткани клеточную суспензию пассировали в среде DMEM, содержащей 10 % фетальной бычьей сыворотки (ФБС) и 1 % гентамицина, для получения первичных двумерных культур. Для изучения способности клеток к сфероидообразованию первичную культуру снимали с культурального пластика и пассировали по 2,0 × 10^4 клеток на лунку 6-тилуночного планшета (n = 6) в среду DMEM, содержащую 0,35 % агарозы и факторы роста EGF (20 нг/мл) и FGF (20 нг/мл). Через две недели культивирования определяли средний размер, количество образующихся сфероидов и частоту сфероидообразования. Для тех культур, которые образовали сфероиды, было проведено иммунофлуоресцентное окрашивание двумерной культуры на маркер CD133, после чего определяли частоту CD133+ клеток.

Результаты. Всего было получено девять первичных культур РЭ, из которых только пять образовали сфероиды спустя две недели культивирования в условиях, не поддерживающих адгезию. В этих культурах наибольшую связь со сфероидообразованием показали небольшие полигональные CD133+ клетки, с которыми ассоциировались наиболее крупные сфероиды (98–110 мкм в диаметре).

Заключение. В смешанных культурах РЭ присутствует большое количество клеток микроокружения, из которого часть клеток может экспрессировать CD133, в том числе нормальные стволовые клетки, также образующие сфероиды в мягком агаре. Требуется более подробное изучение клеточных субпопуляций РЭ в сравнении с нормальным эндометрием для установления связи между наблюдаемым разнообразием клеток в культуре и их способностью к сфероидообразованию и другими характеристиками ОСК.

Ключевые слова: рак эндометрия, первичные культуры, опухолевые стволовые клетки, клеточные сфероиды

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Соблюдение этических стандартов: в работе соблюдались этические принципы, предъявляемые Хельсинкской декларацией Всемирной медицинской ассоциации (World Medical Association Declaration of Helsinki, 1964, ред. 2013). Исследование одобрено Комитетом по биомедицинской этике при ФГБУ «Национальный медицинский исследовательский центр онкологии» Министерства здравоохранения Российской Федерации (выписка из протокола заседания № 25 от 08.09.2022 г.). Информированное согласие получено от всех участников исследования

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INTRODUCTION

Endometrial cancer (EC) is the sixth most common type of cancer in women. Over the past 30 years, the overall incidence of EC has increased by 132 %, reflecting an increase in the prevalence of risk factors, in particular, obesity and aging of the population [1]. In Russia, EC occupies the 2nd rank in the structure of oncological diseases of the female genital organs. The total number of patients with EC reached 195.6 per 100,000 population in 2022, which is 31.8 % more than in 2012 [2]. Thus, there is still a need to develop new therapeutic approaches to significantly improve the prognosis in women with recurrent EC or in the later stages of the disease. In this context, tumor stem cells (TSC) of endometrial cancer, capable of self-renewal and differentiation into mature tumor cells, as well as contributing to tumor recurrence, metastasis, heterogeneity, multidrug and radiation resistance, represent a potential target for drug development [3].

TSC were first identified in patients with acute myeloid leukemia in 1994, and have since been considered potential therapeutic targets in the treatment of oncological diseases, including solid tumors [4]. TSC in EC were first described by Hubbard in 2009 [5]. The discovery of endometrial OSCs has radically changed the views on the study of the biology of EC and the development of approaches to the treatment of this disease. As a rule, EC TSC is identified by the expression of specific antigens, by the weak accumulation of the Hoechst 33342 nuclear dye (the so–called side population – "side population"), by the ability to form colonies under conditions of reduced adhesion and initiate the growth of a tumor containing TSC and differentiated cells originating from them with the same phenotype as "parental", in mice with immunodeficiency [3].

A number of markers associated with the TSC of solid tumors have been studied in EC. Aldehyde dehydrogenase 1 (ALDH1) is one of 19 different enzymes involved in aldehyde oxidation. This enzyme is highly active in the early stages of stem cell differentiation. Atypical EC cells with a high level of ALDH1 expression are more tumorogenic, invasive and resistant to cisplatin than cells with a low level of ALDH1 expression. Also, a high level of ALDH1 expression correlates with a worse prognosis in patients with EC [6]. Receptor tyrosine kinase c-Kit or CD117 is a receptor for the Stem Cell Factor (SCF) and, upon activation, triggers a number of intracellular signaling cascades regulating cell survival, migration and proliferation, including TSC [7]. When studying the cellular composition of EC, it was shown that $CD117^+$ cells isolated from Ishikawa and MFE280 EC cultures exhibit greater proliferative ability, as well as the ability to form colonies in soft agar in the presence of SCF. A high level of CD117 expression was also recognized as an independent prognostic factor correlating with the progression of EC [8]. The CD55 antigen is a complement decay acceleration factor and is expressed at a high level in the TSC of endometrioid ovarian cancer and EC. It has been shown that $CD55^+$ cells are able to regulate cell self-renewal and their resistance to cisplatin to a greater extent than CD55 cells [9]. A link with TSC has also been established for the transmembrane glycoprotein CD44, which plays the role of an adhesion molecule. Cells overexpressing CD44 possess such characteristics of TSC as the ability to self-renew and epithelial-mesenchymal transition (EMF), as well as resistance to chemotherapy and radiation therapy [10]. Probably, this marker is also related to TSC EC, since oncospheres obtained on soft agar from cells of EC cultures with stem properties are CD44 positive [3]. In addition, a number of studies have noted the co-expression of CD44 and another TSC marker CD133 in the EC tissue [11, 12]. The transmembrane glycoprotein of the cell surface prominin-1 or CD133 has attracted considerable attention due to the fact that its expression is often observed in various subpopulations of somatic stem cells. Usually, this glycoprotein is observed in the area of various microvilli and protrusions of the plasma membrane, where CD133 can act as a regulator of the lipid composition of membranes or participate in the mechanisms of cellular polarity and migration [13]. In a study by Rutella et al. (2009) subpopulations of cells with the $CD133^+/CD44^+$ phenotype isolated from permanent endometrial cancer cell lines showed the ability to form tumor spheres, increased chemoresistance and were able to initiate the formation of a tumor with the same phenotype as the original tumor when transplanted to immunodeficient mice [14]. Gao (2012) also investigated the AN 3CA line and showed that $CD133^+$ cells express stem markers, demonstrate greater mobility and invasive ability than CD133 cells [15]. In Friel's work, the expression of CD133 in the cells of primary EC tumors and the mechanism for

controlling the expression of this marker in them were investigated [16]. The authors showed that $CD133^+$ cells accounted for 5.7–27.4 % of the total population of tumor cells in the analyzed primary tumors. These cells had increased tumorigenicity in immunodeficient mice, which suggests that these cells belong to the TSC. Similar results were obtained in the work of Sun (2017), where $CD133^+CD44^+$ cells showed a stronger association with all the classical properties of TSC than for other markers studied in the work, such as CD24, CD47, CD29, CXCR4, SSEA3 and SSEA4. The efficiency of spheroid formation in soft agar was 11.7 % for $CD133^+$ cells and 1.7 % for CD133 cells [17]. In addition, $CD133^+CD44^+$ cells showed an increased expression of stem cell transcription factors Myc, Sox-2, Nanog and Oct4 compared with other subpopulations [16], for which a direct relationship with the degree of malignancy of endometrioid carcinoma was established [18].

Despite the established connection between CD133 and EC TSC in a number of studies, data on the relationship between the expression of this marker and the prognosis of the disease course remain quite contradictory. Thus, the work of Elbasateeny (2016) indicates a more pronounced association of CD133 expression with the early stage of the tumor (I–II) and a decrease in the expression of this marker at later stages of the disease. The authors suggested that CD44 and CD133 may be involved in the development of endometrial cancer in the early stages, and their overexpression may contribute to the early diagnosis of endometrial cancer [11]. These results are supported by the data obtained in Mancebo (2017), in which the authors found that tumors in which CD133 expression was high were less likely to have vascular invasion and more likely to be highly differentiated, and were also associated with higher overall and recurrence-free survival [19]. However, there is also the opposite data. Thus, Nakamura (2010) showed a negative correlation between CD133 expression in tumor tissue and life expectancy without recurrence [20]. The negative prognosis of the course of EC with increased CD133 expression in tumor tissue was confirmed in the work of Park in 2019 [12]. The observed discrepancies in the data of different authors may be related to the fact that CD133 expression is observed not only in the EC TSC, but also in normal cells of the glandular epithelium of the endometrium [21]. Thus, CD133 in endometrial tissue can act simultaneously as a marker of epithelial differentiation and as a marker of OSC.

To study the dynamics, functioning and regulation of stem cells, experimental methods are needed to clearly distinguish between stem cells and their offspring. Due to the lack of unique cell surface markers specific only to stem cells and a distinct morphological phenotype, stem cells are usually identified based on functional criteria. Stem cells from various tissues are usually cultured *in vitro* in the form of spheroids under conditions excluding adhesion [22]. According to the literature data, the study of TSC in EC by the method of counting spheroids in conditions that do not support cell adhesion, both on simple samples obtained after sorting by any marker [14, 17] and in whole samples of the primary culture of EC [23]. In the latter case, the quantitative analysis was reduced to measuring the size of spheroids, while the assessment of the quantitative content of TSC and the establishment of a link with the clinical and pathological characteristics of the sample was not carried out.

The purpose of the study: to study the possibility of using the method of spheroid formation in culture to assess the content of EC TSC in complex samples containing various tumor cells and microenvironments. To achieve this goal, we compared the morphological characteristics of primary EC cultures under conditions of adhesive growth and in soft agar, and also studied the expression of the CD133 marker in two-dimensional cultures.

MATERIALS AND METHODS

Nine primary cultures of EC were obtained from fragments of tumors removed during surgery performed as the first stage of the treatment of EC. Patients with EC were treated in the Oncological Gynecology department of the National Medical Research Centre for Oncology, in 2023. The histological diagnosis was confirmed in the pathoanatomical department of the of the National Medical Research Centre for Oncology. The patients were aware of their participation in the scientific study and signed an informed consent for the collection of biological material. The pathologist isolated a 0.5 cm^3 fragment corresponding to the malignant component of the tumor within 20 minutes after extraction of

the drug and placed it in a Hanks solution (Gibco, USA) containing 1 % gentamicin (Biolot, Russia). Next, the sample was fragmented with a scalpel to a size of 1–2 mm^3, after which 300 u/ml of collagenase I (Thermo Fisher Scientific, USA) was added in a DMEM medium (Biolot, Russia) and incubated for an hour at a temperature of 37 °C with constant stirring. At the end of fermentation, the sample was additionally crushed by pipetting and passed through a sterile nylon filter (d = 70 μm) (Beckton Dickinson, USA). The resulting suspension was washed twice in a phosphate buffer and passed onto a culture vial in a DMEM medium containing 10 % FBS (Biolot, Russia) and 1 % gentamicin and cultured under standard conditions at 37 °C and 5.0 % CO_2.

To study the ability of cells to spheroid formation, a two-dimensional culture was removed from culture plastic using a standard technique using a 0.1 % trypsin solution (Biolot, Russia). The resulting cell suspension was mixed with 0.35 % agarose solution in a serum-free DMEM medium with the addition of insulin-transferin and growth factors EGF (20 ng/ml) and FGF (20 ng/ml) and layered on a base of 0.7 % agarose in the same medium. In total, 2.0 × 104 cells were added in this way to the well of a 6-hole tablet. There were 6 repetitions for each culture. The plates with cells were cultured for 2 weeks, during which time the spheroids were photographed. At the end of cultivation, the average size was determined and the spheroids in the well were counted using a Lionheart FX imager (BioTek, USA) using embedded software. The frequency of spheroid formation was calculated as the ratio of the number of spheroids larger than 40 μm in diameter to the total number of cells passioned into the well of the tablet.

To carry out immunophenotyping on CD133, primary cultures were planted on cover glasses. After the formation of the cellular monolayer, the glasses were fixed in a 4 % paraformaldehyde solution for 15 minutes at room temperature, after which permeabilization was carried out in a 0.5 % Triton X-100 solution on a phosphate buffer for 10 minutes. After washing, the glass was blocked for an hour in a solution of 5 % normal goat serum (Gibco, USA) in a phosphate buffer, after which it was kept overnight at 4 °C in a solution of primary polyclonal rabbit antibody to CD133 (ab19898, Abcam, USA) in a phosphate buffer (1/100 dilution) with the addition of 1 % goat serum. After washing the glass three times, it was kept at room temperature for an hour in a solution of secondary goat antibody conjugated with Alexa Flour®594 (ab150080, Abcam, USA) (1/500 dilution), after which they were washed with a phosphate buffer, repainted in a solution of the nuclear dye DAPI (ab228549, Abcam, USA) and mounted on slides in an Anti-Fade Fluorescence Mounting Medium (ab104135, Abcam, USA). The proportion of CD133 positive cells was determined on the Lionheart FX imager (BioTek, USA) using embedded software. 3 glasses were examined for each culture.

The values of the diameter of the spheroids, the frequency of spheroid formation and the proportion of $CD133^+$ cells are given as a sample mean ± standard deviation.

STUDY RESULTS

We received 9 primary cultures of EC from 9 patients who were treated in the Department of Oncological Gynecology at the of the National Medical Research Centre for Oncology in 2023. The results of the pathological examination showed that 2 tumors belonged to the histological type of highly differentiated (G1), 6 to moderately differentiated (G2) and one tumor to the low–differentiated type G3 of endometrioid adenocarcinomas. At the same time, lympho-vascular invasion was detected in three cases, but none of the patients had metastases in regional lymph nodes. All cases were attributed to stage I–II of the disease.

All primary cell cultures formed a monolayer on day 3 of cultivation under conditions that support adhesion. The EC cells in the monolayer culture were relatively small in size and polygonal in shape, often forming "islands" and "rosettes" that were located among the more elongated stroma cells, presumably fibroblasts. When cultured in conditions that do not support cell adhesion on agarose in the medium for TSCs, the formation of spheroids was noted in 5 out of 9 cultures, starting from the 5th day of cultivation. In four other cultures, spheroids did not form even after 2 weeks of cultivation. 1 culture obtained from a highly differentiated tumor and 3 cultures from moderately differentiated tumors did not form spheroids. Thus, there was no obvious relationship between the frequency of spheroid formation and the degree of tumor differentiation. For cultures that formed spheroids in soft agar, additional staining of

the two-dimensional culture on the CD133 marker was performed.

EC cell culture No. 1. Highly differentiated G1 endometrioid adenocarcinoma without signs of vascular invasion. In the primary culture, single large cells of polygonal or rounded shape were observed among numerous elongated cells, presumably fibroblasts (Fig. 1A).

CD133 expression is weak, and rare marker granules are observed in all cells (Fig. 1b). The frequency of $CD133^+$ cells ranged from 0.2 to 1.0 % (0.8 ± 0.15 %). In the culture in agarose, a very slow growth of cellular spheroids from single large cells was observed. On the fifth day of cultivation, spheroids had not yet been detected (Fig. 1B), and two weeks later small spheroids of 4–8 cells were formed (Fig. 1G). The frequency of spheroid formation on the 14th day ranged from 0.1 to 0.7 % (0.5 ± 0.1 %).

EC cell culture No. 2. Low-grade G3 endometrioid adenocarcinoma without signs of vascular invasion. Under cultivation conditions supporting cell adhesion, the cells of the primary culture formed a monolayer of two types of cells, similar in appearance to the culture of EC No. 3 (Fig. 3A): small polygonal cells united in islands among larger and elongated cells similar to fibroblasts (Fig. 2A).

At the same time, islands of polygonal cells could form spheroid-like structures, within which increased CD133 expression was observed. Also, among the monolayer, there were individual large cells expressing CD133 above the general level (Fig. 2B). The frequency of $CD133^+$ cells ranged from 2.7 to 8.0 % (4.8 ± 1.5 %). On the fifth day of cultivation in conditions that do not support adhesion, in the medium

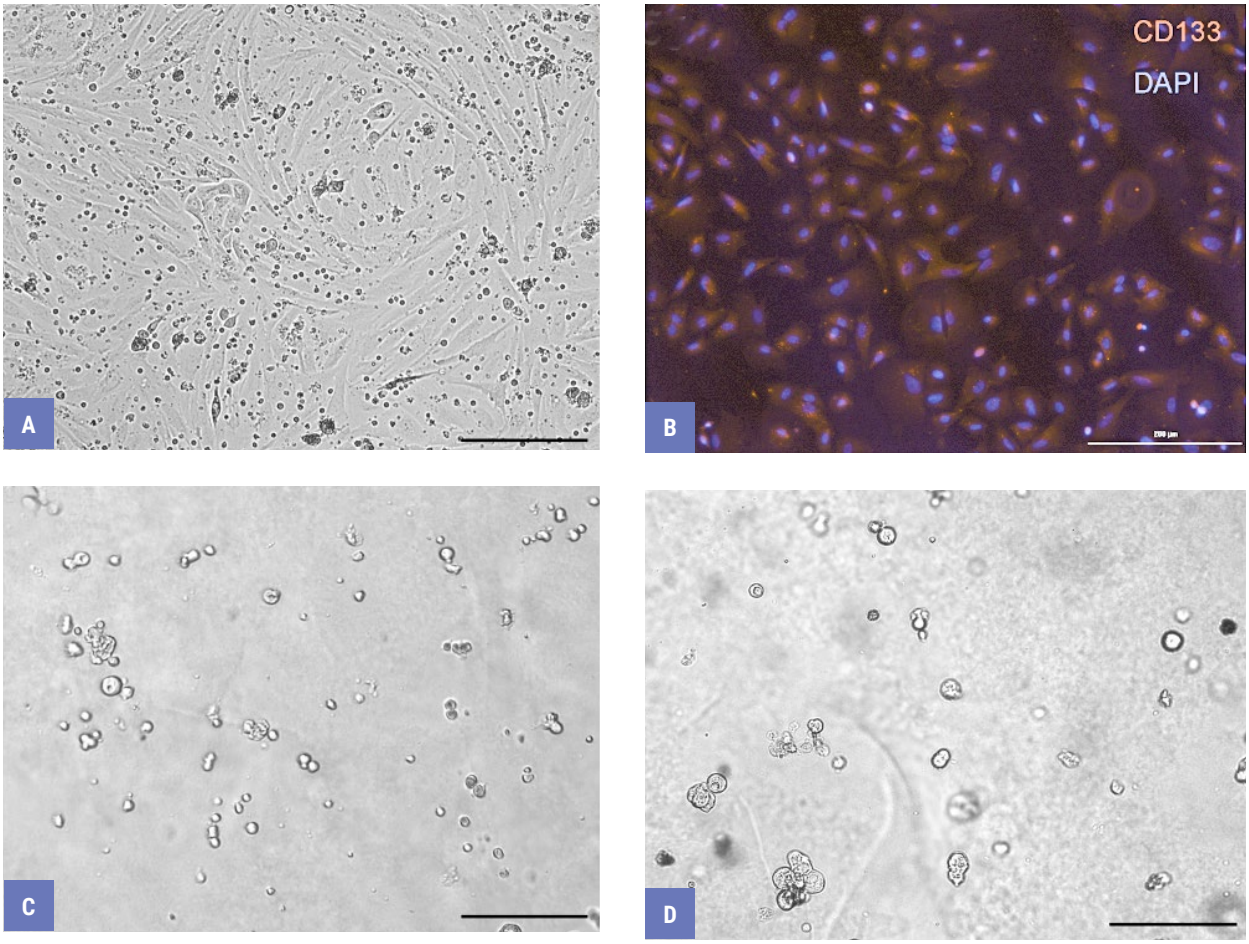

Fig. 1. Primary culture of endometrial cancer No. 1. A – general view of monolayer culture; B – staining of monolayer culture on CD133; C – view of cellular spheroids in agarose on the 5th day of cultivation. G – a type of cellular spheroids in agarose on the 14th day of cultivation. The size of the scale ruler is 200 μm

for TSC, the culture of EC No. 3 formed small cellular spheroids of 4–8 cells with a frequency of about 15 % (Fig. 2B). Further, some of the cell spheroids showed rapid growth and after two weeks of cultivation reached 80–150 μm in diameter (110.3 ± 32.7 μm). The frequency of spheroid formation on the 14th day ranged from 0.3 to 2.5 % (1.5 ± 0.8 %) (Fig. 2G).

EC cell culture No. 3. Moderately differentiated G2 endometrioid adenocarcinoma with signs of vascular invasion. The culture in the monolayer had a pronounced division into small polygonal cells, united into islands among larger and elongated cells similar to fibroblasts (Fig. 3A).

Immunofluorescence staining showed an increased content of the CD133 marker in small polygonal cells, while individual cells showed a particularly bright label (Fig. 3B). The frequency of $CD133^+$ cells ranged from 5.8 to 16.9 % (11.2 ± 5.2 %). When grown on agarose in a medium for TSC on the 5th day in culture, about 15 % of the cells formed spheroids 5–30 μm in diameter (Fig. 3C). After two weeks of cultivation in agarose, individual cellular spheroids increased in size to 30–150 μm in diameter, the remaining spheroids degraded (98.3 ± 51.4 μm). The frequency of spheroid formation on day 14 ranged from 0.3 to 10.1 % (5.7 ± 4.1 %) (Fig. 3D).

EC cell culture No. 4. Moderately differentiated G2 endometrioid adenocarcinoma without signs of vascular invasion. In the monolayer culture, large polygonal cells were observed separately or assembled in small groups against the background of elongated cells of various sizes (Fig. 4A).

When stained with the CD133 EC TSC marker, a positive reaction was shown not only by large po-

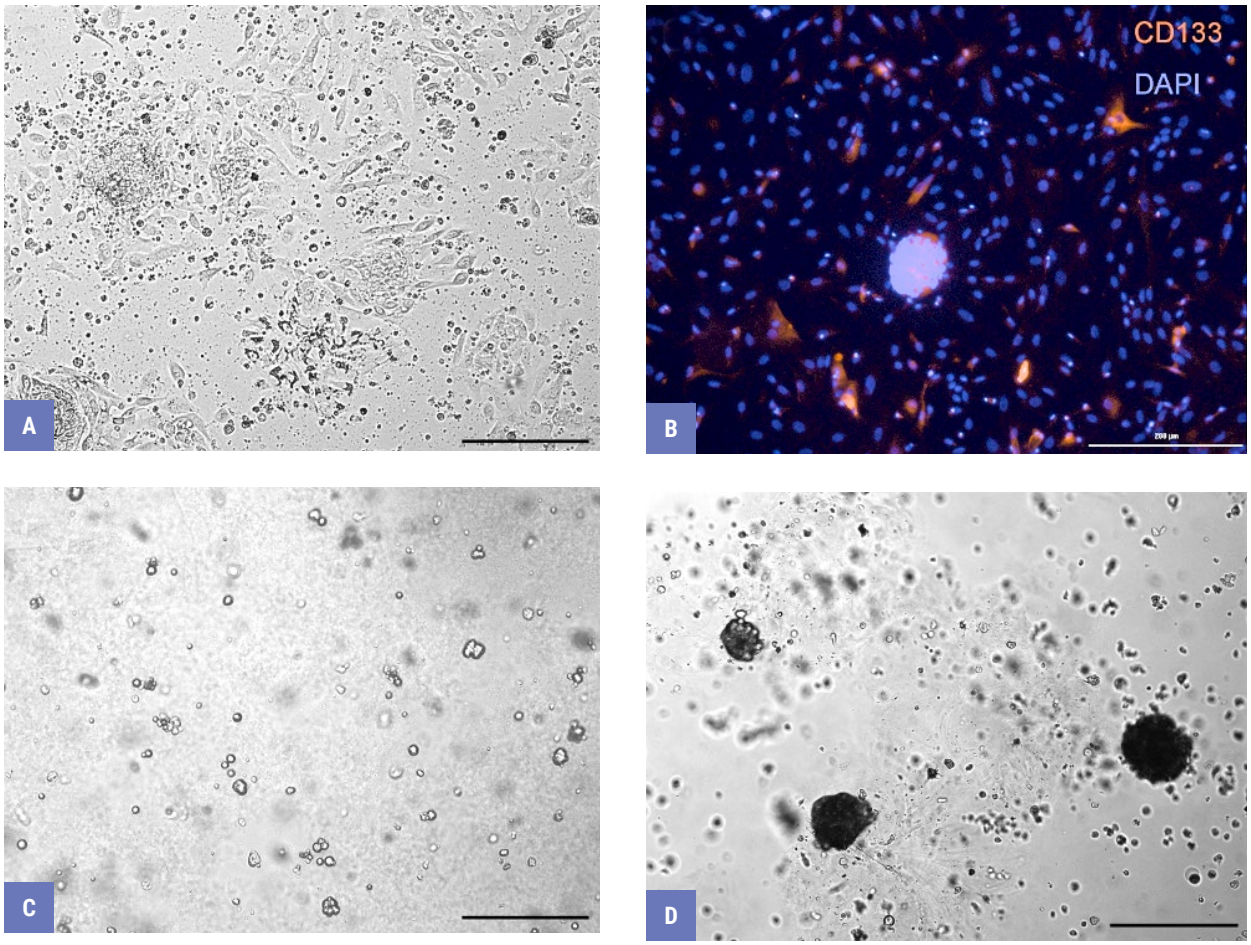

Fig. 2. Primary culture of endometrial cancer No. 2. A – general view of the monolayer culture; B – staining of the monolayer culture on CD133; C – type of cellular spheroids in agarose on the 5th day of cultivation; D – type of cellular spheroids in agarose on the 14th day of cultivation. The size of the scale ruler is 200 μm

lygonal cells and their clusters, but also by numerous small elongated cells. Large multinucleated cells positive for CD133 were also found in the preparation (Fig. 4B). The frequency of CD133⁺ cells ranged from 40.1 to 65.2 % (average 52.2 ± 10.2 %). After five days of cultivation in agarose, about 5–10 % of the culture cells formed small loose spheroids of 4–16 cells (Fig. 4C), two weeks later the spheroids increased to 20–80 μm in diameter (54.7 ± 28.4 μm), the frequency of spheroid formation on the 14th day ranged from 2.1 to 8.4 % (5.1 ± 2.7 %) (Fig. 4D)

EC Culture No. 5. Moderately differentiated G2 endometrioid adenocarcinoma without signs of vascular invasion. In the monolayer culture, islands of rather large polygonal cells of epithelial morphology were found among elongated cells of stromal origin (Fig. 5A).

The reaction to CD133 was similar to the EC culture No. 2, namely, positive staining was demonstrated by individual large cells of elongated or epithelial morphology with a certain group of small elongated cells, which, due to staining, stand out against the background of larger elongated cells negative for CD133 (Fig. 5B). The frequency of CD133⁺ cells ranged from 22.4 to 51.2 % (35.5 ± 12.7 %). On the fifth day of cultivation in conditions that do not support adhesion, EC culture No. 5 formed small cellular spheroids of 2–4 cells with a frequency of about 5 % (Fig. 5C). Further, some of the cell spheroids showed rapid growth and reached 30–80 μm in diameter after two weeks of cultivation (average 55.4 ± 25.1 μm). The frequency of spheroid formation on day 14 ranged from 0.5 to 2.8 % (1.8 ± 0.9 %) (Fig. 5D).

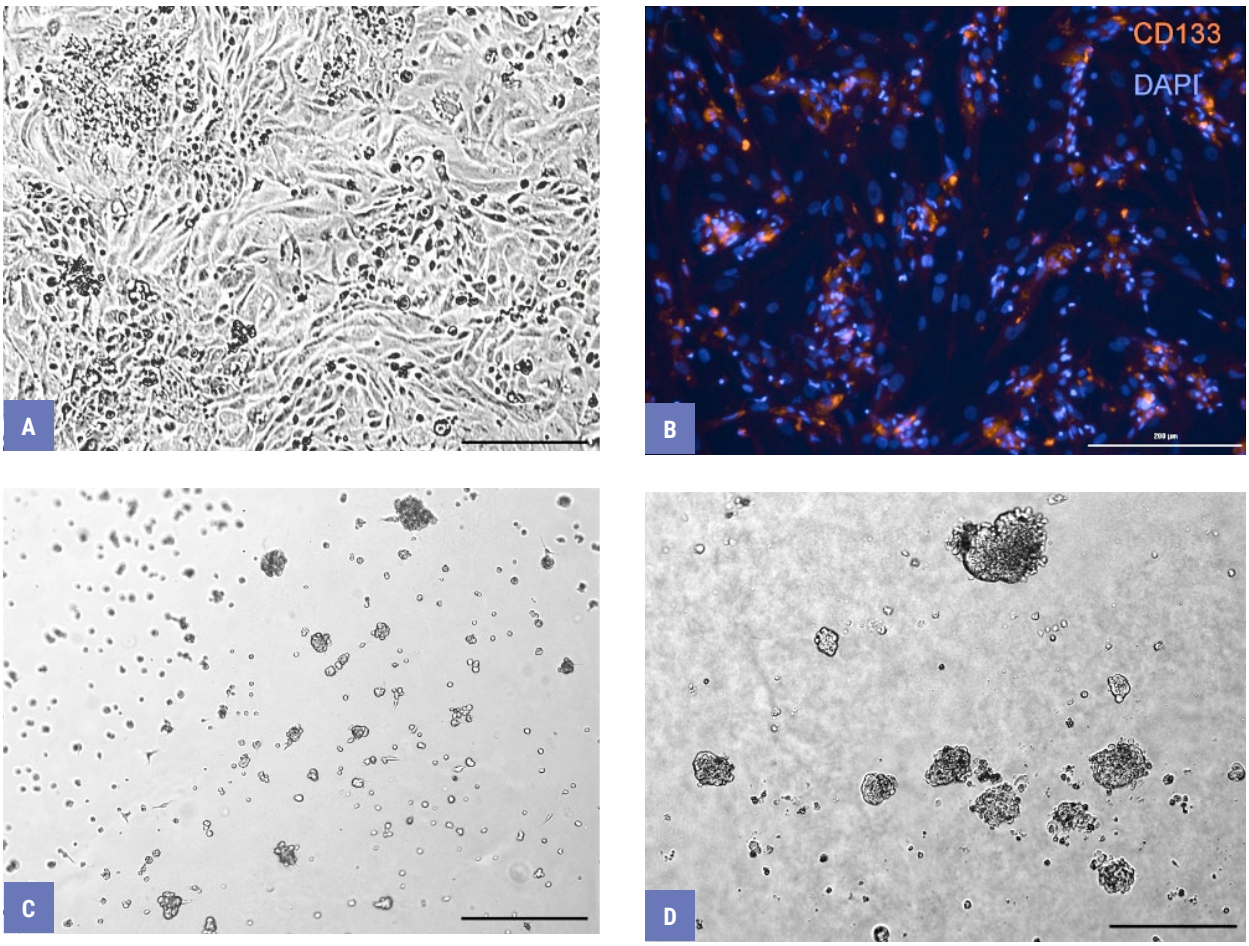

Fig. 3. Primary culture of endometrial cancer No. 3. A – general view of the monolayer culture; B – staining of the monolayer culture on CD133; C – type of cellular spheroids in agarose on the 5th day of cultivation; D – type of cellular spheroids in agarose on the 14th day of cultivation. The size of the scale ruler is 200 μm

The characteristics of primary EC cultures are combined in the table (Table 1).

The lowest frequency of spheroid formation, as well as the lowest frequency of $CD133^+$ cells, were observed in a culture obtained from a highly differentiated tumor.

DISCUSSION

In all the obtained primary cultures, the presence of a stromal component is noted, represented by elongated cells of different sizes, demonstrating a negative (No. 1, No. 2, No. 3) up to medium and high CD133 expression (No. 4, No. 5). In addition, polygonal cells are noted in cultures, which occur as islands among stromal cells. Their sizes vary between cultures, and on this basis, cultures can be divided into two groups – including small polygonal cells that assemble into dense colonies-"domes" (cultures No. 1 and No. 2), and including larger polygonal cells that can form flat islands of different sizes (cultures No. 1, No. 4 and No. 5). The expression of the CD133 marker in these cells is quite pronounced, especially against the background of a weakly colored stromal component consisting of large cells (cultures No. 4 and No. 5).

The presence of elongated or process-shaped cells resembling fibroblasts in primary EC cultures and capable of forming spheroids under conditions that do not support cell adhesion was also noted in the work of Helweg (2022) [23]. The picture of the composition of cell cultures obtained by us is also similar in cell morphology to the results obtained in the work of Chan et al. (2004) [24]. In this study, the

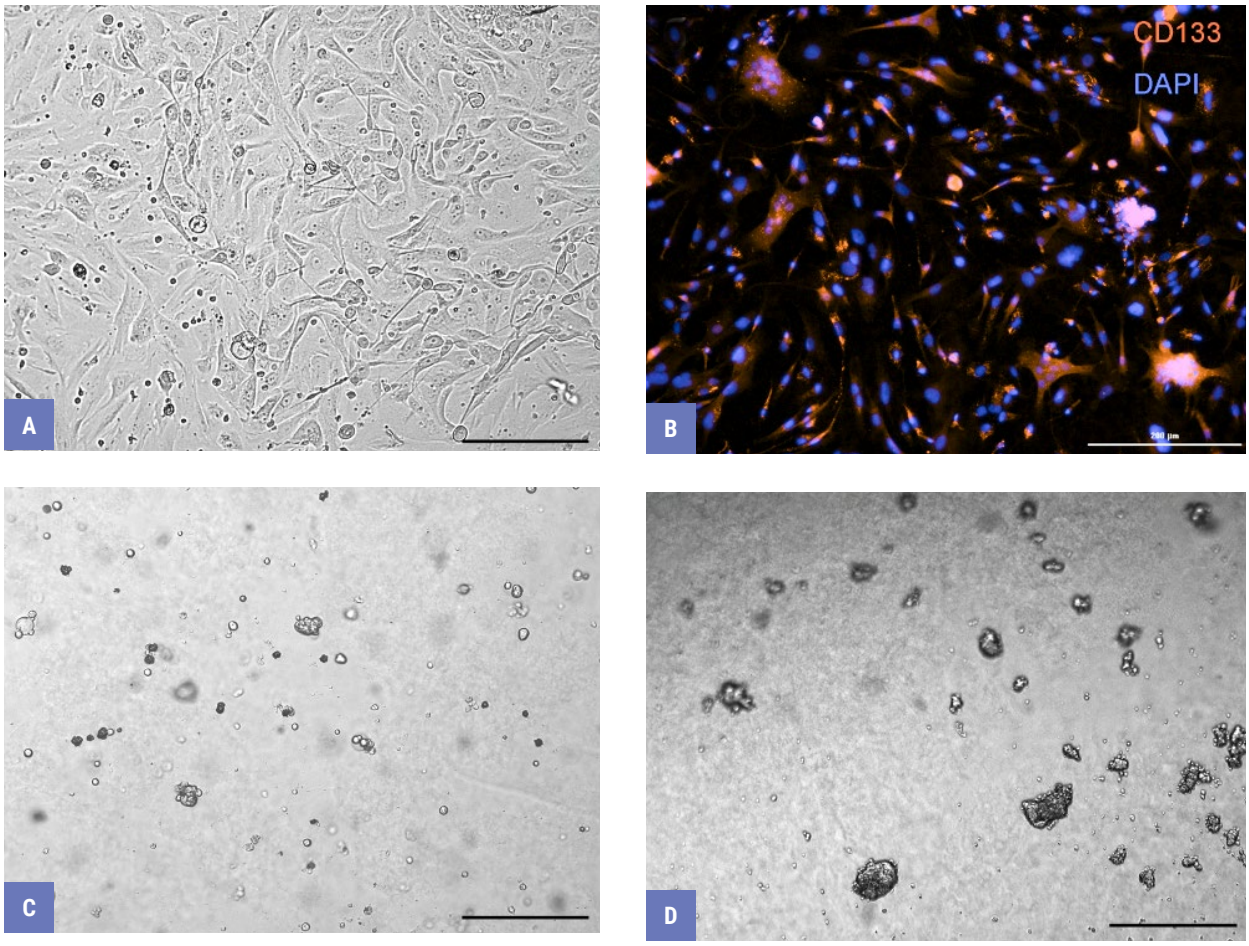

Fig. 4. Primary culture of endometrial cancer No. 4. A – general view of the monolayer culture; B – staining of the monolayer culture on CD133; C – type of cellular spheroids in agarose on the 5th day of cultivation; D – type of cellular spheroids in agarose on the 14th day of cultivation. The size of the scale ruler is 200 μm

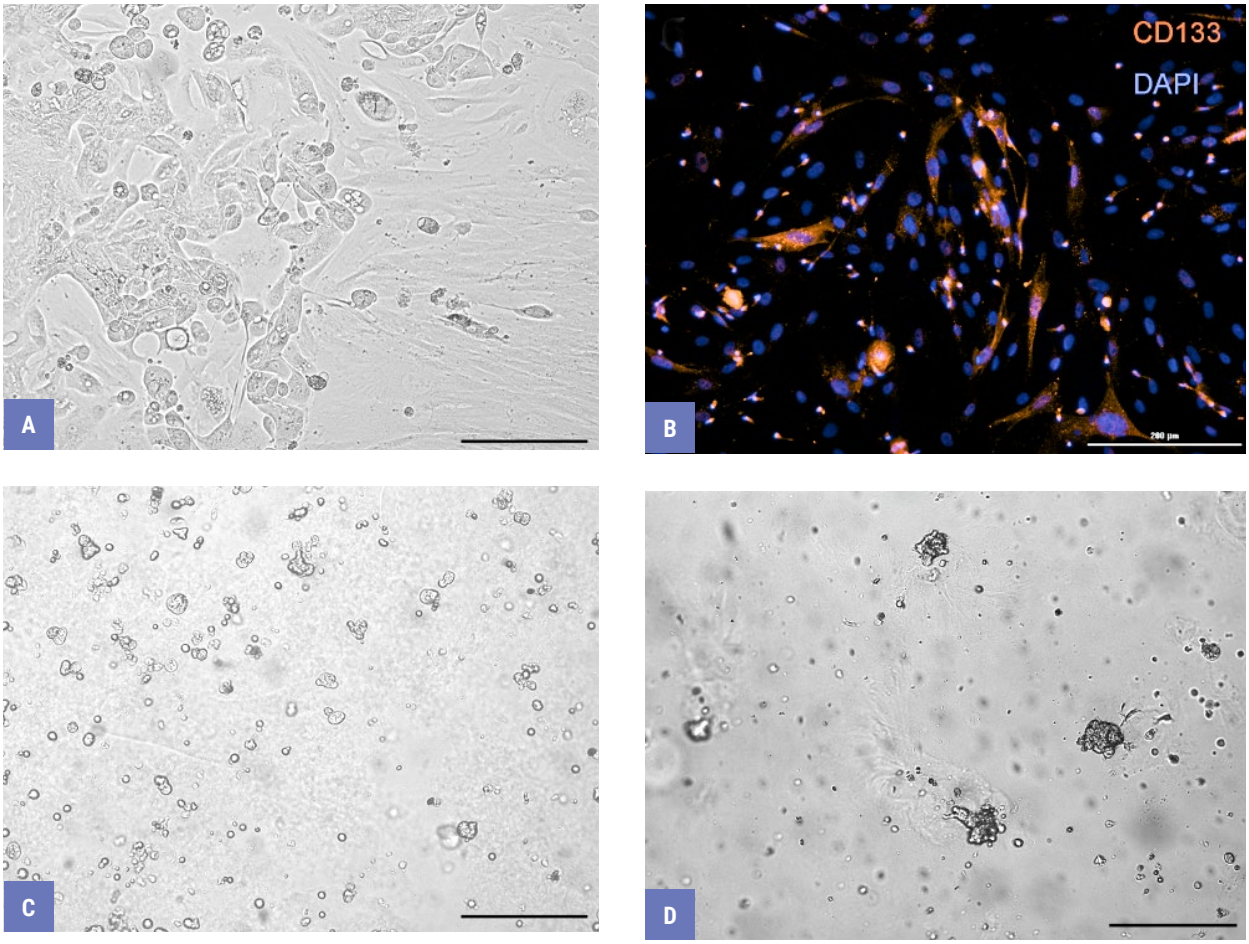

Fig. 5. Primary culture of endometrial cancer No. 5. A – general view of monolayer culture; B – staining of monolayer culture on CD133; C – type of cellular spheroids in agarose on the 5th day of cultivation; D – type of cellular spheroids in agarose on the 14th day of cultivation. The size of the scale ruler is 200 μm

Table 1. Summary characteristics of primary endometrial cancer cultures

Culture No.	Grade	Lympho-vascular invasion	Reaction to CD133, proportion of CD133+ cells, mean ± SD %	The frequency of spheroids ≥ 40 μm per 14 days, average ± SD, %	The diameter of the spheroids for 14 days, average ± SD, μm
No. 1	G1	n/p	0.8 ± 0.15 %, weak reaction in some large stromal cells, single polygonal cells with bright coloration	0.5 ± 0.1	20 ± 10
No. 2	G3	n/p	4.8 ± 1.5 %, small polygonal cells in dense colonies, individual stromal cells	1.5 ± 0.8 %	110.3 ± 32.7
No. 3	G2	p	11.2 ± 5.2 %, small polygonal cells in dense colonies	5.7 ± 4.1 %	98.3 ± 51.4
No. 4	G2	n/p	52.2 ± 10.2 %, polygonal process large multinucleated cells, stromal small elongated cells	5.1 ± 2.7 %	54.7 ± 28.4
No. 5	G2	n/p	35.5 ± 12.7 %, separate large and small elongated stromal cells, separate large polygonal cells	1.8 ± 0.9 %	55.4 ± 25.1

Note: not present – n/p, present – p

authors studied the behavior in culture of various cells of the normal human endometrium isolated from the epithelial and basal layers [24]. According to the authors, the epithelial layer gave two groups of polygonal cells – small cells that gather in close colonies with a high ability to proliferate, and larger cells that form looser colonies on cultural plastic. The stromal component of endometrial tissue consisted of two types of elongated cells: large loosely lying cells and small closely lying cells, giving curls in a monolayer. In addition, human endometrial stromal cells were positively stained for fibroblast markers (CD90, 5B5, type I collagen), which confirms the relationship of these cells to fibroblasts and, possibly, myofibroblasts, which express markers of both fibroblasts and smooth muscle cells. Thus, based on the picture of the two-dimensional culture obtained in our work, it is impossible to unambiguously identify which cells belong to the TSC, since externally they are little distinguishable from normal endometrial cells.

The expression of the TSC CD133 marker in the cultures obtained by us ranged from 0.8 to 52.2 % on average. The data obtained go beyond the range of 5.7–27.4 % indicated by other authors [16, 17]. A nonlinear relationship between CD133 expression and the degree of tumor differentiation was also revealed – both high and low differentiation were associated with reduced immunoreactivity on CD133 compared with average differentiation. No connection was found between the level of CD133 expression, the degree of differentiation and other clinical and pathological characteristics of the tumor also in Nakamura [20]. CD133 is known to occur in normal differentiated endometrial cells. Thus, based on existing data, it is impossible to determine to what extent CD133 expression reflects the degree of cell malignancy in primary EC cultures and the level of their differentiation. To solve this problem, more extensive studies are required, including a comparison of normal endometrial tissue and RE.

The frequency of spheroid formation in our cultures ranged from 0.5 to 5.7 % on average. It is known that the frequency of cells with SC properties in the normal endometrium is 0.02–0.1 % [24]. It would not be too much of an assumption to accept these levels as indicative for estimating the frequency of spheroid formation in cultures of normal endometrium. In the scientific literature, we have not found data on the frequency of spheroid formation in conditions that do not support cell adhesion in whole EC cultures, since individual subpopulations purified by marker expression or nuclear dye retention are usually the object of research. However, taking into account the fact that the frequency of spheroid formation is the highest among $CD133^+$ EC cells and amounts to 11.7 % in this subpopulation [17], as well as the fact that the total content of these cells in EC ranges from 5.7 to 27.4 % [16], we can multiply these indicators to obtain an approximate the frequency of spheroid formation in a mixed culture of EC, which in this case will be 0.7–3.2 %. Thus, our results lie in the range corresponding to the known data on EC, therefore, the main part of the spheroids in our cultures is presumably formed by malignant cells with the TSC phenotype.

Our data show that in EC cultures, the overall response to CD133 does not show a clear relationship with the frequency of spheroid formation. Thus, in cultures No. 4 and No. 5, the frequency of spheroid formation is an order of magnitude lower than the content of $CD133^+$ cells. In this regard, the question arises as to which cells are the sources of spheroids in EC cultures? Comparison with the morphological features and phenotype of the normal endometrium [21, 24, 25] indicates a number of patterns. Thus, in culture No. 1, obtained from a highly differentiated tumor, in a monolayer we see large cells similar to the limited dividing progenitor cells of the stromal and epithelial components of the normal endometrium [24]. The expression of the CD133 marker in these cells is very weak, while this culture formed small lymphoid (loose) spheroids on soft agar with a frequency of less than 1 %. Lymphoid colonies in conditions that do not support adhesion can form immune cells, namely, T lymphocytes and NK in the presence of specific cytokines such as IL-2, IL-15 or IL-7 [26]. Nevertheless, their characteristic appearance can be considered a reliable difference between lymphocytes and other cells – even in a stimulated state, these are small cells (6–15 μm) with a high nuclear-cytoplasmic ratio [27]. In our case, the cells in the colonies have larger sizes (about 40 μm in diameter), which means that they are not lymphocytes with a high probability. At the same time, the spheroids of culture No. 1 are similar to the lymphoid colonies that were obtained from the EC tissue in Tabuchi's work [28]. Culture No. 2, obtained from a low-grade tumor (G3), also contains cd133 fibro-

blast-like cells, however, a subpopulation of small polygonal cells stands out well against their background, demonstrating bright immunoreactivity on CD133, which morphologically most resembles cells with stem properties, in the work of Chan et al. [24]. Also in this culture, when grown on soft agar, large (about 90 μm in diameter) spheroids were observed, albeit with a small frequency (about 1.5 %). Despite the fact that no special marker selection was carried out, it would be a small assumption to assume that these CD133⁺ cells form spheroids, since large fibroblast-like cd133 cells did not produce similar large spheroids in culture No. 1. Confirmation of this assumption may also be the fact that culture No. 3, similar in phenotype to culture No. 2, with a subpopulation of small CD133⁺ cells forming dense colonies clearly standing out against the background of a weakly colored stromal component, also formed large (about 115 μm in diameter) spheroids in soft agar with a frequency of about 10 %. Another source of spheroids may be small spindle-shaped cells brightly colored on CD133, found in cultures No. 5 and No. 4. The morphological features of these cells correspond to tissue stem cells, of which CD133 express, for example, epithelial or endothelial SC, which are normally present in the endometrium [30, 31]. According to the literature data, the size and appearance of colonies formed on soft agar by such normal stem cells do not differ from spheroids obtained from TSC [31]. Finally, for a subpopulation of relatively large polygonal CD133⁺ cells that are present in cultures No. 4 and No. 5, the connection with spheroids cannot be traced, although these cells can be confidently attributed to the malignant component, since they sometimes show signs of multinucleation. It can also not be argued that CD133-negative cells do not produce spheroids on soft agar, since in the work of Ding [29], in particular, they showed that both CD133⁺ and CD133 cells sorted from endometrial tumor tissue by this marker can form spheroids, but in the second case less effectively.

Thus, it is impossible to unambiguously determine which of the cells of the primary EC cultures obtained by us took part in the formation of spheroids. Some presumptive relationship can be established only for small epithelial and fibroblast-like CD133⁺ cells, of which only the first can be conditionally classified as malignant. To identify exactly TSC EC, it is necessary to conduct a comparative study of cell cultures on soft agar obtained from tumor tissue and the corresponding normal tissue, in combination with cell sorting using markers CD133, CD44, CD117, CD24, CD47 and others, for which a connection with TSC and normal stromal stem cells has been established [32].

CONCLUSION

We were able to obtain and characterize the culture of cellular spheroids from the postoperative EC material. However, the indicators of the frequency of spheroid formation and the average size of spheroids in this culture cannot serve as a marker of the amount of TSC in tumor tissue without comparing these data for tumor tissue and normal endometrium. A more detailed study of the cellular subpopulations of EC in comparison with normal endometrium is required to establish a link between the observed diversity of cells in culture and their ability to spheroid formation and other characteristics of TSCs.

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