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ESTABLISHMENT AND CHARACTERIZATION OF NEW BREAST AND OVARIAN CANCER CELL LINES AS A MODEL FOR STUDYING CELLULAR PLASTICITY *IN VITRO*

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Aim: To compare biological properties of primary tumor cells isolated from malignant effusion of cancer patients with the same cells of permanent lines established during their long-term cultivation *in vitro* and to assess the impact of phenotypic conversion that was caused by changes in their microenvironment on their behavioral characteristics. *Materials and Methods*: The study was performed on primary cell cultures from pleural effusion or ascites of breast and ovarian cancer and permanent cell lines derived from them, namely permanent ovarian cancer cell line I, permanent ovarian cancer cell line II and permanent breast cancer cell line I. Biological characteristics were studied using standard cell culture methods and immunocytochemical assays. *Results*: Three new cell lines were established from breast and ovarian cancer and cell morphology, migration activity, the kinetics of growth, colony forming activity in semisolid agar and sensitivity to anticancer drug were examined. These characteristics were compared with those of the primary tumor cells. It has been shown that among the primary tumor cells from malignant effusion, cells with mesenchymal characteristics were the most prevalent. Cultivation of primary cancer cells *in vitro* leads to a phenotypic change of their population: it becomes more homogeneous in morphology with predominantly epithelial-like cells. Also, later after a number of cell doublings *in vitro,* the cell population changes to include cells primarily with immunophenotypic properties characteristic of epithelial cells. These changes include increase in number of E-cadherin-positive cells and a decrease in number of vimentin and α-smooth muscle actin-positive cells. It was found that significant changes in expression of epithelial-mesenchymal transition associated proteins in cells during their cultivation *in vitro* in new microenvironment are accompanied by a rapid change in their sensitivity to anticancer drugs. *Conclusions*: The new breast and ovarian cancer cell lines were established and characterized. The induction of phenotypic transdifferentiation in malignant cells from pleural effusion and ascites can be an important approach for suppressing the progression of neoplastic process.
Key Words: cell line, epithelial-mesenchymal transition, tumorigenicity, anticancer drug.

Cell lines as pharmacogenomics models have the following advantages: they are easy to culture, relatively inexpensive, and amenable to testing of various therapeutic agents. Data generated from cell lines can then be used to link cellular drug response to genomic and proteomic features, where the ultimate goal is to build predictive signatures of patient outcome. Despite decades of improvements in methods for establishing cancer cell lines it is extremely difficult to routinely establish "quality" permanent cell lines from human primary tumors with high efficiency, limiting the number and diversity of cell lines available for study. Additionally, in many types of tumors, only specific subtypes of cells from a tumor biopsies yield a viable cell line and this has resulted in availability of a collection of cell lines that do not accurately reflect the true heterogenic spectrum of tumor cells in neoplasia and tumors encountered in the clinic; at the same time, the original tumor is not available for analysis with the modern technologies [1]. Therefore, it is important to develop more effective platform for drug investigation with a more efficient method of establishing human tumors as cultures that reflect the

heterogeneity of human malignancies. The critical first step in the pre-clinical cascade of cancer drug discovery depends almost entirely on the use of *in vitro* models, however, *in vitro* models of human cancers that are currently used in cancer biology and drug discovery do not incorporate complexity or heterogeneity of tumors [2]. Cancer is a complex and heterogeneous pathological growth that continuously and dynamically interacts with its host. Among the different phenotypic properties acquired by cancer cells during tumor progression markers of epithelial-mesenchymal transition (EMT) have been extensively described. EMT allows an epithelial polarized cell to acquire a more mesenchymal phenotype with increased mobility and invasiveness. In the neoplastic context in many tumors EMT is associated with a more aggressive tumor phenotype including local invasion and distant metastasis. Despite the fact that many EMT markers have been associated with poor disease prognosis in different studies and, while an EMT phenotype seems to be clearly associated with an increased metastatic phenotype, the use of such markers has not yet been translated into clinical practice. Any prognostic markers should be reproducible among different laboratories and have to display great robustness. EMT markers have not yet been used in clinical practice for several reasons and tumor heterogeneity is one of the main reasons [3]. Such heterogeneity can be the result of cell intrinsic genomic differences or interaction with the microenvironment [4]. Investigations into the role of the tumor

microenvironment and the mechanisms of stromal cell recruitment have also provided insights into a distinct aspect of tumor biology: cancer progression may also be directed by the systemic responses of organism to malignancy and by the involvement of organ systems located at sites distant from primary tumor. Many of the factors have dual or ambivalent roles [5].

Dissemination of tumor cells in abdominal or pleural cavities as ascitic form or cancer pleural effusion is the indicator of poor prognosis in cancer patients: the spread of the tumor cells to the ascites has been postulated to play a dominant role in metastasis, chemoresistance and, ultimately, an incurable disease. Ascitic form of cancer differentially triggers a dissemination phenotype related to the initial cell features by either allowing the proliferation and the formation of spheroids and the extension of colonies for cells that present an initial epithelial intermediate phenotype, or favoring the migration of cells with a mesenchymal intermediate phenotype. There are data that ascites induces a shift toward an unstable intermediate state of the epithelial-mesenchymal spectrum and confers a more aggressive cell behavior that takes on a different pathway based on the initial epithelial-mesenchymal cell features. A thorough understanding of the biology of the ascites microenvironment is essential for developing effective therapeutic intervention for metastatic ovarian, breast, lung and many other cancers. Cancer cell lines, originally isolated from ascites, are readily available, immortalized, highly autonomic and provide a low-cost option to assess tumor cell behavior [6].

Heterogeneity of metastatic cancer cell population is one of the reasons why some patients are more responsive to some treatments over others. EMT, while favoring the formation of more metastatic cancer cells, also provides signals for increasing survival which may cause drug resistance in some if not all the cells present in a tumor. Since this is a very complex and dynamic mechanism, thorough investigation is necessary to fully understand the mechanism of drug action and drug resistance [7].

MATERIALS AND METHODS

Primary cell cultures. Ascites or pleural effusion were collected at the time of paracentesis for palliative reasons from patients with ovarian cancer and breast cancer. The cell biomass was separated from the cancer effusion and placed into 75 cm^2 tissue culture flasks (TPP, Italy) in culture medium RPMI-1640 (PAA, Austria) supplemented with 15% fetal bovine serum (FBS) (PAA, Austria) and 40 mg/ml gentamicin (Sigma, USA). Flasks were incubated at 37 °C and with 5% CO_2. The medium was removed after 3–4 days and replaced with fresh RPMI-1640 medium supplemented with 15% FBS, 2 mM L-glutamine, 40 mg/ml gentamicin. Subsequently, the media was replaced twice a week.

Permanent cell lines. The human breast cancer and ovarian cancer cell lines designated as BCC/Permanent (BCC/P) and OCC/Permanent II (OCC/PII), respectively were maintained in RPMI-1640. Human ovarian cancer cell line OCC/Permanent I (OCC/PI)

was cultured in DMEM. All media were supplemented with 10% FBS and 40 mg/ml gentamicin. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells were detached with EDTA solution (Sigma, USA) using standard method [8]. All permanent cell lines were placed in collection of the Bank of Cell Lines from Human and Animal Tissues of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine.

Study of cell morphology. The flasks with BCC/P, BCC/Primary (BCC/Pr), OCC/PI, OCC/Primary I (OCC/PrI), OCC/PII and OCC/Primary II (OCC/PrII) cells were viewed in inverted microscope Axiovert 25 (Carl Zeiss, Germany) equipped with digital camera (Canon PowerShot A640, UK) at 100–320-fold magnification. The cultures of live cells were photographed and the images were analyzed using Shortcut to Remote Capture software. Additionally, cells were stained using standard methods with Romanowsky — Giemsa stain [8]. Stained slides were photographed and the images were analyzed.

Growth kinetics of tumor cells. All experiments were initiated using stock cultures maintained in exponential phase of growth. Cells ($1 \cdot 10^4$/ml) were seeded in 24-wells plate in 1 ml of DMEM or RPMI-1640 medium supplemented with 10% FBS and 40 mg/ml gentamicin. The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 7 days. Every day cells were detached with EDTA solution from three wells at each time point, stained with trypan blue solution (HyClone, USA) and counted using hemocytometer. All experiments were repeated twice.

Semisolid agar colony assay. The assay was conducted in parallel wells in 6-well plates. Base layers of 3 ml of 0.5% agar (Difco, USA) were prepared on RPMI-1640 medium with 10% FBS. A 1 ml overlayer of 0.33% agar containing BCC/P, OCC/PII or OCC/PI cells ($5 \cdot 10^3$) in RPMI-1640 medium with 10% FBS and 40 mg/ml gentamicin was then applied and the plates were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 14 days of the incubation, the plating colonies were stained with 0.2% solution of methylthiasoliletetrazolium (Sigma, USA) at 37 °C for 3 h. After that, the number of colonies was counted in each well using binocular magnifier BM-51-2 (LOMO, Russia). Images of live colonies were acquired on the inverted microscope Axiovert 25 equipped with a digital camera. Colony-forming activity (CFA) of these cells was determined using the formula:

$$CFA = (A/B) \cdot 100\%,$$

wherein A — the number of colonies per well; B — the number of cells plated in semisolid agar per well.

Migration assay. The BCC/P, OCC/PI and OCC/PII cells ($1 \cdot 10^5$/ml) were seeded in 12-well plates (TPP, Italy) in DMEM or RPMI-1640 complete medium and incubated at 37 °C in a humidified atmosphere with 5% CO_2 until cells reach 100% confluence to form a monolayer. We used a p200 pipet tip to create a scratch in the cell monolayer. In each well the medium was changed to remove debris and to smooth the edges

of the scratch. After that, the migration of cells across the "scratch" was photographed with phase contrast microscope. The first image was made 3 h later and the following images were taken at intervals of 6 h. The images were analyzed and the time required to close the scratch was determined [9].

Immunocytochemical assay. The phenotype of BCC/P, OCC/PI and OCC/PII cells was determined by immunocytochemical staining with monoclonal antibodies against E-cadherin, Vimentin, α-sm-actin (Thermo Scientific, USA) and N-cadherin (BioLegend, USA). Cells were grown on coverslips for 48 h and analyzed using standard method [10]. For visualization, Ultra Vision LP Value Detection system was used. DAB Quanto (3,3-diaminobenzydyne; Thermo Scientific, USA) was used as a chromogen. Immunocytochemical reactions were assessed semiquantitavely using H-score accounting for the proportion and intensity of the stained cells. The percentage of the stained cells was multiplied by the score number corresponding to the staining intensity (0 = none, 1 = weak, 2 = moderate, 3 = strong). The resulting score ranged from 0 (no stained cells) to 300 (diffuse intense staining of cells), and H score was calculated as (% of cells stained at intensity 1 × 1) + (% of cells stained at intensity 2 × 2) + (% of cells stained at intensity 3 × 3). H-score between 0 and 300 was obtained where 300 corresponded to 100% of intense staining [11].

Sensitivity to the anticancer drugs. All experiments were initiated using stock cultures maintained in exponential phase of growth. Cell suspensions (1 · 10⁴ cells/ml) were seeded into 96-well culture plates, 100 ml per well and incubated for 24 h at 37 °C. Then the medium was replaced with fresh medium containing different concentrations of anticancer drugs: fluorouracil, platinum and camptothecin. Plates were incubated for 72 h at 37 °C and 5% CO_2. Cells were counted by colorimetric assay with crystal violet [12]. All experiments were performed in triplicate and the results were expressed as inhibitory rate (IR):

$$IR = (1 - A540\,(experiment)/A540\,(control)) \cdot 100\%.$$

Statistical analysis. Statistical data processing was performed by the Student *t*-test using Statistica 6.0 computer program (StatSoft Inc., USA).

RESULTS AND DISCUSSION

In order to better understand the behavior of cancer cells *in vitro* we investigated certain biological characteristics of these cells, such as EMT signature and sensitivity to anticancer drugs, under conditions permissive to phenotypic plasticity. We derived two permanent cell lines from ovarian cancer ascites (OCC/PI and OCC/PII) and one cell line from breast cancer pleural effusion (BCC/P). Morphological analysis of primary cell lines obtained from pleural fluid of the patient with breast cancer BCC/Pr showed that cell population consisted mainly of two types the cells, round shaped and elongated fibroblasts (which constitute the majority of cell population) with small number of polygonal cells (Fig. 1).

We obtained the permanent BCC/P cell line from primary cell line BCC/Pr by long-term passages *in vitro* (more than 20–30 cell divisions). The study of morphological features of BCC/P cells showed that they formed a homogeneous culture of cells smaller than the BCC/Pr cells with mainly epithelial-like morphology (Fig. 1, *d*, *e*).

From primary cell lines from ovarian cancer ascites (OCC/PrI and OCC/PrII) we obtained 2 permanent cell lines — OCC/PI and OCC/PII by long-term passages *in vitro* more than 20–30 cell divisions (Fig. 2).

The study of morphological features of cell lines derived from ovarian cancer showed that the primary culture OCC/PrI is heterogeneous and contains adherent cells with fibroblast-like morphology and cells that are substrate independent and grow in suspension
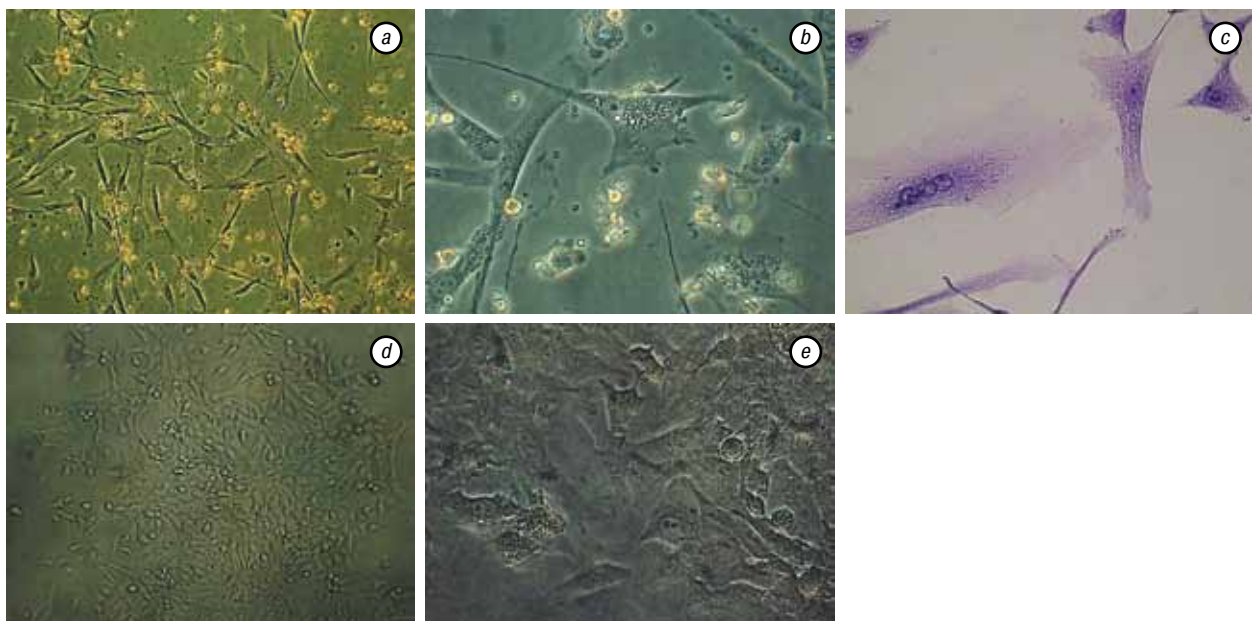
Fig. 1. The morphology of primary BCC/Pr cells (*a–c*) and a permanent BCC/P cell line (*d*, *e*): live cells (*a*, *b*, *d*, *e*), cytological preparation (stained by Romanowsky — Giemsa (*c*)). × 100 (*a*, *d*), × 320 (*b*, *e*), × 400 (*c*)
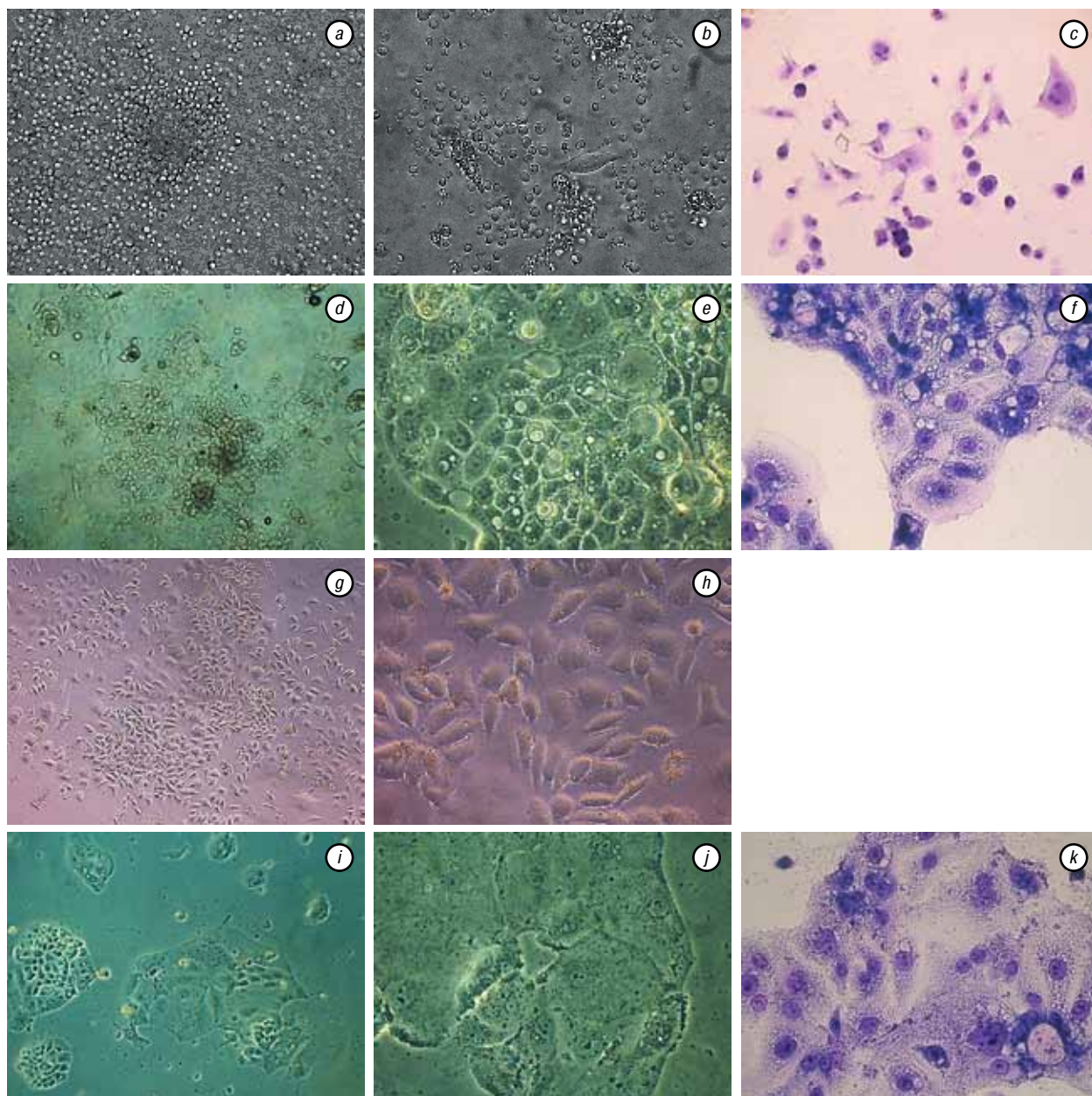
Fig. 2. Morphology of primary ovarian cancer OCC/PrI (*a–c*) and OCC/PrII (*d–f*) cells and OCC/PI (*g, h*), OCC/PII (*i, j, k*) cell lines: live cells (*a, b*), cytological preparation (stained by Romanowsky — Giemsa (*c, f, k*)). × 100 (*a, d, g, i*), × 320 (*b, e, h, j*), × 400 (*c, f, k*)

(Fig. 2, *a–c*). Permanent cell line OCC/PI is characterized by homogeneity of the population that grows as a substrate dependent culture and acquires morphology similar to epithelial cells.

Morphological analysis of primary cell lines OCC/PrII revealed that primary cell cultures formed "cell islands" consisting of big epithelial-like cells with large vacuoles (Fig. 2, *d–f*). In addition, we observed a significant number of typical epithelial cells that form the structure of "cobblestone" type. Through 30–40 cell doublings we obtained the permanent cell line OCC/PII that consists from two cell types: smaller-sized cells that form the epithelial islands and large cells (more than 100–200 micrometers) with 1 or 2 nuclei (Fig. 2, *j*).

Analysis of the kinetics of growth of derived permanent cell lines revealed a significant difference in their growth characteristics: cells of BCC/P line quickly reached the maximum number (4 days) and entered the plateau phase of the rapid aging of the cell culture and reduction in the number of live cells; whereas cells of OCC/PI and OCC/PII lines grew slowly with a gradual doubling of the cells (Fig. 3).
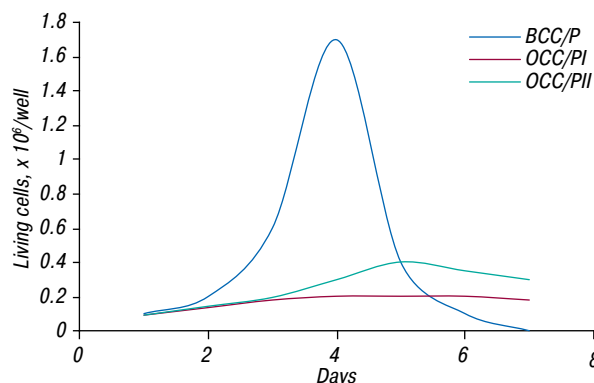

Fig. 3. Kinetics of growth of permanent cell lines derived from pleural effusion or ascites of patients with breast and ovarian cancer
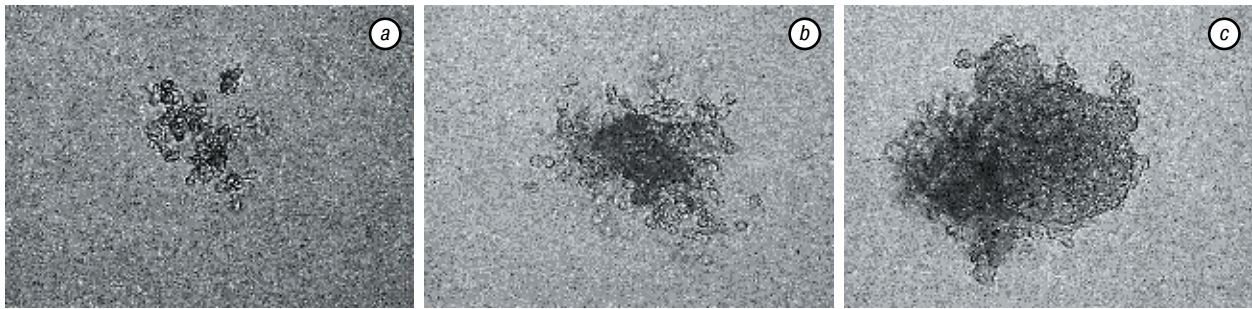
Fig. 4. Colonies of BCC/P cells of different size in semisolid agar: *a* — small (10–30 cells), *b* — medium (30–100 cells), *c* — large (> 100 cells)

Normal cells need a variety of internal and external signals to support their proliferation and differentiation as compared to malignant cells that acquire the capacity for autonomous (independent) growth through a series of genetic and epigenetic changes. It is one of the main features of cellular malignant transformation. The ability of tumor cells to form colonies in semisolid agar is considered the most accurate method of assessing the "level of malignancy" of cells *in vitro* and correlates with the level of their tumorigenicity and metastatic potential *in vivo*. We analyzed the colony forming ability of primary and permanent cell lines BCC and OCC and showed that only BCC/P and OCC/PI are able to form colonies in semisolid agar. This indicates the acquisition of certain new biological characteristics by permanent cells when compared to their original source as a result of certain selective actions during their growth *in vitro*. Colony forming activity in semisolid agar of BCC/P cell line was 8.0 ± 0.1%, OCC/PI — 3.4 ± 0.7% (Fig. 4). We also calculated the number of colonies formed by BCC/P cells of different size: 17.6 ± 1.6% of large colonies (> 100 cells), 16.4 ± 0.9% of small colonies (10–30 cells) and 66.0 ± 0.8% of medium colonies (30–100 cells).

One of the important parameters of malignancy of tumor cells is their ability to migrate. This, together with the invasion, are key characteristics of the manifestation of "metastatic behavior" of malignant cells, such as their ability to proliferate and form secondary metastatic lesions. One of the most simple and effective methods for analyzing the migration ability of tumor cells *in vitro* is the scratch assay. This method can be used to study the mechanisms of regulation of cell migration during the interaction of cells between themselves and with the intercellular matrix [13]. Therefore, we investigated the cell migration activity of BCC/PI and OCC/PI cells using scratch assay. OCC/PII was excluded from this analysis because it failed to consistently reproduce the results of the assay. The analysis showed that total time of the monolayer restoration for BCC/PI line was 48 h (Fig. 5) and for OCC/PI — 60 h.

Molecular markers of EMT are an important characteristic of tumors. EMT plays an important role in the acquisition of mesenchymal characteristics by epithelial cells and is associated with an increased invasive and migratory ability of cells and the formation of malignant metastatic phenotype. In addition, tumor cells characterized by predominantly mesenchymal markers are less sensitive to anticancer drugs and therefore constitute a resistant phenotype [14–16]. It has been shown that increase in E-cadherin expression (epithelial cell marker) is associated with increased sensitivity of re-
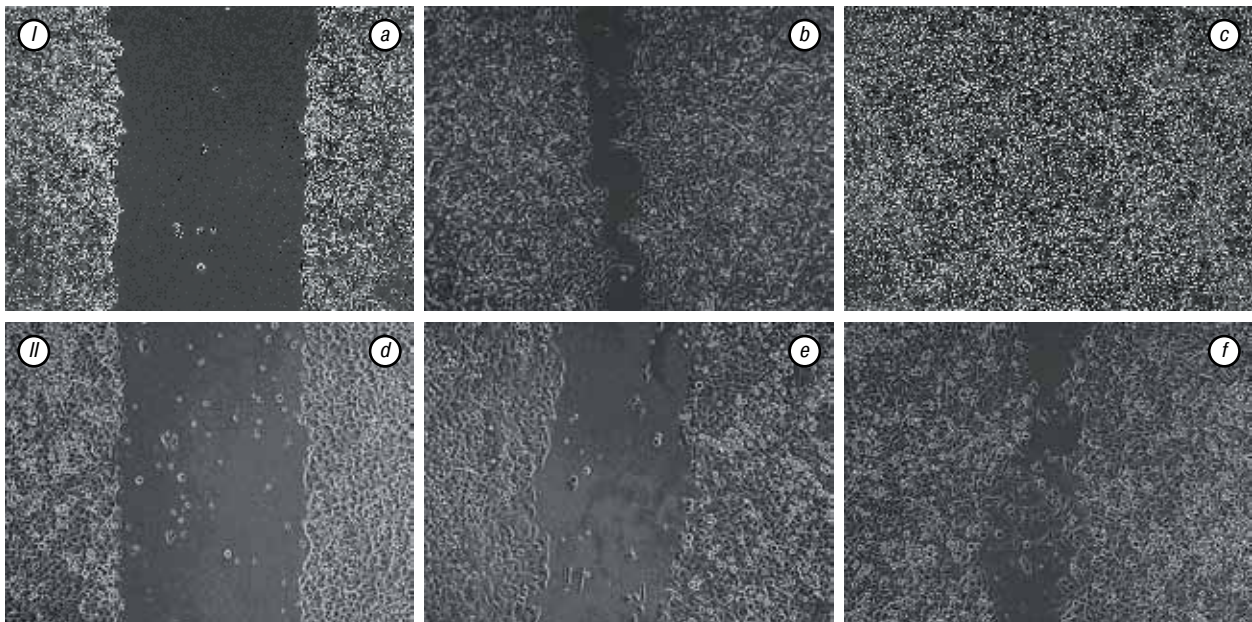

Fig. 5. Migration activity of the BCC/PI (I) or OCC/PI (II) cells: *a, d* — 3 h; *b, e* — 24 h; *c, f* — 48 h after damage of their monolayer

sistant human lung cancer cells (with mesenchymal characteristics) to inhibitor of epidermal growth factor receptor [14]. In addition, it has been reported that tumor cells with mesenchymal phenotype have a "protective effect" from DNA-damaging antineoplastic factors at the level of Zeb2/SIP1 expression, which is inversely correlated with the expression of E-cadherin [17]. Recently, cancer stem cells have been shown to be resistant to anticancer drugs and it was suggested that the occurrence of cancer stem cells is partly a consequence of EMT [18, 19].

This led us to investigate some EMT markers in primary *versus* permanent cells of BCC and OCC lines in conditions of their unstable microenvironment: after growth in "spheroidal/suspension" conditions in ascites and the following *in vitro* cultivation — the substrate "extracellular environment". We show that after a number of cell doublings *in vitro* cell immunophenotypic characteristics changed to reflect the properties of epithelial cells. This includes an increase in the number of E-cadherin-positive cells and a decrease in the number of vimentin and α-smooth muscle actin-positive cells. These changes were especially dramatic (and statistically significant) in the breast cancer cells (Fig. 6).
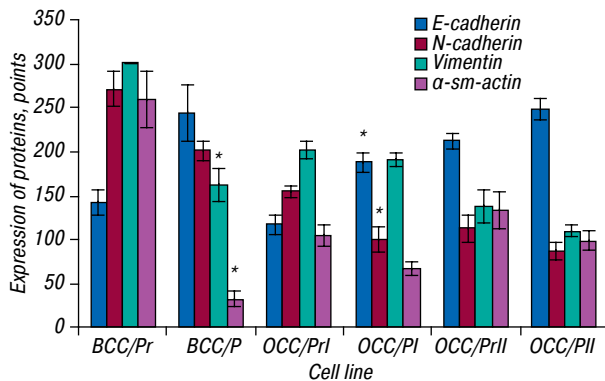

Fig. 6. EMT markers in primary and permanent cell lines of breast and ovarian cancer. The level of antigen expression is presented in points of H-score system. *Differences in marker expression levels in primary cells *vs* permanent cell line are statistically significant ($p < 0.05$)

The course of prescribed anticancer therapy is based on the main phenotypic characteristics of the tumor and prevalence of disease. Recent studies have focused on finding ways to individualize a choice of therapy based on understanding of the mechanisms of carcinogenesis and molecular markers. The cells from the original tumor and newly formed metastatic lesions often differ in sensitivity to chemotherapy. Thus it is extremely important to understand the sensitivity of tumor cells to chemotherapeutics with different mechanism of action during tumor progression and phenotypic conversion of tumor cells. We found that the sensitivity of primary tumor cells from ascites quickly changes after cells are cultured as the permanent cell lines (Fig. 7).

CONCLUSIONS

In conclusion, we have obtained new tumor cell lines from pleural effusion and ascites from patients with breast or ovarian cancer and described some of their growth characteristics, immunophenotype features and sensitivity to certain anticancer drugs. The results indicate that primary mesenchymal-like tumor cells quickly (during 5–10 doublings) transform into cells with epithelial phenotype. Also, we have established that cells as permanent cell lines lose their EMT-signature while their sensitivity to anticancer drugs increases significantly. We found rapid transformation and the acquisition of new properties in a changing microenvironment (intercellular and substrate contacts, soluble factors, etc). This suggests that the induction of phenotypic transdifferentiation in malignant cells from pleural effusion and ascites can be an important approach for suppressing the progression of neoplastic process. Our results demonstrate that change in phenotype of tumor cells causes a change in their behavior and, consequently, the effects of anticancer agents. These results also highlight the necessity to further study the association of EMT of tumor cells with their response to anticancer agents and the importance of monitoring the phenotypic plasticity of malignant cells.
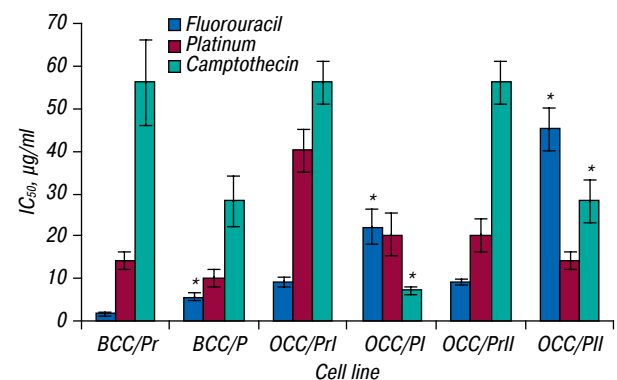

Fig. 7. Half maximal inhibitory concentration (IC_{50}) of anticancer drugs with different mechanisms of action for primary and permanent cell lines from pleural effusion or ascites of patients with breast/ovarian cancer. *Difference in IC_{50} for primary cells *vs* permanent cell line is statistically significant ($p < 0.05$)

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