Exp Oncol 2014
36, 2, 72–78

Experimental ONCOLOGY

IMPACT OF STROMAL CELL COMPONENTS OF TUMOR MICROENVIRONMENT ON EPITHELIAL-MESENCHYMAL TRANSITION IN BREAST CANCER CELLS

N. Bezdenezhnykh[1,], N. Semesiuk[1], O. Lykhova[1], V. Zhylchuk[2,3], Y. Kudryavets[1]*
[1]R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine, Kyiv 03022, Ukraine
[2]Rivne Region Oncology Hospital, Rivne 33001, Ukraine
[3]Danylo Halytsky Lviv National Medical University, Lviv 79010, Ukraine

Background: **Cell and tissue homeostasis results from the dynamic balance of cell – cell and cell – extracellular component crosstalk that regulates proliferation, differentiation, and apoptosis of cells as well as secretion and activation of soluble factors and/or deposition of extracellular matrix (ECM) components.** ***Aim:*** **The aim of the work was to study the crosstalk between tumor cells and stromal cell components using noncontact co-cultivation *in vitro* system.** ***Materials and Methods:*** **Human and rat breast cancer (BC) cell lines, normal human fibroblasts (NHF) and endothelial cells, and aspirates of bone marrow (BM) of BC patients with different clinical course of the disease (groups "Remission" (BM-R) and "Progression" (BM-P)) were used in noncontact co-cultivation system *in vitro*. The cell growth, expression of epithelial-mesenchymal transition (EMT) and tumor stem cell markers (E-cadherin, vimentin, CD44), Ki-67, p21 and Slug were investigated using immunocytochemical analysis.** ***Results:*** **Analysis of expression of E- and N-cadherin, vimentin and Slug in BC cells has shown that T-47D and MRS-T5 cells possess mesenchymal phenotype, while MCF-7 and MRS cells possess mostly epithelial phenotype with a part of cells with mesenchymal patterns. Upon noncontact co-cultivation of fibroblasts with T-47D or MRS-T5 cells, BC cells acquired higher proliferative activity compared to the control cells ($p < 0.05$) or MCF-7 and MRS cells co-cultivated with fibroblasts. Upon noncontact co-cultivation of T-47D cells with normal fibroblasts and BM cells from BC patients from group "Progression" there were observed increased quantity of $CD44^+$ T-47D cells (by 26%), decreased quantity of E-$cadherin^+$ T-47D cells, and appearance of vimentin-positive cells. In co-cultivation variant T-47D + NHF + BM-R ("Remission") the quantity of $CD44^+$ T-47D cells significantly decreased ($p < 0.005$) and E-cadherin expression remained unaltered compared to control cells. At the same time, in NHF cell population (co-cultivation variant T-47D + NHF + BM-P) there was detected significant increase of quantity of p21+-cells ($p < 0.005$), cytoplasmic localization of p21, and nuclear localization of Slug. Expression of vimentin did not alter in any variant of co-cultivation.** ***Conclusion:*** **The new integration cell system for investigation of the mechanisms of interaction between tumor cells and the tumor microenvironment *in vitro* was developed. The significant changes in proliferative activity of TC dependently on its EMT-status were detected after their interaction with fibroblasts and endothelial cells in noncontact co-cultivation system. BM cells of BC patients had different modifying influence on TC dependent on clinical BC course. The activation of EMT program was revealed in TC upon noncontact co-cultivation with BM cells of BC patients with progression of the disease.**
Key Words: **breast cancer, epithelial-mesenchymal transition, microenvironment, bone marrow, co-cultivation.**

In most cases the cancer-related mortality is caused by haematogenous spread of cancer cells into distant organs and their subsequent growth to overt metastases. After surgical removal of the primary tumor, minimal residual disease (MRD) is defined as the presence of tumor cells (TC) that are not detectable by the current routine diagnostic procedures used for tumor staging in cancer patients, but become apparent after a period of time [1]. The crosstalk between tumor and cells of its microenvironment crucially determines the fate of tumor progression. The stroma is a very heterogeneous milieu including various cell types and adhesion molecules, both contributing to the functional activity and structural support of the tumor microenvironment (TME) [2]. S. Paget laid the foundations of the TME research area by formulating the seed and soil theory. The tumor is directed into one or several possible molecular evolution pathways by signals originating in native and/or modified microenvironmental factors. Many of these pathways may lead to metastasis. The TME has some characteristics: 1) the molecular composition of the TME is established by TC and by resident/infiltrating non-tumor cells; 2) intercellular interactions between TC and components of their microenvironment are crucial in determining cancer progression; 3) tumor — microenvironment interactions are bidirectional; 4) certain tumor — microenvironment interactions may initiate tumor progression ; 5) microenvironmental factors play opposing roles in tumor progression by either promoting or alternatively antagonizing this process [3].

The TME was recognized as the product of a developing crosstalk between different cells types: endothelial cells, carcinoma-associated fibroblasts (CAFs), adipocytes, mesenchymal cells, mesenchymal stem cells (MSCs; bone marrow derived — (BM-MSCs), or carcinoma-associated (CA-MSCs)), and cells from the immune and inflammatory systems (tumor-associated macrophages (TAM), regulatory T cells, etc.). The stromal cells interact not only with TC but also with each other. TC induce changes in fibroblasts, which contact with them [4], transforming them into myofibroblasts, which activate program of epithelial-mesenchymal transition (EMT) in TC resulting in the domination of mesenchymal features

Submitted: May 8, 2014.
*Correspondence: E-mail: beznalia@mail.ru
Abbreviations used: BC – breast cancer; BM – bone marrow; ECM – extracellular matrix; EMT – epithelial-mesenchymal transition; MC – mononuclear cells; MET – mesenchymal-epithelial transition; MRD – minimal residual disease; TAM – tumor-associated macrophage; TC – tumor cells; TME – tumor microenvironment; TNF – tumor necrosis factor.

and development of highly metastatic phenotype [5]. All these processes take place with involvement of soluble (humoral) factors of microenvironment (cytokines, growth factors, etc.), which, in turn, activate other links of interaction, involved in "malignant transition", in particular, transcriptional factors. In the result of this, epithelial cells lost the capacity to form dense intercellular contacts, their adhesive properties decrease while capacity for invasion and migration develop. However, when such cells achieve their "destination point", the inverse process takes place — mesenchymal-epithelial transition (MET), during which they again acquire epithelial phenotype with increased adhesion that gives opportunity to them to fasten onto the substrate and to give growth to secondary metastatic focus [6]. Thus, a major TAM-derived inflammatory cytokine shown to be highly expressed in breast carcinomas is tumor necrosis factor alpha (TNF-α) which is a multifactorial cytokine. TNF-α activity varies under different physiological conditions and in a cell-type-dependent manner contributes to a sense of ambiguity regarding its antitumor effects. Indeed, the permanent expression of TNF-α in breast tumors actually supports tumor growth and plays a role in the metastatic behavior of breast carcinomas [7]. Furthermore, in BM of breast cancer (BC) patients with advanced cancer significantly higher TNF-α concentration was detected [8]. Therefore, the further study of intercellular interaction and cell transdifferentiation may reveal new targets in antimetastatic therapy.

It is well known that certain organs and tissues are typical as dominant sites of metastasis for many types of tumor. For example, bone marrow (BM) and bones often become such distant sites in BC. Today is well known the important role of stromal cells, in particular fibroblasts, as elements of TME, in control of their phenotype and biological behavior [9]. However, the role of BM cells as components of TME is poorly understood. Therefore, the determination of influence of BM cells, especially in comparative aspect with fibroblast elements, on the phenotypic conversion and proliferative activity of cells of BC at their co-cultivation, is of special interest.

In routine practice for *in vitro* research of compounds of different nature (antitumor drugs, cytotoxic factors) isolated cell cultures (primary cell cultures and stable cell lines) are used. Experimental systems *in vitro* with use of human cell lines represent important instrument for estimation of the level and mechanisms of toxicity of antitumor drugs with the aim of precise definition of their possible antitumor action *in vivo*. The main disadvantage of such *in vitro* system is an absence of numerous components of interaction, which exist *in vivo*. The principally new approach for determination of intercellular interaction *in vitro* has been developed by A.P. Li and co-authors [10] who created a system of integrated discrete cultures of normal cells of different histogenesis and BC cells for the study of simultaneous combined toxic action of drugs on the TC-targets and on the normal cells of organism (liver, kidneys, lungs, vascular endothelium, etc.). The same scheme with some modification has been developed by us for testing the toxicity of chemical compounds, in particular, heavy metal salts, *in vitro,* so-called multi-organ system of toxicity (MOST) [11]. For investigation of cellular interaction in progression of disease, we have developed in this work new cellular system with use of primary and stable cell lines of different genesis: human and rat BC TC, normal human fibroblasts (NHF) and normal rat fibroblasts (NRF), normal porcine endothelial cells (PAE), cells from BM aspirates from BC patients with different disease stage.

The aim of the work was to study *in vitro* the crosstalk between TC and stromal cell components using noncontact co-cultivation *in vitro* system.

MATERIALS AND METHODS

Clinical materials. In co-cultivation *in vitro* system, mononuclear cells (MC) isolated from BM of BC patients with II and III stages of the disease (n = 6), were used. BC patients were cured in Rivne Regional Oncological Hospital (Rivne, Ukraine). The patients were informed about the survey and provided written consent for participation in the research. The study was carried out with approval of the local Ethics Committee (R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine).

After conducted treatment (radical surgical intervention and adjuvant chemotherapy) BC patients have been distributed into 2 groups: "Progression" (n = 3) and "Remission" (n = 3), at the basis of their clinical state, presence of TC in BM and high level of tumor-associated cytokines in plasma of BM and peripheral blood [12, 13].

The BM aspirates (3–4 ml) were taken from sternum with an incision in the skin to avoid contamination with epithelial cells. BM samples were obtained from BC patients prior to therapy.

MC were isolated by Ficoll-Hypaque density gradient LSM 1077 (PAA, Austria) centrifugation at 1,500 rpm for 20 min. MC were washed three times with RPMI-1640 and were cryopreserved ($3–5 \cdot 10^6$ cells per vial).

Cell lines. In our co-cultivation study the following cell lines were used: NHF, NRF of Wistar rats, normal porcine endothelial cells (PAE), human BC cell lines — T-47D and MCF-7 and their sublines modified by long-term action of interferon (IFN) (MCF-7 + IFN and T-47D + IFN), rat mammary carcinoma — MRS [14, 15], which are characterized by domination in population of low tumorigenic cells with epithelial phenotype as well as highly tumorigenic subline MRS-T5, which is characterized by the presence of cells of only mesenchymal phenotype. All cell lines have been obtained from Bank of Cell Lines from Human and Animal Tissues of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of National Academy of Sciences of Ukraine (Kyiv, Ukraine).

The modeling system of noncontact cell co-cultivation. The cells of different type which were cultured separately, serve as control (Fig. 1, *a*). For co-cultivation experiment, the wells were combined with each other via specially made channels in the wall of wells in such a way

that interaction of cells through their nutrient medium could occur (Fig. 1, *b*).

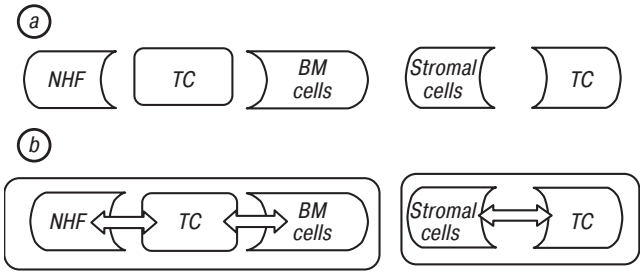

Fig. 1. Scheme of noncontact co-cultivation *in vitro*: *a* — control wells — isolated; *b* — wells with noncontact co-cultivation

Cells were cultivated in plastic glassware ("TPP", Italy) in DMEM medium ("PAA", Austria), which contained 2 mM of L-glutamine and $NaHCO_3$ with 10% of FBS ("PAA", Austria) in humidified atmosphere at 37 °C and 5% CO_2. Quantity of cells after their co-cultivation has been determined by staining with crystal violet ("Sigma", USA) with further registration of optical density of well content using multiwell spectrometer (Labsystems Multiskan PLUS, Finland) [16].

Immunocytochemical analysis. The slides of cytospins were fixed in methanol + acetone (1:1) solution for 2 h at –20 °C, and then incubated with 1% BSA solution for 20 min. For immunocytochemical analysis, the following monoclonal antibodies have been applied: anti-E-cadherin (Thermo Scientific, UK), anti-CD325 (N-Cadherin) (BioLegend, USA), anti-vimentin (Diagnostic BioSystems, USA), anti-CD44 (Diagnostic BioSystems, USA), Slug (GeneTex, USA), p21/waf1 (NeoMarkers, USA), Ki-67 (Thermo Scientific, UK). Primary antibodies were used according to the instructions of manufacturers. For visualization, Poly Vue system (Thermo Scientific, UK) was used. The slides were also stained with Mayer's hematoxylin ("Sigma", USA) and examined using microscope AxioVert ("Carl Zeiss", Germany) with ×320 magnification. Data were analyzed by calculation of positive (+) cells using classical H-score method:

$$S = 1B \cdot A + 2B \cdot B + 3B \cdot C,$$

where S — H-score index, which value lies within the limits from 0 (no expression) to 300 (intensive expression in 100% of cells); A — percentage of poorly stained cells; B — percentage of moderately stained cells; C — percentage of strongly stained cells.

Statistics. Statistical processing of obtained results has been carried out using mathematical program STATISTICA 6.0. Significance of differences between mean values has been conducted with use of Student's *t*-criterion.

RESULTS AND DISCUSSION

First of all, the expression of EMT markers (E- and N-cadherin, vimentin and Slug) was analyzed in TC which were cultured separately as a control and later were used in noncontact co-cultivation system. It was found that the mesenchymal antigen expression profile was the most typical for cells of T-47D and MRS-T5 lines. MCF-7 and MRS cell lines are characterized by the prevalence of cells with epithelial phenotype with the presence of cells with mesenchymal features (Table).

Then, TC and stromal elements (fibroblasts, endothelial cells) were co-cultivated *in vitro* using the system described above. In the case of co-cultivation of TC and fibroblasts, the proliferation of fibroblasts was close to that of control cells but the quantity of TC varied dependently on domination of epithelial or mesenchymal phenotype of these cells. In particular, the quantity of TC with domination of mesenchymal features (T-47D, MRS-T5 cells) in noncontact co-cultivation with fibroblasts was more prominently increased compared to control cells ($p < 0.05$), than quantity of TC with prevalence of epithelial phenotype (MCF-7, MRS) (Fig. 2). In the case of co-cultivation of fibroblasts (RNF) with rat TC with mesenchymal or epithelial features (variant of co-cultivation MRS + RNF + MRS-T5) significant inhibition of MRS-T5 cells with mesenchymal phenotype in presence of epithelial MRS cells was detected (see Fig. 2). So, the cells with more differentiated epithelial phenotype may significantly repress growth potential of cells with mesenchymal phenotype [17].

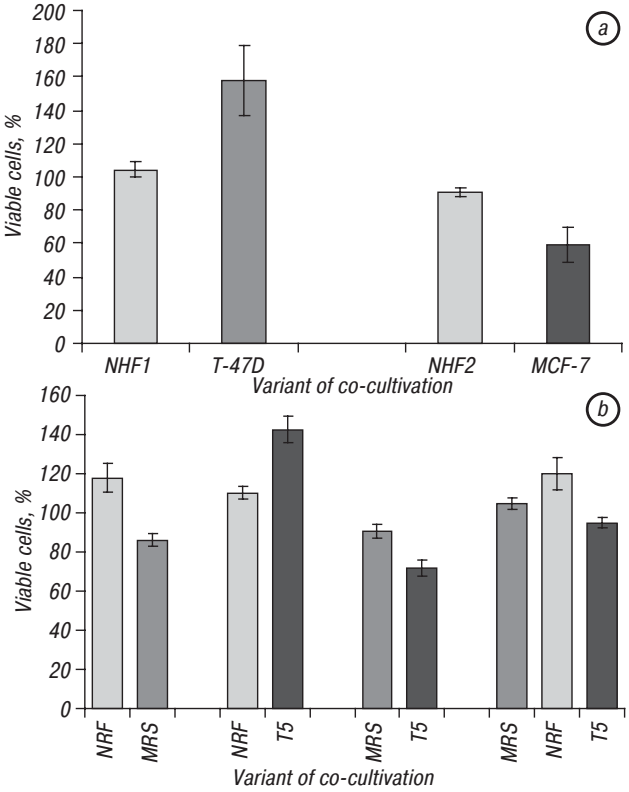

Fig. 2. Quantity of viable NHF and human TC (*a*) and NRF and rat TC (*b*) upon their combined noncontact co-cultivation. OY axis represents the percent of viable cells compared to respective control cells cultured in separate wells (taken as 100% of viable cells)

It is well known that tumor and stromal cells in microenvironment can interact and crosstalk between them might be bidirectional. Tumor angiogenesis is a prerequisite for tumor progression and metastasis. It is a complex process

Table. EMT marker expression in human BC cell lines

Antigens	E-cadherin					N-cadherin					Slug				
TC	T-47D	MCF-7	MRS		T5	T-47D	MCF-7	MRS		T5	T-47D	MCF-7	MRS		T5
			E	M				E	M				E	M	
Scores (H-score system)	98±11	126±7	270±21	22±9	39±4	81±11	53±3	72±8	221±28	174±16	136±21	54±10	156±26	224±32	269±11

that requires cooperative reciprocal interaction of tumor and endothelial cells and involves soluble and cellular components. This requires a coordinated expression of proangiogenic factors and suppression of antiangiogenic factors, which leads to endothelial cell proliferation, migration and vessel formation. M. Buess et al. [18] suggest that the interaction of endothelial cells with TC that express the $CD44^{+}/CD24^{-}$ signature (indicating a low proliferative potential) might explain association of the $CD44^{+}/CD24^{-}$ signature with highly proliferative tumors that have an unfavorable prognosis.

The differences in proliferative activities of human BC cells (control cells and IFN-modified cells) in co-cultivation with cellular TME components (fibroblasts and endothelial cells) were identified. For this study, we used the following design of noncontact cultivation cell system:

- set 1: 1) MCF-7/MCF-7 (IFN modified) + NHF; 2) MCF-7/MCF-7 (IFN modified) + PAE;
- set 2: 1) T-47D/T-47D (IFN modified) + NHF; 2) T-47D/T-47D (IFN modified) + PAE.

So, we obtained the following results: using MCF-7 cell line and MCF-7 + IFN subline, we observed an inhibition the BC proliferation independently on variant of stromal cells (NHF or PAE). This inhibition was more significant in the case with MCF-7 + IFN cells (20–25%) compared with control cells (Fig. 3).

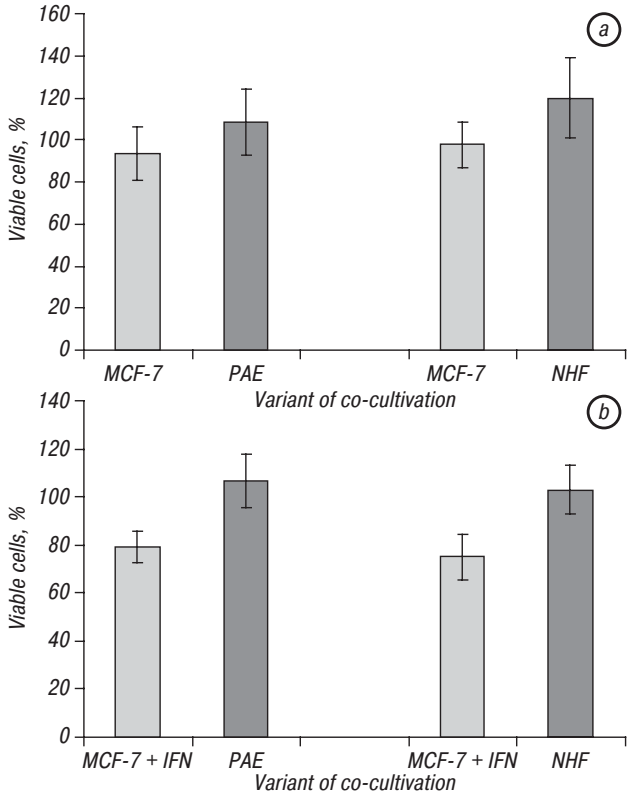

Fig. 3. Proliferative activities of MCF-7 (*a*) and IFN-modified MCF-7 (*b*) cells in noncontact co-cultivation system with NHF and PAE cells. OY axis represents the percent of viable cells compared to respective control cells cultured in separate wells (taken as 100% of viable cells)

Interestingly, proliferative activity of NHF and PAE was changed, too: quantities of stromal elements were higher (about 10% in case co-cultivation with PAE and 3–20% in case co-cultivation with NHF). To explain this fact we will investigate the phenotype of these cells. It is known that tumor can induce phenotypic changes associated with transcriptional reprogramming of endothelial cells, which can be detected by expression profiling: production of growth factors by TC that induce endothelial cells to express specific ligands and their cognate receptors coordinately [19].

In another set of our study, the proliferative activities of BC and stromal cells were different indicating a significant interplay of cells. In particular, cultivation of T-47D cells with NHF or PAE as well as cultivation of IFN-modified T-47D cells with NHF or PAE resulted in increased proliferation of both cell types (Fig. 4).

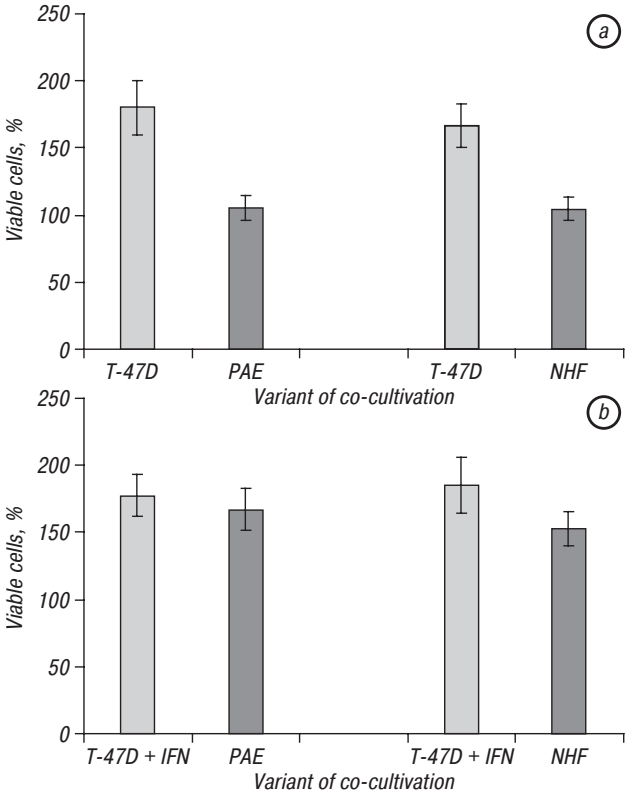

Fig. 4. Proliferative activities of T-47D (*a*) and IFN-modified T-47D (*b*) cells in noncontact co-cultivation system with NHF and PAE cells. OY axis represents the percent of viable cells compared to respective control cells cultured in separate wells (taken as 100% of viable cells)

BM is considered to be depot for TC and is direct participant of MRD [20–22]. So, the influence of BM components to TC as an additional factor was studied. Cells from aspirates of BM of BC patients were used in new integrated system *in vitro*. The significant changes of phenotypic features of both tumor and normal cells in noncontact co-cultivation system with addition of BM cells were detected (Fig. 5): variant of co-cultivation T-47D + NHF + BM ("Progression") and T-47D + NHF + BM ("Remission"). To investigate changes at the cellular and molecular level we decided to analyze the expression of some protein markers associated with EMT.

The investigation of expression of cell cycle regulator p21 has shown that the quantity of p21-positive T-47D cells as well as intracellular localization of p21 remained unchanged in different variants of co-cultivation (see Fig. 5, *a*). The subcellular localization of p21 is very

important because this protein performs opposite functions in nucleus and cytoplasm. In particular, if p21 localizes in nucleus, it acts as regulator of cell cycle through inactivation of transcriptional factors E2F1, c-Myc, STAT3 [23], as well as inhibitor of replication due to oppression of subunit of DNA-polymerase of δ-protein PCNA. When p21 localizes in cytoplasm, it performs formation of stress-fibrin and focal contacts that assists the migration of cells. Moreover, p21 in cytoplasm demonstrates antiapoptotic functions through decrease of proapoptotic kinases activity (ASK-1, JNK, p38) and inhibition of proxapase-2.

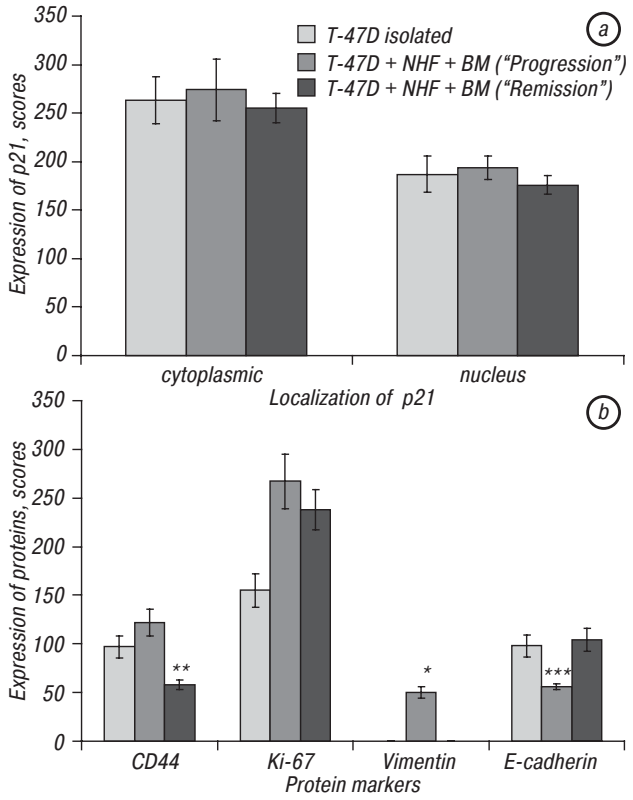

Fig. 5. Expression of p21 (*a*), and CD44, Ki-67, vimentin, E-cadherin (*b*) in T-47D cells in noncontact co-cultivation system *in vitro* with NHF and BM cells. *p ⩽ 0.005; **p ⩽ 0.05; ***p; evaluation by H-Score, scores

Next, we investigated CD44 expression. CD44 is adhesion molecule, which plays important role in interaction "tumor — microenvironment" and is involved in processes of migration and invasion of TC. Moreover, CD44 is used as marker of progression and metastasis at different types of cancer, in particular, BC. TCs with phenotype $CD44^+CD24^-$ are considered as cancer stem cells (CSC). In our study, the quantity of CD44-positive ($CD44^+$) T-47D cells were increased by 26% at their co-cultivation with BM cells (variant T-47D + NHF + BM ("Progression")) compared with control of cells in isolated wells. Interestingly, the quantity of $CD44^+$-cells was significantly decreased in variant of co-cultivation T-47D + NHF + BM ("Remission") compared to isolated control ($p < 0.005$) and compared to these in co-cultivation variant with BM cells of patients from "Progression" group ($p < 0.005$) (see Fig. 5, *b*).

The next step was to study of E-cadherin expression in T-47D cells in different variants of co-cultivation: the quantity of $E\text{-cadherin}^+$ T-47D cells in co-cultivation variant T-47D + NHF + BM ("Progression") was decreased by 1.8 times compared to isolated control and was not changed compared to these in co-cultivation variant T-47D + NHF + BM ("Remission") (see Fig. 5, *b*). E-cadherin is a marker of epithelial phenotype. Loss of the E-cadherin molecule is thought to enable metastasis by disrupting intercellular contacts — an early step in metastatic dissemination and prominently associated with tumor invasiveness and poor prognosis [24].

Vimentin is a protein of cytoskeleton normally expressed in cells of mesenchymal origin [25]. It is used widely as a marker of the EMT and can induces changes in cell shape, motility, and adhesion during of this process [26]. Its expression was investigated in T-47D cells which were included in different variants of co-cultivation. Thus, this protein was not detected in control T-47D cells (isolated) as well as in T-47D cells of variant co-cultivation T-47D + NHF + BM ("Remission"), but the expression of vimentin was observed in T-47D cells in co-cultivation with NHF and BM cells ("Progression") (see Fig. 5, *b*).

Such changes of phenotypic profile of T-47D cells are the results of their co-cultivation with components of microenvironment (NHF and BM cells of BC patients with different BC course). These indicate the significant influence of BM cells and humoral factors, which they produce, on the BC cells which depended on the state of tumor process in BC patients (progression or remission of the disease). The phenotypic profile of TC in co-cultivation variant with BM cells of BC patients of "Remission" group did not differ from control cells. But the activation of EMT program were observed in TC in co-cultivation variant with BM cells of BC patients of "Progression" group, which was associated with obtaining of more aggressive features by cells and possible increase of their metastatic potential.

The phenotypic profile of NHF after their co-cultivation with T-47D and BM cells of BC patients with different courses of tumor process was changed too. The expression of vimentin, p21 and Slug was investigated. So, changes of expression of vimentin were not detected in NHF in any variants of co-cultivation. Changes of expression of transcription factor of mesenchymal cells Slug were detected. In co-cultivation NHF with TC and BM cells of BC patients Slug was relocalized to the nucleus of NHF ($p < 0.002$). Interestingly, the presence of p21 protein only in cytoplasm of NHF was observed (Fig. 6). The quantity of p21+ NHF cells was significantly increased upon co-cultivation with BM cells of BC patients ("Progression" group) compared with control ($p < 0.005$).

These results in some way explain the well-known data about differentiation of fibroblasts into myofibroblasts in co-cultivation with TC [27]. Stromal elements, which include also fibroblasts, may be inductors of transcription factors in TC that support the domination of mesenchymal features in cells. Such modulations of intracellular processes occur through the secretion by cells of series of factors (cytokines, factors of growth, etc.). One of such fac-

tors may be epithelial-stromal interaction 1 (EPST1). EPST1 has been identified as interferon response gene exactly in co-cultivation of BC cells with stromal fibroblasts [28, 29]. Its expression was increased in postsurgical tumor material of BC patients in contrast to control mammary gland tissues. The highest intensity of expression of EPST1 was determined exactly in regions of tumor, which closely contacted with stroma [29].

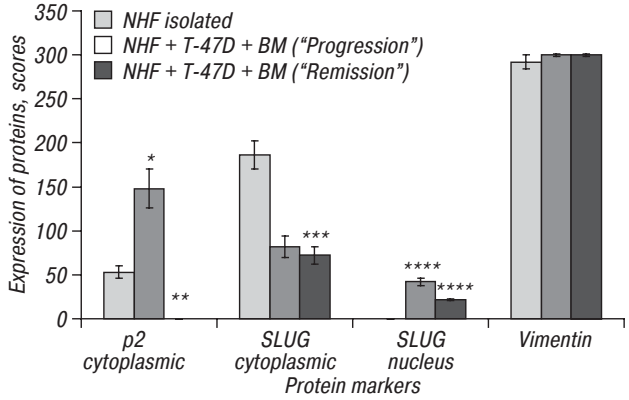

Fig. 6. Expression of p21 (*a*), Slug and vimentin in NHF cells upon noncontact co-cultivation with TC and BM cells from BC patients. *p < 0.005; **p < 0.005; ***p < 0.02; ****p < 0.002; evaluation by H-Score, scores

In conclusion, the new integration cell system for investigation of the mechanisms of interaction between TC and TME *in vitro* was developed. The significant changes in proliferative activity of TC dependently on its EMT-status were detected after their interaction with fibroblasts and endothelial cells in noncontact co-cultivation system. BM cells of BC patients had different modifying influence on TC dependent on clinical BC course. The activation of EMT program was revealed in TC upon noncontact co-cultivation with BM cells of BC patients with progression of the disease.

ACKNOWLEDGEMENTS

This study was supported with the grant NAS of Ukraine for Young Scientists "Development of new integration cell system for investigation of the mechanisms of interaction TC with microenvironment *in vitro*", № 2.2.5.383 from 01.07.2013.

REFERENCES

1. Castaco Z, Tracy K, McAllister SS. The tumor macroenvironment and systemic regulation of breast cancer progression. Int J Dev Biol 2011; **55**: 889–97.

2. Schiavoni G, Gabriele L, Mattei F. The tumor microenvironment: a pitch for multiple players Front Oncol 2013; **3**: 90.

3. Witz IP. The tumor microenvironment: the making of a paradigm cancer microenvironment. Cancer Microenviron 2009; **2**: 9–17.

4. Tran-Thanh D, Done SJ. The role of stromal factors in breast tumorigenicity. Am J Pathol 2010; **176**: 1072–74.

5. Castells M, Thibault B, Delord J-P, ***et al.*** Implication of tumor microenvironment in chemoresistance: tumor-associated stromal cells protect tumor cells from cell death. Int J Mol Sci 2012; **13:** 9545–71.

6. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest 2009; **119**: 1420–28.

7. Ben-Baruch A. Host microenvironment in breast cancer development: inflammatory cells, cytokines and chemokines in breast cancer progression: reciprocal tumor-microenvironment interactions. Breast Cancer Res 2003; **5**: 31–6.

8. Semesiuk N, Zhylchuk A, Bezdenezhnykh N, ***et al.*** Disseminated tumor cells and enhanced level of some cytokines in bone marrow and peripheral blood of breast cancer patients as predictive factors of tumor progression. Exp Oncol 2013; **35**: 295–302.

9. Strell C, Rundovist H, Ostman A. Fibroblasts — a key host cell type in tumor initiation, progression and metastasis. Upsala J Med Sci 2012; **117**: 187–195.

10. Li AP, Bode C, Sakai Y. A novel *in vitro* system, the integrated discrete multiple organ cell culture (IdMOC) system, for the evaluation of human drug toxicity: comparative cytotoxicity of tamoxifen towards normal human cells from five major organs and MCF-7 adenocarcinoma breast cancer cells. Chem-Biol Interact 2004; **150**: 129–36.

11. Trahtenberg MI, Kudryavets Yu, Marchenko ML, ***et al.*** Improved model of multiorgan system toxicity of xenobiotics from co-cultivation human cells *in vitro*. Such Probl Toksykol 2011; **1–2**: 60–4 (in Ukrainian).

12. Semesiuk NI, Zhylchuk AV, Bezdenezhnykh NO, ***et al.*** Tumor necrosis factor in peripheral blood and bone marrow of breast cancer patients as a marker of the tumor progression. Onkologiya 2012; **4**: 293–7 (in Ukrainian).

13. Semesiuk NI, Zhylchuk AV, Bezdenezhnykh NO, ***et al.*** Colony stimulating activity in peripheral blood and bone marrow breast cancer patients and its prognostic value. Zbirnyk. Hematol Perelyv Krovi 2012; **36**: 232–7 (in Ukrainian).

14. Kudryavets YuI, Bezdenezhnykh NO, Trehubova ON, ***et al.*** The new model of experimental oncology: cell line MRS from spontaneous rat breast cancer tends to modulate the transformed phenotype. XI congress of oncologists Ukraine. Sudak, 2006, 29 p.

15. Kudryavets YuI, Bezdenezhnykh NO, Kovaleva OA, ***et al.*** Cell line MRS from breast adenocarcinoma rat as a model to study mechanisms of epithelial-mesenchymal transition of tumor cells. Klin Onkol 2011; **2**: 218 (in Ukrainian).

16. Vega-Avila E, Pugsley KM. An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells. Proc West Pharmacol Soc 2011; **54**: 10–4.

17. Bezdenezhnykh NO, Semesiuk NI, Lykhova OO, ***et al.*** Modification of epithelial-mesenchymal transition in breast cancer cells by means of their co-cultivation with fibroblasts and bone marrow cells. Onkologiya 2013; **15**: 191–6 (in Ukrainian).

18. Buess M, Rajski M, Vogel-Durrer BML, ***et al.*** Links the $CD44^{+}/CD24^{-}$ phenotype with poor prognosis in early-stage breast cancer. Neoplasia 2009; **11**: 987–1002.

19. Khodarev NN, Yu J, Labay E, ***et al***. Tumour-endothelium interactions in co-culture: coordinated changes of gene expression profiles and phenotypic properties of endothelial cells. J Cell Sci 2002; **116**: 1013–22.

20. Krishnamurthy S, Cristofanilli M, Singh B, ***et al.*** Detection of minimal residual disease in blood and bone marrow in early stage breast cancer. Cancer 2010; **116**: 3330–7.

21. Bischoff J, Rosenberg R, Dahm M, ***et al.*** Minimal residual disease in bone marrow and peripheral blood of patients with metastatic breast cancer. Recent Res Cancer Res 2003; **162**: 135–40.

22. Diel IJ, Cote RJ. Bone marrow and lymph node assessment for minimal residual disease in patients with breast cancer. Cancer Treat Rev 2000; **26**: 53–65.

23. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. Nat Rev Cancer 2009; **9**: 400–14.

24. Onder TT, Gupta PB, Mani SA, ***et al.*** Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer Res 2008; **68**:3645–54.

25. Gilles C, Polette M, Zahm J-M, ***et al.*** Vimentin contributes to human mammary epithelial cell migration. J Cell Sci 1999; **112**: 4615–25.

26. Mendez MG, Kojima S-I, Goldman RD. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. FASEB J 2010; **24**: 1838–51.

27. de Neergaard M, Kim J, Villadsen R, ***et al.*** Epithelial-stromal interaction 1 (EPSTI1) substitutes for peritumoral fibroblasts in the tumor microenvironment. Am J Pathol 2010; **176**: 1229–40.

28. Nielsen HL, Ronnov-Jessen L, Villadsen R, ***et al.*** Identification of EPSTI1, a novel gene induced by epithelial-stromal interaction in human breast cancer. Genom 2002; **79**: 703–10.

29. Honeth G, Bedahl PO, Ringner M, ***et al.*** The $CD44^{+}$/$CD24^{-}$ phenotype is enriched in basal-like breast tumours. Breast Cancer Res 2008; **10**: R53.