N.O. Bezdenezhnykh¹
N.I. Semesiuk¹
O.O. Lykhova¹
V.E. Zhylchuk²
Yu.I. Kudryavets¹

¹ R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine, Kyiv
² Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

Key words: breast cancer, epithelial-mesenchymal transition, microenvironment, bone marrow, co-cultivation.

MODIFICATION OF EPITHELIAL-MESENCHYMAL TRANSITION IN BREAST CANCER CELLS BY MEANS OF THEIR CO-CULTIVATION WITH FIBROBLASTS AND BONE MARROW CELLS

Summary. Objective: the goal of our study was to investigate the interaction between tumor cells and some components of their microenvironment for identification of leading factors in tumor progression. Object and methods: we used biopsies of bone marrow breast cancer patients with different stages of disease, human and rat breast cancer cell lines and normal fibroblasts, methods of immunocytochemical and statistical analysis. The proliferative potential and immunophenotypical features of tumor and normal cells were revealed in our new co-cultivation system in vitro. The analysis included cell growth control, detection of expression of epithelial—mesenchymal transition (EMT) and tumor stem cell markers (adhesion and cytoskeleton proteins — E-cadherin, Vimentin, CD44), protein of cell cycle p21 and transcription factor Slug. Results: it was revealed the great differences in growth and phenotypic features of these cells which depended on EMT status of tumor cells and version of co-cultivation (using the bone marrow cells of breast cancer patients with progression or remission of the disease). Conclusion: Cell growth and EMT-signature of cancer and normal cells significantly changes depending on microenvironment, which formed during progression or remission of cancer process. In particular, the EMT stimulation of tumor cells exposed to factors produced by bone marrow cells of patients in stage of disease progression was determined.

INTRODUCTION

It is known that tumors include several structural elements: malignantly transformed cells, blood vessels, cells of immune system, fibroblasts, which usually are combined by extracellular matrix, and form so called tumor stroma [1]. Till this time, there is a great amount of information about role and peculiarities of interaction between “tumor stroma” with neoplastic cells. However, there are still many questions without answers remain. In particular, there are data on strengthening of “malignant properties” of human breast cancer cells (HBC), including their invasiveness and mobility at interaction with stromal elements [2]. Tumor cells (TC), in turn, induce changes in fibroblasts, which contact with them [2], transforming them into myofibroblasts, which activate program of epithelial-mesenchymal transition (EMT) in TC that causes in latter the domination of mesenchymal features. Activation of these features forms highly metastatic phenotype and makes it at the most closer to stem cells with high level of drug resistance. All these processes take place with involving of soluble (humoral) factors of microenvironment (cytokines, growth factors and so on), which, in turn, activate other links of interaction, involved in “malignant transition”, in particular, transcriptional factors, EPST1 (epithelial-stromal interaction 1) and others. In the result of this, epithelial cells loose capacity for formation of dense intercellular contacts, their adhesive properties are decreasing; they obtain capacity for invasion and migration. 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 [3]. The development of metastasis remains the main reason of cancer patients’ death, that is why the further study of the role of intercellular interaction in complicated and extremely important biological process of transdifferentiation would allow significantly deepen the understanding of mechanisms of its control and realization that, in the upshot, may reveal new targets in anti-metastatic therapy.

Clear understanding of mechanisms of so called homing-effect and conditions of formation and long-lasting survival of remote micrometastases is fundamentally important for the development of approaches to the anti-metastatic therapy. It is well known that for many localizations of tumor process certain organs and tissues of organism as target of prevailed metastasis are typical. For HBC bone marrow (BM) and bones often become such targets. Today is well known the important role of stromal cells, in particular, fibroblasts, as elements of microenvironment of TC, in control of their phenotype and biological behavior [4]. However, the role of BM cells as components of tumor microenvironment is little-studied. At the same time, BM is a focus of significant amount of cells of different level of differentiation and different commitment. These are not just hematogenic cells, but also cells, which are able to form other tissue structures, for example, vessels and capillaries. All these cells are active producers of cytokines and chemokines, which, without a doubt, influence the part of disseminated TC. Moreover, these very dissolving components often stipulate localization of metastases. That is why the determination of influence of BM cells, especially in comparative aspect with fibroblast elements, on the phenotypic conversion and proliferative activity of cells of HBC at their cocultivation, is logical.

In routine practice for research of influence of compounds of different nature (antitumor drugs, cytotoxic factors) are used isolated cell cultures (primary cell cultures and permanent cell lines) in vitro. 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 system in vitro is absence of numerous components of interaction, which exist in united organism, between different types of cells, which represent every certain organ. Ideally, such cellular systems in vitro shall as much as possible reflect conditions in vivo, in particular, with saving both species and tissue specificity. The principally new approach for determination of intercellular interaction in vitro has been developed by A.P.Li and co-authors. They have created system of integrated discrete cultures of normal cells of different histogenesis (IdMOC) and cells of HBC for study of simultaneous combined toxic action of chemodrugs on the TC-targets and on the normal cells of organism (liver, kidneys, lungs, vascular endothelium, etc.) [5]. 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 and has got the name of multi-organ system of toxicity (MOST) [6]. For investigation of cellular interaction in progression of disease we have developed in this work new cellular system with use of primary and permanent cell lines of different genesis (human breast cancer TC and rat breast cancer TC, normal fibroblasts of human – NFH and of rat – NFR, cells from punctates of BM of patients with HBC with different course of disease).

The aim of the work was to study in vitro the interaction between TC and some components of microenvironment for determination of possible role of the latter in modification of phenotype of TC in development of disseminated tumor process with involvement of BM.

**PATIENTS AND METHODS**

**The modeling of system of noncontact co-cultivation of cells.** As material for co-cultivation were used mononuclears, which were extracted from BM of patients with HBC, who were treated in Rivne regional oncological dispensary. The punctates of BM have been obtained by the puncture of sternum through the insection of skin for avoidance of contamination by epithelial cells from patients with HBC (stages T1–4N1–2M0–1 and T1–4N0–2M0, histological type of tumor – infiltrated duct carcinoma) before surgical intervention. After conducted treatment (radical surgical intervention and adjuvant chemotherapy) patients have been separated into two groups: “progression” and “remission”. The basis of separation into the groups was clinical state of patients, presence of TC in BM and high level of tumor-associated cytokines in plasma [7, 8]; at that the current standards of full, partial remission or progression were followed. In researches were used samples of BM of 3 patients with progression of the disease, and samples of 3 patients without signs of progression. At that, along with detection
of cytokeratin-positive cells in BM of patients with the progression of process, has been marked high level of necrosis of
tumors (> 150 pg/ml) in BM and TC, colony-stimulating factor 1 (> 300 un./ml) in TC, transforming factor of growth β1
(> 10 ng/ml) and interleukin 6 (> 50 pg/ml) in TC [7, 8]. All patients have been informed about examination and have given
consent to the use of material for research purposes.

Mononuclears have been extracted in density gradient LSM 1077 (“PAA”, Austria), have been twice washed out by
centrifugation in DMEM environment (“PAA”, Austria) during 10 min. at 1000 rev./min, their quantity has been calculated
in Goryaev’s camera and used cryopreservation (2,5–4×10⁶ cells/ampule).

Among cell lines were used NFH and NFR lines Wistar and cells of lines of breast cancer of human— T-47D, MCF-7 and
rat – MRS [9, 10], which are characterized by domination in population of cells with epithelial phenotype as well as highly
endogenous subline MRS-T5, which is characterized by presence of cells of only mesenchymal phenotype. All cell lines have
been obtained from cell bank of lines of human and animal tissue of R.E. Kavetsky Institute of Experimental Pathology,
Oncology and Radiobiology of National Academy of Sciences of Ukraine. In carrying out the experiment, cells of different
type were physically isolated one from another (Fig. 1 a) and served as control, but studied wells in variant of co-cultivation
were combined with each other with the help of specially made canals in wall of wells in such way that interaction of cells
through their nutrient medium could take place (Fig. 1 b). For characteristics of phenotypic profile of cells were chosen
antigens, which are markers of EMT (proteins of adhesion and cytoskeleton – E-cadherin, vimentin, transcriptional factor
Slug – EMT-profile), antigens of stem tumor cells CD44 and regulator of cellular cycle p21WAF (endogenous inhibitor of
cyclin-dependent kinases).

Fig. 1. General scheme of noncontact co-cultivation in vitro: a — control wells – isolated; b — wells with noncontact co-cultivation.

**Cultivation of cells in vitro.** Cells have been cultivated in plastic glassware («TPP», Italy) in DMEM environment («PAA»,
Austria), which contained 2 mM of L-glutamine and NaHCO₃ with 10% of embryos/new born calf serum (“PAA”, Austria)
in moisture atmosphere at t 37 °C and 5% CO₂. Quantity of cells after their co-cultivation has been determined by staining
with crystal violet («Sigma», USA) with further registration of optical density of content of wells with the help of multi-well
spectrometer (Labsystems Multiskan PLUS) [11].

**Immunocytochemical analysis.** At conduction of immunocytochemical (ICC) analysis, cells have been fixing in fixing
solution (methanol + acetone: 1:1) during 2 hours at t -20 °C, incubated with 1% solution of bovine serum albumin (BSA)
during 20 min. Afterwards monoclonal antibodies in solutions according to the instructions of manufacturers have been applied:
anti-E-cadherin (Thermo Scientific), anti CD325 (N-Cadherin) (BioLegend), anti-Vimentin (Diagnostic BioSystems), anti-
CD44 (Diagnostic BioSystems), SLUG (GeneTex), p21/waf1 (NeoMarkers) during 60 min., whereupon was used system of
visualization Poly Vue (Thermo Scientific), conjugated with peroxidase, and detected the activity of enzyme with use of 3,3´-
diaminobenzidine (Thermo Scientific) as substrate. After conduction of ICC reaction, specimens were washed out by water and
stained with Mayer’s hematoxylin («Sigma») (15–30 s.). Analysis of results was carried out by calculation of positive (+) cells
with the help of microscope AxioVert («Carl Zeiss», Germany) with x320 magnification and estimated with the help of classical
H-score method:

\[
S = I \cdot A + 2B + 3C,
\]

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.

Statistical processing of obtained results has been carried out with the help of mathematical program of medical-biological
statistics STATISTICA 6.0. Calculation and comparison of significance of difference between mean values has been
conducted with use of Student’s t-criterion.
RESULTS AND DISCUSSION

In order to determine EMT-profile of TC, which were included in system of noncontact co-cultivation, has been determined expression of markers of the most typical for EMT-transition, in particular, expression of E- and N-cadherin, vimentin and transcription factor Slug of mesenchymal cells (Table 1). It has been detected that in human TC of line T-47D, the mesenchymal antigen profile is more apparent, than in cells of line MCF-7, which is characterized by epithelial phenotype. Cells of line MRS-T5, which differentiate by mesenchymal morphology, also were characterized by domination of mesenchymal signs compared with cells of MRS line, which is the culture with prevalence of cells with epithelial phenotype, but also with presence of cells with mesenchymal features (see Table 1).

<table>
<thead>
<tr>
<th>Antigens</th>
<th>T-47D</th>
<th>MCF-7</th>
<th>MRS</th>
<th>T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin</td>
<td>98 ± 11</td>
<td>126 ± 7</td>
<td>270 ± 21</td>
<td>22 ± 9</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>81 ± 11</td>
<td>53 ± 3</td>
<td>72 ± 8</td>
<td>221 ± 28</td>
</tr>
<tr>
<td>Slug</td>
<td>174 ± 16</td>
<td>136 ± 21</td>
<td>54 ± 10</td>
<td>156 ± 26</td>
</tr>
</tbody>
</table>

Table 1

In the study of influence on proliferative activity of TC and fibroblasts, has been detected that in co-cultivation the quantity of fibroblasts almost do not change (in respect to isolated control), while the quantity of TC essentially varies dependently on domination in them of features of epithelial or mesenchymal phenotype, that is quantity of TC is essentially influenced exactly by their EMT-profile (Fig. 2). It has been determined that quantity of TC with domination of mesenchymal features (T-47D, MRS-T5) at their noncontact co-cultivation with fibroblasts, increases more in respect to control (p < 0.05), than quantity of TC with prevalence of epithelial phenotype (MCF-7, MRS).

Fig. 2. Quantity of viable normal fibroblasts and TC of human (a) and rat (b) with different phonotypical features at their combined noncontact co-cultivation

Thus the essential mutual influence of TC and normal fibroblasts has been detected. The results of this mutual influence significantly depended on phenotypic profile of TC. Data concerning combined co-cultivation of fibroblasts with TC of rat with mesenchymal or epithelial features (variant of co-cultivation MRS+NFR+MRS-T5) are considered interesting (see Table 1). In this case took place significant inhibition of cells of mesenchymal phenotype of line (MRS-T5) in presence of epithelial cells MRS. To be exact, cells of more differentiated epithelial phenotype may oppress growth potential of cells of mesenchymal phenotype. It is the evidence of opportunity of control the cell proliferation on the clonal-population level with involvement in this process of certain cellular factors, which are forming phenotypic profile of cells.

The next stage of work was the study of influence of additional factor in new integrated system in vitro, exactly – cells, obtained from punctates of BM of patients with HBC, since BM is considered to be depot for TC and is direct participant of minimal residual disease [12–14].

The essential changes of phenotypic features of both tumor and normal cells in system of noncontact co-cultivation with addition of cells of BM, obtained from patients with different course of process, have been detected (Table 2). In investigation of protein-regulator of cellular cycle p21 the essential changes of quantity of positive cells were observed only in normal fibroblasts at their co-cultivation with TC. At the same time, in latter the level of expression and subcellular localization of protein p21 did not change in different variants of influence. The localization of p21 in cells was important to take into account, since in nucleus and cytoplasm this protein performs the contrary functions. In particular, if p21 localizes in nucleus, it acts as regulator of cellular cycle through inactivation of transcriptional factors E2F1, c-Myc, STAT3 [15], as well as inhibitor of replication due to oppression of subunit of DNA-polymerase of δ-protein PCNA. If p21 localizes in cytoplasm, it performs formation of stress-fibrin and focal contacts that assists the migration of cells. Moreover, p21 performs in cytoplasm antiapoptotic functions through the oppression of activity of proapoptotic kinases ASK-1, JNK, p38 and inhibition of proapapase-2.
The interesting data are obtained in investigation of expression CD44 — molecule of adhesion, which plays important role in correlation “tumor-environment” and is involved in processes of migration and invasion of TC. Moreover, CD44 is used as marker of progression and metastasis at many oncological diseases, in particular, HBC, cells with phenotype CD44⁺CD24⁻ are considered stem TC. We have detected the 26% increase of quantity of CD44-positive (CD44⁺) T-47D cells at their co-cultivation with cells of BM (variant “Progression”) compared with control of cells in isolated wells. At this, at use of cells of BM (patients from “Remission” group) as additional factor of influence has been registered opposite result — significant decrease of quantity of CD44⁺-cells (р < 0,005) in respect to isolated control and significant decrease of quantity of CD44⁺-cells in respect to variant with co-cultivation of cells of BM of patients from “Progression” (р < 0,005) (see Table 2).

In study of expression in T-47D cells of one more protein, which characterizes intercellular adhesion and adhesion of cells to substrate and reside cells of “epithelial phenotype”, - E-cadherin — has been detected the essential quantity of positive cells T-47D in variant of their co-cultivation with cells of BM of patients from group “Progression” compared with control; co-cultivation with cells of BM of patients from group “Remission” did not change the quantity of E-cadherin⁺-cells of HBC (see Table 2).

In study of expression of protein of cytoskeleton of vimentin reside for cells of mesenchymal nature, has been defined that cells of T-47D do not express this protein. However, at co-cultivation of them with BM of patients from “Progression” group the cells with its expression arise, in contrast to the full absence of vimentin⁺-cells in isolated control and in variant of co-cultivation with cells of BM of patients from “Remission” group.

Such changes of phenotypic profile of T-47D TC in consequence of their co-cultivation with components of microenvironment (in the research with normal fibroblasts and cells of BM of patients with different course of tumor process) are the evidence of essential influence of cells of BM and factors, which they produce, on the HBC cells. At this, dependently on the state of tumor process of patients — progression or remission of the disease — the result of influence of BM cells on the TC essentially differs. In variant with co-cultivation with cells of BM of patients from “Remission” group the phenotypic profile of TC almost did not differ from control cells, while in variant with use of cells of BM of patients from “Progression” group has been observed launch of EMT program in TC, which is associated with obtaining of more aggressive features by cells and possible increase of their metastatic potential.

### Table 2

<table>
<thead>
<tr>
<th>Variant of co-cultivation of cells</th>
<th>Proteins markers</th>
<th>p21</th>
<th>CD44</th>
<th>Vimentin</th>
<th>E-cadherin</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-47D isolated (without influence)</td>
<td></td>
<td>263 ± 24</td>
<td>187 ± 19</td>
<td>97±11</td>
<td>0</td>
</tr>
<tr>
<td>T-47D Variant of co-cultivation + NFH + BM from “Progression” group</td>
<td></td>
<td>274 ± 32</td>
<td>194 ± 12</td>
<td>122 ± 14</td>
<td>50 ± 6*</td>
</tr>
<tr>
<td>T-47D Variant of co-cultivation + NFH + BM from “Remission” group</td>
<td></td>
<td>255 ± 15</td>
<td>176 ± 10</td>
<td>58 ± 5**</td>
<td>0</td>
</tr>
</tbody>
</table>

*p < 0.005; ** p < 0.05; †evaluation by H-Score method, scores.
At characterizing of phenotypic profile of NFH after their co-cultivation with T-47D TC and cells from BM of patients with different variants of tumor process, the expression has been studied: vimentin, p21 and Slug. At that changes of expression of vimentin was not detected in any of variants of co-cultivation, while in research of expression of transcription factors of mesenchymal cells Slug were detected changes of both quantity of Slug+-cells and localization of this protein in cells. In particular, the re-localization of Slug in nucleus of NFH at their co-cultivation with TC and cells of BM of patients that proves the known facts of activation of fibroblasts in action of TC with obtaining by them of myofibroblasts features. Also the interesting fact was found: the presence of p21 protein only in cytoplasm of normal fibroblasts (Table 3). At that, the quantity of p21+-cells in co-cultivation with cells of BM of patients from “Progression” group has significantly increased, compared with control (p < 0.005).

<table>
<thead>
<tr>
<th>Cells</th>
<th>p21</th>
<th>Studied markers*</th>
<th>Vimentin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cytoplasmic localizatio n</td>
<td>Nucleus localizatio n</td>
</tr>
<tr>
<td>Isolated NFH (without influence)</td>
<td>53 ± 7</td>
<td>0</td>
<td>186 ± 16</td>
</tr>
<tr>
<td>NFH variant of co-cultivation</td>
<td>148 ± 22**</td>
<td>0</td>
<td>82 ± 12****</td>
</tr>
<tr>
<td>+ T-47D + BM from “Progression” group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFH variant of co-cultivation</td>
<td>0***</td>
<td>0</td>
<td>72±10****</td>
</tr>
<tr>
<td>+ T-47D + BM from “Remission” group</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*evaluation by -Score method, scores; ** p < 0.005; *** p < 0.005; ****p < 0.02; *****p < 0.002.

Thus obtained by us facts confirm and in some way explain the well-known data concerning activation of fibroblasts and their conversion in myofibroblasts in co-cultivation with TC [2], whereupon the “activated” fibroblasts start to stimulate TC. Stromal elements, which include also fibroblasts, may be inducers of transcription factors of TC that in future assists the domination in cells of mesenchymal features. Such modulations of intracellular processes occur through the secretion by cells of series of factors (cytokines, factors of growth, etc.). One of such factors, in particular, may be EPST1 (epithelial-stromal interaction 1). EPST1 has been identified as interferon response gene exactly in co-cultivation of HBC cells with stromal fibroblasts [16, 17], and in immunohistochemical analysis of postsurgical tumor material of HBC patients has been detected its increased expression exactly in tumor material (in contrast to control mammary gland tissues); and “the highest intensity of expression” of EPST1 has been determined exactly in regions of tumor, which closely contacted with stroma [17].

**CONCLUSION**

1. With the help of created by us system of noncontact co-cultivation of cells of different genesis has been detected essential difference in quantity of TC after their interaction with fibroblasts dependently on EMT-status of TC.
2. Different modifying influence of mononuclears of BM of patients with HBC on TC dependently on clinical course has been detected (group “Remission” or “Progression”).
3. The fact of stimulation of EMT of HBC TC by influence of dissolvent factors on them, which probably, are produced by cells of BM of patients with HBC in the stage of progression of the disease.

The given results were obtained due to financial support of President of Ukraine grant for gifted youth.
REFERENCES


8. Семесюк Ні, Жильчук АВ, Бездєнєжних НО та ін. Прогностичне значення рівня колонієстимулюючого фактора 1 в периферичній крові та кістковому мозку хворих на рак молочної залози. Зб. Клін онкол 2011, Спец вып II: С. 218.


