The Effect of the Cell Microenvironment on Cell Functions Associated with Tumor Promotion and Progression

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Abstract—To study the role of the cellular microenvironment in tumor promotion and progression, transformed cells of rat embryonic fibroblasts (CL-1 clone) were transplanted into transgenic immunodeficient mice and cells of the formed tumor were converted into culture (CL-1-1 cells). Cells before and after transplantation were compared in morphology, growth rate, and permeability of intercellular gap junction. CL-1-1 cells were shown to have a changed morphology, to grow faster than the CL-1 cell, and to have no contact inhibition. In the G\textsubscript{1} phase of the cell cycle, there were many more CL-1 than CL-1-1 cells, whereas, in the G\textsubscript{2} and M phases, CL-1-1 cells were predominant. The permeabilities of the gap junction in the CL-1 and CL-1-1 cells were approximately the same. It was concluded that the cell microenvironment can stimulate tumor promotion and tumor progression upon transplantation of transformed cells into immunodeficient animals.

Keywords: tumor promotion, tumor progression, proliferation, intercellular gap junction communication

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INTRODUCTION

The formation of a tumor is a complex, multistep process, in which three stages are identified: initiation, promotion, and tumor progression (Rakitsky et al., 2000; Bock and Köhle, 2005). At the stage of initiation, there occur irreversible inherited changes of the cell genetic apparatus. At the stage of promotion, no structural changes take place in the DNA molecule. As a result of epigenetic actions of environmental factors (promoting agents), the “release” of initiated cells from regulatory action of normal cells occurs. This is expressed in stimulation of proliferation of initiated cells, disturbance of intercellular interactions that lead to an increase of the number of cells, and formation of a tumor conglomerate capable of self-maintained existence. Tumor promotion is accompanied by numerous phenotypical changes connected with changes in their microsurroundings, owing to growth of tumor cells.

Numerous studies have shown the microenvironment to be one of the important factors in the process of tumor development and progression (Schedin and Elias, 2004; Hu and Polyak, 2008; Molognoni et al., 2011). The microenvironment can produce epigenetic effects by affecting intercellular interactions or through the factors stored in the microenvironment (Abbott et al., 2008). The molecular composition of the tumor microenvironment in vivo is established together both by tumor cells and by constantly present and infiltrating nontumor cells (Witz, 2009). They include cells of stroma (connective tissue) and cells of the immune system, as well as of blood and lymphatic vessels and extracellular matrix (Berezhnaya, 2009; Calorini and Bianchini, 2010). Changes in the microenvironment in the cell culture have been shown to cause changes in cell behavior; however, these effects as a rule disappear upon transition of cells into standard conditions (Kobliakov et al., 1993; Sultatos and Vesell, 1980, Ahn et al., 2001). Under conditions in vivo, the changes produced by the action of the microenvironment can be fixed and connected with tumor progression. In normal animals, this may be due to the cell selection connected with functioning of the immune system, while in the immunodeficient ones it may be connected exclusively with the effect of the microenvironment.

The goal of this work was comparison of morphology, growth rate, and functioning of intercellular gap junction (IGJ) in transformed embryonic fibroblasts before and after their transplantation to immunodeficient mice. Rat embryonic transformed cells (clone CL-1) were transplanted into transgenic immunodeficient mice. Rat embryonic transformed cells (clone CL-1) were transplanted into transgenic immunodeficient mice, and cells of the formed tumor again were transplanted into the culture (cells CL-1-1). The cells CL-1-1 were shown to grow significantly faster than the cells CL-1, and their contact inhibition is absent,
while the number of cells in the state of rest (phase G_1 of the cell cycle) is significant lower than in the cells CL-1. Various authors have shown cell-growth acceleration to be accompanied as a rule by a fall in the IGJ level (Koffler et al., 2000; Zhang et al., 2003). Therefore, to find out whether this rule is also true for our model, we studied the IGJ level in the cells CL-1 and CL-1-1. It turned out that, in both cell types, the level of intercellular overflowing of Lucifer yellow characterizing the IGJ activity was equal. The enumerated changes in cell behavior characterize them as more “advanced” along the pathway of development of malignancy than is the initial cell culture. Thus, it can be concluded that the microenvironment is the factor stimulating the tumor promotion and progression by producing changes in cell phenotype. These changes are also preserved after elimination of the action.

MATERIALS AND METHODS

In this work, we used the CL-1 cell clone transformed by benzo/a/pyrene and obtained from rat embryonic fibroblasts; the clone was kindly given to us by Dr N.P. Shcherbak, for which we express great gratitude. The CL-1-1 cells were obtained by transplantation of the transformed clone CL-1 of the thymusless mouse. To obtain the tumor, the suspension of the CL-1 cells (5 mln cells/mL) was administered into the mouse axillary area. After 4 weeks, the formed tumor was removed in a sterile fashion, minced, placed into a chilled mixture of trypsin and Versen (1:1), kept for 24 h at 4°C, washed with Hanks’ solution, added to the medium (a mixture of DMEM and RPMI, 1:1) containing 10% embryonic serum (Flow, Austria) and 100 µg/mL gentamicin, and seeded into Correl flasks. After 24 h, the medium was changed for removal of unattached cells. The attached cells were then cultivated in the standard medium used for both cell types: a mixture of the media DMEM and RPMI (1:1) containing 10% fetal calf serum.

Distribution of cells for cell cycle phases was studied with the aid of flow cytometry. The cells were seeded (40 thousand cells per 1 Petri dish (6 cm)) and studied after 3 and 5 days. The cells were removed from the dish surfaces by treating them alternately with Versen and trypsin. The cells were then suspended in 1 mL of the buffer solution PBS and centrifuged at 1000 rpm for 20 min. After centrifugation, supernatant was discarded and 0.4 mL of water buffer containing 0.1% sodium citrate, 0.3% NP40, 100-µg/mL RNase A, and 50 µg/mL propidium iodide was added to the cell sediment. The mixture was then carefully suspended and stirred on a vortex at maximal revolutions. Analysis of cell distribution for the cell cycle phases was performed on a BD Facs Canto II flow cytometer (United States) with the BD FacsDiv software. The data of the apparatus were processed with the aid of the software WinMDI (version 2.9). The proliferation rate of the cells was estimated by using the growth curves. The number of cells was counted in Goryaev’s chamber every 24 h for 5 days.

To determine the morphology of the cells, they were stained directly in Petri dishes (6 cm). For this, the cells were fixed in 2 mL of the dye-fixative May-Grünwald for 20 min in a closed dish. The cells were then washed with distilled water and stained in 2 mL of Romanovskii azure–eosin solution for 20 min. After staining, the preparations were washed with distilled water and dried. The stained preparations were studied and photographed using a Nicon Eclipse TS 100 microscope (Japan) with a photostiching at a magnification of objective 40×. Microphotographs were made by using the software NIS—Elements F 3.0.

Permeability of IGJ cells was determined as described earlier (Sharovskaja et al., 1988). One of the monolayer cells was injected with the fluorescent dye Lucifer yellow CH (Sigma, United States), and then its spreading into neighbor cells was recorded. We used an AxioLab fluorescent microscope (Carl Zeiss, Germany) with phase contrast optics and water immersion objective 40× and a CCTV videocamera (Panasonic, Japan) fed to a computer. The number of stained cells was counted 2 min after injection of the dye.

RESULTS AND DISCUSSION

Figure 1 presents microphotographs of cell cultures of rat embryonic transformed fibroblasts prior to transplantation into thymusless mice (CL-1) and culture of the same cells after their transplantation into thymusless mice with subsequent transition of cells of the formed tumor into the culture (CL-1-1), stained with azure–eosin by Romanovskii. In microphotographs, it is seen that cells of the transformed clone CL-1-1 have a typical fibroblast-like morphology, whereas CL-1-1 cells have a more flattened appearance characteristic of epithelium-like cells (Figs. 1a, 1d). In both cultures, we revealed polyploid cells that are observed more often in the CL-1-1 culture and in most cases have fused nuclei (Figs. 1b, 1e). Unlike the CL-1 culture, in the CL-1-1 culture cells, with the complete monolayer, contact inhibition is absent and, as seen from Fig. 1f, the cells begin to creep over each other, which is not observed in the CL-1 culture (Fig. 1c).

A change of cell morphology after growth in an animal is also accompanied by functional changes. As seen in Fig. 2, the CL-1-1 cells grow significantly faster than the initial CL-1 culture. After 5 days of cultivation, the number of the CL-1-1 cells is about double that of the CL-1 cells.

Data are presented in Fig. 3 on distribution of cells for cycle phases. It is seen that, after both 3 and after 5 days of cultivation, many more CL-1 cells are in the state of rest (the G_0/G_1 phases) as compared with the CL-1-1 cells, their number being also higher in the synthetic phase of the cell phase (S). At the same time, in the premitotic and mitotic phases (G_2/M),