Theses of Ph.D. dissertation

HOMOLOGOUS AND HETEROLOGOUS INTERCELLULAR COMMUNICATION IN CELL CULTURE MODELS OF TUMOR-HOST INTERACTIONS

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1. INTRODUCTION

During the past few decades, the intra-, inter-, and extracellular events leading to malignant transformation, tumor growth and progression have been partially elucidated. In vivo and in vitro studies have clearly shown that qualitative and quantitative changes in the intercellular communication have crucial role in tumor progression. The cell to cell interactions include: (1) the release of modulating factors into the extracellular fluid that influence neighboring cells, (2) responses of cells to components on neighboring cell plasma membranes and that of the extracellular matrix, and (3) direct signalling between cells through gap junctional channels. In solid tumors around and between the malignant parenchymatic cells there is a well organized stroma which always contain different host cells with special characteristics and an altered extracellar matrix. The complex interactions between this special microenvironment and the malignant cells have crucial role in the progression of malignant diseases.

Gap junctional intercellular communication (GJIC) between the neighboring cells is an important component of tissue homeostasis and integrity. Many cell types express connexin proteins on their surface and show high extent of GJIC. The GJIC shows characteristic changes during cell proliferation and decreases for the effect of tumor promoters (e.g. phorbol esthers). The partial decrease or complete loss of GJIC and the subsequent izolation of malignant cells is a prerequisite for the uncontrolled growth of these cells. Many intra-, and extracellular factor has characteristic effect on the GJIC. These hormones, growth factors and chemicals can act on two different ways: by the control of connexin protein expression and by controlling the permeability of the gap

junction channels. The growth factors released by neoplastic cells have diverse effect on GJIC: the epidermal growth factor (EGF), the transforming growth factor-α (TGF-α), the platelet derived growth factor (PDGF) decrease, the transforming growth factor-β (TGF-β) and the basic fibroblast growth factor (bFGF) increase the GJIC depending on the target cell type.

The extracellular matrix (ECM) deposited in the microenvironment of the malignant cells show characteristic changes during the tumor progression. Elevated level of fibrin, fibronectin, type I. and type III. collagen and proteoglicans can be found in the ECM deposited around the tumor cell nests and around the individual cells. Marked tenascin deposition have been found in the connective tissue matrix of many malignant tumors. Tenascin-C is a hexameric glycoprotein found in embrional tissues, wound healing and neoplastic processes. Tenascin-C expression in normal adult tissues is restricted but marked reexpression was found in tumor tissues. Both, normal and malignant cells are able to produce tenascin but the intense tenascin deposition in pathological conditions is a result of the special activity of fibroblasts. In cell cultures tenascin inhibits the attachment of the cells to the surface. Tenascin expression always colocalized with physiological and pathological conditions where there is an intense migration of the cells. Based on these results it is supposed that tenascin is able to facilitate the local invasion of the malignant cells. The EGF like repeats in the tenascin molecule can induce mitotic response in EGF receptor expressing target cells. Immunohistochemical studies showed that a well organized tenascin deposition can be seen at the interface of the proliferating epithelial cells and the mesenchyme. This phenomenon suggests that the intercellular communication have an important role in the modulation of tenascin expression.

These results show that the malignant cell-fibroblast interaction is an important aspect of the complex tumor-host interaction. The tumor cell-fibroblast interaction can influence

directly and indirectly (by the changes in tumor matrix deposition) the different steps of tumor growth and progression. These homologous and heterologous (with malignant cells) interactions of the fibroblasts result in a supportive microenvironment for the tumor progression.

Present work is a summary of those studies, which were done to the better understanding of homologous and heterologous intercellular communication in malignant tissues. We have studied the changes of intercellular communication and tenascin production of fibroblast cultures treated with malignant cells and tumor conditioned medium.

2. AIMS OF THE STUDY

We have investigated the following questions in in homologous and heterologous cell culture models of tumor cell-fibroblast interactions:

A. In tumor conditined medium treated fibroblast cultures (TCM)

2.1. We followed up and characterized the change in the intercellular communication of target fibroblasts,

2.2. We studied the change of fibroblast proliferation parallel to the GJIC and the possible relationship between these two parameters.

B. In fibroblast-melanoma cell cocultures

2.3. Does tenascin reexpression occur during the culture for the effect of malignant cells?

2.4. If there is any special pattern of the tenascin deposition or it is randomly distributed?

2.5. Which signalling pathway is involved in the increased production of the tenascin?

3. MATERIALS AND METHODS

3.1. Mono- and cocultures

WiDr (colon adenocarcinoma) and Int 407 (normal gastrointestinal) cell were cultured at standard conditions for 4 days in DMEM supplemented with 10 % fetal bovine serum. After confluency the medium was harvested, centrifuged to remove cellular debris and then dialysed against 10-20 volumes of DMEM 10% FBS to return nutrients. The mediums prepared by this procedure were applied for the treatment of the Balb/c 3T3 cells as tumor conditioned medium (TCM) and normal cell conditioned medium according to the cell type. Tumor conditioned medium was prepared in the same way from the medium of HT-168 M1 higly metastatic melanoma cell line, and was used for the treatment of HFFF2 human fetal fibroblast culture. The pattern and intensity of tenascin reexpression was investigated in the monocultures and in the coculture of these two cell lines also.

3.2. Measurement of intercellular communication

Intercellular communication was measured as spreading of microinjected Lucifer Yellow CH to the neighboring cells. The injected fluorescent marker is able to spread to the neighboring cell just through the gap junction channels, therefore the extent of spreading is represents the physiological status of the gap junction channels. The number of neighboring cells containing dye was determined 10 min after injection.

3.3. Proliferation assay

In order to determine the proliferative fraction of the fibroblasts 10 μmol/l bromodeoxyuridine (BrdU) was added to the culture medium for 1 hour. The BrdU incorporated into the DNA during the S-phase of the cell cycle was visualized by indirect immunohistochemical staining using mouse anti-BrdU and FITC labelled rabbit anti mouse antibodies. Parallely propidium iodide staining was applied. Cell proliferation was expressed as the ratio of the FITC labelled and the total number of nuclei.

3.4. Visualization of the tenascin by indirect immunofluorescent labelling

The produced tenascin was visualized in the monolayer of TCM treated and control monocultures and in the cocultures by rabbit anti-human tenascin, goat anti-rabbit IgG and Texas red-streptavidin immunoreagents. In the cocultures anti-human prolyl-4-hydroxylase fibroblast marker reaction was applied to distinguish the two different cell types.

3.5. Qualitative and quantitative determination of the tenascin deposition by image analysis

Qualitative and quantitative fluorescent microscopy was done using an Axioplan fluorescent microscope (Zeiss, Germany) equipped with a 50 W mercury arc lamp, connected to a black and white intensified charge coupled device (CCD) camera, an image analysis processor and a microcomputer system (ISIS, Metasystems, Germany). Red fluorescence (tenascin) intensity was measured on the images and referred to the number of cells on the capture. The cell numbers were determined by the DAPI labeling of cell nuclei. On the coculture colocalisation of the tenascin with fibroblasts was also verified by the simultaneous detection of tenascin

and the fibroblast marker enzyme. Quantitative analysis on 20-70 images captured for 1 second from randomly selected areas of the cultures was carried out and the results obtained on different cultures were compared as well as different areas of the coculture were analyzed.

4. RESULTS

4.1. Effect of tumor conditioned medium on the intercellular communication and proliferation of fibroblasts

The possible role of tumor derived factors in the regulation of gap junctional intercellular communication and proliferation was investigated in tumor conditioned medium, normal cell conditioned medium and control medium treated Balb/c 3T3 fibroblast cultures. The time course of intercellular communication and cell proliferation was followed up from the subconfluent stage to the postconfluent stage of the treated fibroblast cultures (36 h period). At 0 h, just before the media change the subconfluent culture of Balb/c 3T3 cells showed evidence of well developed gap junctional intercellular communication (15-25 coupled cells). We had observed a slight, not significant increase of the GJIC in both (normal cell conditioned medium treated and control medium treated) of the control cultures at the beginning of the treatment. At the 30^{th} h the intercellular communication decreased in all of the treated fibroblast cultures. We observed a significant (49 %) increase of the intercellular communication at the 6^{th} hour of the TCM treatment which increase remained in a variable extent (15-40 %) during the full period of the TCM treatment of fibroblast cultures.

Parallel to the GJIC, the cell proliferation was determined by BrdU incorporation assay. In the control medium treated cultures the cell proliferation was nearly constant until the 24^{th} hour of the treatment. At the 30^{th} h there was a sharp (20 %) decrease in the cell proliferation of the control medium treated fibroblast cultures. In the TCM treated fibroblast cultures the cell proliferation had a decreasing trend during the treatment. The difference between the cell

proliferation of control medium treated and TCM treated fibroblast cultures was statistically significant in the 24^{th} and 30^{th} hour.

4.2. Effect of melanoma cells on the tenascin production of fibroblasts

We had investigated the tenascin production in melanoma cell and fibroblast monocultures and in the coculture of the two cell types. In the melanoma monoculture as well as in the fibroblast monoculture there was no labeling for tenascin. In contrary very intense tenascin reaction was observed in the cocultures showing characteristic distribution pattern. Tenascin labeling appeared in pericellular localization around fibroblasts being in contact with mealnoma cells. An apparent correlation and linear relationship (correlation coefficient $r=0.83$) were observed between the intensity of staining for tenascin and the ratio of melanoma cells to fibroblasts in different areas of the coculture. For TCM treatment tenascin reexpression could not be observed. These phenomena suggest the role of an insoluble (membrane anchoraged) factor in the induction of tenascin gene reexpression.

5. SUMMARY AND DISCUSSION

We have investigated the intercellular communication and tenascin reexpression in tumor conditioned medium treated fibroblast monocultures and in the coculture of melanoma cells/fibroblasts:

We have found that,

5.1. The soluble factors produced by the malignant cells and released to the medium are able to increase the gap junctional intercellular communication and to decrease the cell proliferation of the target fibroblasts.

5.2. We observed a significant tenascin reexpression in melanoma cell-fibroblast cocultures. The tenascin deposition was heterogenous in the coculture and the intensity of pericellular tenascin staining linearly correlated to the number of melanoma cells being in contact with fibroblasts.

5.3. In melanoma conditioned medium treated fibroblast cultures tenascin production could not be observed.

Our results suggest the role of contact signalization between the two cell types in the induction of tenascin synthesis. While the increase of GJIC and decrease of cell proliferation in tumor conditioned medium treated fibroblast cultures probably attributable to the effect of active factors secreted by the malignant cells. Our results contributed to the better understanding of some phases of the complex tumor-host interactions.

6. PUBLICATIONS

Tóth L, Pásti G, Sárváry A, Balázs M, Ádány R: Effect of tumor conditioned medium on intercellular communication and proliferation of Balb/c 3T3 cells. Cancer Letts, 151: 57-61 (2000)
IF: 1,518

Szűcs S, Vámosi Gy, Póka R, Sárváry A, Bárdos H, Balázs M, Kappelmayer J**, Tóth L**, Szöllősi J, Ádány R: Single-cell measurement of superoxide anion and hydrogen peroxide production by human neutrophils with digital imaging fluorescence microscopy.
Cytometry, 33: 19-31 (1998)
IF: 2,317

Tóth L, Pásti G, Ádány R: Contact stimulation of fibroblasts for tenascin production by melanoma cells (submitted for publication)

ABSTRACT

Szűcs S, Vámosi Gy, Póka R, Sárváry A, Bárdos H, Balázs M, Kappelmayer J, **Tóth L**, Szöllősi J, Ádány R: Single-cell measurement of granulocyte respiratory burst with digital imaging fluorescence microscopy. Br. J. Haematol., 102., 58, 1998. (abstract)