CHARACTERIZATION OF BASIC FUNCTIONS OF CULTURED HUMAN GASTRIC MYOFIBROBLASTS

Ph. D. THESIS

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Articles related to the subject of the thesis

I.

Czepán M, Rakonczay Z Jr, Varró A, Steele I, Dimaline R, Lertkowit N, Lonovics J, Schnúr A, Biczó G, Geisz A, Lázár G, Simonka Z, Venglovecz V, Wittmann T, Hegyi P.

NHE1 activity contributes to migration and is necessary for proliferation of human gastric myofibroblasts.

Pflugers Archive – European Journal of Physiology. 2012 Mar;463(3):459-75. **IF: 4.463**

II.

Holmberg C, Quante M, Steele I, Kumar JD, Balabanova S, Duval C, **Czepán M**, Rakonczay Z Jr, Tiszlavicz L, Németh I, Lázár G, Simonka Z, Jenkins R, Hegyi P, Wang TC, Dockray GJ, Varró A.

Release of TGFβig-h3 by gastric myofibroblasts slows tumor growth and is decreased with cancer progression.

Carcinogenesis. 2012 Aug;33(8):1553-62. **IF: 5.702**

Summarized impact factor: 10.165

Introduction

Myofibroblasts, or also known as activated fibroblasts, are dynamic, spindle-like cells sharing the functional characteristics of both fibrocytes and smooth muscle cells. They actively migrate and proliferate in the subepithelial matrix, secrete extracellular matrix proteins and promote wound healing. Myofibroblasts are found in low density in healthy tissue but are abundant in wounded tissue. As the unspecific inflammatory response advances in wounded tissue, different mixtures of bioactive compounds (such as growth factors, cytokines, chemokines and cyclo-oxygenase products) are released by the injured mucosa, endothelial cells and even by the fibroblasts causing a regulatory cascade and transformation of the resident fibrocytes into myofibroblasts.

Under pathophysiological conditions, in case of a permanent noxa the normal remodelling process is damaged. Chronic gastritis due to *Helicobacter pylori* infection has been extensively studied in recent decades and the connection between the pathogen and gastric cancer is now proven. Most investigations focused their research on epithelial cells and little is known about the connective tissue cells in tumors, though they are important in tumor expansion. In gastric cancer, the tumor stroma can make up to 80% of the whole tumor mass and the dominating cell type within is the fibroblast.

Myofibroblasts take part in the chronic inflammation-adenoma-carcinoma sequence by altering the microenvironment around epithelial cells. In the tumor, the boosted metabolic pathways generate

lots of acidic metabolites which are exported from the cells via various plasma membrane transporters. As upregulated ion transporters excrete the excess protons from the cells, the cytoplasm becomes more alkaline, while the environment becomes more acidic. In recent years this phenomenon became a hallmark of cancer and provided the researchers a new, thermodynamic approach to cancer research. Many authors have investigated the regulation of acid/base transporters and their role in migration and proliferation. Generally, they found that certain transporters are implicated in upregulated migration and proliferation of sancer cells. Acid/base transporters are well characterized in gastric epithelial cells, but there is scarce information about the transporters of gastric myofibroblasts which surround malignant cells. The alterations in the epithelial microenvironment in the evolution of cancer are poorly understood, yet they are of great importance. Data indicate that myofibroblasts may be the pioneer cells at the site of distant invasion, called niche, thus aiding malignant cells in forming metastases. Cancer-associated myofibroblasts (CAMs), a sub-class of myofibroblasts, are important stromal cells with distinct properties and recent work indicates that differences in gene expression in the stromal compartment predict clinical outcome and response to therapy. The changes on epigenetic level have also been detected in CAMs from gastric cancer compared with myofibroblasts derived from normal tissue myofibroblasts. We also know that TGF-β1 levels in the tumor mass

and myofibroblast numbers correlate with worse survival of gastric cancer.

Aims

Our primary objective was to characterize the acid/base transporters of cultured human gastric myofibroblasts and to search for a connection to cell function.

Materials and methods

The study was approved by the Ethics Committee of the University of Szeged, Hungary (study number 12/2006). All patients gave infomed consent. Gastric tissue specimens were collected intra-operatively from patients who underwent gastric tumor resection or from multiple organ cadaver donors.

Then specimens were cultured to establish monolayer myofibroblast cultures and they also were analyzed histologically. The cultures were stained for myofibroblast markers with immunocytochemistry.

The resting pH_i, basic acid/base characteristics, the buffering capacity and the acid/base transporters of the human gastric myofibroblasts (HGMs) were then investigated with microfluorometry using pH-sensitive dye and a specific sodium/hydrogen exchanger (NHE) inhibitor.

We used RT-PCR, immunocytochemistry and immunoblot analysis to show isoforms of NHE.

Migration and proliferation assays were utilized to examine the connection between NHE activity and migration-proliferation of HGMs.

Statistical analyses (Students' t-test or ANOVA) were applied to test significance of results. Results were considered significant at $p<0.05$.

Results

Morphology of the myofibroblasts and purity of HGM cultures

We histologically processed the same gastric samples from which we took of our specimens to investigate myofibroblast morphology. We showed increased number of myofibroblasts, disordered cell morphology and damaged cyto-architecture in the samples from gastric cancer compared to the normal tissue. We also showed that the purity of our HGM cultures was ~100%.

Investigation of acid/base transporters with microfluorometry

Our experiments showed that the resting pH_i of HGMs in standard HEPES solution was 7.09 ± 0.02.

We found using ion withdrawal techniques that an active Na^+-dependent H^+-efflux mechanism is present in HGMs indicating the

presence of NHEs. In HCO_3^- /CO_2-containing solutions, we showed certain pH regulating mechanisms indicating the presences of the Na^+/HCO_3^- cotransporter (NBC) and the anion exchanger (AE). The above findings were confirmed by using the ammonium pulse technique. We also determinded the buffering capacity of HGMs. Our data suggest that NHE and NBC are very active in HGMs.

Next we focused on NHEs and charcterized the functional acitivities of NHE isoforms using selective NHE inhibitor HOE-642. Our data indicate that NHE1 is responsible for about 85% of the Na^+/H^+ exchange activity, whereas NHE2 activity is around 10 % and the remaining NHE activity is approximately 5 %.

Then we investigated the effects of insulin-like growth factor II (IGF-II) and carbachol on the activities of NHEs. Importantly, both IGF-II and carbachol dose-dependently stimulated NHE activity. 10 µM carbachol had the greatest effect on NHE activity (two-fold increase). 100 ng/ml IGF-II was more effective (one and a half-fold increase) than 10 ng/ml in increasing NHE activity.

mRNA and protein expression of NHE1-3

Based on the results of the functional measurements, we investigated the presence of NHE transporters at the mRNA and protein levels. RT-PCR confirmed the expression of NHE1, NHE2 and NHE3. We also analysed the expression of NHE isoforms in HGMs by Western blot. We found that NHE1 is present in HGMs, but we were unable to

show NHE2 and NHE3 expression. Using immunocytochemistry, we demonstrated NHE1-3 localization to the plasma membrane of HGMs.

IGF-II increases proliferation in an NHE1-dependent manner

Finally, we tested the effects of HOE-642, IGF-II and carbachol on HGM proliferation. Ethynyl deoxy uridine incorporation assays showed that 100 ng/ml IGF-II increased cell proliferation over two-fold. Carbachol and/or HOE-642 did not affect proliferation. However, NHE1 inhibition by 1 μM HOE-642 completely blocked the stimulatory effect of IGF-II on cell proliferation.

TGFβig-h3 restrains tumor growth *in vivo*

We showed in our parallel work (article related to the subject of the thesis II.) that proliferation of gastric myofibroblasts is even more stimulated by IGF-II from sepcimens of gastric cancer (CAMs). Those data demostrate that the proteome and secretome of normal myofibroblasts and CAMs are different by Metacore analyses. The differences are exaggerated in CAMs from patients with short survival. Dominant changes are mostly at the level of ECM (extracellular matrix) proteins, cytoskeletal re-arrengement and actin filaments.

By focusing on CAM secretomes, we have identified an unexpected role for myofibroblasts in restraining tumor migration and

proliferation in early disease through secretion of a certain ECM molecule, TGFβig-h3, which not only inhibits proliferation and migration of myofibroblasts and gastric cancer cells, but also restrains tumor growth *in vivo* in xenograft experiments.

This molecule is down-regulated in CAMs enabling more aggresive expansion and better response to IGF-II. We demonstrate, that TGFβig-h3 expression is greatly decreased in CAMs originating from patients with poor survival.

Depression of TGFβig-h3 secretion by myofibroblasts occurs with tumor progression and could provide a novel functional biomarker for stromal cell properties in cancer. Moreover, it may also be possible to develop novel therapeutic strategies based on the observation that stromal cell-stimulated tumor growth *in vivo* is prevented by restoration of TGFβig-h3.

Summary and new findings

Myofibroblasts play central roles in wound healing, deposition of the extracellular matrix and - via extensive neurohumoral communication - in the epithelial function and cancer development as well. Their functions depend on migration and proliferation within the subepithelial matrix, which results in boosted cellular metabolism. Upregulated metabolic pathways generate acidic metabolites which need to be excreted to maintain intracellular pH (pH_i). It has been reported that acid/base transporters have a great impact on cell function and they also may have specific contributions to cancer development.

We isolated human gastric myofibroblasts (HGMs) from surgical specimens of 5 patients. Our data show that HGMs originating from gastric cancer are greatly increased in number, their morphology is distorted and the architecture is damaged compared to those originating from nomal tissue. Then we characterized, for the first time, the expression and functional activities of the Na^+/H^+ exchanger (NHE) isoforms 1, 2 and 3, and the functional activities of the Na^+/HCO_3^- cotransporter (NBC) and the anion exchanger (AE) in cultured HMGs using microfluorimetry, immunocytochemistry, RT-PCR and immunoblot analysis.

We showed that NHE1-3, NBC and AE activities are present in HMGs and that NHE1 is the most active of the NHEs. In scratch wound assays we also demonstrated (using the selective NHE

inhibitor HOE-642) that carbachol and IGF-II partly stimulate migration of HMGs in a NHE1-dependent manner. EdU incorporation assays revealed that IGF-II induces proliferation of HMGs which is inhibited by HOE-642.

The results indicate that NHE1 is necessary for IGF-II-induced proliferation response of HMGs. Overall, we have characterized the pH_i regulatory mechanisms of HGMs. In addition, we demonstrated that NHE1 activity contributes to both IGF-II- and carbachol-stimulated migration and that it is obligatory for IGF-II-induced proliferation of HGMs

Additionally, we demonstrated in our parallel work, that HGMs secrete TGFβig-h3 that restrains tumor growth *in vivo* and this function is damaged in CAMs.

Our results contribute to the better understanding of the epithelial and tumor microenvironment and may have further significance in the future of targeted cancer therapy.

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