

Stromal-epithelial interaction in regenerative processes and tumorigenesis in the colon

PhD thesis

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INTRODUCTION

The stromal-epithelial interactions have fundamental role in pathological processes of the colon, such as the regenerative phase of inflammatory bowel diseases and tumorigenesis. These processes include both migration and phenotype changes of cells and diffusion of regulatory ligands between the epithelium and the stroma. In the setting of increased epithelial injury, the capacity of tissue stem cells is not enough for the perfect repair, in this case bone marrow-derived stem cells (BMDCs) may be also involved in the regeneration. The lymphoid aggregate is thought to be an intermediate station in the homing of BMDCs. These cells migrate to the stroma (lamina propria) where they differentiate to mesenchymal cells (e.g. fibroblast, myofibroblast) or enter into the epithelium where they differentiate to epithelial-like cells via mesenchymal to epithelial transition (MET). During stromal migration BMDCs can commit to epithelial lineage as a consequence of altered pressure and/or tissue specific regulators. The epithelial commitment of BMDCs and the potential role of lymphoid aggregates in their migration are unclear in the scientific literature.

During colorectal carcinogenesis, epithelial to mesenchymal transition (EMT) is a typical cell phenotypic change. During EMT epithelial cells loss their epithelial characteristics (e.g. cuboid cell shape, apical-basal polarity, cytokeratin expression) and adopt mesenchymal-like properties (e.g. α -smooth muscle actin/ α -SMA expression, spindle cell-shape, acquisition of motility). The epithelial originated mesenchymal cells play a role in the building of abnormal microenvironment of cancer stem cells. The main regulator of EMT is transforming growth factor- β (TGF- β) via its receptor TGF- β RII, but unmethylated CpG motifs can also cause EMT via Toll-like receptor 9 (TLR9) activation.

The altered expression of regulatory molecules of epithelial cells is also involved in the building of abnormal microenvironment via changing normal to pathological cell ratio (e.g. fibroblast to myofibroblast) in the stromal microenvironment. In these processes, expression of proteins secreted by epithelial cells (e.g. TGF- β and/or osteopontin) may have fundamental role. No univocal data are available in the scientific literature in reference to the changes of percentage of EMT events and its main regulators the TGF- β and the TLR9 during colorectal adenoma – carcinoma sequence.

OBJECTIVES

The aim of this work was to examine cell phenotypic changes (MET, EMT) in intraepithelial location and protein expression that influence these changes, and may play a role in the building of tumor microenvironment.

During my PhD work I had the following objectives:

1. To examine the role of bone marrow-derived cells and lymphoid aggregates in the colonic epithelial regeneration.
2. To detect the early epithelial commitment of bone marrow-derived stem cells during their stromal migration using CDX2 epithelial marker and Musashi-1 stem cell marker.
3. To detect the frequency of epithelial to mesenchymal transition processes in intraepithelial location with the help of α -SMA (myofibroblast marker) and cytokeratin (CK) (epithelial marker) during colorectal adenoma – carcinoma sequence.
4. To examine different proteins (i.e. TGF- β RII, TLR9 and OPN) expression that can influence epithelial to mesenchymal transition (processes) during colorectal adenoma – carcinoma sequence.

MATERIALS AND METHODS

The experiments presented in this thesis were all conducted in the Cell Analysis Laboratory of the 2nd Department of Internal Medicine. For the examination of bone marrow-derived stem cells, samples from female patients transplanted with male bone marrow (healthy n=5, aspecific colitis n=5) were used. *Fluorescence in situ hybridization* (FISH) was performed using alpha-satellite probes specific for X-, Y-chromosome centromeres and the slides were digitalized. Then coverslip was eliminated and the slides were immunostained with epithelial marker cytokeratin, lymphocyte marker CD45 and slides were digitalized again. In this way two digital slides from one glass slide contained different information, that could be analyzed parallelly with digital microscopy and CD45-, Y-FISH+ cells were identified. For the detection of early, epithelial commitment of stem cells dual fluorescent staining (e.g. epithelial marker CDX2 and stem cell marker Musashi-1) was used.

Epithelial to mesenchymal transition was detected in different histological stages of colorectal carcinogenesis (healthy n=8, low grade adenoma n=8, colorectal cancer n=8). α -SMA (myofibroblast marker) and CK (epithelial marker) monoclonal antibodies were used to detect intraepithelial transition processes. Proliferating transitioned cells were detected with Ki-67/ α -SMA dual immunofluorescent staining. Slides were digitalized and analyzed with digital microscope. An included module called marker counter was used to determine the exact cell number. Both E-cadherin and osteopontin were semiquantitatively detected on light microscopic slides using a scoring scheme. TLR9 was also detected with conventional immunohistochemistry, and the ratio of positive/negative cells was counted using digital microscopy. TGF β RII protein expression was confirmed on mRNA level with Affymetrix whole genomic microarray. OPN immunohistochemistry was evaluated with a semiquantitative scoring scheme on digital microscope.

	XY-FISH, CD45/CK protein	α-SMA/CK protein	E-cadherin protein	TGFβRII protein	TGFβRII mRNA	TLR9 protein	OPN protein
N	5	8	8	6	6	8	13
I	5	-	-	-	-	-	-
Ad	-	8	8	6	6	8	13
CRC	-	8	8	6	6	8	13

Number of samples according to their histologic stages used in different experiments.

Chromosome detection was performed with FISH; protein detection with immunohistochemistry; measurement of mRNA level with Affymetrix whole genomic microarray.

Histology stages: N: normal, I: aspecific inflammation, Ad: adenoma, CRC: colorectal carcinoma.

RESULTS

CD45-, Y-FISH+ cells in colonic epithelium

Significantly higher ($p < 0.01$) intraepithelial CD45-, Y-FISH+ cell proportion was found adjacent to lymphoid aggregates ($1.0685 \pm 0.240\%$) compared to healthy ($0.017 \pm 0.017\%$) and diffuse colitis ($0.043 \pm 0.005\%$) samples.

CDX2 and Musashi1 expression of stromal cells

CDX2 (epithelial marker) positive cells were found in the stroma, and not in the epithelial layer of crypts. The low percentage of these cells showed Y-FISH positivity. Some CDX2 positive cells showed positivity on Musashi1 stem cell marker, as well.

Change of percentage of intraepithelial α -SMA positive cells during colorectal ACS

Intraepithelial CK positive cells with myofibroblast-like properties showed dot-like α -SMA expression mainly in nuclear or perinuclear area. Significantly higher intraepithelial α -SMA+/CK+ cell percentage was found in CRC samples ($3.34 \pm 1.01\%$) compared to healthy ($1.94 \pm 0.69\%$) and adenoma ($1.62 \pm 0.78\%$) samples.

Change of E-cadherin expression during ACS

The expression of E-cadherin showed continuously decreasing trend during carcinoma formation. During the semiquantitative evaluation, strong E-cadherin expression was found both in cell membrane and plasma (typical scoring value: +2) in healthy samples. In adenoma decreased membrane and plasma staining were found compared to healthy samples (typical scoring value: +1). The CRC samples did not show cytoplasmic E-cadherin expression, the membrane staining was mild and fragmented (typical scoring value: -2).

Alteration of TGF- β receptor II protein expression during ACS

In healthy samples, both cell membrane and cytoplasmic TGF- β RII protein expression were found (typical scoring value: 0 and +1). The TGF- β RII expression confined to the apical part of epithelial cells, in some cases it disappeared. In adenoma, the membrane staining was fragmented (typical scoring value: -2). CRC samples showed stronger membrane and cytoplasmic expression than the normal samples (typical scoring value: +2).

Alteration of TGF- β receptor II mRNA expression during ACS

In independent samples, we found an increased TGF- β RII mRNA expression both in adenoma and CRC compared to the healthy samples using whole genomic microarray. Significant difference was found between healthy and CRC samples and between healthy and adenoma samples ($p < 0.05$).

Alteration of OPN protein expression during ACS

The expression of osteopontin protein localized to the cytoplasmic region of epithelial cells in all histological stages, no OPN positivity in the nuclear area was detected. Healthy samples showed mild, diffuse cytoplasmic OPN expression. In adenoma, the OPN expression was stronger than in healthy samples. In CRC strong diffuse cytoplasmic OPN expression was found.

THE MOST IMPORTANT NEW STATEMENTS

- Under healthy circumstances and during mild, aspecific inflammation the migration and proliferation of BMDCs into the epithelium is a rare process.
- Lymphoid aggregates may play a role in the regenerative processes of colonic epithelium as an intermediate station of BMDCs' homing.
- BMDCs showed early epithelial commitment during their stromal migration, probably because of altered tissue pressure and altered composition of regulatory molecules
- Transition processes can appear in normal circumstances but their percentage is significantly increased during tumorigenesis. These processes are well characterized by a dot-like α -SMA expression. Diffuse, cytoplasmic α -SMA expression was rarely detected. Initial phase of EMT (i.e. migration from the epithelial layer) do not require intense cell motility. Probably, the main inducer of EMT is the extended intraepithelial pressure caused by increased epithelial proliferation.
- Dot-like intranuclear α -SMA expression may refer to the initial phase of EMT that can cause an altered cell or nucleus shape and may initiate further transcription (mechanotransduction) processes.
- High percentage of intraepithelial α -SMA positive cells are in proliferative phase (Ki-67+).
- Expression of TGF- β receptor II protein showed parallel expression with the frequency of EMT during ACS, which is another evidence that the main inducer of EMT is this receptor.
- Increased TLR9 expression was found in adenoma, but it was not followed by an increased percentage of intraepithelial α -SMA+ cells, probably due to the deficiency of tumor originated CPG-ODNs, which may activate these receptors only in CRC.

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