The effect of human tissue-specific DNA sequences in cell culture models

Doctoral thesis

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1 Introduction

1.1 The discovery of cell-free DNA

Cell-free DNA (cf-DNA) was discovered by Mandel and Metais in 1948. However, this discovery was not fully appreciated until 40 years later Leon et al. detected increased amount of cell-free DNA in various cancers. From this point it was believed that cell-free DNA could serve as a 'liquid biopsy' for the detection of various diseases. Due to the above mentioned analysis, the main goal was to find a diagnostic tool for the identification of prognostic patterns in cf-DNA.

1.2 The role of cell-free DNA

According to several studies tumor-derived DNA fragments (21-500 bases long DNA molecules) are creating a microenvironment supporting the proliferation and spreading of the tumor and preventing tumor-specific immune response. The circulating DNA primary origins from apoptotic / necrotic tumor cells, but live cells also emit cf-DNA into the extracellular space. The tumor-derived cf-DNA is detectable in plasma or serum, and can serve as a biomarker for detection of various types of cancer. Cf-DNA contains numerous cancer-specific motives, including mutated oncogenes, tumor suppressor genes, aberrant microsatellites and chromosomal DNA, as well as tumor-specific methylation patterns. Recent studies have confirmed the tumor-specific uptake of DNA sequences into the intracellular compartment of the recipient cells. These tumor-specific DNA motifs may play a role in the cell cycle of the recipient cells due to chronic activation of signaling pathways (e.g. MAPK and AKT signaling pathways), enhancing cell growth and morphological transformation.

1.3 The role of Toll-like receptor 9 (TLR9) in carcinogenesis

TLR9 as the main DNA sensing receptor recognizes endogenous or exogenous pathogen associated molecular patterns (DAMPs and PAMPs), specifically non- methylated DNA sequences. The global hypomethylation of cancers (including colorectal tumors) is a well-known phenomenon, which suggests a different effect of hypo- and hypermethylated DNA on the recipient cells gene expression via TLR9 signaling pathway. Increased expression of this DNA sensor in several tumor types (e.g. prostate, esophagus and cervical cancer) has been associated with higher metastatic potential.

2 Aims

The goals of my PhD study

1. To test the effect of fragmented and hypermethylated DNA sequences on TLR9 signaling pathway in HT-29 colon carcinoma cells.
2. To determine the 5-methylcytosine (5-mC) ratio of DNA from colon carcinoma cell lines, primary healthy and colon cancer tissue samples used for DNA treatments.
3. To perform whole genome expression analysis of primary healthy and colon cancer tissue DNA treated HT-29 colon cancer cells.
4. To map the effect of healthy and colon cancer tissue DNA on TLR9 signaling pathway in HT-29 colon cancer cells by qRT-PCR.
5. To test the effect of primary healthy and colon cancer tissue DNA sequences on the cell cycle of HT-29 cell colon cancer cells.
6. To perform whole genome expression analysis of primary healthy and colon cancer tissue DNA treated human PBMCs.

3 Methods

3.1 Cell culture

HT-29 colon adenocarcinoma cells were purchased from ATCC. Cells were cultured in a specific pathogen-free cell culture laboratory. HT-29 cells were maintained in RPMI 1640 (Sigma-Aldrich, Saint Louis, USA) and supplemented with 10% (v/v) fetal bovine serum (FBS; StandardQuality; PAA Laboratories GmbH, Pasching, Austria), 50 mg/ml gentamycin (Sandoz, Sandoz GmbH, Austria), and 2.5 µg/mL amphotericin B (Sigma-Aldrich).

The isolation of genomic DNA was performed by using High Pure PCR Template Preparation Kit (Roche GmbH, Germany) from surgically removed healthy and colon tumor tissue, which were provided by Transplantation Clinic (Semmelweis University, Budapest, Hungary). Patients suffering from colon cancer had definitive histologic diagnosis (Dukes' B and C stage) and have not received chemotherapy or radiotherapy before surgery. The isolated protein-free DNA was incubated at 37 °C for 1 hour with RNase A/T1 mix (Thermo Fisher Scientific Baltics UAB, Lithuania). The purity and quantity of the isolated DNA was determined by NanoDrop 1000 Spectrophotometer (Thermo Scientific, Germany).

Artificial hypermethylation of the DNA isolated from HT-29 colorectal cell line was performed by M.SssI CpG methyltransferase (New England Biolabs, Ipswich, USA), which uses as methyl donor SAM (S-adenosylmethionine) to hypermethilate cytosine (C5) molecules of the double-stranded DNA.

During the treatment by HT-29 genomic DNA sequences, artificially modified HT-29 DNA sequences and primary tumor and healthy DNA sequences 15 µg of purified DNA was used in 200 µl of sterile PBS. The gene expression primers for TLR9 signaling pathway and interleukin 8 (IL-8) gene were designed by Primer3 program. After NanoDrop 1000 Spectrophotometer quantification, capillary electrophoresis was performed on the isolated RNA samples (Bioanalyzer Pico 6000 RNA chip) to ensure the quality control of the isolated RNA.

The transcription of cDNA from 1 µg total RNA was performed by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems , USA) using the manufacturer 's instructions.

The real-time RT–PCR experiments were performed on LightCycler 480 qRT-PCR instrument (Roche, Switzerland) using LightCycler 480 Probes Master (Roche GmbH, Germany) reagent and SYBR Green (Roche GmbH, Germany) intercalating dye.

3.2 Whole genome microarray expression studies

Microarray experiments were performed on healthy and tumor tissue DNA treated HT-29 colorectal carcinoma cells and PBMCs from healthy volunteers. As a first step in the statistical process we did pre-processing, which consisted of background correction and normalization. Subsequently, differentially expressed genes between different groups were determined by SAM (Significance Analysis of Microarray) analysis method. After normalization performed with gcRMA software PAM fine threshold determination (soft tresholding) were used with the aim to make comparisons between the two groups (healthy tissue DNA treated vs. control, and tumor tissue DNA treated vs. control) and to determine the highly distinct gene expression changes between the compared groups.

3.3 Cell viability experiments

In cell viability experiments 15 µg of DNA dissolved in 200 µl of sterile PBS isolated from surgically removed healthy and tumor fresh-frozen tissue was used for DNA treatments,. To the control samples, 200 µl of sterile PBS were added. After 72 h treatment, the cells were harvested by using 0.25% trypsin-EDTA (Sigma-Aldrich, USA) solution, washed twice in 1 ml of sterile PBS and fixed in 1 ml of -20C˚70% ethanol at room temperature for 15 minutes.

The samples were stored at -20 °C till the flow cytometry cell viability analysis. The measurements were carried out in three independent biological replicates.

Further 72 hour viability assays were performed on HT-29 cells using trypane blue staining method. The numbers of living and dead cells were determined using a haemocytometer counting where we compared the live and dead cell numbers at primary healthy and colon cancer tissue DNA treated HT-29 samples to PBS treated HT-29 controls. The average number of live and dead cells in three independent calculations of three biological replicates were determined using the Student's t-test as statistical analysis.

3.4 Quantitative determination of 5-methylcytosine ratio from HT-29, SW480 and Caco2 colon carcinoma cell line genomic DNA and from primary healthy and colon cancer tissues

The 5-mC ratio was determined from genomic DNA isolated from HT-29, SW480, Caco2 colon carcinoma cell lines and the surgically removed tumor and healthy colon tissues. 100 ng of genomic DNA was used for the determination of the 5-mC ratio.

In our studies 5-mC DNA ELISA kit was used (Zymo Research Corp., USA) according to the manufacturer's instructions, which included an anti-5-methylcytosine monoclonal antibody, 5-mC coating buffer (5-mC Coating Buffer) 5-mC ELISA buffer, secondary antibody and horseradish peroxidase (HRP).

The values of 5-mC ratio were determined from the absorbation value using standard curve from the positive controls at 450 nm.

3.5 Experiments performed on peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers

20 ml of peripheral blood was taken from healthy volunteers. The blood was layered over Ficoll-Histopaque-1077 gradient and centrifuged at 1600 rpm for 20 minutes. The buffy coat was collected from the interphase, disperged in 5 ml of Hank's medium (Biomed, Poland) and centrifuged at 1600 rpm for 10 minutes. The recovered PBMCs were two times washed in sterile PBS by centrifugation at 1100 rpm for 5 minutes,

3.6 LPS and DNA treatment performed on PBMCs

$1x10^6$ PBMCs were plated on a six-well plate in RPMI 1640 medium supplemented with 10% FBS, gentamicine and amphotericin B. DNA treatment was performed using 15 µg of primary healthy and tumor tissue DNA in 200 µl of sterile phosphate buffered saline (PBS). In the combined LPS-DNA therapy, 100 ng of LPS (O26: derived from B6 serotype of Escherichia coli lipopolysaccharide, Sigma-Aldrich, USA) was used. To the untreated control samples, 200 µl of sterile PBS were added. The cells were grown at 37 °C, 5% CO2 and 95% humidity. Total RNA isolation from the PBMCs was performed by RNeasy Mini Kit (Qiagen, USA) using manufacturer´s recommendation. The isolated RNA was stored at -80°C.

4 Results

4.1 The effect of artificially modified (hypermethylated, fragmented) DNA sequences on the TLR9 signaling pathway in HT-29 adenocarcinoma cells

The unmethylated, non-fragmented (nMnF) DNA treatment resulted in significant overexpression at TLR9 gene, in contrast with MyD88 and TRAF6 genes in which the treatment caused decreased gene expression ($p \leq 0.05$) .

The treatment with unmethylated, fragmented (NMF) DNA sequences resulted in overexpression of TLR9, MyD88 and TRAF6 genes ($p \leq 0.05$).

Methylated, non-fragmented (MnF) DNA treatment showed increased expression of IRAK2, NFKB and IL-8 genes while decreased TRAF6 expression ($p \leq 0.05$) .

The treatment with methylated, fragmented (MF) DNA sequences showed overexpression of MyD88, and TRAF6 genes, while resulted decreased in gene expression at IRAK2, TNFSF10, and IL-8 genes ($p \leq 0.05$).

4.2 Quantitative determination of the 5-methylcytosine ratio in HT-29, SW480 and Caco2 colon carcinoma cell cultures, in healthy and colon cancer tissues

Using ELISA-based 5-mC assay in healthy colon tissue we found 13.35% of 5-methylcitosine, while in colon cancer tissues we detected 8.35% methylated cytosine. The colon carcinoma cell lines reflected very low methylation ratio (1.54%). Based on these results we concluded that the colon cancer tissue is characterized by general hypomethylation, while in healthy colon tissue general hypermethylation was detected.

4.3 Healthy and tumor tissue derived DNA effect of HT-29 colon carcinoma cells

After healthy and tumor tissue DNA treatment of HT-29 colon cancer cells, overexpression of methallothionen genes (MT1H, MT1G, MT1X, MT1P2 and MT2A) was observed.

In tumor tissue DNA treated HT-29 cells using whole genomic microarray, we identified overexpression of metastasis related genes, including the metastasis-associated molecule 1 in the colon tumors (MACC1), lung adenocarcinoma-associated metastasis transcript 1 (MALAT1), genes regulating cell adhesion, such as carcinoembryonic antigen related cell adhesion molecule

5 (CEACAM5) and metabolic genes, such as the insulin-induced gene 1 (INSIG1), endothelial lipase (LIPG) and messenger molecules genes, such as dual adapter phosphotyrosine and 3-phosphoinositides (DAPP1) and cAMP messenger battery 3-like protein 2 (CREB3L2).

4.4 Examination of the main elements of TLR9 pathway in HT-29 cells after the treatment by primary healthy and colon cancer tissue DNA by qRT -PCR

Both healthy and tumor tissue DNA treatment induced overexpression at interleukin 1β (IL-1β). ($logFc \geq 1$; $p \leq 0.05$). This element of the TLR9/MyD88 -dependent signaling pathway acts as a pro-inflammatory cytokine at the end of the signaling pathway. After tumor tissue DNA treatment we detected higher mRNA levels of IL-1β compared to healthy DNA treated samples.

4.5 Cell viability experiments in HT-29 cells treated by primary healthy and colon cancer tissue DNA

In support of our RNA expression results we carried out cell viability assays to determine the effect of increased gene expression on the cell cycle of the cancer cells. PI (propidium iodide) cell cycle analysis showed significantly higher live (G1 + S + G2 + M cell phase) cell population in tumor DNA treated group compared with the control group (average live cell population is measured in three independent samples of primary tumor DNA treated live cell population (39.11±0:57%) compared to the control group (32.00±2:50%; $p \leq 0.05$). Primary healthy tissue DNA did not affected the cell cycle of cancer cells.

(At the cell viability assays) we examined HT-29 cell´s viability by trypane blue staining method. The cell counts after counting in a haemocytometer were statistically analyzed. The tumor tissue DNA therapy induced significantly higher cell viability compared to the PBS control group (average number of viable cells in the tumor DNA treated group 54.22±3.03 in the control group 42.00±3.77 x 10 000 cells / ml ; $p \leq 0.05$) healthy tissue DNA treatment induced significantly lower cell viability compared to the PBS -treated control group (the average number of cells after healthy tissue DNA treatment 28.67±3.08; the control group 42.00±3.77 x 10 000 cells / ml); ($p \leq 0.05$).

4.6 The healthy and tumor tissue DNA effect on PBMCs

To determine the long-term (24 hours) effect of primary healthy and tumor tissue DNA on PBMCs we employed Affymetrix microarray HGU 133 2.0 system to map whole genome expression changes in the PBMCs from healthy volunteers. Primary healthy tissue DNA caused expression changes in 48 genes compared to PBS treated control group, while after primary tumor tissue DNA treatment we observed altered gene expression at 12 genes.

LPS treatment affected the expression of 1479 genes compared to the PBS treated control group.. LPS - primary healthy tissue DNA co-treatment caused expression changes at 129 genes, while LPS - primary tumor tissue DNA co-treatment caused expression changes in 21 genes.

5 Discussion

The tumor tissue DNA acts as a biologically active molecule depending on the DNA methylation and the lengths of the fragments. The biological effect of DNA sequences is dependent on the methylation status. Hypomethylated sequences acts via MYD-88 dependent pathway, whereas hypermethylated sequences activates MyD88-independent pathways.

Our experimental results showed that tumor tissue DNA increases the expression of multiple metastases associated genes and genes playing a significant role in the metabolism of cancer cells.

We also performed cell cycle assays, where we found increased absolute number and viability of cancer cells, which could have clinical prognostic role.

Furthermore, we examined the immunomodulatory effect of primary tumor and healthy DNA on peripheral blood mononuclear cells of healthy volunteers. After primary healthy tissue DNA treatment high IL-2 gene expression was observed and the expression level of other genes regulating T-cell proliferation (e.g. CD1d) Th1/Th2 differentiation (e.g. IL-2, IL-4, IL-6) was increased.

The 24-hour LPS treatment induced the overexpression of acute phase inflammatory cytokines (e.g. IL-6), immunoregulatory genes (e.g. IRG1), monocyte marker (CD 163) and several chemokines (e.g. CXCL1, CXCL5). Primary healthy tissue DNA reduced the expression of the LPS sensing receptor (TLR4), and genes encoding several chemokines (CXCL6, CXCL9 and CCL13) expression. Our results demonstrate the possible antagonistic effect of TLRs in PBMCs depending on the origin of the TLR ligands.

6 Conclusion

DNA isolated from HT-29 cells acts as a biologically active molecule inducing gene expression changes in the case of re-treatment of the same cell culture.

Artificial methylation of the isolated DNA induces overexpression of the elements of Myd88 independent pathway and IL-8 in the contrast with hypomethylated DNA induced signaling pathways.

DNA isolated from primary colon cancer tissues induces the overexpression of pro-metastatic such as metastasis-associated molecule 1 in the colon tumors (MACC1) and lung adenocarcinoma-associated metastasis transcript 1 (MALAT1), metabolic genes, such as insulin induced gene 1 (INSIG1) and endothelial lipase (LIPG) and second messenger molecules, such as the dual adapter phosphotyrosine and 3-phosphoinositides (DAPP1) and cAMP messenger battery 3-like protein 2 (CREB3L2).

Colon cancer tissue DNA isolated from primary colon tissue increased the HT-29 colon carcinoma cell viability.

In peripheral blood mononuclear cells of healthy volunteers (healthy tissue) DNA resulted in increased expression of IL-2 gene. This gene encoding a cytokine has several functions regulating T-cell proliferation and Th1 / Th2 differentiation. During long-term treatment healthy tissue as a TLR9 ligand reduced the overexpression of LPS sensing receptor (TLR4) and the expression of multiple chemokines Our results can point the possible antagonism of TLR receptors (TLR4 and TLR9) on PBMCs.

7 List of publications

Publications related to the PhD dissertation published in international journals

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