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Crosstalk between the microbiome and cancer cells by quorum sensing peptides

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ABSTRACT

To date, the precise role of the human microbiome in health and disease states remains largely undefined. Complex and selective crosstalk systems between the microbiome and mammalian cells are also not yet reported. Research up till now mainly focused on bacterial synthesis of virulence factors, reactive oxygen/nitrogen species (ROS/RNS) and hydrogen sulphide, as well as on the activation of exogenous mutagen precursors by intestinal bacteria. We discovered that certain quorum sensing peptides, produced by bacteria, interact with mammalian cells, *in casu* cancer cells: Phr0662 (*Bacillus* sp.), EntF-metabolite (*Enterococcus faecium*) and EDF-derived (*Escherichia coli*) peptides initiate HCT-8/E11 colon cancer cell invasion, with Phr0662 also promoting angiogenesis. Our findings thus indicate that the human microbiome, through their quorum sensing peptides, may be one of the factors responsible for cancer metastasis.

Introduction

Studies of the human microbiome revealed an individual and age-related diversity of microbes, occupying different habitats like skin, mouth, mammary gland, vagina and gut [1–5]. The beneficial effects of the gastrointestinal microbiota are currently being exploited in daily life by the use of probiotics. These products, most often positioned as functional foods, claim to restore the gut microbiota composition, possibly preventing gut inflammation or other intestinal or systemic disease phenotypes [6]. The most commonly studied organisms for probiotic therapies in the treatment of gastrointestinal diseases include organisms of the genera *Bacillus*, *Enterococcus*, *Escherichia*, *Faecalibacterium* and *Propionibacterium*. *Enterococcus faecium*, together with *Bacillus subtilis* and *Lactobacillus* spp., were investigated as probiotics for acute gastroenteritis, while *Streptococcus* spp., *B. subtilis* and *Escherichia coli* were explored for their use as probiotics in irritable bowel syndrome; a reduced duration or decreased abdominal pain of both gastrointestinal diseases was established. Manipulation of the microbiota with rationally selected pre- or probiotics can inhibit pathogens, strengthen epithelial barrier functions and supply the host with key nutrients (*e.g.* vitamins) [7]. Despite these claimed health benefits, it has also to be cautioned that certain safety aspects should be taken into account when using probiotics: taxonomic identification of the probiotic strain is necessary to avoid pathogenicity, as well as inhibiting the risk of infection and antimicrobial resistance [8]. The use of probiotics is indeed already associated with diverse side effects, *e.g.* bacteremia, fungemia and gastrointestinal ischemia; critically ill patients and immune-compromised individuals are the most-at-risk populations [9].

However, the interactions with the host can affect metabolic, neurological, inflammatory and immunological functions as well, and also the development of cancer can directly or indirectly be promoted [10]. For example, the progress of colorectal cancer

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can directly be initiated by DNA-damaging superoxide radicals or genotoxins, both produced by gut (mucosa-associated) bacteria. Indirectly, bacteria can induce cell proliferation or pro-carcinogenic pathways by T-helper cells or Toll-like receptors, respectively [11]. Human microbiome studies have revealed significant differences between cancer patients and healthy controls regarding the relative abundance of certain microbes. In colon cancer patients, an increased population of *e.g. E. coli* was observed in feces, inducing colitis and colibactin synthesis and thereby promoting inflammation and cancer [12].

Although the quorum sensing process within Gram-positive bacterial colonies is already extensively described in literature, the direct link between quorum sensing peptides and tumor development remains unexplored. Recent investigations revealed that the quorum sensing process is activated in the human gut: a set of acylhomoserine lactone (AHL) molecules, *i.e.* signaling molecules produced by Gram-negative bacteria, were identified in human feces of gastrointestinal disease patients as well as healthy subjects [13]. Moreover, bacterial quorum sensing molecules are likely to play a role in bacterial colonization of mucosa, thus requiring quorum sensing-mediated biofilm formation [14]. Finally, Casula and Cutting showed the germination of *B. subtilis* spores in the murine gastrointestinal tract, thereby probably requiring signaling peptides for quorum sensing pathway activation [15]. Although thus not yet investigated, it is very likely that also quorum sensing peptides are found in the human intestinal tract.

In this study, we utilize a collagen invasion assay, transcriptome assay, Chick Chorioallantoic Membrane (CAM) assay, cytokine profiling and phospho-receptor tyrosine kinase array to investigate the influence of quorum sensing peptides on mammalian cancer cells. Our preliminary observations unexpectedly reveal that quorum sensing peptides stimulate metastasis behavior of human colon cancer cells, thereby opening new perspectives on the role and applications of the microbiome on the guest's health, with the possibility of translating these findings into other biological and applied medical fields as well.

Materials and methods

Cell culture

An epithelial subclone from human ileocecal colorectal adenocarcinoma cells (ATCC® CCL-224), *i.e.* HCT-8/E11, was grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen/GIBCO, Gent, Belgium) in a humidified atmosphere of 5% CO_2.

Collagen type I invasion assay

Morphology changes of HCT-8/E11 cells were investigated using previously described methods [16]. In brief, 10 000 cells were seeded per well in a 48-well plate, containing 150 μl of collagen type I gel per well, thereby investigating cell morphology 24 h post-treatment (Leica DMI3000B phase contrast microscope). Peptide solutions (Supplementary Table 1, all from GL Biochem, Shanghai, China) were prepared using ultrapure water (with or without DMSO), obtaining final peptide concentrations of 1 μM, 100 nM and 10 nM after 1:10 dilution using growth medium; the placebo sample solely contained ultrapure water. Two independent morphology 'scorings' were obtained for each of the 3 replicates; peptides were found positive if collagen-invasion or cell-stretching at minimum 2 consecutive concentrations was established at minimum 2 out of the 3 replicates.

Supplementary Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.peptides.2014.12.009.

Human Transcriptome Array

Peptide (EDF-analog: 10 nM, Phr0662 and EntF-metabolite: 100 nM and 1 μM) treated cells were analyzed in duplicate (independent treatment and analysis) for whole transcriptome expression using Affymetrix GeneChip Human Transcriptome Array 2.0 by AROS Applied Biotechnology A/S (Aarhus, Denmark); RNA expression was compared with placebo (ultrapure water) treated samples. Data analysis was performed using Transcriptome Analysis Console (Affymetrix) and MetaCore (Thompson Reuters) software programs.

Chick Chorioallantoic Membrane (CAM) assay

The Chick Chorioallantoic Membrane (CAM) assay was performed as described by Sys et al.: 6 days after (pre-treated, 100 nM) tumor cell transfer to the fertilized eggs, CAM was microscopically scored and histologically examined after H&E staining [17,18]. For quantification of microscopically observed neovascularization, an average CAM score was calculated: the number of blood vessels in the 1 mm diameter ring around the 2 mm radius tumor center was determined. Significant differences were evaluated using the Mann–Whitney *U* test.

Cytokine profiling

Cytokine expression was investigated using the Human XL Cytokine Array kit (R&D Systems, Abingdon, United Kingdom), following the instructions of the supplier. In brief, cell supernatant was obtained after 24 h of incubation with Phr0662 (100 nM) and 102 different cytokines analyzed in duplo using spotted capture antibodies, followed by incubation with detection antibodies and chemiluminescent visualization. Membranes were finally exposed to X-ray films for 1–10 min. The mean (blank corrected, $n = 2$) pixel density was calculated using ImageJ software and compared with placebo treatment. Significant differences were evaluated using the independent samples *t*-test.

Phospho-receptor tyrosine-kinase array

A human phospho-receptor tyrosine kinase (RTK) array kit (R&D Systems, Abingdon, United Kingdom) was used to detect phosphorylation levels of 49 different RTK after Phr0662 treatment (100 nM) of HCT-8/E11 cells, following the manufacturer's instructions. Cell lysates were prepared after 5 min of peptide incubation and incubated with capture antibodies (in duplo). Previous experience with the receptor tyrosine kinase assay showed that a 5 min exposure time is a generally acceptable exposure time to obtain a sufficient degree of receptor activation/phosphorylation without dropping down to basal phosphorylation levels due to phosphatase activity. Incubation was followed by treatment with detection antibodies and Horseradish Peroxidase for chemiluminescent visualization. X-ray films were again analyzed for mean (blank corrected, $n = 2$) pixel density and compared with placebo results using the ImageJ software program. Significant differences were evaluated using the independent samples *t*-test.