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Lithocholic acid, a bacterial metabolite reduces breast cancer cell proliferation and aggressiveness☆

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ABSTRACT

Our study aimed at finding a mechanistic relationship between the gut microbiome and breast cancer. Breast cancer cells are not in direct contact with these microbes, but disease could be influenced by bacterial metabolites including secondary bile acids that are exclusively synthesized by the microbiome and known to enter the human circulation. In murine and bench experiments, a secondary bile acid, lithocholic acid (LCA) in concentrations corresponding to its tissue reference concentrations (< 1 μM), reduced cancer cell proliferation (by 10–20%) and VEGF production (by 37%), aggressiveness and metastatic potential of primary tumors through inducing mesenchymal-to-epithelial transition, increased antitumor immune response, OXPHOS and the TCA cycle. Part of these effects was due to activation of TGR5 by LCA. Early stage breast cancer patients, versus control women, had reduced serum LCA levels, reduced chenodeoxycholic acid to LCA ratio, and reduced abundance of the baiH (7α/β-hydroxysteroid dehydroxylase, the key enzyme in LCA generation) gene in fecal DNA, all suggesting reduced microbial generation of LCA in early breast cancer.

1. Introduction

The human body harbors a vast number of symbiotic, commensal and pathogenic bacteria in the bodily cavities and the body surface. The ensemble of these microbes is referred as the microbiota and its collective genome as the microbiome. Recent advances pointed out that changes in the composition of the microbiome and certain bacterial metabolites crucially impact metabolic, behavioral, cardiovascular and immune functions of the host and have pivotal roles in diseases that were previously not associated with bacteria [1–4]. Alterations of the microbiome are associated with certain cancers. Although, the microbiota may have a widespread role in carcinogenesis, the number of directly tumorigenic bacteria is extremely small, some 10 bacterial species fall into this category [5]. It seems more likely that pathological

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changes in the microbiota/microbiome (dysbiosis) determine susceptibility to the disease or influence the progression of the disease [4].

Most of these cancers affect those organs that are directly in contact with microbes such as the urinary tract [6], cervix [7], skin [8], airways [9], and the colon [4]. Such microbiome-host interactions are best characterized in the colon. In the intestine a breach of the biological barrier between the microbes and the underlying tissues enables an adverse physical contact between microbes and host cells, that induces the production of paracrine bacterial metabolites [4]. Through these, the microbiome modulates tumorigenesis, tumor promotion, severity of the disease, and chemotherapy effectiveness in colonic tumors [4]. Direct stimulation of the cancer cells by bacteria probably has role in bacteria-mediated induction of lymphomas [10,11] and possibly prostate cancer [6].

Much less is known of the role of the microbiome in the regulation of those tumors that are located to different compartments and are indirectly connected to the microbiome through the circulation. Changes in the microbiome are associated with metabolic diseases (e.g. obesity or type II diabetes) [12]. These metabolic diseases are risk factors of certain cancers, among them, breast cancer [13,14]. It is likely that similar mechanisms can confer susceptibility to cancer as to metabolic diseases. Blood-borne bacterial metabolites (e.g. short chain fatty acids) mediate human metabolism, hence these metabolites are likely candidates to be transported to a potential tumor by the bloodstream to exert carcinogenic or anti-carcinogenic effects in distant tumors. For hepatocellular carcinoma, lipopolysaccharide [15] and deoxycholic acid (DCA) [16] have been identified as promoters, while propionate, a short chain fatty acid (SCFA), is an inhibitor [17].

Numerous bacterial metabolites have been identified that are either the microbes' own metabolites (e.g., short chain fatty acids, lactate, pyruvate) or modified products of the host (e.g., secondary bile acids, metabolites of aromatic amino acids, redox-modified sex steroids) [18–20]. These bioactive metabolites act through various pathways that involve the modification of gene expression (e.g., activation of histone deacetylases and other lipid-mediated transcription factors) or the modulation of signal transduction in the host. Our aim with the current study was to investigate a potential causal link between changes in the microbiome, microbiome-derived metabolites and breast cancer.

2. Materials and methods

2.1. Chemicals

All chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Radioactively labelled substrates for the pulse-chase metabolomics experiment were from Cambridge Isotope Laboratories, Andover, MA, USA. The inhibitors and antagonists used in the TGR5 experiments (U73343 (phospholipase C inhibitor), NF449 ($G_{s\alpha}$-selective antagonist), CINPA1 (CAR antagonist), DY268 (FXR antagonist), GSK2033 (LXR antagonist)) were obtained from Tocris Bioscience and were used at the concentration of 5 μM except for U73343 which was used at a final concentration of 1 μM.

2.2. Image based correlation spectroscopy (ImFCS)

After an exponential of polynomial bleach correction, pixel-by-pixel autocorrelation functions (ACFs) were calculated using a multi-tau correlation scheme [21]. To obtain the diffusion coefficient (D) for all pixels ACFs were fitted according to the equation in [21]. To identify and describe the mode of membrane organization by investigating the size-dependency of diffusion coefficient, we used the Imaging FCS type of FCS diffusion law [22]. According to that, the diffusion time (τ_D) of the fluorescent probe depends on the observation area (A_{eff}), as described by

$$\tau_D(A_{eff}) = \tau_0 + \frac{A_{eff}}{D}$$

where A_{eff} is the area of the membrane in which the labelled particle travels across,τ_0 is the intercept of the diffusion law plot on the y-axis of A_{eff}/D vs. A_{eff}. This parameter provides information about the diffusion confinement. A more detailed description of the method can be found among the Supplementary Materials.

2.3. Cell culture

MCF7 cells were maintained in MEM (Sigma-Aldrich) medium supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine at 37 °C with 5% CO_2.

4T1 cells were maintained in RPMI-1640 (Sigma-Aldrich) medium containing 10% FBS and 1% penicillin/streptomycin, 2 mM L-glutamine and 1% pyruvate at 37 °C with 5% CO_2.

SKBR3 cells were maintained in DMEM (Sigma-Aldrich, 1000 mg/L glucose) medium supplemented with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine at 37 °C with 5% CO_2.

Primary fibroblasts cells were maintained in DMEM (Sigma-Aldrich, 1000 mg/L glucose) medium supplemented with 20% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and 10 mM HEPES at 37 °C with 5% CO_2.

2.4. In vitro cell proliferation assays

Sulphorhodamine B assays were described in [23]. For colony formation assays five hundred cells were seeded in a 6-well plate in complete medium and were cultured with the indicated concentrations of LCA for 7 days. At the end of the assay plates were washed twice in PBS. Colonies were fixed in methanol for 15 min, dried and stained according to May-Grünwald-Giemsa for 15 min. Plate was washed with water and the colonies were counted using Image J software [24].

2.5. Detection of cell death

LCA-induced cytotoxicity was determined by propidium iodide (PI) uptake. Cells were seeded in 6-well plate (MCF7 - 200.000 cell/well; 4T1 - 75.000 cell/well) treated with LCA for two days and stained with 100 μg/mL propidium iodide for 30 min at 37 °C, washed once in PBS, and analyzed by flow cytometry (FACSCalibur, BD Biosciences).

2.6. Scratch assay and video microscopy

Cells were grown in 6-well plates until cell confluence reached about 70–80%. The plates were manually scratched with sterile 200 μL pipette tip, followed by washing the cells twice with PBS. Then cells were incubated with vehicle or LCA (0.3 μM) in a thermostate. Cell densities were monitored every hour for one day using JuLi Br Live cell movie analyzer (NanoEnTek Inc., Seoul, Korea).

2.7. Electric cell-substrate impedance sensing (ECIS)

ECIS (Electric cell-substrate impedance sensing) model Zθ, Applied

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