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Mesalamine modulates intercellular adhesion through inhibition of p-21 activated kinase-1

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ABSTRACT

Mesalamine (5-ASA) is widely used for the treatment of ulcerative colitis, a remitting condition characterized by chronic inflammation of the colon. Knowledge about the molecular and cellular targets of 5-ASA is limited and a clear understanding of its activity in intestinal homeostasis and interference with neoplastic progression is lacking. We sought to identify molecular pathways interfered by 5-ASA, using CRC cell lines with different genetic background. Microarray was performed for gene expression profile of 5-ASA-treated and untreated cells (HCT116 and HT29). Filtering and analysis of data identified three oncogenic pathways interfered by 5-ASA: MAPK/ERK pathway, cell adhesion and β-catenin/Wnt signaling. PAK1 emerged as a consensus target of 5-ASA, orchestrating these pathways. We further investigated the effect of 5-ASA on cell adhesion. 5-ASA increased cell adhesion which was measured by cell adhesion assay and transcellular-resistance measurement. Moreover, 5-ASA treatment restored membranous expression of adhesion molecules E-cadherin and β-catenin. Role of PAK1 as a mediator of mesalamine activity was validated in vitro and in vivo. Inhibition of PAK1 by RNA interference also increased cell adhesion. PAK1 expression was elevated in APC^{min} polyps and 5-ASA treatment reduced its expression. Our data demonstrates novel pharmacological mechanism of mesalamine in modulation of cell adhesion and role of PAK1 in APC^{min} polyposis. We propose that inhibition of PAK1 expression by 5-ASA can impede with neoplastic progression in colorectal carcinogenesis. The mechanism of PAK1 inhibition and induction of membranous translocation of adhesion proteins by 5-ASA might be independent of its known anti-inflammatory action.

1. Introduction

Ulcerative colitis (UC) is a chronic inflammatory disease of the rectum that may extend to more proximal parts of the colon in a continuous fashion. Histopathology shows an infiltrate of neutrophils within the lamina propria and within mucosal crypts leading to epithelial destruction [1]. The primary cause(s) of UC is unknown, however, genetic and environmental factors are involved [2]. Patients with UC are at risk of developing colorectal cancer (CRC). Colon carcinogenesis involves multiple mutations or epigenetic modifications followed by changes in gene expression. These changes require several years leaving open a window of opportunity to prevent the transition from normal to malignant cells.

Sulfasalazine, a drug used in the treatment of chronic gut inflammation, was developed to combine antibacterial and anti-inflammatory effects [3]. The anti-inflammatory potential of its metabolite mesalamine (5-amino salicylic acid, 5-ASA), has been investigated in studies involving the arachidonic acid cascade, cyclooxygenase and lipoxygenase metabolites [4,5]. Although,the therapeutic efficacy of mesalamine in inducing and maintaining

Abbreviations: CRC, colorectal cancer; IBD, inflammatory bowel diseases; UC, ulcerative colitis; AJ, adherens junction; PAK1, p21 activated kinase 1.

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remission in UC is well established its role in prevention of colitis-associated carcinogenesis remains controversial [6,7]. Some authors have found inhibitory effects on the growth of colorectal cancer cells and in β-catenin signaling [8].

At the cellular level, 5-ASA reduces oxidative stress [9], inhibits cell cycle progression through activation of a replication checkpoint [10], decreases transcriptional activity of NF-κB [11] while activating PPAR-gamma [12] and interfering with the canonical Wnt pathway [13,14]. Though several potential mechanisms of its action have been investigated, a clear understanding of its interference with cancer development is lacking [8]. Such understanding may help to identify key regulators of colon homeostasis and carcinogenesis. To this end, we identified both known (Wnt-β-catenin) and novel (cell adhesion and MAPK/ERK) cellular mechanisms regulated by mesalamine using differential gene expression analysis. PAK1 (p21-activated kinase-1) turned out to be the consensus target of 5-ASA, orchestrating all three pathways. In this study we investigated the functional relevance of PAK1 as a mediator of mesalamine activity in vitro and in vivo.

2. Materials and methods

2.1. Cell lines and reagents

Human colorectal carcinoma cell lines HCT116, HT29 (obtained from ATCC) were grown in IMDM (Gibco/Invitrogen, Lofer, Austria) containing 10% fetal bovine serum (FBS; Biochrom, Berlin, Germany). 5-ASA (>99.9% pure; a generous gift from Shire Inc., Eysins, Switzerland) was dissolved in the culture medium (pH adjusted to 7.2 with NaOH) as described earlier [10]. IPA3 (Sigma–Aldrich) was dissolved in DMSO.

2.2. Microarray and data analysis

Accession number: The data is open to public at Array Express: http://www.ebi.ac.uk/microarray-as/ae/; experiment **ID E-TABM-768**

Total RNA was linearly amplified and fluorescently labeled using modified protocol of Low RNA Input Linear Amplification Kit (Agilent Technologies). The modifications to the protocol allow for incorporation of aminoallyl-UTP (Epicenter Biotechnologies) into amplified RNA and subsequent labeling by coupling with DY-547 NHS-ester and DY-647 NHS-ester fluorescent dyes (Dyomics). Fluorescently labeled cRNA from control and treated cells were co-hybridized to Human OneArray oligonucleotide microarrays (Phalanx Biotech). Four hybridizations in two replicas were carried out for each time interval (8 and 24 h) for CRC cell lines HCT116 and HT29. Microarrays were scanned with confocal microarray scanner (ScanArray Express, Perkin Elmer), extraction of spot intensities was done using QuantArray software (Packard Biochip). Data was further processed (normalization, filtering) in R statistical environment (http://www.R-project.org) and R Bioconductor [15] and analyzed with MEV software. Significantly up- and down-regulated genes were determined with SAM algorithm implemented in MEV (DFCI, JCVI, and the University of Washington). Gene expressions that were not different between 8 and 24 h were selected to represent the change induced by the treatment (5-ASA, 20 mM) in both the cell lines. Analysis of microarray data was performed using various available programs, including Pathway Studio Ariadne software (Ariadne, Rockville, MD, http://www.ariadnegenomics.com), Onto-Compare (Intelligent Systems and Bioinformatics Laboratory, Wayne State University, http://vortex.cs.wayne.edu), and MAPP-Finder (Gladstone Institutes, University of California, http://www.genmapp.org/and http://www.gladstone.ucsf.edu).

2.3. Cell fractionation, Western blotting and antibodies

Cytosolic and membrane fractions were collected as described elsewhere [16]. Briefly, cells were grown in 15 cm dishes and were collected with 400 μl cold hypotonic buffer (10 mM Tris–HCl pH 7.5, 0.2 mM MgCl2, with protease and phosphatase inhibitors (Roche) with a cell scraper. The extract was dounce homogenized and kept on ice for 30 min. The extract was spun at 15,000 × *g* for 45 min at 4 °C, and the supernatant was collected as the cytosolic fraction. The pellet was washed twice in hypotonic buffer and then resuspended by vortexing in 50 μl lysis buffer (150 mM NaCl, 20 mM Tris–HCl pH 7.5, 1% Triton X-100, and proteinase inhibitors). The extract was vortexed for 10 seconds every 10 min and kept on ice for 30 min. Afterwards, the supernatant was collected as the membrane fraction. Both fractions were incubated with Laemmli sample buffer containing 10% β-mercaptoethanol at 95 °C for 10 min and then analyzed by western blot. Protein concentrations were measured by Bradford assay (Bio-Rad). Proteins were separated by SDS-PAGE and immunoblotted onto a PVDF membrane. The protein bands were visualized with anti-rabbit or anti-mouse antibodies coupled to horseradish peroxidase using the ECL kit (Amersham) or with IRDye coupled antibodies (either or both mouse/rabbit; LI-COR) and scanned on Odyssey imager (LI-COR Biotechnology). Primary antibodies used were as follows: monoclonal antibody, anti-E-cadherin, anti-β-catenin (BD Transduction Laboratories), Rad 6 (Zymed), pan-actin, calnexin, Lamin B1 (Santa Cruz Biotech), FRZB, alpha-tubulin, anti-Oct1, Anti-Fibrillarin (Abcam), phospho-β-catenin, PAK1, Na,K-ATPase, Phospho-p44/42 MAPK, and p44/42 MAPK (Cell signaling).

2.4. Cell adhesion assay

Cell adhesion assay was modified and performed as described previously [17,18]. Cells were treated with 5-ASA for 24 h (5–20 mM; as indicated in figures), washed in PBS, counted and plated equally (40–50,000 cells/well) in 24-well plates for attachment. After 30 min of incubation, each plate was washed with PBS until no floating cells remained and then replaced with the fresh medium (without 5-ASA) and MTT reagent. This washing step is critical for the cell attachment assay. After 4 h, medium was removed and the remaining precipitates were dissolved in DMSO/ethanol mixture (50/50, v/v). The experiment was repeated three times, and for each condition, four wells were scored.

2.5. Transcellular resistance measurement

Real time quantitative technique electric cell-substrate impedance sensing (ECIS) was utilized for measuring cell attachment [18]. The 96 well ECIS plate (Applied Biophysics, 96W10E+) was pre-coated with fibronectin (10 μg/ml; Sigma, F2006-1MG) for 1 h at 37 °C in a CO_2 Incubator with 5% CO_2. Then 80,000 Caco-2 cells (Sigma, 86010202) per well were plated in 200 μl Dulbeco's modified Eagle Medium (DMEM, 1% L-Glutamine, 1% Penicillin/Streptomycin, 10% FCS; PAA, E15-009) and incubated overnight. On the next day the plate was connected with the ECISz instrument (Applied Biophysics) and measured with an AC current of 4 kHz. The substances: control (DMEM), 5-ASA 1 mM and 5 mM were pre- incubated in the 37 °C Incubator with 5% CO_2 for 1 h in a 96well plate. Then the experiment was paused and after removal of the old medium the substances were added to the Caco-2 cells. ECIS plate was re-connected with the instrument and the measurement was continued. Afterwards the impedance (ohm) was normalized by setting the time point 0 h at 1.