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ORIGINAL RESEARCH

Identification of a Novel Substance P–Neurokinin-1 Receptor MicroRNA-221-5p Inflammatory Network in Human Colonic Epithelial Cells

Kai Fang,[1] Aristea Sideri,[1] Ivy Ka Man Law,[1] Kyriaki Bakirtzi,[1] Christos Polytarchou,[2] Dimitrios Iliopoulos,[2] and Charalabos Pothoulakis[1]

[1]Inflammatory Bowel Disease Center and [2]Center for Systems Biomedicine, Division of Digestive Diseases, David Geffen School of Medicine, University of California, Los Angeles, California

SUMMARY

Substance P–neurokinin-1 (NK-1R) microRNA-221-5p (miR-221-5p) network regulates inflammation in human colonic epithelial cells through inhibition of interleukin-6R expression. Because silencing of miR-221-5p exacerbates experimental colitis, the use of miR-221-5p mimics may be a promising approach for colitis treatment.

BACKGROUND & AIMS: Substance P (SP), a neuropeptide member of the tachykinin family, plays a critical role in colitis. MicroRNAs (miRNAs) are small noncoding RNAs that negatively regulate gene expression. We examined whether SP modulates expression of microRNAs in human colonic epithelial cells.

METHODS: We performed microRNA profiling analysis of SP-stimulated human colonic epithelial NCM460 cells overexpressing neurokinin-1 receptor (NCM460-NK-1R). Targets of SP-regulated microRNAs were validated by real-time polymerase chain reaction (RT-PCR). Functions of miRNAs were tested in NCM460-NK-1R cells and the trinitrobenzene sulfonic acid (TNBS) and dextran sulfate sodium (DSS) models of colitis.

RESULTS: SP stimulated differential expression of 29 microRNAs, including miR-221-5p, the highest up-regulated miR (by 12.6-fold) upon SP stimulation. Bioinformatic and luciferase reporter analyses identified interleukin-6 receptor (IL-6R) mRNA as a direct target of miR-221-5p in NCM460 cells. Accordingly, SP exposure of NCM460-NK-1R cells increased IL-6R mRNA expression, and overexpression of miR-221-5p reduced IL-6R expression. Nuclear factor κB and c-Jun *N*-terminal kinase inhibition decreased SP-induced miR-221-5p expression. MiR-221-5p expression was increased in both TNBS- and DSS-induced colitis and in colonic biopsy samples from ulcerative colitis but not Crohn's disease patients compared with controls. In mice, intracolonic administration of a miR-221-5p chemical inhibitor exacerbated TNBS- and DSS-induced colitis and increased colonic tumor necrosis factor-α, C-X-C motif chemokine 10 (Cxcl10), and collagen, type II, α 1 (Col2α1) mRNA expression. In situ hybridization in TNBS- and DSS-exposed colons revealed increased miR-221-5p expression primarily in colonocytes.

CONCLUSIONS: Our results reveal a novel NK-1R-miR-221-5p-IL-6R network that protects from colitis. The use of miR-221-5p mimics may be a promising approach for colitis treatment. *(Cell Mol Gastroenterol Hepatol 2015;1:503–515; http://dx.doi.org/10.1016/j.jcmgh.2015.06.008)*

Keywords: Colitis; Inflammation; MicroRNA; Substance P.

Substance P (SP), an 11-amino-acid peptide member of the tachykinin family, is expressed in many organs including the intestine.[1] SP plays a critical role in colitis pathophysiology by interacting with its high-affinity neurokinin-1 receptor (NK-1R)[2–4] and activating signaling pathways related to nuclear factor-κB (NF-κB)[5–7] and c-Jun *N*-terminal kinase (JNK)[8] in different cell types, including colonocytes.[5,8,9] NK-1R expression is increased in the colon of inflammatory bowel disease (IBD) patients,[10,11] further suggesting an important role in IBD pathogenesis.

MicroRNAs (miRNA, MiR) represent a class of small noncoding single-stranded RNAs that control translation and mRNA degradation by binding to target mRNAs through complementary sequences.[12] Functional studies have demonstrated that miRNAs play critical roles in many physiologic and pathologic conditions, including inflammation[13] and colitis pathogenesis.[14–16] There is limited evidence, however, on the interaction between the SP/NK-1R system and miRNAs. Both miR-130a and miR-206 were found to regulate SP synthesis and release in neuronal cells,[17] and miR-449b and miR-500 modulate NK-1R expression in human astrocytoma cells.[18] Moreover, a miR-203 mimic blocked SP-mediated increased phospholipase-A2 activating protein expression in keratinocytes.[19] However, whether SP

Abbreviations used in this paper: **as, anti-sense; CAPE, caffeic acid phenethyl ester; COL2α1, collagen, type II, α 1; CXCL10, C-X-C motif chemokine 10; DSS, dextran sulfate sodium; IBD, inflammatory bowel disease; IL, interleukin; IL-6R, interleukin-6 receptor; JAK-STAT, Janus kinase/signal transducer and activator of transcription; JNK, c-Jun *N*-terminal kinase; miRNA, microRNA; NF-κB, nuclear factor-κB; NK-1R, neurokinin-1 receptor; RT-PCR, real-time polymerase chain reaction; siRNA, small-interfering RNA; SP, substance P; TNBS, trinitrobenzene sulfonic acid; TNFα, tumor necrosis factor-α; UC, ulcerative colitis; UTR, untranslated region.**

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modulates miRs in colonic epithelial cells and whether this response is related to the ability of SP to regulate colitis is not known. We performed a miRNA expression analysis to detect the miRNA signature upon SP stimulation of human colonic NCM460 epithelial cells.

Materials and Methods

Cell Studies and Reagents

NCM460 human colonic epithelial cells overexpressing NK-1R (NCM460-NK-1R), cultured as previously described elsewhere,[5] were starved in serum-free medium overnight and then stimulated with 0.1 μM SP at specific times. CAPE (caffeic acid phenethyl ester; cat. no. C8221), a specific inhibitor of NF-κB, was obtained from Sigma-Aldrich (St. Louis, MO), and the JNK inhibitor SP600125 (cat no. 8177) was obtained from Cell Signaling Technology (Beverly, MA). SP was purchased from Sigma-Aldrich (cat. no. S6883). Rabbit anti-interleukin-6 receptor (anti-IL6R; SC-661) was purchased from Santa Cruz Biotechnology (Dallas, TX). Mouse anti-β-actin was obtained from Sigma-Aldrich.

Transfection Experiments

Inhibitors of miR-221-5p (cat. no. 4464084), negative anti-miRNAs controls (cat. no. 4464076), a miR-221-5p mimic (cat. no. 4464067), and mimic miRNA controls (cat. no. 4464058) were purchased from Life Technologies (Carlsbad, CA). Mouse anti-miR-221-5p and negative control were purchased from Exiqon (Vedbæk, Denmark); the target sequence of anti-miR-221-5p is TGTAACATACGGTCC, and the target sequence of anti-miR-control is ACGTCTAT ACGCCCA. Lipid-based siPORTNeoFX Transfection Agent was purchased from Ambion (AM4511; Ambion/Life Technologies, Austin, TX), Lipofectamine 2000 was purchased from Life Technologies (cat. no. 52758). NF-κB p65 small-interfering RNA (siRNA; sc-29140), c-Jun siRNA (sc-29223), and control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology.

NCM460-NK-1R cells were transfected with siRNA using Lipofectamine RNAiMAX (Life Technologies). For miR-221-5p silencing or overexpression, the cells were transfected with antisense-miR-221-5p (as-miR-221-5p) or miR-221-5p mimic, respectively. Cells transfected with siRNA-control, antisense-control miR, or miR-mimic control served as controls.

Microarray Analysis of miRNA Expression

The miRNA microarray experiments were performed using the TaqMan low-density array human miRNA v1.0 system, which contains 365 microRNAs. The high-capacity reverse transcription reagent for cDNA was from Applied Biosystems (cat. no. 4368813; Foster City, CA). The real-time polymerase chain reaction (RT-PCR) primers were purchased from Life Technologies, except the miR-221-5p primers which were from Exiqon (cat. no. 204302). The total RNA of the NCM460-NK-1R cells were isolated by using TRIzol reagents, and the RNA concentration were determined by Nanodrop. Data were collected and normalized to nonfunctional small RNA internal controls. The results were validated using quantitative reverse-transcription PCR. The miRNA template for RT-PCR analysis was prepared using Exiqon reagents. RNU1A1 (cat. no. 203909; Exiqon) expression was used as the internal control. The threshold cycle (Ct) value formula was used to calculate the relative expression of selected miRNAs, as we previously reported elsewhere.[20]

Human Inflammatory Bowel Disease Biopsy Specimens

Total RNAs from the colon tissues of patients with active ulcerative colitis (UC) (n = 14), active Crohn's disease (n = 15), and healthy individuals (n = 9–10) were purchased from OriGene (Rockville, MD). These biopsy samples were obtained through strict institutional review board protocols and with full, documented patient consent, all from accredited U.S.-based medical institutions (www.origene.com). Conversion of the cDNA of RNA samples was performed as described earlier, and the levels of NK-1R, IL-6R and miR-221-5p were determined by quantitative RT-PCR analysis.

Luciferase Assays

IL-6R 3′-UTR (untranslated region) containing the two predicted binding sites and mutated sequences were chemically synthesized by GENEWIZ (South Plainfield, NJ). The wild-type and mutants of the IL-6R 3′-UTR sequence were then subcloned into the luciferase reporter vector from SwitchGear Genomics (cat. no. 32011; Carlsbad, CA). NCM460-NK-1R cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with luciferase reporter constructs containing the wild-type or mutant of 3′-UTR of IL-6R and the miR-221-5p mimic. Cell lysates were prepared 24 hours after transfection, and luciferase activity was measured using the LightSwitch Luciferase Assay Kit from SwitchGear Genomics (cat no. LS100) according to the manufacturer's instructions.

Immunoblot Analyses

NCM460-NK-1R cells were washed with ice-cold phosphate-buffered saline and incubated with radiolabeled immunoprecipitation assay buffer containing the protease inhibitors and sodium orthovanadate (Santa Cruz Biotechnology) for 5 minutes on ice. Equal amount of cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes. The membranes were blocked (phosphate-buffered saline, 5% nonfat dry milk, 0.05% Tween-20) and probed with antibodies followed by corresponding horseradish peroxidase-labeled secondary antibodies. Blots were developed with an enhanced chemiluminescence reagent (cat. no. 34080; Thermo Fisher Scientific, Waltham, MA).

In Situ Hybridization

In situ hybridization was performed on mice colon tissue from C57BL/6J mice after treatement with trinitrobenzene sulfonic acid (TNBS) (7 days) or dextran sodium sulfate(DSS) (6 days), as we previously reported elsewhere[21]