Original Paper

miR-135a Protects Dextran Sodium Sulfate-Induced Inflammation in Human Colorectal Cell Lines by Activating STAT3 Signal

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miR-135a • Inflammation • STAT3

Abstract

Background/Aims: miR-135a is reduced in several cancers and has been suggested to mediate immune and inflammatory responses. However, the effect of miR-135a on inflammatory bowel diseases was obscure. This study firstly attempted to investigate the hypothesis that miR-135a alleviates dextran sodium sulfate (DSS)-induced inflammation in colonic cells and potential mechanisms are also studied. **Methods:** Caco-2 and HT-29 cells in this study were treated with DSS, miR-135a mimic, and S3I-201, and then CKK-8 assay was used to test cell viability. Expressions of miR-135a, cytokines, and signal transducers and activators of transcription factors (STATs) were determined by RT-PCR. Also, cytokine productions were further tested by using ELISA kits. Activation or inactivation of STAT3 signal was validated by western blotting analysis. **Results:** The results showed that DSS markedly downregulated miR-135a expression ($P < 0.05$) and induced inflammatory response in Caco-2 and HT-29 cells evidenced by the upregulations and productions of interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) ($P < 0.05$). Transfection with miR-135a mimic significantly alleviated DSS-induced upregulations and productions of IL-1 β and TNF- α in Caco-2 and HT-29 cells ($P < 0.05$). STATs were analyzed and miR-135a mimic treatment reversed STAT3 downregulation in DSS-challenged Caco-2 and HT-29 cells compared with the mimic control ($P < 0.05$). Also, STAT3 phosphorylation was inhibited in DSS-challenged Caco-2 cells and miR-135a mimic activated STAT3 signal ($P < 0.05$). S3I-201, an inhibitor of STAT3 signal, further used to inactivate STAT3 signal and the results showed that S3I-201 blocked the anti-inflammatory effect of miR-135a mimic on Caco-2 and HT-29 cells evidenced by the lowered expressions and productions of proinflammatory cytokines (IL-1 β and TNF- α) ($P < 0.05$). **Conclusion:** Our results indicated that miR-135a alleviated DSS-induced inflammation and activated STAT3 signal in colonic cells. Inhibition of STAT3 reversed the anti-inflammatory function of miR-135a by regulating

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proinflammatory cytokines. Thus, STAT3 signal might serve, at least in part, as the potential mechanism of miR-135a-mediated anti-inflammatory effect in colonic cells.

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Introduction

Inflammatory bowel diseases (IBD) include Crohn disease, ulcerative colitis, and IBD-unspecified and have been considered as immune mediated chronic inflammatory disorders of the gastrointestinal tract [1-2]. Although the pathological mechanisms are multifactorial, such as environmental, genetic, immune mediated, and gut microbial factors, the main targets of therapy focus on colonic inflammation [1, 3, 4]. Cytokines play an important role in the pathogenesis of IBD by mediating multiple aspects of the inflammatory response [5-8]. Thus, targeting cytokines balance has resulted in the development of multiple biological agents in IBD. Also, cytokine-mediated activation of signal transducers and activators of transcription factor (STAT) 3 in the epithelial lining cells has been reported to improve cellular protection, survival, and proliferation in IBD models, including dextran sodium sulfate (DSS) colitis model [9-11]. Compared with other chemicals-induced experimental models, DSS serves as a direct chemical toxin to colonic epithelium leading to epithelial cell injury and the DSS model has been widely used in IBD research due to its rapidity, simplicity, reproducibility and controllability [12].

MicroRNAs (miRNAs) are small endogenous RNA molecules and serve as an important family of transcriptional and post-transcriptional regulators for gene expression [13-17]. In recent decades, various bio-functions of miRNAs have been identified, such as cell proliferation and differentiation, metabolism, inflammation, oxidative stress, and tumor biology [18, 19]. Altered concentrations or dysfunctions of miRNAs are highly associated with the development of IBD [20]. A number of miRNAs are highly associated with the initiation, development, and progression of IBD, but some also exert anti-inflammatory effect and may have the potential to be used as therapeutic targets [21, 22], such as miR-135a. Previous studies indicated that miR-135a abundance was dramatically reduced in diseases and tumor tissues [23, 24], suggesting a novel therapeutic potential in diseases, especially for cancers. For example, miR-135a has been evaluated to predict lymph node metastasis and tumor stage in gastric cancer [25] and acts as a tumor suppressor in gastric cancer through targeting Kinesin family member C1 [26]. In inflammatory diseases, intranasal administration of lentiviral miR-135a strongly inhibited the infiltration of eosinophils and mast cells into the nasal mucosa via influencing GATA binding protein 3 and the balance of Th1 and Th2 cells [27]. Although previous studies suggested an anti-inflammatory effect of miR-135a in cancer and nasal inflammation, the effect of miR-135a on IBD model was obscure. Thus, in this study, we firstly investigated the protective effects of miR-135a on DSS-induced inflammation in the human epithelial colorectal cell lines Caco-2 and HT-29. Both Caco-2 and HT-29 cells are isolated from colon adenocarcinomas and have been widely used for *in vitro* attachment and mechanistic studies [28]. The Caco-2 cell line is characterized by the polarized monolayers in culture and differentiation with high homology to enterocytes in the intestinal epithelium, while HT-29 cells in culture are heterogeneous containing a small proportion of mucus-secreting cells and columnar absorptive cells [28]. To our knowledge, this is a first study to investigate the effects of miR-135a on two colorectal cell lines.

Materials and Methods

Cell lines and cell culture

Caco-2 and HT-29 cells (two of human epithelial colorectal cell lines) were provided by the cell bank of Chinese Academy of Sciences and were seeded in Dulbecco's modified Eagle medium (DMEM)/F12 or RPMI 1640 medium added with 10% FBS (HyClone, Logan, UT) and 50 U/mL penicillin-streptomycin. Cells maintained at 37°C in a humidified chamber of 5% CO₂. Confluent cells (75–90%) were treated with 2% DSS

for 4 days to establish inflammatory model [29, 30]. 100 μ M S3I-201, an inhibitor of STAT3, was purchased from Calbiochem (La Jolla, CA, USA) and incubated for 1 h prior to DSS treatment [31]. The culture medium was renewed every 2 or 3 d.

Transfection of miRNA

Chemically modified double-stranded RNAs designed to mimic the endogenous miR-135a and negative control miRNA (mimic NC) were all brought from Genepharma (Shanghai, China). Cells were seeded in 6-well plates and transfected at 60–70% confluence with miR-135a mimic or the mimic NC using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions [30]. After transfection for 48 h, the transfected cells were treatment with 2% DSS or S3I-201 for 4 days for further assays.

Cell viability

Cell viability was determined using the CKK-8 assay (Sigma-Aldrich). Briefly, cells dispersed evenly in medium were seeded in a 96-well plate with a density of 1×10^4 cells/well. Next day, cells were treated with various concentrations of DSS, miR-135a mimic, and the negative control for 4 days. Then, CKK-8 solution was added to each well, followed by a 2 h incubation. The optical density (OD) in 570 nm was measured by a BioTek multilabel counter. The cell proliferation inhibition index was calculated according to the formula: $(OD_{\text{control}} - OD_{\text{treatment}}) / OD_{\text{control}} \times 100\%$. The result was normalized according to the value of the control at day 4.

ELISA kits

The culture supernatant was collected. Cellular abundances of IL-1 β (CSB-E08053h), IL-10 (CSB-E04593h), and TNF- α (CSB-E04740h) were tested in the medium by ELISA kits (CUSABIO, Wuhan, China) according to the manufacturer's instructions and signals detected at 450 nm [32].

Real-time PCR

Inflammatory cytokines (IL-1 β , IL-10, and TNF- α) were determined by real-time PCR. Approximately 1×10^6 cells/mL cells from each group were collected from six-well plates for RNA extraction and DNA synthesis according to kits (TAKARA). The gene sequences were used to design primers and synthesized by Invitrogen (Table 1). Double-distilled water was used instead of template as a negative control. The number of β -actin transcripts was used as a reference of endogenous RNA, and the quantification of test genes for each sample was standardized relative to the number of β -actin transcripts. miR-135a expression was normalized according to the abundance of U6. The $2^{-\Delta\Delta Ct}$ cycle threshold formula was used to calculate the relative abundance of transcripts.

Western blot

Total proteins from 10-cm dishes (10^{6-7} cells) were extracted using protein extraction reagents (Thermo Fisher Scientific Inc., Waltham, MA, USA). Proteins samples were quantified by BCA Protein Assay Kit (Beyotime, Jiangsu, China) and denaturated with SDS-PAGE Sample Loading Buffer (Beyotime, Jiangsu, China). 30–50 μ g proteins were separated by a reducing SDS-PAGE electrophoresis. Then the proteins were transferred onto a PVDF membrane (Millipore, MA, USA) and blocked with 5% non-fat milk in Tris-Tween buffered saline buffer for 1.5 hour. Anti-beta Actin antibody [Abcam 8226], Anti-STAT3 antibody [EPR787Y] (ab68153), and Anti-STAT3 (phospho S727) antibody (ab30647) were incubated overnight at 4°C and the HRP-conjugated secondary antibodies were subsequently incubated for 2 hours at room temperature. Then the membrane developed the blots using Alpha Imager 2200 software (Alpha Innotech Corporation, CA, USA). We digitally quantified the resultant signals and normalized the data to β -actin.

Table 1. Primers used in this study

Genes	Nucleotide sequence of primers (5'–3')
β -Actin	F: CCGTTGCCCTGAGGCTCTTT R: CCTTCTGCATCCTGTCAGCA
IL-1 β	F: CAGAAGTACCTGAGCTCGCC R: AGATTTCGTAGCTGGATGCCG
IL-10	F: TGTTCCTTGGGGAGCCAACA R: GGGCTCCCTGGTTTCTCTTC
TNF- α	F: CTGGGACAGTCTACTTTGGG R: CTGGAGGCCAGTTGAAT
STAT1	F: CCGTGGCACTGCATACAATC R: ACCATGCCGAATCCCAAAG
STAT3	F: CATCCTGAAGCTGACCCAGG R: TCCTCACATGGGGAGGTAG
STAT6	F: GGGACCTGGGTAGAAGGAGA R: CTGTAGCTCTGTCCAGCGAG
miR-135a	F: TATGGCTTTTATTCTATGTGA R: TATGGCTTTTCTATCTATGTGA
U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT

Statistical analysis

All data were analyzed by SPSS 17.0 software. Difference was tested by student's T test. Data are expressed as the mean $\pm$ standard error of mean (SEM). Values in the same row with * are significant ($P < 0.05$).

Results

DSS inhibited miR-135a expression and induced inflammation in Caco-2 and HT-29 cells

DSS exposure markedly inhibited cell viability at day 3 and 4 compared with the control group in Caco-2 cells ($P < 0.05$) (Fig. 1A). Expressions of miR-135a, IL-1 β , IL-10, and TNF- α were determined via RT-PCR to test the inflammatory response after DSS exposure and the results showed that DSS treatment significantly downregulated miR-135a expression in Caco-2 cells ($P < 0.05$) (Fig. 1B). Also, DSS-challenged Caco-2 cells exhibited marked inflammatory response evidenced by the upregulations of IL-1 β and TNF- α in the DSS group (Fig. 1C and E) ($P < 0.05$). Meanwhile, the expression of IL-10, an anti-inflammatory cytokine, was upregulated in DSS challenged-Caco-2 cells ($P < 0.05$) (Fig. 1D).

DSS-treated HT-29 cells exhibited similar results that DSS exposure reduced cell viability at day 4 ($P < 0.05$) (Fig. 1F) and downregulated miR-135a expression ($P < 0.05$) (Fig. 1G) compared with the control HT-29 cells. In addition, DSS treatment markedly increased cellular mRNA abundances of IL-1 β and TNF- α ($P < 0.05$) (Fig. 1H and K).

Fig. 1. Effects of DSS on cell viability, miR-135a and cytokine expressions in Caco-2 and HT-29 cells. (A) cell viability (%) in Caco-2 cells ($n=8$); (B) miR-135a expression in Caco-2 cells ($n=4$); (C) IL-1 β mRNA abundance in Caco-2 cells ($n=4$); (D) IL-10 mRNA abundance in Caco-2 cells ($n=4$); (E) TNF- α mRNA abundance in Caco-2 cells ($n=4$). (F) cell viability (%) in HT-29 cells ($n=8$); (G) miR-135a expression in HT-29 cells ($n=4$); (H) IL-1 β mRNA abundance in HT-29 cells ($n=4$); (J) IL-10 mRNA abundance in HT-29 cells ($n=4$); (K) TNF- α mRNA abundance in HT-29 cells ($n=4$). Data are expressed as the mean $\pm$ SEM. * means the difference was significant ($P < 0.05$). DSS, dextran sodium sulfate; IL-1 β , interleukin-1 β ; IL-10, interleukin-10; TNF- α , tumor necrosis factor- α .

Fig. 2. Effects of miR-135a on cell viability and cytokine expression and production in Caco-2 and HT-29 cells (n=4). (A) IL-1 β mRNA abundance in Caco-2 cells; (B) IL-10 mRNA abundance in Caco-2 cells; (C) TNF- α mRNA abundance in Caco-2 cells; (D) IL-1 β production in Caco-2 cells; (E) IL-10 production in Caco-2 cells; (F) TNF- α production in Caco-2 cells; (G) IL-1 β production in HT-29 cells; (H) IL-10 production in HT-29 cells; (I) TNF- α production in HT-29 cells. Data are expressed as the mean $\pm$ SEM. * means the difference was significant (P<0.05). DSS, dextran sodium sulfate; IL-1 β , interleukin-1 β ; IL-10, interleukin-10; TNF- α , tumor necrosis factor- α .

Fig. 3. miR-135a mimic upregulated STAT expression in DSS-challenged Caco-2 and HT-29 cells by RT-PCR (n=4). (A) STAT1 mRNA abundance in Caco-2 cells; (B) STAT3 mRNA abundance in Caco-2 cells; (C) STAT6 mRNA abundance in Caco-2 cells; (D) STAT1 mRNA abundance in HT-29 cells; (E) STAT3 mRNA abundance in HT-29 cells; (F) STAT6 mRNA abundance in HT-29 cells. Data are expressed as the mean $\pm$ SEM. * means the difference was significant (P<0.05). DSS, dextran sodium sulfate; STAT, signal transducers and activators of transcription factor.

miR-135a attenuated DSS-induced inflammation in Caco-2 and HT-29 cells

miR-135a has been widely demonstrated to involve in inflammation, thus miR-135a mimic was used to investigate the effect of miR-135a on DSS-induced inflammation in Caco-2 and HT-29 cells. Compared with the negative control, miR-135a mimic treatment markedly downregulated IL-1 β and TNF- α expressions in Caco-2 cells ($P < 0.05$) (Fig. 2A and C). Meanwhile, cytokine productions were determined via ELISA kits and the results showed a similar trend that miR-135a mimic alleviated IL-1 β and TNF- α abundances in the cell medium ($P < 0.05$) (Fig. 2D-F).

The results were further validated in HT-29 cells (Fig. 2G-I) that miR-135a mimic markedly reversed IL-1 β production in the DSS treated HT-29 cells ($P < 0.01$). Meanwhile, TNF- α abundance tended to be decreased in the miR-135a mimic group, but the difference was insignificant ($P = 0.089$). Together, these results suggested an anti-inflammatory effect of miR-135a by inhibiting the expressions and productions of proinflammatory cytokine.

miR-135a mimic activated STAT signal in Caco-2 and HT-29 cells

STATs are highly associated with the expression of proinflammatory cytokines [33]. In this study, we found that DSS exposure markedly reduced mRNA abundance of STAT3 in Caco-2 and HT-29 cells compared with the control group ($P < 0.05$) (Fig. 3). Meanwhile, in DSS-challenged cells, miR-135a mimic markedly upregulated STAT3 expression compared with the inhibitor negative control ($P < 0.05$) (Fig. 3).

Western blotting analysis was further used to validate the effect of miR-135a on STAT3 expression in Caco-2 cells and the results showed that DSS markedly inhibited STAT3 phosphorylation ($P < 0.05$) (Fig. 4), while miR-135a mimic markedly enhanced STAT3 phosphorylation ($P < 0.05$) (Fig. 4), indicating that miR-135a activated STAT3 signal in DSS-challenged cells.

STAT3 inhibition blocked the anti-inflammatory effect of miR-135a in Caco-2 and HT-29 cells

In DSS and miR-135a mimic co-treated cells, we further used S3I-201 to inhibit STAT3 signal and the results showed that S3I-201 markedly reduced STAT3 phosphorylation compared with the DSS and miR-135a mimic co-treated group ($P < 0.05$) (Fig. 5).

Fig. 4. miR-135a mimic enhanced STAT phosphorylation in DSS-challenged cells by western blot ($n = 4$). (A) Ratio of STAT3 phosphorylation; (B) immunoblotting for STAT3 and p-STAT3. Data are expressed as the mean $\pm$ SEM. * means the difference was significant ($P < 0.05$). DSS, dextran sodium sulfate; STAT3, signal transducers and activators of transcription factor 3.

Fig. 5. S3I-201 inhibited STAT3 signal in DSS and miR-135a mimic co-treated cells ($n = 4$). (A) Ratio of STAT3 phosphorylation; (B) immunoblotting for STAT3 and p-STAT3. Data are expressed as the mean $\pm$ SEM. * means the difference was significant ($P < 0.05$). DSS, dextran sodium sulfate; STAT3, signal transducers and activators of transcription factor 3.

Fig. 6. Effect of S31-201 on cellular cytokine expression and production in Caco-2 and HT-29 cells (n=4). (A) IL-1 β mRNA abundance in Caco-2 cells; (B) IL-10 mRNA abundance in Caco-2 cells; (C) TNF- α mRNA abundance in Caco-2 cells; (D) IL-1 β production in Caco-2 cells; (E) IL-10 production in Caco-2 cells; (F) TNF- α production in Caco-2 cells; (G) IL-1 β production in HT-29 cells; (H) IL-10 production in HT-29 cells; (I) TNF- α production in HT-29 cells. Data are expressed as the mean $\pm$ SEM. * means the difference was significant (P<0.05). DSS, dextran sodium sulfate; IL-1 β , interleukin-1 β ; IL-10, interleukin-10; TNF- α , tumor necrosis factor- α .

Moreover, S31-201 treatment markedly upregulated IL-1 β and TNF- α expressions in DSS and miR-135a mimic co-treated Caco-2 cells (P<0.05) (Fig. 6). The results were further confirmed via ELISA analyses that miR-135a blocked the inhibitory effect of miR-135a on IL-1 β and TNF- α productions in Caco-2 cells (P<0.05) (Fig. 6). In DSS and miR-135a co-treated HT-29 cells, S31-201 addition also increased IL-1 β and TNF- α production (P<0.05) (Fig. 6), suggesting that inhibition of STAT3 blocked the beneficial effect of miR-135a on DSS-induced inflammation.

Discussion

miR-135a has been widely demonstrated to involve in inflammatory response and cytokines production, thus targeting miR-135a may serve as a novel therapeutic method for treating inflammatory diseases [27, 34]. In this study, we found that miR-135a was reduced in DSS-induced inflammation and miR-135a mimic alleviated DSS-induced inflammatory response and activated STAT3 signal in Caco-2 and HT-29 cells, while STAT3 antagonist (S3I-201) blocked the anti-inflammatory effect, suggesting that STAT3 might serve as the potential mechanism in the anti-inflammatory role of miR-135a in DSS-induced inflammation.

IBD is chronic and inflammatory disorder primarily involving the mucosa and submucosa of the gastrointestinal tract and patients with IBD represent a major risk factor for the development of colorectal cancer [35-37]. Inflammatory response has been considered as a major characteristic of IBD and it is highly associated with the occurrence of IBD [38]. DSS has been widely used to induce inflammatory response *in vivo* and *in vitro* IBD models [39-41]. In this study, DSS exposure markedly caused inflammation via upregulating expressions of proinflammatory cytokines (IL-1 β and TNF- α) in Caco-2 cells. miR-135a has been reported to exhibit various physiological functions, such as cell proliferation, tumor biology, and immune system [42, 43]. In this study, we found that miR-135a mimic alleviated DSS-induced inflammation by down-regulating IL-1 β and TNF- α expressions and productions in Caco-2 and HT-29 cells. IL-1 β and TNF- α are two major mediators of the inflammatory response and generations of IL-1 β and TNF- α precipitate acute attacks of systemic or local inflammation or chronic diseases [44-46]. Therefore, blocking proinflammatory cytokines (IL-1 β and TNF- α) by targeting miRNA may serve as a novel potential method to treat inflammatory diseases.

STAT proteins play a central role in regulating cytokine-dependent inflammation and immunity [47-49]. 7 STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) are identified and STAT3 is highly associated with inflammatory response. In response to inflammatory stress, STAT3 directly serves as a transcription factor to regulate expression of proinflammatory cytokines [50]. STAT3 activation has been widely reported to maintain epithelial homeostasis and cytokine balance [9, 51]. For example, STAT3 activation is essential to countering the deleterious consequences of sepsis on pneumonia susceptibility [52]. However, other studies also indicated a proinflammatory effect of STAT3 [53], which has been confirmed in a hyperimmunoglobulin E syndrome model that STAT3 revealed pro- and anti-inflammatory functions [54]. Several studies showed that miR-135a can target MAPK and nuclear factor-kappaB pathways [11, 55-58], which might be further interacted with STAT3 signal. In this study, miR-135a mimic treatment markedly activated STAT3 signal, while inhibition of STAT3 blocked the anti-inflammatory effect. Similarly, the phosphorylation level of STAT3 signal was inhibited by the suppressor of cytokine signaling 3 along with the elevated expression of TNF- α , whereas activation of STAT3 indicated a lowered TNF- α expression in 3T3-L1 adipocyte [59]. However, some reports also indicated that STAT3 activation promoted proinflammatory cytokine productions [60]. The difference may be caused by different models that the proinflammatory effect of STAT3 mainly occurs in animal models (mice and rats), while the *in vitro* effect of STAT3 in Caco-2 and HT-29 cells was investigated in the present study. Also, STAT3-driven negative-feedback loop may serve as the potential mechanism, which further limits the formation of pro-inflammatory cytokines [61]. Various reports have indicated that STAT3 serves as a potential target in DSS-induced mouse colitis [62, 63]. For example, Koukos et al. reported that miRNA can mediate the expression of STAT3, which further influences inflammatory response in pediatric patients with ulcerative colitis [20]. Thus, we anticipated that STAT3 serves as the molecular mechanism in the protective effect of miR-135a inhibition on DSS-induced inflammation *in vitro*.

Conclusion

In conclusion, DSS induced inflammation by upregulating of proinflammatory cytokines (IL-1 β and TNF- α) in Caco-2 and HT-29 cells. miR-135a mimic treatment alleviated DSS-induced inflammation by inhibiting IL-1 β and TNF- α expressions and productions in Caco-2 and HT-29 cells. The mechanism might be associated with STAT3 signal as STAT3 inhibitor blocked the anti-inflammatory effect of miR-135a in DSS-challenged Caco-2 and HT-29 cells. However, detailed mechanisms of STAT3-mediated pro- or anti-inflammatory effects should be studied in different models. Together, our findings provided new approaches to ameliorate IBD by targeting miRNA, especially for miR-135a.

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Disclosure Statement

The authors have declared that no competing interests exist.

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