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BRIEF REPORT

Down-regulation of miR-140-3p is a cause of the interlukin-13-induced up-regulation of RhoA protein in bronchial smooth muscle cells

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ABSTRACT

The current study aimed to determine the role of a microRNA (miRNA), miR-140-3p, in the control of RhoA expression in bronchial smooth muscle cells (BSMCs). In cultured human BSMCs, incubation with interleukin-13 (IL-13) caused an up-regulation of RhoA protein concurrently with a down-regulation of miR-140-3p. Transfection of the cells with a miR-140-3p inhibitor caused an increase in basal RhoA protein level. Although a mimic of miR-140-3p had little effect on the basal RhoA level, its treatment inhibited the IL-13-induced up-regulation of RhoA. These findings suggest that RhoA expression is negatively regulated by miR-140-3p, and that the negative regulation is inhibited by IL-13 to cause an up-regulation of RhoA protein in BSMCs.

Increased airway responsiveness to a wide variety of stimuli, called airway hyperresponsiveness (AHR), is a characteristic feature of allergic bronchial asthma. Airway smooth muscles (ASMs) of individual asthmatics exhibited an increased agonist-induced contraction [1–3], indicating that an abnormality of the property of ASM *per se* is involved in the exaggerated airway narrowing. Rapid relief from airway limitation by bronchorelaxants also suggests an involvement of augmented ASM contraction in the airway obstruction. It is thus important for the development of asthma therapy to understand changes in the contractile signalling of ASM cells associated with the disease.

A small GTPase RhoA is a key protein involved in the Ca^{2+} sensitization of smooth muscle contraction, including ASMs *e.g.* [4]. Increasing evidence suggests that the augmented RhoA-mediated Ca^{2+} sensitization of ASM contraction is associated with the AHR in experimental asthma [5–10]. An up-regulation of RhoA in the ASMs has also been demonstrated in animal models of allergic asthma [5–10]. An involvement of interleukin-13 (IL-13), one of the asthma-related cytokines, in the RhoA up-regulation has been suggested [11–13], although the mechanism is not fully understood.

MicroRNAs (miRNAs) are small single-stranded non-coding RNAs that negatively modulate gene expression [14–16]. There is increasing evidence that dysregulation of miRNAs is found in multiple human diseases, including chronic obstructive pulmonary disease (COPD) and asthma [17–20]. Although the miRNA roles in function and pathology of ASM remain largely unknown, increasing evidence suggests miR-140-3p as a candidate miRNA that contributes to dysfunction of ASM in asthma [21–24]. More recently, RhoA has been suggested as one of the targets of miR-140-3p in ASM cells [25]. Here, we show that IL-13 down-regulates miR-140-3p to cause up-regulation of RhoA in bronchial smooth muscle (BSM) cells.

Methods

Human bronchial smooth muscle cell (hBSMC) culture and sample collection

Normal human BSM cells (hBSMCs; Cambrex Bio Science Walkersville, Inc., Walkersville, MD) were maintained in SmBM medium (Cambrex) supplemented with 5% foetal bovine serum, 0.5 ng/mL human epidermal growth factor (hEGF), 5 µg/mL insulin, 2 ng/mL human fibroblast growth factor-basic (hFGF-b), 50 µg/mL gentamicin and 50 ng/mL amphotericin B. Cells were maintained at 37°C in a humidified atmosphere (5% CO_2), fed every 48–72 hours, and passaged when cells reached 90–95% confluence. Then the

hBSMCs (passages five through seven) were seeded in 6-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at a density of 3,500 cells/cm^2 and, when 80–85% confluence was observed, cells were cultured without serum for 24 hours before addition of recombinant human IL-13 (100 ng/mL; PeproTech EC, Ltd., London, UK). At the indicated time after the IL-13 treatment, cells were washed with phosphate-buffered saline, immediately collected and disrupted with 1x SDS sample buffer (150 μL/well), and used for Western blot analyses. Total RNAs containing miRNAs were extracted using Vantage^{TM} total RNA purification kit (Origene Technologies, Inc., Rockville, MD) according to the manufacturer's instructions.

Transfection of hBSMCs with miR-140-3p inhibitor and mimic

The hBSMCs were plated at a density of 2×10^5 cells/ well in a 6-well plate with SmGM medium. The next day, cells were transfected with 100 pmol (final 40 nM) of either an inhibitor of miR-140-3p (Peptide Nucleic Acids (PNAs^{TM}) miRNA inhibitor; Cat. No.: PI-1171; Panagene Inc., Daejeon, Korea), a mimic of miR-140-3p (miCENTURY OX miNatural; Cat. No.: HN0000140A1-2; Cosmo Bio Co., Ltd., Tokyo, Japan), or a control RNA (Cosmo Bio Co., Ltd.) using the Lipofectamine^{TM} 2000 transfection reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. After a 4-hour transfection in Opti-MEM^{TM} I reduced serum medium (Thermo Fisher Scientific), cells were cultured in SmGM medium for 20 hours and then in serum-free SmGM medium. Forty-eight hours after the transfection, IL-13 (100 ng/mL) or its vehicle (PBS) was administered to the cells.

Quantitative RT-PCR analyses

To synthesize cDNAs, 200 ng of total RNAs were poly-adenylated and cDNAs were synthesized using miRCURY LNA^{TM} Universal cDNA Synthesis Kit (#203,300, Exiqon A/S, Vedbaek, Denmark) according to the manufacturer's instructions. Then the RT reaction mixture (1 μL) was subjected to real-time PCR analyses using StepOne^{TM} real-time PCR system (Applied Biosystems, Foster City, CA) with Fast SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. The reactions were incubated in a 96-well optical plate at 95°C for 20 seconds, following by 43 cycles of 95°C for 3 seconds and 60°C for 30 seconds. The primer sets used were: LNA^{TM} PCR primer sets for human miR-140-3p (#204,304, Exiqon A/S) and for human U6 snRNA (#203,907, Exiqon A/S).

Western blot analyses

Protein samples were subjected to 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and the proteins were then electrophoretically transferred to a PVDF membrane (WSE-4051CP; Atto, Co., Tokyo, Japan). After blocking with EzBlock Chemi^{TM} (AE-1475CP; Atto, Co.), the PVDF membrane was incubated with polyclonal rabbit anti-RhoA (sc-418; 1:2,500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibody. Then the membrane was incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (sc-2313; 1:2,500 dilution; Santa Cruz Biotechnology, Inc.), detected by EzWestBlue^{TM} (AE-1490CP; Atto, Co.) and analysed by a densitometry system. Detection of house-keeping gene was also performed on the same membrane by using monoclonal mouse anti-GAPDH (sc-32233; 1:10,000 dilution; Santa Cruz Biotechnology, Inc.) to confirm the same amount of proteins loaded.

Data and statistical analyses

In the real-time PCR analyses, the comparative threshold cycle (C_T) method was used for relative quantification of the target genes. Differences in the C_T values (ΔC_T) between miR-140-3p and U6 snRNA were calculated to determine the relative expression levels, using the following formula: $\Delta\Delta C_T = (\Delta C_T$ of the treated sample) $- (\Delta C_T$ of the control sample). The relative expression level between the samples was calculated according to equation $2^{-\Delta\Delta CT}$.

All the data are expressed as the mean ± S.E. Statistical significance of difference was determined by unpaired Student's t-test or one-way analysis of variance (ANOVA) with post hoc Bonferroni/Dunn (Prism^{TM} 5 for Mac OS X; GraphPad Software, Inc., La Jolla, CA). A value of p < 0.05 was considered significant.

Results

Effects of IL-13 on expression levels of RhoA protein and miR-140-3p in cultured human bronchial smooth muscle cells (hBSMCs)

Since IL-13 is capable of inducing an up-regulation of RhoA protein in hBSMCs [11,12], the effect of IL-13 on

the expression of miR-140-3p was determined in the hBSMCs. Consistent with our previous studies [11,12], treatment of the cells with IL-13 (100 ng/mL) caused an up-regulation of RhoA protein (Figure 1(a)). As shown in Figure 1(b), the real-time RT-qPCR analyses revealed that the expression level of miR-140-3p was significantly decreased in the hBSMCs treated with IL-13 (p < 0.01 by unpaired Student's t-test). The treatment also reduced the 5′-strand of miR-140 duplex, miR-140-5p, significantly (1.06 ± 0.10 in Vehicle *versus* 0.68 ± 0.15 in IL-13, p < 0.05).

Effect of inhibition of miR-140-3p on RhoA protein expression in cultured human bronchial smooth muscle cells (hBSMCs)

To determine the role of miR-140-3p in RhoA expression of the ASM cells, cultured hBSMCs were transfected with its synthetic inhibitor, miR-140-3p inhibitor. A non-targeting 20–25 nt RNA was used as a control RNA. Immunoblot analyses of these cells revealed that RhoA protein expression was significantly increased when the cells were transfected with miR-140-3p inhibitor (Figure 2, 'Control RNA' *versus* '140–3p inhibitor' groups), indicating that miR-140-3p is an endogenous modulator of RhoA protein expression in hBSMCs. On the other hand, transfection of the cells with miR-140-3p mimic had no effect on the expression level of RhoA protein (Figure 2, 'Control RNA' *versus* '140–3p mimic' groups).

Effects of miR-140-3p inhibitor and mimic on the IL-13-induced up-regulation of RhoA protein in cultured human bronchial smooth muscle cells (hBSMCs)

To determine the role of miR-140-3p on the IL-13-induced up-regulation of RhoA protein, the hBSMCs transfected with miR-140-3p inhibitor or mimic were stimulated by IL-13 (100 ng/mL). As a result, the IL-13-induced up-regulation of RhoA protein was slightly but not significantly augmented when the cells were transfected with miR-140-3p inhibitor (Figure 2: 'Control RNA + IL-13' *versus* '140–3p inhibitor + IL-13'). On the other hand, transfection of the cells with miR-140-3p mimic inhibited the RhoA up-regulation induced by IL-13, almost completely (Figure 2): the significant increase in RhoA induced by IL-13 ('Control RNA' *versus* 'Control RNA + IL-13') was not observed in the miR-140-3p-transfected cells ('Control RNA' *versus* '140–3p mimic + IL-13').

Discussion

Our previous study revealed a decrease in the level of miR-140-3p in bronchial smooth muscles (BSMs) of a murine asthma model [25] that possesses BSM hyper-responsiveness together with up-regulations of RhoA protein and IL-13 [11,26]. Analyses using a public database tool suggested that RhoA mRNA is one of the targets of miR-140-3p [25]. The vector-based analyses also demonstrated that miR-140-3p interacts with 3′-UTR of
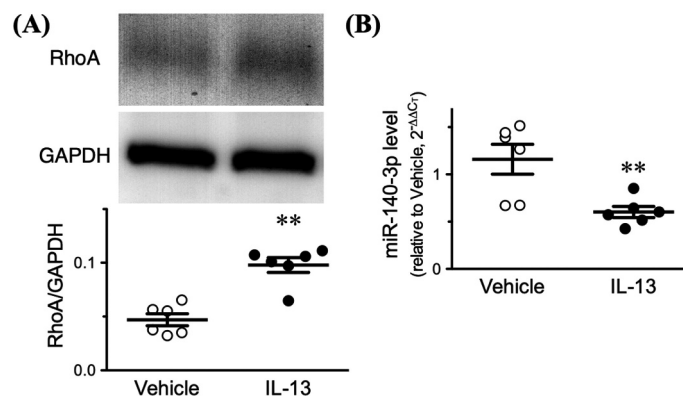

Figure 1. Interleukin-13 (IL-13) causes an up-regulation of RhoA protein concurrently with a down-regulation of miR-140-3p in cultured human bronchial smooth muscle cells. Cells were treated with IL-13 (100 ng/mL) or its vehicle for 24 hours, and proteins and total RNAs including miRNAs were extracted. (*a*) (*Upper panel*) Representative Western blots. The relative expression of RhoA to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) proteins in each sample was calculated and the data are summarized in *lower panel*. (*b*) miR-140-3p expression level determined by quantitative real-time reverse transcriptase-polymerase chain reaction. The relative gene expressions of miR-140-3p to U6 snRNA was calculated by the $2^{-\Delta\Delta CT}$ methods. Results are presented as mean ± S. E. from 6 independent experiments. **p < 0.01 *versus* Vehicle group by unpaired Student's t-test.
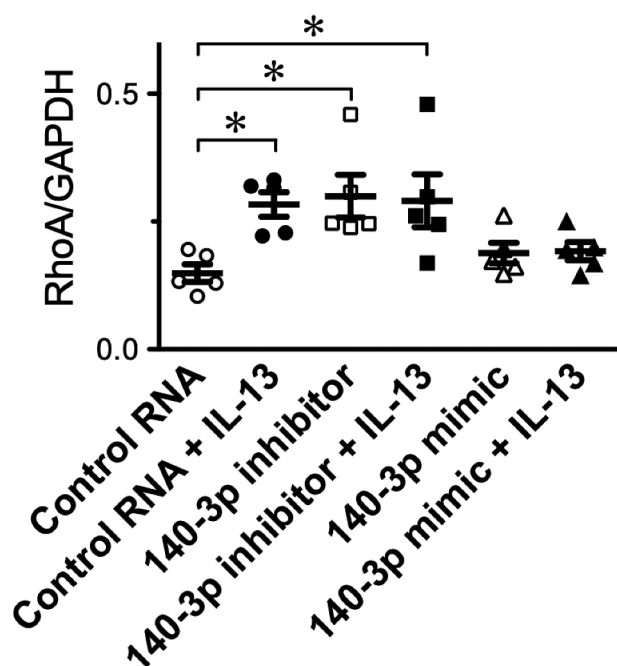
Figure 2. Effects of miR-140-3p inhibitor and mimic on the interleukin-13 (IL-13)-induced up-regulation of RhoA protein in cultured human bronchial smooth muscle cells. Cells were transfected with a non-targeting 20–25 nt RNA (Control RNA), a miR-140-3p inhibitor (140–3p inhibitor), or a miR-140-3p mimic (140–3p mimic), and then treated with IL-13 (100 ng/mL) or its vehicle 48 hours after the transfection. Twenty-four hours after the IL-13 treatment, total protein samples were prepared and immunoblot analyses were performed. The relative expression of RhoA to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in each sample was calculated and the data are summarized. Results are presented as mean ± S.E. from 5 independent experiments. *p < 0.05 *versus* Control RNA only group by Bonferroni/Dunn test. Note that the RhoA up-regulation induced by IL-13 (Control RNA + IL-13 group) was inhibited by the transfection of miR-140-3p mimic (140–3p mimic + IL-13 group).

RhoA mRNA to negatively regulate the RhoA protein expression [25]. In the present study, inhibition of endogenous miR-140-3p by its synthetic inhibitor caused an up-regulation of RhoA protein in cultured hBSMCs (Figure 2). Treatment of hBSMCs with IL-13 caused an up-regulations of RhoA protein together with a down-regulation of miR-140-3p (Figure 1). The IL-13-induced up-regulation of RhoA protein was inhibited when the cells were transfected with miR-140-3p mimic (Figure 2). These observations indicate that the expression level of RhoA is negatively regulated by endogenous miR-140-3p and that IL-13 could induce RhoA up-regulation *via* inhibition of miR-140-3p expression at least in part.

Increasing evidence suggests that dysregulation of miRNA is involved in multiple human diseases including asthma [27,28]. Among the miRNA families, miR-140-3p

has been suggested as a candidate miRNA that contributes to the dysfunction of ASM in asthma [21–25]. Kannan and colleagues demonstrated that miR-140-3p negatively controls the expression of CD38 [22], a regulatory protein of the ASM contractility [29,30]. The current study revealed that miR-140-3p also regulates the RhoA expression in BSM. It is thus possible that miR-140-3p controls the expression of several genes, such as CD38 and RhoA, in the ASM to regulate its contractility.

The ASM is a crucial effector tissue regulating bronchomotor tone. It has been suggested that modulation of ASM by inflammatory mediators such as cytokines plays an important role in the development of AHR [31–34]. Our previous findings that both the increased BSM contractility and the up-regulation of RhoA protein observed in the antigen-induced murine asthma model were reproduced by IL-13 [11] suggest that IL-13 is a key mediator for the induction of AHR. The IL-13-induced up-regulation of RhoA protein was inhibited both by a synthetic inhibitor and a small interfering RNA (siRNA) for STAT6 [26]. However, their inhibitory effects were only partial, whereas complete inhibition of STAT6 activity was observed [26], suggesting that some STAT6-independent mechanisms might also contribute to the IL-13-induced up-regulation of RhoA. We show here that IL-13 induced a down-regulation of miR-140-3p in hBSMCs (Figure 1 (b)). Based on the current result that a miR-140-3p inhibitor caused an up-regulation of RhoA (Figure 2), the IL-13-induced down-regulation of miR-140-3p could be a cause of the IL-13-induced up-regulation of RhoA protein. Our preliminary observations that neither inhibitor nor mimic of miR-140-3p had effect on the mRNA expression of RhoA (data not shown) support an idea that miR-140-3p controls translation of RhoA, although further detailed studies are required.

In conclusion, the current findings suggest that RhoA protein expression is negatively regulated by miR-140-3p in hBSMCs. IL-13 is capable of reducing the miR-140-3p expression in BSMs. The reduced expression of miR-140-3p might cause an up-regulation of RhoA in BSM cells, resulting in an augmentation of the contraction, which is one of the causes of AHR in asthmatics.

Abbreviations

ACh	acetylcholine
AHR	airway hyperresponsiveness
ANOVA	analysis of variance
ASM	airway smooth muscle
BSM	bronchial smooth muscle
cDNA	complementary DNA

COPD	chronic obstructive pulmonary disease
C_T	threshold cycle
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
hBSMC	human bronchial smooth muscle cell
hEGF	human epidermal growth factor
hFGF-b	human fibroblast growth factor-basic
IL-13	interleukin-13
LNA	locked nucleic acid
miRNA	microRNA
mRNA	messenger RNA
nt	nucleotide
PCR	polymerase chain reaction
PNA	peptide nucleic acid
PVDF	polyvinylidene difluoride
RT-PCR	reverse transcriptase-polymerase chain reaction
RT-qPCR	quantitative RT-PCR
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
snRNA	small nuclear RNA
UTR	untranslated region

Author contributions

Conception and design: Y.C., H.S. and M.H. Analysis and interpretation: Y.C., Y.A., Y.K. and H.S. Drafting the manuscript for important intellectual content: Y.C., H.S. and M.H. All authors provided final approval of the final version of the manuscript.

Disclosure of potential conflictof interest

The authors declare that they have no conflict of interest.

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