

Original Article

Mastic gum suppresses secretion of thymic stromal lymphopoietin in the asthmatic airway via NF- κ B signaling pathway

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Received April 29, 2015; Accepted January 25, 2016; Epub August 15, 2016; Published August 30, 2016

Abstract: Mastic gum (MG) is a nature extracted product of the plant *Pistacia lentiscus* var. *chia* and has various effects such as antioxidant and anti-inflammatory effects. Thymic stromal lymphopoietin (TSLP) is increased in the asthmatic airway and induces dendritic cell-mediated activation of T_H2 inflammatory responses. Viral stimuli, a major cause of asthma exacerbations, have been shown to induce overexpression of TSLP in asthmatic epithelium. However, the effect of MG on the production of TSLP has not been investigated. Here we have explored effects of MG on viral surrogate (dsRNA)-induced TSLP in human bronchial epithelial (NHBE) cells. NHBE were stimulated with dsRNA in the presence of IL-4 and IL-13. TSLP release was measured by ELISA. The effects of MG were analyzed. We demonstrated that mastic gum inhibited the production and mRNA expression of TSLP in normal human bronchial epithelial cells or in the asthmatic airway of mice. Mastic gum also inhibited the nuclear factor (NF)- κ B luciferase activity induced by poly I:C, and it prevented dsRNA-induced loss of the NF- κ B repressor protein I κ B α . These results suggest that mastic gum may alleviate inflammatory and atopic diseases through the inhibition of TSLP.

Keywords: Mastic gum, thymic stromal lymphopoietin, bronchial epithelial cells, double-stranded RNA

Introduction

Mastic gum (MG) is a natural resin that is obtained from the stem and leaves of *Pistacia lentiscus* trees. It has been extensively used for centuries in Mediterranean and Middle Eastern countries, both as a dietary supplement and traditional medicine. Medical trials demonstrate that mastic has cytoprotective or anti-acid effects on the gastrointestinal system, such as relief of ulcers [1] and reduction of intensity of gastric mucosal damage caused by antiulcer drugs, with little or no side effects. Moreover, it is already known for its antioxidant [2], antibacterial [3, 4], and antitumor properties [5, 6].

Exacerbations of asthma are the main cause of asthma morbidity and mortality and account for significant healthcare costs [7]. It is now evident that asthma deterioration and exacerba-

tions in children and adults are frequently associated with viral infection, and the bronchial epithelium is the major target the infection [8, 9]. The viral infection of airway epithelial cells induces the production of proinflammatory cytokines and antiviral INF- β [10].

Recent findings indicate that thymic stromal lymphopoietin (TSLP) plays an important role in allergic inflammation [11]. TSLP is highly expressed by airway epithelial cells of patients with asthma [12-14]. The pathophysiology of human TSLP involves innate allergic immune responses with direct effects of TSLP on mast cells and on dendritic cells (DCs). TSLP-activated DCs initiate adaptive allergic immune responses by triggering differentiation of naive T cells into inflammatory T_H2 cells that produce several traditional Th2 cytokines and also large amounts of TNF- α . Thus, TSLP represents a critical factor linking responses at interfaces

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between the body and environment to allergic T_H2 immune reactions [15, 16].

Double-stranded RNA (dsRNA) is produced during viral replication and induces innate immune responses including epithelial generation of interferons and inflammatory cytokines [17]. Recently, it was demonstrated that polyinosinic-polycytidylic acid (poly I:C), a synthetic double-stranded RNA (dsRNA) recognized by dsRNA sensors including Toll-like receptor 3 (TLR3), was a strong trigger for TSLP production in human bronchial epithelial cells, and the TSLP release can be synergistically enhanced with an atopic cytokine milieu [16]. These findings imply that respiratory viral infection, through epithelial TLR3 stimulation, may amplify T_H2 inflammation via the induction of TSLP in the asthmatic airway.

Qiao et al. [18] reported that mastic alleviates allergic inflammation in asthmatic model. However, their direct action on TSLP production in human bronchial epithelial cells is not well understood. Therefore, the objective of this study was to evaluate the effect of MG on the dsRNA-induced TSLP release from human bronchial epithelial cells.

Materials and methods

Agent

Mastic gum (Sigma, St Louis, MO, USA, No G0878) was dissolved in 1% dimethyl sulfoxide (DMSO) in saline.

Cell culture

Normal human bronchial epithelial cells (NHBE) were obtained from Cambrex. NHBE were maintained in serum-free bronchial epithelial cell growth medium (Cambrex). Cells were seeded in flat-bottomed 96-well microculture plates and cultured until they reached 100% confluence, and then the medium was changed to fresh medium. After further cultivation for 24 h, cells were stimulated with poly I:C (GE Healthcare, Buckinghamshire, UK) and T_H2 cytokines (IL-4 and IL-13) in the absence or presence of MG.

ELISA

The concentrations of TSLP protein was measured using diluted (1/2 for TSLP) culture super-

natant collected 24 h after stimulation with ELISA kits (R&D Systems). In this study, the minimum detection limits for TSLP in the supernatant were 3.9 $\mu\text{g/ml}$.

RNA isolation and real-time RT-PCR

RNA was isolated and purified using the RNeasy Mini kit (Qiagen, Hilden, Germany). For real-time RT-PCR analyses, 1 μg of DNase-treated total RNA was reverse transcribed. The amplification of the cDNA was accomplished using the ABI Prism 7900HT sequence detection system in the presence of the commercially available SYBR Green PCR Master Mix (Takara, Dalian, China) in a 40-cycle PCR. The primer sequences were as follows: GAPDH (Forward, 5'-GGT CGG AGT CAA CGG ATT TG-3'; Reverse, 5'-ATG AGC CCC AGC CTT CTC CAT-3'); human TSLP (Forward, 5'-TAT GAG TGG GAC CAA AAG TAC CG-3'; Reverse, 5'-GGG ATT GAA GGT TAG GCT CTG G-3'). The denaturing, annealing, and extension conditions of each PCR cycle were 95°C for 5 seconds, 60°C for 20 seconds, and 72°C for 34 seconds, respectively. The relative expression was calculated using the $2^{-\Delta\text{CT}}$ method. The mRNA levels of each target gene were normalized to the levels of GAPDH and were represented as fold induction.

Transient transfection and luciferase assay

For the transfection, we seeded NBHE cells (5×10^6) in a 100 mm culture dish. We then used Lipofectamine™2000 purchased from Invitrogen (Carlsbad, CA, USA) to transiently transfect pNF- κ B luciferase (LUC) and pSV40-LUC reporter gene constructs into NBHE cells. To measure the luciferase activity, we used a luminometer 1420 luminescence counter purchased from Perkin Elmer (Waltham, MA, USA) in accordance with the manufacturer's protocol. All the transfection experiments were performed in at least three different experiments, with similar results. The relative luciferase activity was defined as the ratio of firefly luciferase activity to renilla luciferase activity.

Western blotting

NHBE cell lysates were separated by SDS-PAGE (10% acrylamide gels) and transferred to polyvinylidene difluoride (PVDF) membranes, following detailed protocols described previously [19]. The blots were probed with phospho-I κ B α , and β -actin antibodies and then proteins were

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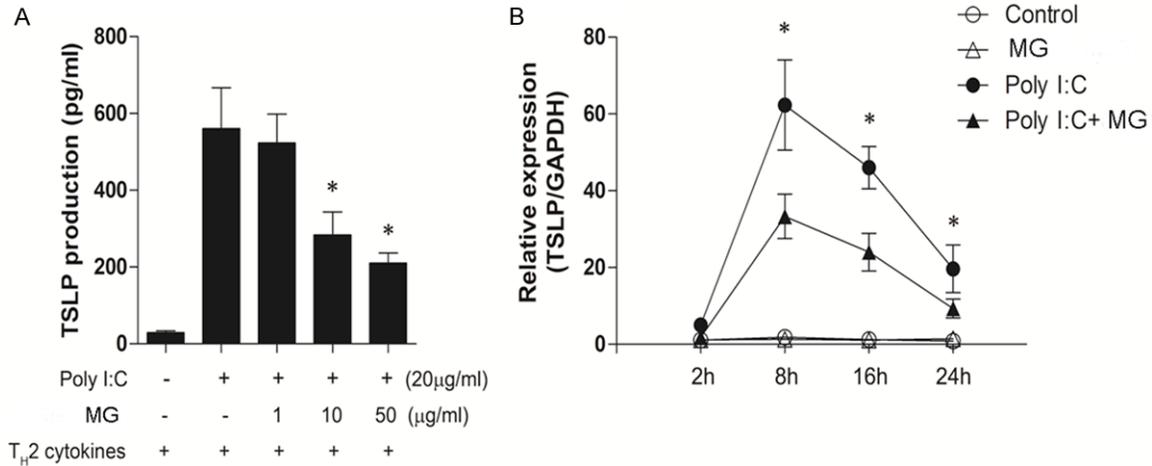


Figure 1. MG decreases dsRNA-induced expression of TSLP in NHBE cells. A. NHBE cells were pretreated with increasing concentrations of MG (0, 1, 10 and 50 µg/ml) for 4 h and subsequently were stimulated with poly (I:C) (20 µg/ml) plus T_H2 cytokine milieu (IL-4 and IL-13) in the presence of MG. Concentrations of TSLP in the culture supernatant collected 48 h after stimulation were measured. Data shown are the means for 2 wells and are representative of 3 independent experiments (*P < 0.05). B. NHBE cells were pretreated with MG (50 µg/ml) for 4 h, and then the cell were stimulated with poly (I:C) (20 µg/ml) for 2, 8, 16 and 24 h in the absence of 100 ng/ml IL-4 and 100 ng/ml IL-13. TSLP gene expression was analyzed by real-time RT-PCR and is presented as the fold change relative to the expression in the negative control at 2 h.

visualized by the ECL Western Blotting System (Pierce, Rockford, IL).

Statistical analysis

Results are expressed as the mean ± SD. A Student's t-test was used to determine significance among the groups. A value of *P* < 0.05 was considered significant. Analyses and graphical representation were performed using Graph-Pad Prism 5.01 software.

Results

Effect of MG on dsRNA-induced expression of TSLP in NHBE cells

To assess the inhibitory effect of MG on the production of TSLP, we pretreated normal NHBE cells with increasing concentrations of MG for 4 h and subsequently stimulated the cells with poly (I:C) plus T_H2 cytokine milieu (IL-4 and IL-13) for 48 h in the presence of MG. As shown in **Figure 1A**, the stimulation with poly (I:C) increased TSLP production from NHBE cells. The levels of TSLP that increased due to poly (I:C) were significantly decreased by MG treatment in a dose-dependent way.

To determine whether MG can modulate poly (I:C)-induced mRNA expression of TSLP, we pretreated the cells with MG for 4 h before the poly

(I:C) stimulation. We then stimulated the cells with poly (I:C) for 2, 8, 16 and 24 h in the absence of IL-4 and IL-13. The dsRNA induced gene expression of TSLP peaked at 8 h, and MG showed an inhibitory effect at 8, 16 and 24 h (**Figure 1B**).

Effect of MG on dsRNA-induced gene expression of IL-8 and IFN-β in NHBE cells

To investigate whether MG suppresses the dsRNA-induced innate inflammatory response, NHBE cells were pretreated with MG in concentrations ranging from 1 to 50 µg/ml for 4 h before they were challenged by poly (I:C) 20 µg/ml for 12 h in the absence of T_H2 cytokines. MG displayed a concentration-dependent inhibitory effect on dsRNA-stimulated mRNA expression of IL-8, whereas the expression of IFN-β was not significantly modified (**Figure 2**).

Effect of MG on the expression of RNA-recognizing receptors in NHBE cells

Several intracellular receptors have been described to bind to dsRNA, such as Toll-like receptor 3 (TLR3), PKR, RIG-I and MDA5 [20]. To reveal the inhibitory mechanisms of MG, we examined the mRNA expression of these RNA-recognizing receptors. As shown in **Figure 3**, stimulation of NHBE cells with poly (I:C) lead to increases in the different receptors at a vari-

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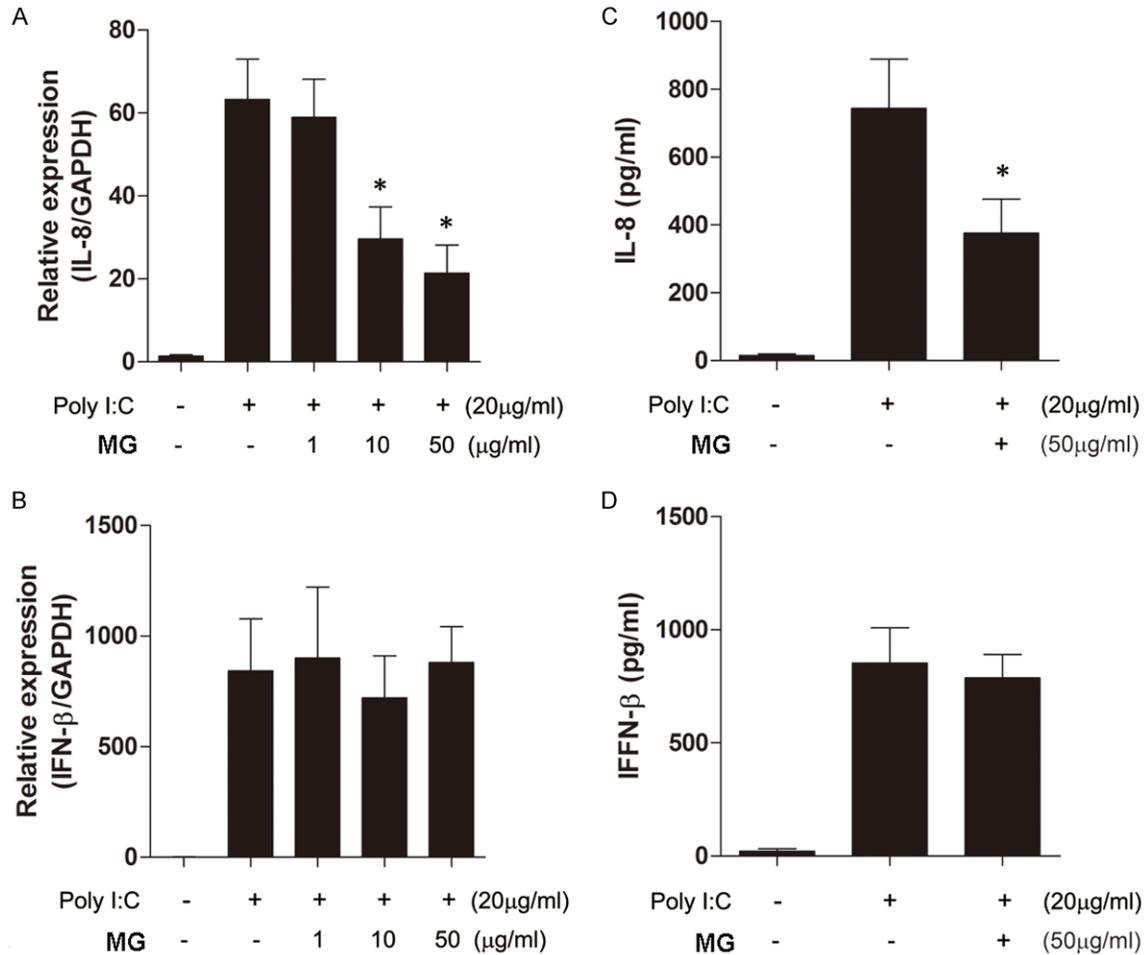


Figure 2. MG inhibits dsRNA-induced upregulation of IL-8 but not IFN- β expression. NHBE cells were pretreated with increasing concentrations of MG (0, 1, 10 and 50 $\mu\text{g/ml}$), and then the cells were stimulated with poly (I:C) (20 $\mu\text{g/ml}$) for 8 h. IL-8 (A) and IFN- β (B) gene expression was analyzed by real-time RT-PCR and is presented as the fold change relative to the expression in the negative control. The data are presented as the mean \pm SD of a total of 2 independent experiments (* $P < 0.05$). (C, D) NHBE cells were stimulated with poly (I:C) (20 $\mu\text{g/ml}$) in the presence or absence of MG (50 $\mu\text{g/ml}$). Concentrations of IL-8 and IFN- β in the culture supernatant collected 48 h after stimulation were measured.

able degree, whereas MG treatment did not affect the mRNA level of these genes.

Suppressive effects of MG on dsRNA-stimulated TSLP production via NF- κ B signaling pathway

NF- κ B is one of the key transcription factors regulating the expression of proinflammatory genes and is known to be involved in mediating TSLP release from NHBE cells stimulated with dsRNA [21]. Next, we examined whether MG could regulate the luciferase expression specifically via NF- κ B activation. As shown in **Figure 3**, the poly (I:C) stimulation increased the reporter gene activity. However, this increased

NF- κ B luciferase activity was significantly decreased by MG treatment (**Figure 4A**).

The activity of NF- κ B is controlled by I κ B, we examined whether MG influenced the degradation of I κ B α by means of western blot analysis. In this study, treatment of NHBE cells with poly (I:C) showed increased degradation of I κ B α in the cytoplasm extracts measured by immunoblotting (**Figure 2**). MG treatment (50 $\mu\text{g/ml}$) restored the level of phospho-I κ B α in dsRNA-stimulated NHBE cells (**Figure 4B**).

Discussion

The current treatment of asthma consists of corticosteroids and/or β_2 -agonists and only

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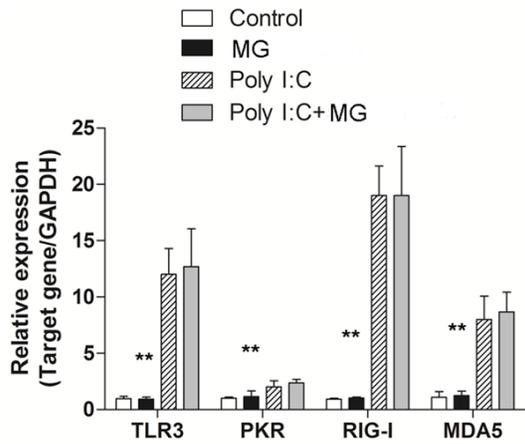


Figure 3. MG does not change the expression levels of TLR3, PKR, RIG-I and MDA-5 mRNA in NHBE cells. NHBE cells were treated or not with MG (50 $\mu\text{g}/\text{ml}$) in the presence or absence of poly (I:C) stimulation (20 $\mu\text{g}/\text{ml}$) for 8 h, and then TLR3, PKR, RIG-I and MDA5 mRNA expression were analyzed by real-time RT-PCR and is presented as the fold change relative to the expression in the negative control.

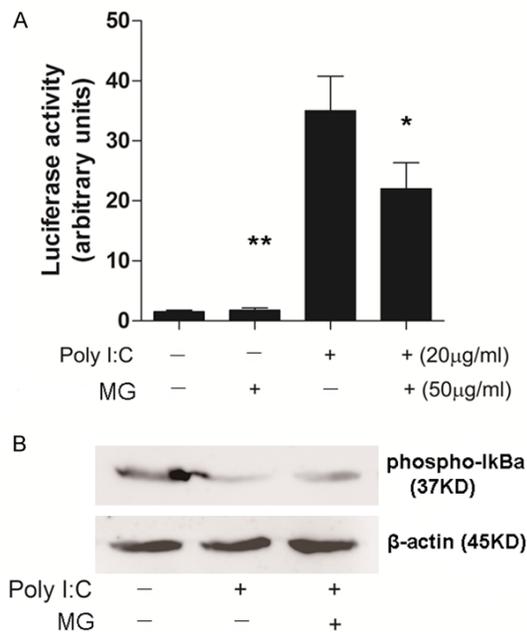


Figure 4. MG inhibits dsRNA-induced NF- κB activation in NHBE cells. A. NHBE cells (1×10^7) were transiently transfected pGL4.32 and treated with or without MG (50 $\mu\text{g}/\text{ml}$) for 2 h and then stimulated with or without poly (I:C) (20 $\mu\text{g}/\text{ml}$) for 48 h. The NF- κB activity was assessed with a luciferase assay. The data are presented as the mean \pm SD of a total of 2 independent experiments (* $P < 0.05$). B. NHBE cells were incubated with or without poly (I:C) (20 $\mu\text{g}/\text{ml}$) in the presence or absence of MG (50 $\mu\text{g}/\text{ml}$) for 18 h, and degradation of phospho-IkBa in cytoplasmic extract from NHBE cells was assessed by western blot and densitometric analysis.

partially prevents asthma exacerbations [22]. A vital need exists for new treatment regimes for this form of asthma. In the present study, we showed that MG inhibited dsRNA-induced TSLP from normal human bronchial epithelial cells by inhibiting NF- κB signaling, while expression of anti-virus cytokine IFN- β was not significantly influenced.

The observation that viral products induce TSLP production by bronchial epithelium may be related to the well-documented aggravating role of infection in allergic as well as asthma [23]. For example, 60-80% of asthma exacerbations in children and adults are caused by rhinovirus infection. During viral replication, single-stranded (ss) RNA viruses such as rhinovirus produce double-stranded (ds) RNA which is detected as a 'danger signal' by the innate immune system. Previous studies have shown that exposure to rhinovirus infection or dsRNA in vitro induces TSLP production in bronchial epithelial cells from healthy subjects [24], suggesting that this TSLP may link the innate antiviral response and the type 2 adaptive immune response. TSLP was originally reported to exert its T_H2 -promoting properties through a dendritic cell-mediated pathway in human beings that involved induction of the OX40 ligand on dendritic cells [25]. There has therefore been great interest in targeting the pathways downstream of TSLP such as TSLP-TSLP receptor interaction and OX40L-OX40 interaction. In the present study, we found that MG inhibited the production and mRNA expression of TSLP. To our knowledge, this is the first study showing an inhibition of TSLP by MG in bronchial epithelial cells.

Induction of immune responses by dsRNA and RNA viruses involves endosomal receptor TLR3 or present in the cytoplasm including the RNA helicases RIG-I and MDA5 and the serine-threonine kinase PKR [20]. Our data demonstrated that none of these cytoplasmic dsRNA-recognition receptors were modulated by MG treatment, however the roles of MDA5, RIG-I or PKR could not be completely excluded. Activation of TLR3 by dsRNA RNA viruses transduces its signals to NF- κB and IRF-3 and induces proinflammatory genes and type I IFNs including IFN- β [26]. Here, we showed that secretion IFN- β which may have protective role in asthma exacerbations, was not compromised by MG treatment.

The transcription factor NF- κ B mediates cytokine gene activation downstream of TLR3 signaling. Drugs that inhibit NF- κ B by stabilizing its binding to I κ B have also been shown to be effective inhibitors of TSLP generation in dsRNA-stimulated bronchial epithelial cells from healthy and diseased donors [27]. Additionally, such drugs have produced a less desirable inhibition of the antiviral IFN- β in these bronchial epithelial cells [28]. The present findings suggest possibilities of separating anti-TSLP and anti-IFN- β effects since MG reduced expression of TSLP without inhibiting the IFN- β production.

In conclusion, we demonstrated that MG suppressed the dsRNA-induced release of TSLP from normal human bronchial epithelial cells. TSLP is considered the master switch for T_H2 responses. Therefore, it is a promising strategy to shut down the release of TSLP in the bronchial epithelial cells of asthma patients. MG could therefore be effective in the treatment of asthma exacerbation through the suppression of TSLP release from bronchial epithelial cells, when exogenous dsRNA is involved in the pathogenesis or exacerbation.

Disclosure of conflict of interest

None.

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