Original Article

Localization and expression of Slingshot-1L in peripheral eosinophils from patients with acute asthma exacerbation

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Abstract: Background Eosinophils play a pivotal role in asthmatic airway inflammation. We previously found a significantly high expression of Slingshot-1L (SSH-1L) in peripheral eosinophils in acute exacerbations of asthma. Objective To investigate the expression and localization patterns of SSH-1L in peripheral blood eosinophils of asthmatic patients and their changes after treatment with inhaled corticosteroids. Methods We recruited 4 outpatients with acute exacerbations of asthma who received no previous corticosteroid treatment and 1 healthy volunteer. From all the subjects 30 ml peripheral venous blood samples were collected before and after a 3-month treatment with inhaled fluticasone. The eosinophils were isolated, purified and counted, and the expressions of SSH-1L in the eosinophils were examined by RT-PCR and Western blotting. The localization of SSH-1L phosphatases in the peripheral eosinophils was detected by immunofluorescence assay in one patient. Results SSH-1L phosphatases distributed diffusely in the cytoplasm, especially dense near the membrane of the peripheral eosinophils. Glucocorticoids treatment resulted in a significant reduction in both the SSH-1L mRNA expression (0.7403 ± 0.1124 vs. 0.4101 ± 0.0363, P=0.001) and SSH-1L protein expression (0.3410 ± 0.1337 vs. 0.1543 ± 0.0551, P=0.039). Conclusions A high expression of SSH-1L in peripheral eosinophils in acute exacerbations of asthma may play a role in the activation and migration of eosinophils. The efficacy of inhaled corticosteroids in asthma control might be partly attributed to a down-regulated expression of SSH-1L.

Key words: Slingshot-1L; asthma; eosinophils; corticosteroids

Introduction

Asthma is a chronic inflammatory condition of the airway characterized by prominent eosinophil infiltration in the bronchial mucosa. Eosinophils are derived from the pluripotential stem cells in the bone marrow, and in asthma attacks, they become activated and migrate into the airway.

SSH was initially identified as a dedicated ADF/cofilin (henceforth referred to as cofilin) phosphatase through genetic studies in Drosophila, where its dysfunction was noted to cause disorganized epidermal cell morphogenesis, including splintered hair bristles (hence the name Slingshot). Three genes were known to encode human SSHs (hSSH-1L, -2 and -3, in which L means a long form). SSH-1L is known to regulate actin filament dynamics by dephosphorylating and activating cofilin, an actin-depolymerizing factor.

We previously identified an array of differentially expressed genes associated with cell adhesion and immune regulation in eosinophils from an asthmatic patient by suppression subtractive hybridization. Among these genes, Slingshot (SSH) gene was found to be up-regulated in the eosinophils. We also found significantly increased expression of SSH-1L in peripheral eosinophils of patients with acute exacerbations of asthma. In this study, we investigated the intracellular expression and localization of SSH1L in the peripheral eosinophils of asthmatic patients, aiming to explore the role of SSH1L in the activation and migration of eosinophils in asthmatic patients.

Methods

Patients

This study was conducted under the approval by the Human Ethics Review Committee of Nanfang Hospital, and written informed consent was obtained from each participant. Four non-smoking out-patients with acute asthma exacerbation were enrolled in this study, including 2 male and 2 female patients with a mean age of 45 years (range 32-66 years), who took β-2-receptor agonist inhalation irregularly without other treatments. All the patients had an established diagnosis of acute asthma exacerbation based on clinical and functional evidences. After enrollment in this study, all the asthmatic patients received a 3-month therapy with salmeterol/fluticasone (Seretide, 50/250 µg twice daily, GlaxoSmithKline, UK). One male healthy volunteer aged 27 years, a non-smoker, was also included. The volunteer was free of asthma or other respiratory diseases with a normal chest X-ray finding, and reported no medications prior to participation in this study. Peripheral venous blood was collected from each...
subject, and the eosinophil count was determined. The eosinophils were isolated and purified from the blood samples from all the subjects following the protocols as previously described\(^3\).

**RT-PCR**

The total cellular RNA was isolated from the eosinophils (purity >95\%) using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer’s instructions. Fifteen second after addition of chloroform (100 μl) in each tube, the samples were centrifuged at 12 000 r/min for 15 min at 4 ℃. The clear aqueous supernatant was transferred to a fresh tube, where 250 μl isopropanol were added. After incubation at 4 ℃ overnight, the samples were centrifuged at 12 000 r/min for 15 min at 4 ℃. The total RNA was washed with 500 μl 75% ethanol and air-dried. The concentration and purity of the RNA samples were examined by measuring the absorbance at 260 and 280 nm, and the integrity of the extracted RNA was confirmed by agarose gel electrophoresis. With β-actin as the internal standard, RT-PCR was performed using Real Time One step RNA PCR ver.2.0 kit (Takara BIO INC. Dalian, China). The primers used for amplification of SSH-1L and β-actin mRNA (synthesized by Invitrogen Life Technologies) were listed below:

β-actin 5’-GGGCTCCAGGCACTCAGTC 3’ (sense),

5’-GCCGCTCAGGCACTCAGTC 3’ (antisense)

SSH-1L 5’-GGAAGAATCTGTCACCCAA-3’ (sense)

5’-CACGCCCTAGAAGAAAGG-3’(antisense).

The PCR reaction was carried out with 35 thermal cycles of denaturation at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s, and extension at 72 ℃ for 1 min, with a final extension at 72 ℃ for 10 min. The PCR products (394 bp) were analyzed by 1.5% agarose gel electrophoresis, and identified on the basis of product size (394 bp for SSH-1L and 303 bp for β-actin). The amplified products were sequenced using Gel Doc 1000 automated sequencer (Bio-Rad Laboratories, Hercules, CA, USA).

**Western blot analysis**

The eosinophils were lysed in NuPAGE LDS sample reducing buffer (NuPAGE, Invitrogen, USA) and then centrifuged at 10 000 × g at 4 ℃ for 30 min to obtain the cellular proteins. The protein concentrations were determined by BCA Protein Assay (Pierce Biotechnology Inc., Rockford, IL), and 20 μg of the total protein from each sample were boiled for 5 min before loading on 10% Bis-Tris gels (Invitrogen, USA). The gels were transferred to nitrocellulose membranes, blocked with 5% skim milk for at least 1 h and probed with rabbit anti-human SSH-1L antibody (a kind present from Dr. James R. Bamburg from Colorado State University), HRP-conjugated mouse anti-rabbit antibody (Santa Cruz Biotechnology, USA) was used as the secondary antibody. The blots were washed and developed using a Western Blot Luminal Reagent (Santa Cruz Biotechnology, USA). Each protein band was scanned using the Bio-Rad Gel Doc system and the density was analyzed with Quantity One 4.4.1 software (Bio-Rad Laboratories, Hercules, CA) and compared by densitometry to the positive control.

**Immunofluorescence assay**

Venous blood (15 ml) was collected from a 40-year-old male patient with acute exacerbations of asthma diagnosed according to NHLBI/NIH report \(^4\) and also from the healthy volunteer. The eosinophils were isolated by Percoll density gradient centrifugation, diluted in PBS to 500 μl and transferred to 3 culture dishes. The cells were fixed with 4% paraformaldehyde for 30 min, rinsed 3 times in PBS, and then incubated with the primary antibody (rabbit anti-human SSH-1L antibody) for 1 h at 37 ℃. After washing with PBS, the cells were incubated with the secondary antibody labeled with FITC for 1 h at 37 ℃. After washing, the cells were scanned with a Meridian ACAS570 interactive laser scanning confocal microscope (Meridian Instruments, Okemos, MI, USA).

**Statistical Analysis**

The data were analyzed using SPSS11.0 software (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used for data comparison (LSD test in case of variance homogeneity, and Dunnett’s-T3 test otherwise). A P value less than 0.05 was considered to indicate a statistically significant difference.

**RESULTS**

**Peripheral eosinophil count**

In the asthmatic patients, the peripheral blood eosinophil count decreased significantly from 0.11×10\(^9\)/L before fluticasone treatment to 0.23×10\(^9\)/L after the 3-month treatment (P<0.05).

**RT-PCR**

The expression levels of SSH-1L mRNA in the peripheral eosinophils are shown in Fig.1. SSH-1L mRNA expression was significantly lowered in the asthmatic patients after treatment with fluticasone (0.7403±0.1124 vs 0.4101±0.0363, P=0.001).

**Western blotting**

The expression levels of SSH-1L protein in the peripheral eosinophils is shown in Fig.2. The relative expression of SSH-1L protein in the asthmatic patients was 0.3410±0.1337 before treatment, and significantly decreased to 0.1543±0.0551 after a 3-month fluticasone treatment (P=0.039).

**Localization of SSH-1L in peripheral eosinophils**

As shown in Fig.3 and Fig.4, SSH-1L phosphatase
distributed diffusely in the cytoplasm, and was especially dense near the membrane of peripheral eosinophils. The distribution pattern of SSH-1L was similar to that of coflin \([2]\).

**Fig. 1** Agarose gel electrophoresis of the PCR product of SSH-1L in peripheral eosinophils before (Lanes 1, 3) and after (Lanes 2, 4) fluticasone treatment. M: DNA marker.

**Fig. 2** Western blot of SSH-1L protein in peripheral eosinophils before (Lanes 1-3) and after (Lane 4-6) fluticasone treatment. M: DNA marker.

**Fig. 3** Localization of SSH-1L phosphatases in the peripheral eosinophils of the healthy volunteer. A: Fluoroscope. Scale bar=11.09 \(\mu\)m. B: Fluoroscope. Scale bar=5 \(\mu\)m. SSH-1L phosphatases were diffusely distributed in the cytoplasm, dense near the membrane.

**Fig. 4** Localization of SSH-1L phosphatases in the peripheral eosinophils of an asthmatic patient. A: Scale bar=20 \(\mu\)m. B: Scale bar=8.25 \(\mu\)m. SSH-1L phosphatases are diffusely distributed in the cytoplasm, dense near the membrane.

**DISCUSSION**

Eosinophilia in the blood and other tissues is a characteristic feature of asthma pathology \([3]\). In the event of asthma attacks, mature eosinophils are released from the bone marrow into peripheral blood and migrate into the tissues, a process initiated by local chemoattractant molecules \([6]\). Massive migration of eosinophils is a hallmark of asthma \([6,3]\).

Cellular migration is driven by the assembly and disassembly of actin filaments \([8-9]\), and polymerized actin is important in the adhesion and movement of human
During cell migration, cofilin functions as an important catalyst for actin filament disassembly to maintain lamellipodia protrusion\(^1\). Cofilin is inactivated through phosphorylation of Ser-3 by LIM-kinases or related TES-kinases, and Slingshot family protein phosphatases reactivate the inactive Ser-3-phosphorylated cofilin (P-cofilin) through dephosphorylation\(^{13-19}\). SSH specifically dephosphorylates and reactivates cofilin, hence playing a key role in the regulation of actin cytoskeleton. We found that the distribution of SSH-1L in EOS was similar to that of cofilin by immunofluorescence and SSH mRNA was highly expressed in the peripheral eosinophils of patients with exacerbations of asthma, which correlated to an increased number of cofilin in the peripheral blood\(^3\), suggesting that SSH promoted the activation and migration of eosinophils by reactivating cofilin.

Oxidative stress plays an important role in the development of airway inflammation and hyperreactivity in asthma\(^9\). Recently Kim et al\(^{17}\) found that the reactive oxygen species regulated cytoskeletal organization and cell migration through a SSH-1L-cofilin pathway.

In the current study, the asthmatic patients received irregular inhalation of β2-receptor agonist before recruitment. After 3 months of treatment with inhaled salbutamol/fluticasone, the peripheral eosinophils of the patients with acute exacerbations of asthma were significantly decreased. The treatment also caused a significant reduction in the expression of SSH at both the mRNA and protein levels in the peripheral eosinophils. Inhaled fluticasone may inhibit the migration of eosinophils by down-regulating the expression of SSH, which in turn inactivates cofilin.

Inhaled glucocorticosteroids are the most effective medications currently available for asthma control. It is clear that eosinophils contain receptors for the glucocorticoids and are important targets of glucocorticoids action. The clinical efficacy of glucocorticoids is attributed to a number of distinct actions. In particular, glucocorticoids can prevent or attenuate the recruitment of eosinophils to the sites of inflammation following allergen provocation\(^{18}\), and reduce the number of eosinophils and the concentration of secretory products of the eosinophils in the blood, BAL, and nasal fluid\(^9\). Glucocorticoids also suppress the migration of eosinophils. Miyamasu et al\(^{20}\) found that glucocorticoids inhibited chemokine production by human eosinophils, Nagase and his colleagues\(^{21}\) found that glucocorticoids could regulate the expression of chemokine receptors in the eosinophils. In vitro as well as in vivo studies\(^{22}\) have clearly demonstrated that glucocorticoids downregulate the production of eosinophil-activating chemokines, such as eotaxin, RANTES, and monocyte chemoattractant protein, but the direct effect of corticosteroids on eosinophil chemotaxis is equivocal. It is now clear that eosinophil-derived mediators constitute a disadvantage because of their cytotoxic effects on a variety of cell types\(^{23}\), hence modulation of eosinophil migration represents a promising therapeutic strategy in the treatment of allergic disorders.

SSH promotes the activation and migration of eosinophils by reactivating cofilin which can disassemble and depolymerize actin filaments, thus playing a role in chronic inflammation of the airway. Furthermore, SSH may also participate in the therapeutic mechanism of corticosteroids, whose ameliorating effect on asthma might be explained, at least in part, by the down-regulation of SSH expression.

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References

Slingshot-1L在哮喘急性发作期外周血嗜酸粒细胞中的定位与表达变化

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背景 嗜酸粒细胞(EOS)在哮喘气道炎症中起着重要作用。我们前期发现Slingshot-1L（SSH-1L）在急性发作期哮喘病人外周血EOS表达明显增高，可能在EOS的激活、迁移中发挥重要作用。目的 探讨SSH-1L在哮喘急性发作期病人外周血EOS的定位及治疗后的表达及功能变化。方法 招募4例未经糖皮质激素治疗的急性发作期哮喘门诊病人。在治疗前及吸入糖皮质激素3个月后抽取外周静脉血各30 ml进行EOS的分离纯化并计数，应用RT-PCR和Western Blotting方法分别在基因及蛋白质水平检测哮喘外周血EOS SSH-1L的表达变化；应用免疫荧光观察其中1例病人SSH-1L在外周静脉血EOS中的定位。结果 免疫荧光显示外周血EOS的SSH-1L定位在胞浆中，在贴近胞壁处丰富表达。急性发作期哮喘病人糖皮质激素治疗前后外周血EOS RT-PCR扩增SSH-1L基因的SSH-1L/β-Actin灰度值比：治疗前0.7403±0.1124、治疗后0.4101±0.0363，治疗前后有显著差异(P=0.001);急性发作期哮喘病人吸入糖皮质激素治疗前后外周血EOS蛋白的SSH-1L/β-actin灰度值比分别为0.3410±0.1337和0.1543±0.0551，治疗后明显降低(P=0.039)。结论 SSH-1L可能在急性发作期哮喘病人外周血EOS的激活、迁移上发挥作用，糖皮质激素可能通过抑制哮喘急性发作期EOS的SSH表达而抑制EOS的激活，迁移起到治疗作用。

关键词:Slingshot-1L；支气管哮喘；嗜酸粒细胞；糖皮质激素