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Role of S100A9 in the development of neutrophilic inflammation in asthmatics and in a murine model

Tae-Hyeong Lee [a,1], Hun Soo Chang [a,1], Da-Jeong Bae [a,1], Hyun Ji Song [a], Myung-Sin Kim [b], Jong Sook Park [c], Ji Ae Jun [a], Si Young Lee [d], Soo Taek Uh [e], Soo Hyun Kim [d], Choon-Sik Park [c,*]

[a] Department of Interdisciplinary Program in Biomedical Science Major, Soonchunhyang Graduate School, Bucheon, Gyeonggi-do 420-767, Republic of Korea
[b] Department of Internal Medicine, Soonchunhyang University Gumi Hospital, Gumi, Gyeongsangbuk-do 39371, Republic of Korea
[c] Division of Allergy and Respiratory Disease, Soonchunhyang University Bucheon Hospital, 1174 Jung-dong, Bucheon, Gyeonggi-do 420-767, Republic of Korea
[d] Laboratory of Cytokine Immunology, Department of Biomedical Science and Technology, Konkuk University, 120 Neungdong-ro, Seoul 143-701, Republic of Korea
[e] Division of Respiratory Disease, Soonchunhyang University Seoul Hospital, YongSan-gu, Seoul 04401, Republic of Korea

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ABSTRACT

S100A9 is an endogenous danger signal that promotes and exacerbates the neutrophilic inflammatory response. To investigate the role of S100A9 in neutrophilic asthma, S100A9 levels were measured in sputum from 101 steroid-naïve asthmatics using an ELISA kit and the levels were significantly correlated with percentages of neutrophils in sputum. Intranasal administration of recombinant S100A9 markedly increased neutrophil numbers at 8 h and 24 h later with concomitant elevation of IL-1β, IL-17, and IFN-γ levels. Treatment with an anti-S100A9 antibody restored the increased numbers of neutrophils and the increased airway resistance in OVA/CFA mice toward the levels of sham-treated mice. Concomitantly, the S100A9 and neutrophil elastase double positive cells were markedly reduced with attenuation of IL-1β, IL-17, and IFN-γ levels by the treatment with the anti-S100A9 antibody. Our data support a role of S100A9 to initiate and amplify the neutrophilic inflammation in asthma, possibly via inducing IL-1β, IL-17 and IFN-γ.

1. Introduction

Asthma is a heterogeneous disease composed of various subtypes, which can be defined by etiology, pathology, severity, physiologic parameters, and response to treatment [1]. In recent years, cluster analysis of asthma cohorts has led to identification of distinct clinical sub-phenotypes, including early-onset atopic asthma with normal lung function, non-atopic older obese females with moderate reductions in FEV1, and adult asthmatics with severe airflow obstruction [2–4]. Other parameters such as patterns of airway inflammation or cytokines have been used to define asthma, because clustering using clinical and demographic parameters is of limited utility for predicting clinical course. Airway inflammation is heterogeneous, with eosinophilic, neutrophilic, mixed cellular and pauci-granuloncytic types, each of which has distinct physiologic and clinical characteristics [5,6].

Early-onset allergic asthmatics have robust eosinophilic airway inflammation with Th2-type cytokine dominancy [7], while 5–10% of late-onset asthmatics have severe manifestations with concomitant neutrophilic airway inflammation [5,6,8]. In addition, a cluster analysis of patients with asthma identified a severe asthma subgroup that showed high levels of sputum neutrophils and the sputum neutrophils was the second most influential variable [9]. Interleukin (IL)-4, IL-5, and IL-13 released from Th2-polarized lymphocytes contribute to the airway eosinophilia of asthma, while IL-8, TNF-α, interferon (IFN)-γ and IL-17 are associated with the neutrophilic inflammation [10]. However, the causative molecules of the airway neutrophilia in asthmatics have not been determined. Thus, we screened candidate proteins in neutrophil-dominant sputum, and found elevated S100A9 levels in uncontrolled severe asthmatics under treatment with high doses of inhaled steroid [11].

S100A9 is a small calcium-binding protein recognized as an alarmin released by stressed cells: an endogenous danger signal that promotes and exacerbates the inflammatory response. S100A9 induces activation of Toll-like receptor 4 [12], neutrophil chemotaxis [13], neutrophilic inflammation [14], and augmentation of IL-8 production [15]. Thus,

Abbreviations: ELISA, Enzyme-linked immunosorbent assay; S100A9, S100 calcium binding protein A9; MMP, Matrix metalloproteinases; FEV1, Forced expiratory volume in one second; FVC, Forced vital capacity; TNF, Tumor necrosis factor; IFN, Interferon; IL, interleukin; LPS, Lipopolysaccharide; BAL, Bronchoalveolar lavage; CFA, Complete Freund's Adjuvant; OVA, Ovalbumin; PBS, Phosphate-Buffered Saline; TBS, Tris Buffered Saline; DAPI, 4′,6-Diamidino-2-phenylindole; PE, Phycoerythrin; FITC, Fluorescein Isothiocyanate; BMI, Body mass index.

* Corresponding author at: Division of Allergy and Respiratory Medicine, Department of Internal Medicine, Soonchunhyang University Bucheon Hospital, 1174, Jung Dong, Bucheon, Gyeonggi Do 420-021, Republic of Korea.
E-mail address: mdcspark@hanmail.net (C.-S. Park).

[1] Tae-Hyeong Lee, Hun Soo Chang contributed equally as the first authors.

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S100A9 is speculated to induce airway neutrophilia in asthma. Thus, additional clinical evidence of the association of S100A9 with airway neutrophilia is needed in order to define the role of S100A9 in the neutrophilic subtype of asthma. Furthermore, the contribution of S100A9 to neutrophilic asthma should be clarified in animal models. In the present study, S100A9 protein levels were measured in sputum from steroid-naïve asthmatics to evaluate the association between S100A9 protein and neutrophilic inflammation. In addition, the role of S100A9 was elucidated in a murine model of asthma with neutrophilic inflammation.

2. Materials and methods

2.1. Subjects and sputum collection

Asthma was diagnosed by physicians based on the Global Initiative for Asthma (GINA) guidelines [16]. At the baseline visit, demographic information, such as enrollment age, sex, BMI, asthma-onset age, asthma duration, and smoking amount was collected. At the same time, all patients underwent a standardized assessment, which included analyses of induced-sputum specimens, complete blood cell count with differential counts, total IgE, chest radiography, and allergy skin prick tests with 24 common inhalant allergens (Bencard Co, London, UK). Informed written consent forms were obtained from the participants, and the protocol was approved by the ethics committee of the hospital (SCHGM 2014–16). We enrolled the asthmatics, who did not use systemic or inhaled corticosteroids for at least 4 weeks or more before sputum examination. Sputum was induced using isotonic saline that contained a short-acting bronchodilator. Sputum was prepared as follows: all portions with visibly greater solidity were carefully selected and placed in a pre-weighed Eppendorf tube. The samples were treated by adding eight volumes of 0.05% dithiothreitol (Calbiochem, San Diego, USA) to Dulbecco's phosphate-buffered saline (PBS). One volume of protease inhibitor (0.1 M methylenediaminetetraacetic acid and 2 mg/mL phenylmethylsulfonylfluoride) was added to 100 volumes of homogenized sputum, and the total cell count was determined using a hemacytometer. The sputum cells were collected by cytocentrifugation and 500 cells were examined after staining with Diff-Quick (American Scientific Products, Chicago, USA). Sputum samples that contained >10% squamous epithelial cells were excluded from the study. The remainder of the homogenized sputum sample was centrifuged at 1000*g* for 5 min, and the supernatant was collected and stored at −70 °C for subsequent protein analyses. Sputum samples were obtained from 34 normal controls (NC) and 101 asthmatics (BA) who had never smoked or quit smoking (<10 pack-years). The asthmatics were categorized into 4 inflammatory subtypes [17,18]: neutrophil dominant (≥60%, $n = 42$), eosinophil dominant (≥3%, $n = 27$), co-dominant (≥60%, ≥3% $n = 22$), and pauci-granulocytic (<60%, <3% $n = 10$) subtypes. The characteristics of the study subjects are summarized in Table 1.

2.2. Quantitative measurement using ELISA

S100A9 concentrations in sputum were measured using a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, San Diego, USA) according to the manufacturer's protocol and normalized to the total protein concentration of the samples, which was determined using a BCA Kit (Pierce Biotechnology, Rockford, USA). The detection limit for S100A9 was 0.1 ng/ml. Levels below the detection limit were considered 0 ng/ml. In addition, S100A8 was measured concentration using an ELISA kit (MyBioSource, San Diego, USA) in neutrophilic and eosinophilic sputum from uncontrolled, partially controlled and controlled asthmatics.

2.3. Production and delivery of recombinant S100A9 protein into the airways of mice

S100A9 protein was synthesized and purified using the pCMV6-XL4/S100A9 (Origene, Rockville, USA) human cDNA clone was purchased and amplified with a forward primer (5′-GAC GGA TCC ATG ACT TGC AAC ATG TCG-3′) that contained a *Bam*HI restriction enzyme site and a reverse primer (5′-GAC CTC GAG TTA GGG GGT ACC CTC CCC -3′) that contained an *Xho*I restriction enzyme site. The amplified S100A9 cDNA product was digested with *Bam*HI and *Xho*I restriction enzymes (New England Biolabs, Ipswich, USA) and the digested products were ligated into a pPRO-EX HTb protein expression vector (Life Technologies, Grand Island, USA). At each step, the sequences were confirmed by

Table 1
Clinical and laboratory characteristics of the study subjects.

	NC	BA	BA subtypes				p-value *
			Co-dominant	Neutrophilic	Eosinophilic	Pauci-granulocytic	
No.	34	101	22	42	27	10	
Enrollment age (year)	51.55 ± 3.2	50.68 ± 1.6	54.82 ± 3.6 §‖	54.97 ± 2.3§ ‖	43.44 ± 3.1	43.7 ± 3.2	0.007
Onset of asthma (year)	NA	47.20 ± 1.6	50.18 ± 3.9 §‖	51.40 ± 2.6§ ‖	40.26 ± 2.7	41.9 ± 3.5	0.002
Duration of asthma (year)	NA	5.65 ± 0.5	6.31 ± 1.3	5.93 ± 0.8	4.71 ± 0.7	5.56 ± 1.4	0.424
Smoke (NS/ES/CS)	22 / 12 / 0	87 / 14 / 0	18 / 4 / 0†	38 / 4 / 0 §	22 / 5 / 0 §	9 / 1 / 0	0.012
FVC(% pred.)	90.15 ± 2.4	84.48 ± 1.7†	84.73 ± 3.5	83.95 ± 3.2	82.81 ± 2.6	90.6 ± 3.4	0.615
FEV1(% pred.)	102.94 ± 2.6	85.19 ± 2.0†	86.95 ± 4.3	84.88 ± 3.7	82.15 ± 3.0	90.8 ± 5.1	0.609
FEV1/FVC (%)	84.32 ± 1.2	76.03 ± 1.1†	77.45 ± 1.8	75.14 ± 1.8	75.44 ± 2.1	78.2 ± 2.9	0.922
BMI(kg/m^2)	26.26 ± 1.4	24.37 ± 0.4	25.04 ± 0.8	24.11 ± 0.6	23.81 ± 0.7	25.37 ± 1.2	0.507
Atopy(N/Y/ND)	17 / 7 / 10	58 / 40 / 3‡	12 / 10 / 0	25 / 15 / 2	16 / 11 / 0	5 / 4 / 1	0.034
Sputum total cell count (x10^5/ml)	6.80 ± 1.4	8.96 ± 1.5	6.67 ± 1.5 §	14.98 ± 3.2§ ‖	3.55 ± 0.8	3.34 ± 0.7	0.027
Neutrophils (%)	51.65 ± 4.0	65.83 ± 2.6‡	76.03 ± 2.3 § ¶ ‖	86.87 ± 1.5§ ‖	37.27 ± 2.6	32.15 ± 5.4	<0.001
Eosinophils (%)	1.31 ± 0.9	11.96 ± 1.8†	12.59 ± 1.6 ¶ ‖	0.61 ± 0.1§	33.44 ± 4.2‖	0.19 ± 0.1	<0.001
Macrophages (%)	45.31 ± 4.0	16.81 ± 2.0‡	7.40 ± 1.7 § ‖	8.47 ± 1.3§‖	21.28 ± 3.2‖	60.47 ± 5.4	<0.001
Lymphocytes (%)	0.68 ± 0.2	1.67 ± 0.3†	1.10 ± 0.4	1.31 ± 0.3	2.63 ± 0.8	1.85 ± 0.7	0.179
Columnar cells (%)	1.04 ± 0.6	3.71 ± 0.7†	2.87 ± 1.2	2.72 ± 0.9	5.35 ± 1.8	5.31 ± 1.7	0.082

NC: normal controls, BA: asthmatics, NS: non-smoker, ES: ex-smoker, CS: current smoker, NA: not applied.
Patients were categorized into four inflammatory subtypes based on the percentages of neutrophils and eosinophils in sputum; co-dominant (percentage of neutrophils and eosinophils: ≥60% and ≥3%), neutrophil dominant (≥60%), eosinophil dominant (≥3%) and pauci-granulocytic subtypes. The normality of the distribution was evaluated using the Shapiro-Wilk test. Comparisons of the variables among the subtypes were performed using ANOVA (* indicates *p*-value) and then post hoc analyses using Tukey-HSD test was performed. The chi-squared test was used to compare qualitative variables. Data are presented as mean ± SEM.

† $P < 0.01$, compared with NC.
‡ $P < 0.05$, compared with NC.
§ $P < 0.05$, compared with eosinophilic BA.
¶ $P < 0.01$, compared with neutrophilic BA.
‖ $P < 0.01$, compared with pauci-granulocytic BA.