Expression of neuronal protein Kidins220/ARMS in the spleen and peripheral blood of mice following airway allergen challenge

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Abstract. Nerve growth factor (NGF), combined with the high-affinity tyrosine kinase receptor A (TrkA), has been reported to be involved in the pathogenesis of asthma. Ankyrin-rich membrane spanning/transmembrane substrate of protein kinase D (ARMS/Kidins220), a TrkA-binding protein, modulates the NGF signaling pathway. The aim of the present study was to investigate the expression of Kidins220/ARMS and the effect NGF has on the protein in the spleen and peripheral blood, following airway allergen challenge in mice. BALB/c mice were sensitized and challenged with ovalbumin. The effects of NGF on Kidins220/ARMS in the spleen and peripheral blood of mice were assessed by administering anti-NGF antibody. Expression of ARMS, interleukin (IL)-1β and IL-4 in the spleen and peripheral blood was observed by reverse transcription-polymerase chain reaction, western blot analysis and immunohistochemistry. Pathological changes in the bronchi and lung tissues were examined by hematoxylin and eosin staining. Results showed that Kidins220/ARMS, IL-1β and IL-4 were overexpressed in the spleen and peripheral blood following allergen challenge, compared with the control mice. Moreover, following treatment with anti-NGF, the levels of Kidins220/ARMS, IL-1β and IL-4 in the mice were downregulated. Therefore, the results of the present study showed that Kidins220/ARMS is expressed in the spleen and peripheral blood of normal BALB/c mice and may participate in the immuno-inflammation of asthma through the NGF-mediated signaling pathway.

Introduction

Airway inflammation is an essential characteristic of allergic asthma (1). Findings of previous studies have demonstrated that a number of cell types and cytokines contribute to this inflammation and increase bronchial sensitivity to various stimuli. Nerve growth factor (NGF) is a well-studied mediator in allergic asthma (2-5). NGF plays a critical role in neuro-immune mechanisms of asthma, which induce airway inflammation by causing neurogenic inflammation and enhancing the effects of immune cells. NGF may originate from the infiltrating inflammatory cells and it has been reported that NGF formation has been found in various immune organs, including the spleen, lymph nodes, thymus and immune cells, such as mast cells, eosinophils, and B and T cells (6). The NGF-mediated tyrosine kinase receptor A (TrkA) pathway has been reported to participate in the pathogenesis of asthma. Interleukin (IL)-1β is important in airway responsiveness and inflammation in bronchial asthma (7,8). Moreover, IL-4 is a T-helper (Th)2 cytokine that has a crucial role in allergic inflammation and airway remodeling (9,10).

The ankyrin-rich membrane spanning protein (ARMS), originally identified as a substrate for protein kinase D (Kidins220), serves as a novel downstream target of Trk receptor tyrosine kinases. A previous study showed that TrkA and ARMS association persisted for a number of hours (2). NGF-induced neuronal differentiation is mediated by TrkA receptor and ARMS association directly, which leads to sustained mitogen-activated protein kinase activation, resulting in neurite outgrowth (11,12). Previous studies have shown that expression of ARMS in the lung increased in asthmatic mice, compared with controls (13,14). Taken together, results of those studies suggest that ARMS plays a fundamental role as a signaling molecule in the NGF pathway. NGF and TrkA participate in the pathogenesis of asthma. However, the effect of Kidins220/ARMS in the immuno-inflammation of asthma has not been adequately addressed.

The aim of the current study was to investigate the role of the NGF-mediated Kidins220/ARMS pathway in immunoenflammation of asthma. Results indicate that Kidins220/ARMS was overexpressed in the spleen and peripheral blood of asthmatic BALB/c mice and may participate in immuno-inflammation of asthma through the NGF-mediated signaling pathway.

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Materials and methods

Animals. Female BALB/c mice (age, 6-8 weeks; weight, 18-22 g) were purchased from Beijing Laboratory Animal Research Center (Beijing, China) and maintained under specific pathogen-free conditions. All animal experiments were approved by the Ethics Committee for Animal Use and Care at the Institute of Education of China Medical University (Shenyang, China).

Sensitization and challenge of animal model. In total, 42 BALB/c mice were randomly divided into four groups: Control, ovalbumin (OVA), anti-NGF and anti-ARMS groups. Groups contained 10 mice, with the exception of the OVA group which had 12 mice. An animal model of asthma was established according to previous studies (15,16) with specific modifications. In the OVA group, mice were sensitized to 20 µg OVA absorbed in 2.0 mg aluminium hydroxide, per injection, administered intraperitoneally on day 1. On days 8, 15 and 22, mice were sensitized again to 10 µg OVA absorbed in 1.0 mg aluminium hydroxide, per injection. On day 23, mice were administered inhaled aerosols of 4% OVA in phosphate-buffered saline (PBS) for 25-30 min (until the onset of bronchial obstruction) daily for 7 consecutive days. Mice in the anti-NGF group were also subjected to OVA sensitization and asthma induction in the same manner. Intranasal application of 50 µl (1:50 dilution) polyclonal goat antimouse NGF antibody (1:4,000 dilution blocks bioactivity of 5 ng/ml NGF; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) dissolved in sterile PBS was performed 3 h prior to each airway allergen challenge in the anti-NGF group. Intranasal treatment with anti-NGF antibody was performed according to the methods of Braun et al (17), Choi et al (18) and Nagai et al (19), with minor modifications. Mice in the anti-ARMS group underwent intranasal application of goat polyclonal anti-ARMS antibody that had been diluted 1:25 in PBS (Santa Cruz Biotechnology, Inc.) (14). Mice in the control group were administered PBS alone by injection and were challenged with PBS. All the animals were humanely sacrificed within 24 h following the last OVA or PBS exposure.

Immunohistochemical detection of ARMS in the spleen. Excised spleen tissue was infused with 4% paraformaldehyde and postfixed in sucrose for 24 h. The spleens were then immersed in 30% sucrose in PBS for an additional 24 h, frozen in liquid nitrogen and stored at -80˚C. Frozen sections were blocked with rabbit serum for 30 min at room temperature and then incubated with goat polyclonal anti-ARMS antibody (1:50 dilution; 0.01 mol/l PBS; pH 7.4) at 4˚C overnight. After being washed twice with PBS, the sections were incubated with 1:200 antigen IgG conjugated to fluorescein isothiocyanate (Santa Cruz Biotechnology, Inc.) for 30 min at room temperature. For the negative control, PBS was used instead of primary antibodies. The slides were then observed and images were captured using a microscope (Olympus, Tokyo, Japan).

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) for ARMS mRNA in the spleen and peripheral blood. RT-PCR was performed using the Takara RNA PCR kit (AMV) 3.0 (Takara Bio, Inc., Dalian, China). Total RNA was extracted from the spleen tissue and white blood cells (WBCs) of peripheral blood and purified using RNAout (Takara Bio, Inc.), according to the manufacturer’s instructions. The primers for ARMS were 5'-AGG AGG ATG GGC GGA AGT C-3' (forward) and 5'-CAT AAA CAG GCT ACG GGA GG-3' (reverse) and primers for GAPDH were 5'-AGC TGG CAA AGT GGA GAT TGT TG-3' (forward) and 5'-CAG TCT TCT GGG TGG CAG TGA T-3' (reverse). The reaction was carried out for 30 cycles using a 94˚C, 30 sec denaturing step, a 54˚C, 40 sec annealing step and a 72˚C, 1 min extension step. The procedures conformed to a standard protocol. For the semi-quantitative measurement of ARMS density, the involved slides were photographed and assessed using computer-aided image analysis (Motic Images Advanced 3.2; Motic, Xiamen, China).

Western blot analysis for ARMS, IL-1β and IL-4 in the spleen and peripheral blood. Blood was collected from the eyeball using heparin, then diluted 2-fold with lymphocyte separation medium (ICN-Flow Laboratories, Costa Mesa, CA, USA) and centrifuged for 20 min at 9,690 x g. WBCs were collected and stored at -70˚C. Protein homogenates from the samples of WBCs and spleen tissues were prepared by rapid homogenization in 10 volumes of ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Protein concentrations of the cell and tissue lysates were determined using the Enhanced BCA Protein assay kit (Beyotime Institute of Biotechnology) and the supernatants were boiled in sodium dodecyl sulfate sample buffer for 5 min. Equal amounts of lysate proteins were separated using 7.5 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). Following blocking, the blots were incubated with specific primary antibody overnight at 4˚C and then incubated for 1 h with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence kit with a Lumino-Image analyzer (Tai tec, Tokyo, Japan). Integrated density values were analyzed using a computerized image analysis system (Fluor Chen 2.0; Bio-Rad, Hercules, CA, USA) and normalized to those of β-actin.

Statistical analysis. Data were analyzed using statistical software (SPSS 15.0; SPSS Inc., Chicago, IL, USA). Data are expressed as mean ± SEM. Differences between groups were analyzed, as appropriate, using t-tests and one-way analysis of variance followed by the Fisher’s least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

Results

Immunohistochemical detection of ARMS in the spleen. Expression of ARMS, mainly in the cytoplasm, was visible as brownish yellow particles (Fig. 1A). Optical density analysis revealed that the expression of ARMS in the OVA group increased when compared with the control group. Expression of ARMS in anti-NGF group was lower than that in the asthmatic model group (P<0.05; Fig. 1B).

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Expression of ARMS mRNA in the spleen and peripheral blood. RT-PCR was used to detect the mRNA expression levels of ARMS in the spleen (Fig. 2A) and WBCs of the peripheral blood (Fig. 2B). In the control group ARMS mRNA expression levels were low, but were markedly increased in the OVA group (P<0.01). However, anti-NGF treatment significantly decreased ARMS mRNA upregulation caused by OVA (P<0.01; Fig. 2C and D).

Expression of ARMS protein in the spleen and peripheral blood. Protein levels of ARMS in the spleen (Fig. 3A) and WBCs of peripheral blood (Fig. 3B) were detected by western blot analysis. In the control group ARMS protein was expressed at low levels, but these levels were markedly increased in the OVA group (P<0.01 or P<0.05). However, anti-NGF or anti-ARMS treatment significantly decreased ARMS upregulation caused by OVA (P<0.01 or P<0.05; Fig. 3C and D).

Expression of IL-1β and IL-4 protein in the spleen and peripheral blood. Expression of IL-1β (Fig. 4A) and IL-4 (Fig. 4B) in the spleen was detected. In the control group IL-1β and IL-4 proteins were expressed at low levels, but these levels were markedly increased in the OVA group (P<0.01 or P<0.05). However, anti-NGF or anti-ARMS treatment significantly decreased IL-1β and IL-4 upregulation caused by OVA (P<0.01 or P<0.05; Fig. 4C and D).

Discussion

Kidins220/ARMS is a multifunctional scaffold protein that interacts with a number of transmembrane receptors, including Trks and p75NTR (11,20,21). Intracellular scaffolding proteins have a key role in the association between various receptors, which induce cross-talk of downstream signaling (22). Kidins220/ARMS functions as a unique downstream adaptor protein of Trk receptor tyrosine kinases (23), which activates downstream enzymes by binding with NGF-TrkA (24). Furthermore, NGF-TrkA is important in bronchial hyper-responsiveness and inflammation (25,26) in asthma. Although it has been previously demonstrated that the expression of ARMS in the lung increased in asthmatic mice compared with that in control mice (8,9), little was known about the involvement of Kidins220/ARMS in the spleen and blood.

In the present study, the expression of ARMS in the spleen and WBCs of peripheral blood, as measured by RT-PCR, western blot analyses and immunohistochemistry, increased in the OVA group compared with the control group. Intervention
with anti-NGF antibody resulted in a significant reduction in ARMS expression in the spleen and WBCs of peripheral blood in the OVA group. ARMS blockade was also associated with a reduction of inflammatory cytokines, including IL-1β and IL-4, in the spleen of OVA-sensitized mice. Previously, studies indicated that Th2 cells overproduced cytokines (e.g., IL-4, 5 and 13) and histamines in allergic asthma (27,28). Th1-associated IL-1β and TNF-α, as well as Th2-associated IL-4, are considered to be the key cytokines that induce airway hyper-responsiveness, infiltration of eosinophils into the lungs and mucus secretion in allergic asthma (29,30). The immunopathological hallmark of allergic asthma is the infiltration of eosinophils and Th2 cells in affected tissue. Th2 cells are prominent for immune deviation in allergic asthma (31,32), but the immunological mechanism requires further investigation.

Allergic asthma is a Th2-dominant disease characterized by increased airway inflammation and IgE production, as well as airway hyper-reactivity. It has been reported that NGF may affect the viability of peripheral blood eosinophils from healthy donors (33,34). Moreover, previous studies have revealed elevated serum levels of NGF in subject responses to allergic asthma, particularly in patients with severe allergic asthma (35). There was a regular expression of ARMS in the spleen and WBCs of peripheral blood, while a higher expression was shown in the asthmatic group as compared to the control group. Th1/Th2 cytokine immune imbalance may indirectly induce airway neurogenic inflammation by regulating NGF expression. Furthermore, NGF may promote and magnify immunological inflammation. IL-1, a lymphocyte-activating factor, enhances adhesion and gasification.
of inflammatory cells to the airway inflamed regions (36), inducing the endosomatic immunological process. This finding indicates that NGF-mediated ARMS signaling may regulate immune-activation by upregulating IL-1β and IL-4 in the spleen and WBCs of peripheral blood in asthma. Thus, the present study on Kidins220/ARMS in the spleen and peripheral blood may clarify the mechanism involved in asthma. In addition, targeting Kidins220/ARMS on immune cells may be a new promising therapeutic option for the treatment of allergic asthma.

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References


