Effects of Corticosteroids on Osteopontin Expression in a Murine Model of Allergic Asthma

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Key Words

Osteopontin • Corticosteroid • Mouse • Dendritic cells • CD11c • Bronchial asthma

Abstract

Background: Osteopontin (OPN) contributes to the development of T helper 1 (Th1)-mediated immunity and Th1-associated diseases. However, the role of OPN in bronchial asthma is unclear. Corticosteroids reduce airway inflammation, as reflected by the low eosinophil and T-cell counts, and the low level of cytokine expression. We investigated OPN production and the inhibitory effects of corticosteroids on OPN production in a murine model of allergic asthma. **Methods:** BALB/c mice were sensitized by intraperitoneal injections of ovalbumin (OVA) with alum. Some mice received daily injections of dexamethasone (DEX) or phosphate-buffered saline for 1 week. All OVA-challenged mice were exposed to aerosolized 1% OVA for 30 min an hour after these injections. After the OVA challenge, the mice were killed, and bronchoalveolar lavage (BAL) fluid and lung tissue were examined. **Results:** The levels of OPN protein in BAL fluid

and OPN mRNA in lung tissue increased after OVA challenge. Most OPN-expressing cells were CD11c+ cells and some were T cells. DEX decreased the levels of OPN protein in the BAL fluid, and those of OPN mRNA and OPN protein in lung tissue. **Conclusions:** OPN may play an important role in allergic bronchial asthma. Corticosteroids inhibit OPN production in mice with allergic asthma. The beneficial effect of corticosteroids in bronchial asthma is partly due to their inhibitory effects on OPN production.

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Introduction

Osteopontin (OPN, also known as SPP) is a secreted phosphorylated acidic glycoprotein that performs numerous functions. It contains the amino acid domains RGD and SVVRGLR (SLAYGLR in mouse OPN) [1, 2], which bind various types of integrins, and it facilitates the adhesion and migration of several inflammatory cells [3–7]. Many studies have demonstrated that OPN contributes to the development of T helper (Th1)-mediated

immunity and Th1-associated diseases [8, 9]. Increased levels of OPN expression have been observed in several Th1-associated diseases, including rheumatoid arthritis [10, 11], sarcoidosis [12, 13], tuberculosis [14, 15], and multiple sclerosis [16, 17].

Asthma is an inflammatory disease characterized by chronic infiltration of the airway by several types of inflammatory cells, including lymphocytes, eosinophils, and dendritic cells (DCs) [18, 19]. T lymphocytes have been suggested to play a role in the pathogenesis of bronchial asthma. Th2 cells produce cytokines such as interleukin (IL)-4, IL-5, and IL-13, that induce immunoglobulin (Ig) E production and eosinophil activation. The number of Th2 cells in the airway increases in allergic asthma and further increases after allergen challenge [20, 21]. DCs are crucial for determining the functional outcome after exposure of lung tissue to allergens. Antigen presentation by myeloid DCs leads to the sensitization of Th2 cells, which is a typical characteristic of allergic diseases [22].

However, the role of OPN in Th2-associated allergic asthma has not yet been elucidated thus far. In the present study, we examined the effects of corticosteroids on OPN production in a murine model of allergic asthma.

Animals and Methods

Animals

Five-week-old BALB/c male mice were purchased from San-kyo Co. Inc. (Tokyo, Japan). All experiments were performed in accordance with the guidelines for animal experimentation issued by Showa University (Tokyo, Japan).

Antigen Sensitization and Challenges

The protocol for antigen sensitization and challenge has been previously described [23, 24]. Mice were actively sensitized by intraperitoneal injection of 50 µg ovalbumin (OVA) (grade V; Sigma, Tokyo, Japan) with 1 mg alum (Sigma) on days 0, 7, 14, and 21 of the experiment. Mice were exposed to aerosolized 1% OVA (MIPS Co. Inc., Osaka, Japan) for 30 min on days 28–35 for antigen sensitization. On day 38, the mice were challenged with aerosolized 1% OVA. Following this, they were then killed, and 24 h after the challenge, bronchoalveolar lavage (BAL) fluid and lung tissue samples were obtained. Control mice were sensitized and challenged with phosphate-buffered saline (PBS).

Study Design

Mice were divided into three groups: (1) PBS-sensitized, PBS-challenged, and PBS-treated mice (control group); (2) OVA-sensitized, OVA-challenged, and PBS-treated (OVA group), and (3) OVA-sensitized, OVA-challenged, and dexamethasone (DEX)-treated mice (DEX group).

DEX Injection

The mice were administered an intraperitoneal injection of 3 mg/kg of water-soluble DEX (Sigma) in PBS 1 h before OVA administration on days 28–35 and on day 38. The control mice received injections of 0.2 ml of PBS.

BAL

To perform BAL, the trachea was cannulated under anesthesia, and PBS (0.6 ml) at room temperature was injected three times into the lung via the trachea. The BAL fluid was withdrawn and stored on ice. The cells in the BAL fluid were counted, and a cytopsin sample (Shandon Scientific, Runcorn, UK) was prepared. Cells were stained with Diff-Quick (Kokusai Shinyaku Co., Kobe, Japan), and a differential count of 200 cells was performed using standard morphologic criteria. The remaining BAL fluid was centrifuged at 300 g for 10 min, and the supernatant was collected and stored at –80°C until OPN measurement. The level of OPN in the BAL fluid was measured using a mouse OPN enzyme immunoassay kit (Assay Designs, Ann Arbor, Mich., USA) according to the manufacturer's instructions.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from lung specimens using Isogen reagent (Nippon Gene Co. Ltd, Tokyo, Japan). First-strand cDNA was synthesized using a cDNA-extraction kit (Amersham Biosciences, Little Chalfont, UK). OPN expression was evaluated by real-time quantitative polymerase chain reaction (PCR), which was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, Calif., USA). An oligonucleotide probe was designed to anneal to the target gene between the two PCR primers. The probe was fluorescently labeled with 6-carboxyfluorescein (reporter gene) on the 5' end and with 6-carboxytetramethylrhodamine (quencher dye) on the 3' end. A similar probe and PCR primers were purchased for 18S ribosomal RNA (Applied Biosystems). This second probe incorporated VIC™ as the reporter dye (Applied Biosystems). In the case of both genes, PCR was performed using the primers and probes as well as cDNA prepared from the cells. As the polymerase moved across the gene during the reaction, it cleaved the dye from one end of each probe. This resulted in the emission of fluorescence, which was measured using the Sequence Detection System. Emissions were recorded for each cDNA and can then be converted to determine the gene expression level after normalization to the expression of 18S ribosomal RNA.

Data analysis was performed using the 7900HT Sequence Detection System software (version 2.1; Applied Biosystems). Data from real-time PCR were plotted as the ΔRn fluorescence signals versus the cycle number. ΔRn was calculated using the following equation: $\Delta Rn = (Rn+) - (Rn-)$, where $Rn+$ is the fluorescence signal of production and $Rn-$ is the fluorescence signal of the baseline emission. Ct was defined as the cycle number at which the ΔRn crossed threshold. Fold changes in OPN cDNA were determined as follows: fold change = $2^{\Delta Ct}$ [23, 25].

Immunohistochemical Examination

Lung tissue specimens were fixed in 4% neutral buffered formalin and embedded in paraffin. The paraffin-embedded specimens were sectioned, and the sections were deparaffinized for 20 min in xylene, dehydrated for 5 min each in 100, 95, 90, and 70% ethanol, and then washed with PBS for 10 min.

Fig. 1. OPN protein in BAL fluid and the effect of DEX on the OPN protein. The OPN protein levels in BAL fluid were significantly higher at 24 h after antigen challenge in the OVA-challenged mice than in the control mice. DEX significantly decreased the OPN protein levels in BAL fluid at 24 h after OVA challenge. ** OVA compared with control ($p < 0.01$). + DEX compared with OVA ($p < 0.05$).

Fig. 2. OPN mRNA in the lungs and the effect of DEX on OPN mRNA. OPN mRNA expression in the lungs was significantly higher at 24 h after antigen challenge in the OVA-challenged mice than in the control mice. DEX significantly reduced OPN mRNA expression in the lungs. ** OVA compared with control ($p < 0.01$). + DEX compared with OVA ($p < 0.05$).

After the slides were deparaffinized, they were immersed in 10 mM citric acid buffer (pH 6.0) and heated in an autoclave at 121 °C for 10 min. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide in methanol for 30 min.

OPN Staining

Tissue sections were incubated overnight with goat anti-mouse polyclonal OPN antibody (dilution, 1:100; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at 4 °C overnight. Secondary antibodies were detected by incubation with Histofine Simple Stain mouse MAX-Po (goat) reagent (Nichirei Co., Tokyo, Japan) for 30 min at room temperature. Peroxidase activity was detected with 3-amino-9-ethyl carbazole solution (Nichirei). Sections were counterstained with Mayer's hematoxylin.

Immunofluorescence Staining for OPN, CD11c, and CD4

Lung tissue specimens were submerged in 4% formaldehyde in PBS at 4 °C for 2 h. They were incubated overnight with 18% sucrose in PBS. They were then blotted dry and embedded in optimal cutting temperature compound, and frozen at -80 °C. Frozen tissues were cut sectioned and mounted on slides. The slides were covered with goat blocking solution, 10% goat serum, 1% bovine serum albumin (BSA), 0.5% Tween 20 in PBS, for 1 h at room temperature. They were then covered with rabbit anti-mouse OPN antibody (Assay Designs) diluted in Triton/BSA PBS (PBS containing 0.3% Triton X-100, 1% BSA). They were also covered with one of the following two antibodies: mouse anti-mouse CD11c antibody (Miltenyi Biotec, Auburn, Calif., USA) or mouse anti-mouse CD4 antibody (BD Bioscience Pharmingen, San Diego, Calif., USA) diluted overnight in Triton/BSA PBS at 4 °C. The slides were covered with fluorescent-labeled anti-primary antibody (goat anti-mouse IgG Alexa 488, and goat anti-rabbit IgG Alexa 594) (Molecular Probes, Eugene, Oreg., USA) diluted in

Triton/BSA PBS for 2 h at room temperature. They were then rinsed with Tris-buffered saline (pH 8.6) for 15 min. The tissues were mounted in antifade glycerol (antifade kit Fluoro-mount; Molecular Probes) and coverslipped. Similar sections were imaged for fluorescence detection (Alexa 488 and Alexa 594).

Statistical Analysis

The results are expressed as the means $\pm$ SE. Significant differences between results were detected using Wilcoxon's test. Differences were considered significant at $p < 0.05$.

Results

OPN Protein in BAL Fluid and the Effect of DEX on OPN Protein

The OPN protein levels in BAL fluid were significantly higher at 24 h after antigen challenge in the OVA-challenged mice than in the control mice. DEX significantly decreased the OPN protein levels in BAL fluid at 24 h after the OVA challenge (fig. 1).

OPN mRNA in the Lung and the Effect of DEX on OPN mRNA

OPN mRNA expression in the lung was significantly higher at 24 h after antigen challenge in the OVA-challenged mice than in the control mice. Furthermore, DEX significantly inhibited the expression of OPN mRNA in the lungs (fig. 2).

a Control

b OVA

c DEX

d OPN+ cell

e CD11c+ cell

f OPN+ CD11c+ cell

g OPN+ cell

h CD4+ cell

i OPN+ CD4+ cell

Fig. 3. Immunohistochemical staining for OPN in lung tissues and the effect of DEX on OPN expression. In the control mice, only the bronchial epithelial cells were strongly positive for OPN (**a**). In contrast, in the OVA-challenged mice, OPN staining was predominately observed in the infiltrating cells, and to a lesser extent, in the bronchial epithelial cells 24 h after OVA challenge (**b**). Immunohistochemical staining for OPN was significantly reduced in the DEX-treated mice (**c**). **a** Control mice; **b** OVA mice;

c DEX mice. Most OPN-expressing cells that stained positively were mainly CD11c cells in mice (**d–f, j**). In contrast, a few OPN-expressing cells belonged to the T-cell population (**g–i, k**). **d** OPN (red); **e** CD11c (green), and **f** double-positive cells (yellow). **g** OPN (red); **h** CD4 (green), and **i**, double-positive cells (yellow). **j** The numbers of CD11c-, OPN- and double-positive cells were counted. **k** The numbers of CD4-, OPN- and double-positive cells were counted.

Immunohistochemical Staining for OPN and the Effect of DEX on OPN Expression

Only the bronchial epithelial cells were strongly positive for OPN in control mice (fig. 3a). In contrast, the infiltrating cells were predominately positive for OPN, whereas bronchial epithelial cells were positive to a lesser extent in the OVA-challenged mice, as determined at 24 h after the OVA challenge (fig. 3b).

To identify the various types of infiltrating cells, we performed double immunofluorescence staining for OPN and CD11c, OPN and CD4.

Most OPN-expressing cells stained positively for CD11c (fig. 3d–f, j). In contrast, a few OPN-expressing cells belonged to the T-cell population (fig. 3g–i, k).

Significantly fewer cells stained positive for OPN in the DEX mice group (fig. 3c).

Discussion

In the present study, we showed that OPN expression in the lungs increases in mice with allergic asthma. In addition, we found that most OPN-expressing cells are pos-

itive for CD11c, and a few are T-cells. Kohan et al. [26] reported that eosinophils also express OPN.

DCs are primary antigen-presenting cells involved in interactions with T cells leading to the proliferation of Th1 or Th2 cell types [27]. The findings of many studies conducted on animals and humans strongly suggest that DC function is critical to the development and regulation of the allergic state [28, 29].

We previously reported that significant upregulation of OPN expression is associated with successful venom immunotherapy (VIT). We evaluated OPN expression by using a gene chip microarray and clustering analyses and reported that OPN is a potential biomarker for VIT. OPN expression in monocytes and monocyte-derived DCs is significantly increased in subjects who successfully completed immunotherapy and show clinical tolerance compared to those who are hypersensitive to allergens and have not undergone immunotherapy [30, 31].

Previous studies have investigated the potential involvement of the Th2-associated allergic responses in airway inflammation [32] and mast cell degranulation [33]. We reported that OPN is expressed in the airways of asthmatic patients, and the level of OPN protein is positively

correlated with the percentage of eosinophils in induced sputum collected from asthmatic patients. Anti-OPN antibodies have been shown to significantly attenuate OVA-induced eosinophilic airway inflammation in mouse models of allergic asthma [34]. Xanthou et al. [32] reported that OPN inhibited this process because they found that the administration of anti-OPN antibodies enhances allergic airway inflammation. In contrast, our results indicate that OPN plays a role in the pathogenesis of asthma by mediating migration of eosinophils into the airways [34]. Kohan et al. [26, 35] reported that OPN expression is significantly correlated with the structural changes observed in a murine model of chronic allergen-induced airway remodeling. Thus, OPN plays a role in the airway remodeling observed in asthma. In the present study, we showed that OPN performs important functions in allergic bronchial asthma.

Corticosteroids reduce airway inflammation through multiple mechanisms. First, they directly inhibit several types of inflammatory cells including the numbers of eosinophils and the quantity of cytokines, such as GM-CSF and IL-5 [36, 37]. Second, corticosteroids inhibit the activation of T lymphocytes and their release of cytokines (IL-4, IL-5, and IL-13) and chemokines (RANTES, eotaxins) by T lymphocytes [36, 37]. Many inflammatory genes are expressed in asthmatic states. Corticosteroids produce their effects on responsive airway cells in asthma by activating glucocorticoid receptors to regulate the transcription of target genes. Glucocorticoid receptors may interact with the transcription factors AP-1 and nuclear factor- κ B, which may be important for mediating the inflammatory effects of cytokines and chemokines in the lung [38, 39]. Corticosteroids inhibit several aspects of the inflammatory process by decreasing gene transcrip-

tion and inhibit the transcription of most cytokines and chemokines that are important in the pathogenesis of asthma. Third, corticosteroids have strong inhibitory effects on DC function. They not only prevent the differentiation of monocytes into immature DCs and block maturation of DCs, but also induce apoptosis of DCs and may affect recruitment of DCs to the airway [27, 40, 41].

Vascular calcification is present in many pathological conditions and is recognized as a strong predictor of future cardiovascular events. Kirton et al. [42] reported that DEX downregulated the postulated calcification-inhibitor gene OPN in pericytes. DEX treatment results in a decrease in nuclear factor- κ B protein in the nucleus.

In this study, we have demonstrated in a murine model of allergic asthma that OPN expression in the lungs is upregulated after antigen challenge. We also found that corticosteroids inhibited OPN production. We therefore conclude that the beneficial effects of corticosteroids in bronchial asthma are partly due to their direct inhibitory effects on OPN production.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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