Increased galectin-9 expression in alveolar macrophages in interstitial lung disease

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Abstract

Background: Galectin-9 is a multifunctional β-galactoside-binding lectin, which acts as an eosinophil chemoattractant, an inducer of apoptosis, and a negative regulator of T helper type 1 immunity. The purpose of this study was to examine the expression of galectin-9 in interstitial lung diseases.

Methods: Murine anti-human-galectin-9 monoclonal antibodies were developed for this experiment. We enrolled 15 patients with idiopathic pulmonary fibrosis, 5 with hypersensitivity pneumonitis, 9 with drug-induced pneumonitis, 8 with eosinophilic pneumonia, 31 with sarcoidosis, and 5 healthy volunteers as controls. The localization of galectin-9 in lung tissues was evaluated by immunohistochemical analysis and the protein levels of galectin-9 were measured in bronchoalveolar lavage fluid (BALF) by sandwich ELISA. The galectin-9 levels were also evaluated in THP-1 cells by sandwich ELISA and Western blotting in the presence and absence of phorbol 12-myristate 13-acetate (PMA) stimulation.

Results: The expression of galectin-9 was demonstrated immunohistochemically in alveolar macrophages. In BALF, the levels of galectin-9 were approximately 7-fold higher in patients with IPF ($p=0.032$), hypersensitivity pneumonitis ($p=0.016$), and drug-induced pneumonitis ($p=0.006$) in comparison with healthy individuals. In patients with IPF, the protein level correlated with serum lactate dehydrogenase (LDH) ($r=0.57$, $p=0.033$) and serum KL-6 ($r=0.60$, $p=0.026$). PMA-stimulated THP-1 cells released large quantities of galectin-9.

Conclusions: Our study showed that galectin-9 is highly expressed in macrophages in interstitial lung diseases, suggesting a role in immunological response in the development of these diseases.

Introduction

Galectin-9 is a 35 kDa β-galactoside-binding lectin belonging to an ever-growing family of animal lectins. It was first cloned from tumor tissues of Hodgkin's disease, and has been frequently associated with blood and tissue eosinophilia. This lectin belongs to the tandem-repeat subfamily that is characterized by the presence of two distinct carbohydrate recognition domains connected by a linker peptide.

Galectin-9 has been reported to possess a variety of biological functions. Previous studies have shown to be an eosinophil chemoattractant, and it was named egalectin. In addition, galectin-9 has been shown to induce apoptosis in a variety of cells including activated T cells. Furthermore, recent investigations have disclosed that galectin-9 is closely involved with negative regulation of T helper type 1 immunity. Thus, the role of galectin-9 in inflammation has recently become clearer. In vivo studies have reported galectin-9 gene
expression in diseases such as nephrotic serum nephritis, experimental autoimmune encephalomyelitis, and lipopolysaccharide-induced inflammation. While the galectin-9 gene is normally expressed in these organs, these reports showed that the administration of large amount of galectin-9 led to inhibition of the progression of diseases. These reports suggest that galectin-9 plays a role in suppressing inflammation.

In mice, galectin-9 gene expression has been found in various tissues, such as the thymus, small intestine, liver, lung, kidney, spleen, and cardiac and skeletal muscle. The expression of galectin-9 in humans has been shown in drug-induced liver injury and mucosa of nasal polyp. However, knowledge concerning the expression levels of galectin-9 in the human lung remains limited.

Eosinophilic pneumonia is a disease where the pulmonary parenchyma is infiltrated by inflammatory cells comprising conspicuous eosinophils, and is characterized by the increased levels of expression of IL-5, RANTES, and Eotaxin in bronchoalveolar lavage fluid (BALF). RANTES and Eotaxin are chemokines implicated in the migration and activation of eosinophils. IL-5 is a cytokine which plays a role in the regulation of eosinophils.

To gain insight into the role of galectin-9, we evaluated its expression levels in human tissues from normal lung and from interstitial lung diseases, including eosinophilic pneumonia, idiopathic pulmonary fibrosis (IPF), hypersensitive pneumonitis, drug-induced pneumonitis, and sarcoidosis using newly developed murine anti-human-galectin-9 monoclonal antibodies.

**Methods**

**Study population**

The study population included 15 patients with IPF, 5 with hypersensitive pneumonitis, 9 with drug-induced pneumonitis, 8 with eosinophilic pneumonia, 31 with sarcoidosis, and 5 healthy volunteers as control, who underwent bronchoalveolar lavage (BAL) in Tokyo Medical University Hospital. The characteristics of the study population are shown in Table 1. This study was conducted in accordance with the guidelines of the Declaration of Helsinki, which was approved by the ethics committee of the Tokyo Medical University. All patients provided written informed consent. The diagnosis of IPF was established based on the American Thoracic Society (ATS)/European Respiratory Society (ERS) criteria. In the category of hypersensitivity pneumonitis, there were 3 patients with Japanese summer-type hypersensitivity pneumonitis, 1 with bird fancier’s lung disease, and 1 with humidifier lung. The pathogens of hypersensitivity pneumonitis were determined by challenge test or examination of precipitating antibody. The details of drug-induced pneumonitis were as follows: Shosaikoto (n=2), Saireito (n=1), bleomycin (n=1), pyrimidine fluoride (n=1), gefitinib (n=1), carbamazepine (n=1), isoniazid (n=1), and aspirin (n=1). The diagnoses of drug-induced pneumonitis were made by meticulously excluding all other possible causes. The eosinophilic pneumonia category consisted of 5 patients with chronic eosinophilic pneumonia and 3 with acute eosinophilic pneumonia, all of which were diagnosed by pathological findings. The diagnosis of sarcoidosis was based on previously published criteria.

**Bronchoalveolar lavage**

BAL was performed from the affected segment of lung using the standard procedure. Briefly, three fractions of 50 ml each of sterile saline were instilled and recovered. Cell-free supernatants obtained by centrifugation were stored at -80°C before use.

**Production of anti-Galectin-9 monoclonal antibodies**

The production of CHO-K1 cells expressing galectin-9 has already been described. Recombinant galectin-9 was obtained from harvested supernatants of these cells. Forty-nine mAbs, which were named ECA1 to 49, were raised in 4-week-old female BALB/c mice (Charles River Japan, Yokohama, Japan) by immunization with

**Table 1**

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the recombinant galectin-9. In brief, the recombinant human galectin-9 was injected 3 times at weekly intervals into footpads of mice. At the first immunization, Freund's complete adjuvant (Valeant (ICN), Costa Mesa, CA) was used. Popliteal, iliac, and inguinal lymph node cells were fused with mouse myeloma cell lines using polyethylene glycol 1,500 (Roche Diagnostics, Basel, Switzerland). Hybridomas were screened for their ability to react with human galectin-9 in ELISA. The selected hybridomas were cultured for approximately two weeks. The supernatants were harvested and purified. Specific mAbs to galectin-9 were selected by ELISA using galectin-9 coated plates. The immunological specificities of ECA-8, ECA-42, and ECA-30 are not described here.

**Immunohistochemistry**

Immunohistochemical analysis was performed in lung tissues obtained by trans-bronchial lung biopsy or video-assisted thoracoscopy. We obtained uninvolved lung tissues from the resected lobes from two patients with lung cancer, who had received lobectomy, as control lung tissue. Immunohistochemical staining was performed using previously described procedures. In brief, 3 μm sections of paraflin-embedded tissue were deparaffined and hydrated. Antigenic activity was retrieved by treatment in citric acid buffer at 98°C for 1 h, and endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol. Nonspecific staining was prevented by incubation with 1% normal horse serum. Sections were incubated with the primary antibody of the mAb ECA30, and mouse IgG1k (BD Pharmingen, San Diego, CA) which was used as a negative control. After washing, immunoreactivity was determined with a biotinylated horse universal secondary antibody and the avidin-biotin-peroxidase complex (ABC), both of which were supplied as the Vectastain Elite ABC kit (Vector Lab, Burlingame, CA). Color was developed with diaminobenzidine tetrahydrochloride hydrogen peroxide medium. Sections were counterstained with hematoxylin.

**Cell culture and stimulation with PMA**

The human monocytic cell line, THP-1, (Japanese Cancer Research Resources Bank, Tokyo, Japan) was cultured in RPMI 1,640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal calf serum (Sigma-Aldrich) and antibiotics at 37°C under 5% CO₂ atmosphere. Various doses of phorbol 12-myristate 13-acetate (PMA) were added to the medium for stimulation of the THP-1 cells. After 48 h, supernatants were collected and stored at -80°C before analysis. The THP-1 cells were destroyed in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM sodium chloride, 1% NP-40, 2X Complete EDTA-free (Roche Diagnostics, Basel, Switzerland). After centrifugation, the supernatants were stored at -80°C.

**Sandwich ELISA**

The wells of an EIA titration plate (Corning, Corning, NY) were coated with 50 μl of mAb ECA-8, at 1 μg/ml in PBS for 1 h and treated with 200 μl Block Ace (Dainihon Pharmaceutical Co., Osaka, Japan) for 2 h. After washing with 200 μl PBS containing 0.05% Tween-20 (PBS-T) (Bio-Rad Lab., Hercules, CA), 50 μl of samples were added to the wells, and incubated for 1 h. After washing, 50 μl of biotinylated ECA42 at 0.5 μg/ml diluted with 0.5% BSA/PBS-T was added. The plates were incubated for 1 h. After washing, incubation was performed for 30 min with 50 μl of an ABC Elite solution (Vector Lab., Burlingame, CA) in PBS-T. For color development, 100 μl of 3,3', 5,5'-tetramethylbenzidine (TMB) peroxidase substrate solution (Bio-Rad, Hercules, CA), containing nine parts of TMB and one part H₂O₂ was added and incubated for 30 min. The color reaction was stopped by adding 50 μl 0.5 M H₂SO₄. Concentrations were determined by OD 450 nm vs OD 620 nm using an Immunoreader NJ-2000 (Nunc InterMed, Roskilde, Denmark).

**Western blotting**

Western blotting was performed as previously described. In brief, the samples added by β-mercaptoethanol (β-ME) Sample Treatment for Tris-SDS (Cosmo Bio, Tokyo, Japan) were boiled and subjected to 10–20% gradient SDS-PAGE. Proteins on the gel were electroblotted onto a PVDF membrane (Millipore, Billerica, MA) by a semi-dry electroblotter (Owl Separation Systems, Portsmouth, UK). The membrane was immersed in blocking solution, followed by incubation with 1 μg/ml dilutions of mAb ECA30, and biotinylated-anti-mouse Ig (Amersham Biosciences, Piscataway, NJ). After washing, ABC Elite solution (Vector Laboratories, Burlingame, CA) was added. Finally, the membrane was washed and immersed in ECL Western blotting detection reagents (Amersham Biosciences) and visualized by exposing the membrane to Hyperfilm ECL (Amersham Biosciences).

**Statistical analysis**

The statistical analysis was carried out with Statcel software (OMS publishing, Saitama, Japan). Mann-Whitney tests were used to compare the data of two other diagnostic categories. The correlation coefficients between BALF galectin-9 levels in BALF and clinical parameters were determined by Spearman's rank correlation test. P values less than 0.05 were taken to indicate statistically significant differences.

**Results**

**Immunohistochemistry**

ECA30 was used for immunohistochemical assay to determine the localization of galectin-9 in lung tissues.
Galectin-9 expression was observed in alveolar macrophages in the control lung tissues (Fig. 1A, B). In contrast, no reaction was observed using mouse IgG1k as a primary antibody in the same lung tissues (Fig. 1C, D).

Alveolar macrophages in the lung tissues from patients with IPF (Fig. 1E, F) and chronic eosinophilic pneumonia (Fig. 1G, H) showed high expression of galectin-9. In the patients with sarcoidosis, epithelioid cells in
granuloma and alveolar macrophages showed high expression of galectin-9 (Fig. II, J).

**Development of quantitative sandwich ELISA system**

Fig. 2 shows the standard curve of the sandwich ELISA. By this ELISA system, we successfully detected recombinant galectin-9 in a dose-dependent manner above 0.8 ng/ml (total protein 40 pg). The specificity of this assay was confirmed by the absence of signal production using an isotype-matched control mAb as a primary antibody in the system (data not shown).

**In vitro Expression of Galectin-9**

We performed quantitative analysis of intracellular and extracellular expression of galectin-9 in THP-1 cells, with and without stimulation by PMA. THP-1 cells were cultured for 48 h in various concentrations of PMA. The expression of galectin-9 was increased in both supernatants (Fig. 3A) and cell lysates (Fig. 3B) by stimulation with PMA. Its maximum expression level was detected by sandwich ELISA at a concentration of 25 ng/ml PMA, at which an approximately 8-fold increase was observed in the supernatants and an approximately 3-fold increase in the cell lysates. The increased expression of galectin-9 in the cell lysates was consistent with Western blotting.

**Levels of Galectin-9 in BALF**

Fig. 4 shows galectin-9 expression levels in the BALF from patients with interstitial lung diseases. Galectin-9 was detectable in the BALF supernatants from healthy volunteers (median 1.6 ng/ml, range 1.2–3.0 ng/ml). The levels of each category were as follows; IPF: median 6.5 ng/ml, range 1.1–20.2 ng/ml; hypersensitivity pneumonitis: median 6.9 ng/ml, range 3.0–9.5 ng/ml; drug-induced pneumonitis: median 6.7 ng/ml, range 1.7–18.8 ng/ml; eosinophilic pneumonia: median 5.2 ng/ml, range 0.4–13.8 ng/ml; and sarcoidosis: median 2.6 ng/ml, range 0.9–10.7 ng/ml. Galectin-9 levels in BALF were significantly increased in IPF (p=0.032), hypersensitivity pneumonitis (p=...
Relationship between Galectin-9 levels in BALF and clinical parameters in IPF

Based on the data showing that galectin-9 levels in BALF supernatants were increased in the IPF patients, we evaluated the correlation between the expression of galectin-9 and clinical parameters of IPF. Significant correlations were found in the serum levels of lactate dehydrogenase (LDH) ($r_s=0.57$, $p=0.033$) (Fig. 5A) and KL-6 ($r_s=0.60$, $p=0.026$) (Fig. 5B).

Discussion

In this study, we demonstrated that galectin-9 was expressed in alveolar macrophages and its expression levels in BALF were significantly higher in patients with IPF, hypersensitivity pneumonitis and drug-induced pneumonitis than in control subjects and sarcoidosis. One of our initial purposes performing this study had been to gain insight into the association between the eosinophil chemotaxis effect of galectin-9 and eosinophilic lung diseases such as eosinophilic pneumonia. To achieve this purpose, we investigated the expression of galectin-9 in BALF and lung tissues from patients with eosinophilic pneumonia. Unexpectedly, the levels of galectin-9 in BALF in eosinophilic pneumonia were not as high as those of other interstitial lung diseases. Interestingly, high levels of galectin-9 were found in BALF from patients with IPF, and the levels correlated with clinical parameters such as serum LDH and serum KL-6. Galectin-9 could potentially play a role in the development of IPF.

We established anti-human-galectin-9 mAbs by immunizing BALB/c mice and made a reliable sandwich ELISA system. Our newly developed ELISA system can detect galectin-9 at a minimum sensitivity of 40 pg in 50 μl (0.8 ng/ml).

This study shows that galectin-9 is expressed in alveolar macrophages in control lung tissue, IPF, eosinophilic pneumonia, and sarcoidosis, and epithelioid cells in sarcoidosis granuloma. Galectin-9 has been reported to be expressed in several immune cell lines including Jurkat (T lymphocyte), BALL-1 (B lymphocytes), THP-1 (monocytes/macrophages) and HMC-1 (mast cell), the levels of which were increased by activation of these cells[10]. Furthermore, galectin-9 has been found in Kupffer cells and histiocytes in drug-induced liver injury[11] and in macrophages in nasal polyps in humans[12]. It is possible that macrophages are one of the main sources of galectin-9 in the human body.

To evaluate the synthesis and release of galectin-9 by macrophages, we performed quantitative analysis of intracellular and extracellular expression of galectin-9 in THP-1 cells. PMA-stimulated THP-1 cells in this experiment resulted in increased expression of galectin-9 in both cell lysates and supernatants. THP-1 cells act as activated macrophages when stimulated with PMA[13]. PMA-stimulated THP-1 cells have been shown to produce a variety of mediators such as IL-1, IL-6, TNF-alpha, IL-1Ra, and metalloproteinases[14][15]. These results support the concept that the expression of galectin-9 in BALF is originated from activated macrophages, and may explain the low levels of galectin-9 in the BALF in healthy volunteers.

The medians of the levels of galectin-9 in BALF in comparison to controls in this study were about 7-fold higher in patients with IPF, hypersensitivity pneumonitis and drug-induced pneumonitis. No statistical differences were found among the four types of interstitial
l lung diseases: IPF, hypersensitivity pneumonitis, drug-induced pneumonitis, and eosinophilic pneumonia. Interestingly, the levels in BALF in IPF correlated with clinical parameters such as serum LDH and serum KL-6. KL-6 is a high-molecular-weight glycoprotein, expressed on the surface of alveolar type II cells, that is usually employed as a marker to predict the outcome of rapidly progressive IPF in Japan. Activated macrophage-derived molecules such as IL-8, TNF-alpha, insulin-like growth factor-I and galectin-3 have been identified in BALF from patients with IPF, suggesting the involvement of activated macrophages in the progression of IPF. Together with these findings and our results, elevated galectin-9 levels in BALF from patients with progressive IPF are suggested to play a certain role in development of IPF.

Interestingly, there was a discrepancy between the expression of galectin-9 in immunostaining and the levels in the BALF in sarcoidosis, as shown in Fig. 4. Granuloma formation in sarcoidosis is frequently characterized by increased expression of IFN-gamma and TNF-alpha, which induce activation and fusion of alveolar macrophages. Recently, strong expression of myeloid-related protein (MRP)-14 has been shown in sera and granuloma tissue in sarcoidosis. MRP-14 was found to be an epithelial cell-derived molecule that plays a role in the inactivation of macrophages. In the context of discordant expression of galectin-9 between intracellular immunostaining and quantitative values in BALF, the activation of macrophages in sarcoidosis is assumed to be regulated by the balance of activators and inactivators, leading to the low level of galectin-9 in BALF.

The aim of this study was to clarify the expression pattern of galectin-9 in interstitial lung diseases. It has been reported that galectin-9 possesses anti-inflammatory effects, and can be considered as a probable therapeutic agent, in animal models such as nephrotoxic serum nephritis, experimental autoimmune encephalomyelitis, and lipopolysaccharide-induced inflammation. Galectin-9 seems to be a peculiar protein, which has the possibility of suppressing inflammation among the numerous molecules derived from activated macrophages. Hence, galectin-9 may play a role in suppressing inflammation in some lung disorders. However, the precise action of galectin-9 in various types of interstitial lung diseases remains unclear. Further studies are required to explain the precise biological role of galectin-9 in the lung.

In conclusion, we have shown that galectin-9 is strongly expressed by alveolar macrophages in interstitial lung diseases. High levels of galectin-9 in BALF were observed in IPF, hypersensitivity pneumonitis and drug-induced pneumonitis.

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References


間質性肺疾患の肺胞マクロファージにガレクチン-9 は過剰に発現する

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【要旨】 背景：ガレクチン-9 は β ガラクトシドに結合するレクチンであり、好酸球の遊走、アポトーシスの誘導、Th1 免疫の抑制など様々な生物学的活性を有する。今回、我々は間質性肺疾患におけるガレクチン-9 の発現を検討した。

方法：本研究のために我々はガレクチン-9 のモノクローナル抗体を作製した。対象は特発性肺線維症 15 例、過敏性肺細胞炎 5 例、薬剤性肺炎 9 例、好酸球性肺炎 8 例、サルコイドーシス 31 例、健常者 5 例。肺組織におけるガレクチン-9 の局在を免疫組織化学的に検討し、気管支肺胞洗浄液中のガレクチン-9 濃度を Sandwich ELISA 法で測定した。また Sandwich ELISA 法、Western-blot 法を用いて、phorbol 12-myristate 13-acetate (PMA) 刺激下における THP-1 細胞株のガレクチン-9 の発現を検討した。

結果：免疫組織化学的にガレクチン-9 は肺胞マクロファージに発現していた。気管支肺胞洗浄液中ガレクチン-9 濃度は、特発性肺線維症 (p = 0.032)、過敏性肺細胞炎 (p = 0.016)、薬剤性肺炎 (p = 0.006) の患者において健常者に比べ有意に上昇していた。また特発性肺線維症患者では、気管支肺胞洗浄液中ガレクチン-9 濃度は血清 LDH 値 (rs = 0.57, p = 0.033)、血清 KL-6 値 (rs = 0.60, p = 0.026) に統計学的な相関が認められた。THP-1 細胞株は PMA 刺激下で多量のガレクチン-9 を放出した。

結論：ガレクチン-9 は間質性肺疾患におけるマクロファージに強く発現しており、これらの疾患における免疫学的反応に関与する可能性が示唆された。

〈キーワード〉 肺胞マクロファージ、気管支肺胞洗浄、ガレクチン、間質性肺疾患