ORIGINAL ARTICLE

Role of Galectin-3 in Human Pulmonary Fibrosis

Yumiko Nishi[1], Hideki Sano[1,2], Tatsuo Kawashima[1], Tomoaki Okada[1], Toshihisa Kuroda[1], Kyoko Kikkawa[1], Sayaka Kawashima[1], Masaaki Tanabe[1], Tsukane Goto[1], Yasuo Matsuzawa[1], Ryutaro Matsumura[1,2], Hisao Tomioka[1], Fu-Tong Liu[3] and Koji Shirai[1]

ABSTRACT

Background: Galectin-3 is a β-galactoside-binding protein which is implicated in diverse physiological and pathological processes including human liver cirrhosis and a mouse lung fibrosis model. The aim of this study is to determine whether galectin-3 is involved in human lung fibrosis.

Methods: We measured galectin-3 concentration in bronchoalveolar lavage fluid (BALF) and examined its expression in alveolar macrophages from patients with interstitial lung disorders using ELISA and immunohistochemical staining, respectively. Using monocyte/macrophage cell lines in vitro, we examined the effect of cytokines on galectin-3 expression, and the opposite similarly by RT-PCR and Western blotting. Finally, we performed Micro Boyden chamber assay and Sircoll assay to determine whether galectin-3 induces migration and collagen synthesis, respectively, in fibroblasts.

Results: Galectin-3 was specifically increased in BALF from patients with idiopathic pulmonary fibrosis (IPF) and interstitial pneumonia associated with collagen vascular disease (CVD-IP). Galectin-3 levels in BALF seemed to be lower in IPF and CVD-IP patients receiving corticosteroid therapy. Alveolar macrophages from IPF patients expressed more galectin-3 compared with those from control. Galectin-3 expression was induced by tumor necrosis factor-alpha (TNF-α) and interferon (IFN)-γ in a monocytic cell line U937. Galectin-3 also induced mRNA expression and protein production of TNF-α and interleukin (IL)-8 in a macrophage cell line THP-1. This lectin stimulated NIH-3T3 fibroblast to induce migration and collagen synthesis in vitro.

Conclusions: These results suggest that galectin-3 is involved in the pathogenesis of human IPF and CVD-IP by activating macrophages and fibroblasts.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a chronic interstitial lung disorder with high lethality. Current conventional therapies by corticosteroids and immunosuppressive agents usually have little effect on the natural course of IPF. The pathophysiology is described as usual interstitial pneumonia (UIP), characterized by endothelial or epithelial cell injury with accumulation of inflammatory cells, followed by deposition of extracellular matrix (ECM) and fibroblast activation, resulting in end-stage fibrosis.[1-3] These responses are mediated by various cytokines, chemokines, and growth factors, including tumor necrosis factor-alpha (TNF-α),[4-7] transforming growth factor-beta (TGF-β),[8,9] and platelet-derived growth factor (PDGF).[10,11] Recent studies demonstrated that angiogenic C-C chemokines and T helper type 2 (Th2) cytokines also play important roles in IPF.[12-14] Dysregulation of the expression of these factors is speculated to be responsible for the maintenance of tissue injury and fibrosis of the disease, although the precise mechanism remains uncertain.

Galectin-3 belongs to a family of β-galactoside-

[1]Department of Internal Medicine, Toho University Sakura Medical Center, [2]Department of Allergy and Rheumatology, Chiba-East Hospital, National Hospital Organization, Chiba, Japan and [3]Department of Dermatology, University of California, Davis, School of Medicine, Sacramento, CA95817, USA.
Correspondence: Hideki Sano, M.D., Ph.D., Department of Allergy and Rheumatology, Chiba-East Hospital, National Hospital Organization, 673 Nitonamachi, Chuou-ku, Chiba 260–8712, Japan.
Email: ryu-ma@ka2.so-net.ne.jp

binding animal lectins.[15] It is secreted by various cell types including monocytes, macrophages and epithelial cells.[16,17] The released protein can function as an extracellular molecule to activate cells,[18-23] mediate cell-cell and cell-ECM interactions,[24-26] induce migration of various types of cell,[27,28] and negatively regulate T cell receptor signaling.[29] This protein is also found abundantly inside cells and has been shown to play critical roles in some biological responses through its intracellular actions.[30-35] There are only a few reports which suggest a possible role of this lectin in human pathological fibrotic remodeling such as human liver cirrhosis.[36] Nevertheless, galectin-3 was shown to be increased in a mouse model of bleomycin-induced lung fibrosis,[37] suggesting participation of the protein in the pathogenesis of human lung fibrosis by its variegated effects. Therefore, we aimed to determine the role of galectin-3 in human lung fibrosis, and its biological activity on macrophages and fibroblasts.

METHODS

Materials. Recombinant human galectin-3,[38] mouse mAb against galectin-3 (B2C10), and polyclonal goat anti-galectin-3 Ab[39] were prepared as described previously. Recombinant TNF-α, IL-4, IL-13, IFN-γ, TGF-β, lipopolysaccharide (LPS), and all other reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated.

Preparation of human bronchoalveolar lavage fluid and alveolar macrophages. Human bronchoalveolar lavage fluid (BALF) and alveolar macrophages were obtained using a bronchoscope according to a previously described protocol.[6] Samples were collected from 41 patients who were treated for various lung disorders in the Department of Internal Medicine, Toho University Sakura Medical Center, after obtaining informed consent (patients' mean age 57.9, 26−86 years; male : female = 20 : 21). Diagnoses of IPF/UIP and cryptogenic organizing pneumonia/bronchiolitis obliterance organizing pneumonia (COP/BOOP) was made by the respiratory disease group of the hospital, according to the criteria of the American Thoracic Society (ATS)/European Respiratory Society (ERS) International Multidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias (IIPs).[1] Non-IIP diffuse parenchymal lung diseases including interstitial pneumonia associated with collagen vascular disease (CVD-IP), hypersensitive pneumonia (HP), and *Pneumocystis jiroveci* infection were diagnosed clinically, radiologically, and pathologically. In the case of CVD-IP, we only examined the samples from patients who showed non-specific interstitial pneumonia (NSIP) patterns with typical radiological features and high resolution computed tomography (HRCT) findings (peripheral ground glass and reticular opacity without eminent honeycombing) according to the ATS/ERS Classification.[1]

Immunohistochemistry cell staining. Alveolar macrophages from BALF adhering on coverslips were used in immunofluorescence microscopic analysis according to previously described methods.[35] The purity of the macrophages was more than 90%, and the viability was more than 99%. Briefly, after fixation with 4% paraformaldehyde in PBS, the cells were permeabilized with 0.2% Triton X-100/PBS, incubated with goat anti-galectin-3 Ab followed by rabbit anti-goat Ab conjugated to Alexa 488 (Molecular Probes Inc. Eugene, OR, USA). The coverslips were mounted on glass slides and digital images from a fluorescence microscope were captured.

Preparation of cultured cell lines. The human monocytic THP-1 and U937 cell lines (American Type Culture Collection, Manassas, VA, USA) and NIH-3T3 fibroblast cell line were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Gaithersburg, MD, USA) and 2% L-glutamine at 37℃ in a humidified atmosphere of 5% CO_2. For the induction of the THP-1 cells to differentiate into macrophages, the cells (5×10^5 to 10^6 per ml) were seeded in the same medium containing 10 nM phorbol myristate acetate (PMA) for 24 hours. After removing the nonattached cells, adhered cells were washed with RPMI 1640 three times. These cells were incubated overnight in RMPI 1640 medium with 0.1% FCS before use.

RT-PCR for mRNA detection. To examine mRNA expression, cells were stimulated with various reagents for 1 hour, washed with cold PBS three times, and total mRNA was extracted using ISOGEN Poly (A) + Isolation Pack (Wako Pure Chemical Industries, Ltd., Osaka, Japan). After cDNA was prepared using a cDNA synthesis kit (Roche Diagnostics Ltd, Basel, Switzerland), RT-PCR was performed as described previously.[39] Briefly, 2 μl of cDNA was added to a mixture of 1 × PCR buffer (Perkin-Elmer, Wellesley, MA, USA), 2 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each specific primer, and 1.25 U Taq polymerase (Perkin-Elmer) in a final volume of 50 μl. The PCR primer pairs for cytokines were as follows: TNF-α sense oligonucleotide 5'-GAGTGACAAG CCTGTAGCCCATGTTGTAGCA-3', and antisense oligonucleotide 5'-GGCAATGATGATCCCAAAGTA GACCTGCCCAGACT-3'; IL-8 sense nucleotide 5'-A TGACTTCCAAGCTGGCCGTGGCT-3' and antisense nucleotide 5'-TTCTCAGCCCTCTTCAAAAACTTCT C-3'; an internal control G3PDH sense nucleotide 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3' and antisense nucleotide 5'-CATGTGGGCCATGAGGTCCA CCAC-3'. The PCR cycle consisted of denaturation at 94℃ for 45 seconds, annealing at 60℃ for 45 seconds, and extension at 72℃ for 2 minutes, for 25 cycles. The DNA products obtained were separated by electrophoresis in 3% agarose gel containing ethidium bromide for visualization.

Western blotting and ELISA assay. Concentration of

Table 1 Properties of the BALF from patients.

	Patients n	Total Cells (×10⁴/ml)	Cell Differentiation (%)			
			AM	Ly	PMN	Eo
IPF/UIP	8	3.16 ± 0.44	91.1 ± 2.27	4.67 ± 1.38	2.81 ± 0.99	1.36 ± 0.44
CVD-IP (NSIP pattern)	17	3.67 ± 0.68	61.4 ± 8.06	20.0 ± 5.65	6.96 ± 2.56	11.7 ± 5.33
COP/BOOP	5	5.30 ± 1.20	61.1 ± 10.9	34.3 ± 12.0	3.76 ± 1.68	0.76 ± 0.26
HP	3	12.2 ± 4.07	27.3 ± 13.0	68.6 ± 13.0	1.93 ± 1.16	1.53 ± 0.74
P. jiroveci	2	6.95 ± 4.65	70.1 ± 15.2	27.6 ± 16.5	0.80 ± 0.80	0.70 ± 0.30
control	6	1.46 ± 0.55	91.2 ± 2.50	7.00 ± 1.78	1.50 ± 0.65	0.17 ± 0.17

Definition of abbreviations: IPF = idiopathic pulmonary fibrosis; UIP = usual interstitial pneumonia; CVD-IP = interstitial pneumonia associated with collagen vascular disease; NSIP = non-specific interstitial pneumonia; COP = cryptogenic organizing pneumonia; BOOP = bronchiolitis obliterance organizing pneumonia; HP = hypersensitive pneumonia; P. jiroveci = *Pneumocystis jiroveci infection*.

galectin-3 in BALF was measured by ELISA assay using anti-galectin-3 Ab according to previously described methods.[41] For *in vitro* experiments, U937 cell suspension (5 × 10⁶/ml) was stimulated with various cytokines including TNF-α, IFN-γ, IL-4, IL-13, or TGF-β. After incubation at 37℃ for 24 hours, cells were centrifuged, placed on ice, washed twice with cold PBS, and lysed in NP-40 lysing buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 10 μg/ml leupeptin, 10 μg/ml aprotinin) on ice for 30 minutes. After the lysates were centrifuged at 10,000 *g* for 10 minutes at 4℃, the protein concentration was measured using DC Protein Assay (Bio-Rad, Hercules, CA, USA). For immunoblotting, 30–100 μg of total protein in each lysate was applied to SDS-PAGE and the separated proteins were transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membranes were blocked with skim milk, immunoblotted with B2C10, an anti-galectin-3 mAb, and visualized using the ECL system (Amersham Pharmacia Biotech Inc, Piscataway, NJ, USA). In the case of adhered THP-1 cells, the supernatant was also collected after the indicated time of galectin-3 stimulation, and the concentrations of several cytokines and chemokines were measured by ELISA kit.

Migration assay in vitro. Fibroblast migration was examined using 96-well modified micro-Boyden chambers with 5 μm pore size filters (Neuro Probe, Gaithersburg, MD, USA) as described previously.[27] Briefly, after the indicated concentrations of galectin-3 or LPS prepared in HBSS with Ca²⁺ and Mg²⁺ were applied to the lower chambers, NIH-3T3 fibroblast suspensions (2.5–5.0 × 10⁴ cells/well) were applied to the upper chambers. After incubation for 1 hour in a humidified incubator at 37℃ in 5% CO₂, the membranes were washed once with PBS. After the upper side was wiped with paper, the membranes were processed with Wright stain to visualize migrated fibroblasts to the bottom side. The numbers of migrated fibroblasts on the filters were counted in 5–10

high power fields. Fibroblast migration was calculated from the average numbers of the counted cells and expressed as a percentage of seeded cells in each well.

Detection of collagen synthesis. To examine the effect of galectin-3 on collagen synthesis by fibroblasts, Sircol collagen assay (Biocolor Ltd., Newtownabbey, Northern Ireland) was performed according to the manufacturer's instructions. Briefly, adhered NIH-3T3 cells were stimulated with galectin-3 or rhIL-4 (positive control) for 48 hours, and the supernatant was collected and assayed in triplicate.

Data analysis. Data are presented as means ± SD unless otherwise stated. Statistical analyses of the results were performed by ANOVA using Fisher's protected least significant difference test for multiple comparisons. *P* values less than 0.05 were considered to indicate significant differences.

RESULTS

Galectin-3 was increased in BALF from patients with lung fibrosis. To determine the relationship between galectin-3 and fibrotic lung disease, we first measured the galectin-3 concentrations in BALF from patients with various interstitial lung disorders. As shown in Table 1, BALF samples were collected from healthy sites of the lung in 6 patients with lung cancer as control, and from those of pathological changes in 35 patients with interstitial lung disorders including IPF, CVD-IP, COP/BOOP, HP and *Pneumocystis jiroveci* infection. We only examined BALF samples from CVD-IP patients who showed typical NSIP patterns as described in METHODS.

We found that the concentrations of galectin-3 in BALF were significantly higher in patients with IPF and CVD-IP than in the control [control (*n* = 6): 3.19 ± 1.6; IPF (*n* = 8): 24.3 ± 14.4, *p* < 0.005 *vs.* control; CVD-IP (*n* = 17): 12.0 ± 5.44 ng/ml, *p* < 0.001 *vs.* control] (Fig. 1A). CVD-IP consisted of various entities and showed similar galectin-3 concentrations [vasculitis syndrome (*n* = 5): 15.3 ± 9.51; dermatomyositis
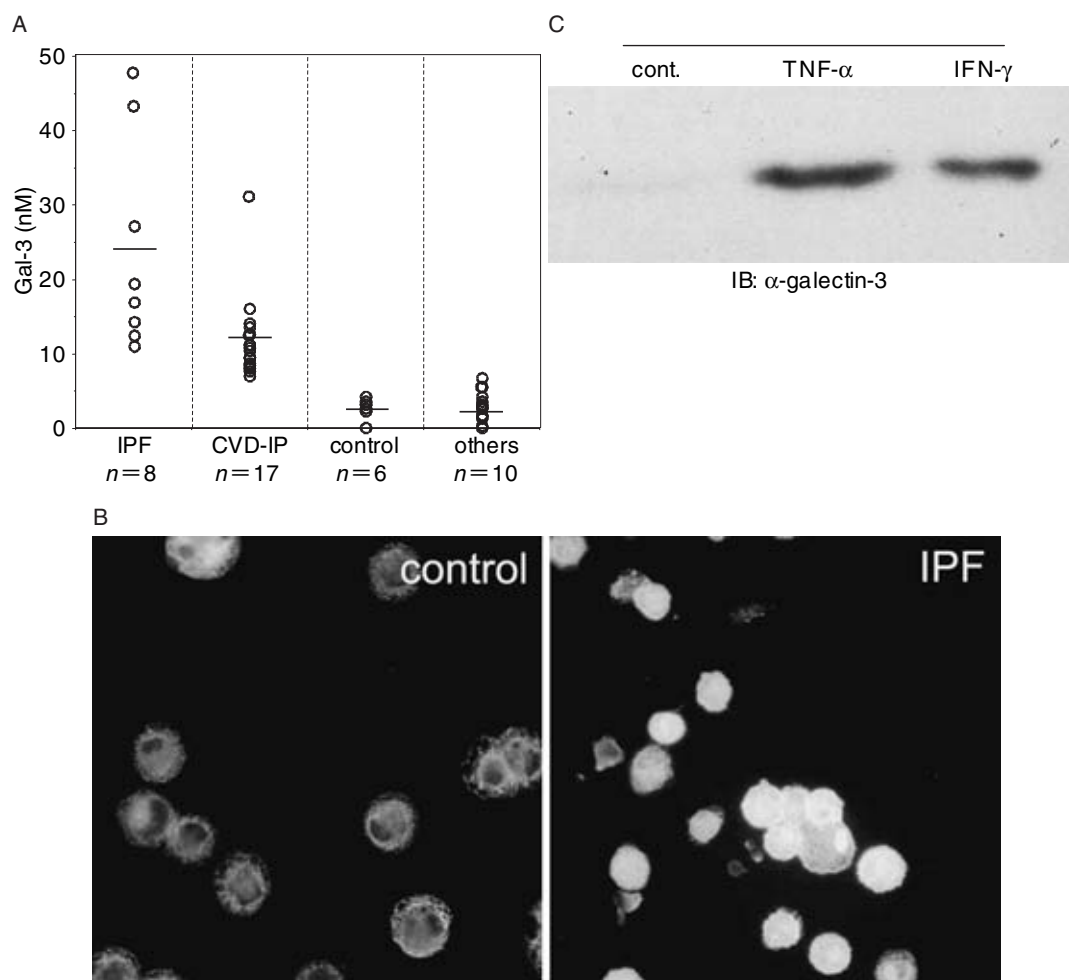
Fig. 1 (**A**) Levels of galectin-3 in the BALF from patients with IPF ($n = 8$), CVD-IP ($n = 17$), control [healthy site of lung cancer] ($n = 6$), and other interstitial lung diseases ($n = 10$). Galectin-3 concentration was measured by specific ELISA as described in METHODS. (**B**) Immuno-fluorescence microscopic analysis of galectin-3 expression in alveolar macrophages from the BALF of patients. Figure 1B shows representative distal images of the cells from control and IPF patients. (**C**) Immunoblot analysis of galectin-3 expression in U937 cells. Galectin-3 was induced by stimulation with TNF-α and IFN-γ.

($n = 4$): 12.6 ± 1.55; undifferentiated connective tissue disease (UCTD) ($n = 4$): 10.4 ± 2.67; rheumatoid arthritis (RA) ($n = 2$): 11.3 ± 4.46; systemic sclerosis (SSc) ($n = 2$): 10.2 ± 1.86; Sjogren's syndrome (SjS) ($n = 2$): 9.18 ± 0.689 ng/ml]. Patients with other interstitial lung diseases including COP/BOOP ($n = 5$), hypersensitive pneumonia ($n = 3$) and *Pneumocystis jiroveci* infection ($n = 2$) showed no significant differences in galectin-3 concentration (3.13 ± 2.05 ng/ml, $n = 10$; Fig. 1A) compared with control. We rectified galectin-3 concentration by total protein concentration, total cell number, and macrophage number of BALF, and found that this protein was significantly increased independent of these factors in IPF and CVD-IP patients (data not shown).

Immunofluorescence microscopic analysis revealed that alveolar macrophages from IPF patients expressed more galectin-3 inside the cells than control subjects (Fig. 1B), suggesting that the increased galectin-3 concentration in BALF was, at least partly, produced by alveolar macrophages. An increase in galectin-3 inside the cells was also observed in CVD-IP, but it was not always significant (data not shown). To determine whether galectin-3 expression is affected by disease activity, we also measured its concentration in BALF from patients with IPF ($n = 3$) and CVD-IP ($n = 2$; dermatomyositis and UCTD), who had improved or showed no obvious progression of the disease after corticosteroid therapy (0.3−1.0 mg/ kg of prednisolone) for more than 4 weeks. We found that galectin-3 concentration seemed to be lower in these patients than in the untreated group [treated
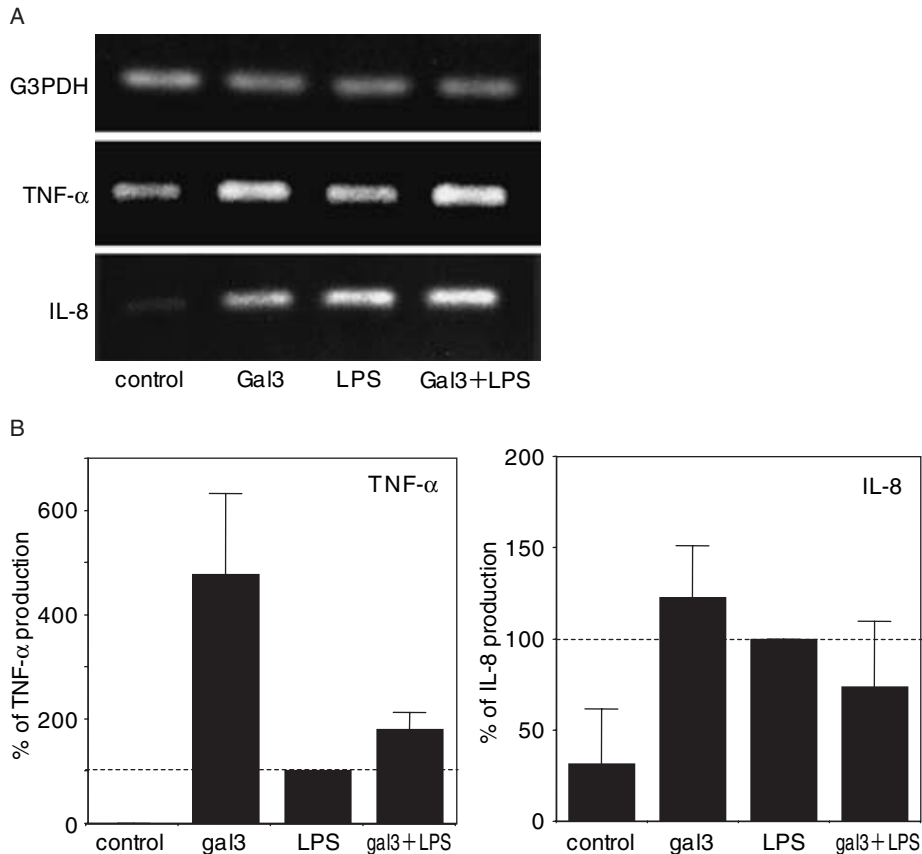
Fig. 2 Detection of TNF-α and IL-8 mRNA (**A**) and protein secretion (**B**) in human macrophage cell line, THP-1 using RT-PCR and ELISA assay, respectively. Cytokine secretion was adjusted by LPS-induced protein concentration, and expressed as % of production.

CVD-IP (n = 2): 1.93 ± 2.73; treated IPF (n = 3): 4.82 ± 2.21; total: 3.66 ± 2.61 ng/ml], although it was not statistically significant.

To determine whether galectin-3 is inducible, we stimulated a monocytic cell line U937, which does not express detectable amounts of galectin-3 in resting state, with several cytokines. We also used another macrophage cell line THP-1, but it was hard to examine, because galectin-3 was constitutively expressed (data not shown). Western blotting showed that stimulation of U937 by TNF-α induced galectin-3 expression after 24 hours (Fig. 1C). Interestingly, an anti-fibrotic cytokine IFN-γ also stimulated the cells to express galectin-3 (Fig. 1C). In contrast, profibrotic cytokines including IL-4, IL-13 or TGF-β did not affect galectin-3 expression in this assay (data not shown). These results suggest that the specific increase of galectin-3 in the BALF and alveolar macrophages from patients with IPF and CVD-IP is probably a result of stimulation by proinflammatory cytokines.

Galectin-3 induces TNF-α and IL-8 production in macrophages. We hypothesized that increased lung galectin-3 may in turn stimulate resident cells by autocrine and paracrine mechanisms to produce profibrotic factors in the lung. We used PMA-differentiated human macrophage cell line THP-1 on a more actual state of the cells as described in METHODS. By using a RT-PCR, we found that galectin-3 stimulated THP-1 macrophages to increase TNF-α and IL-8 mRNA after 1 hour as well as LPS did (Fig. 2A). Galectin-3 and LPS seemed to act additively to induce mRNA expression after 1 hour. ELISA assay also showed that galectin-3 significantly increased secretion of TNF-α and IL-8 from THP-1 compared with control, and the amounts of released cytokines in the supernatant after 24 hours were 4.8 and 1.2 times, respectively, higher than the level induced by LPS [TNF-α; 476 ± 156%; IL-8; 120 ± 28.8% (n = 4)] (Fig. 2B). Interestingly, stimulation of the cells with a combination of galectin-3 and LPS did not reach the levels of cytokine production induced by galectin-3 alone [TNF-α; 96.5 ± 3.65%; IL-8; 180 ± 32.1%, n = 4] (Fig. 2B). We also found that galectin-3 did not induce detectable amounts of IL-4, IL-13 or TGF-β production in this cell line within 24 hour-stimulation (data not shown). These results suggest that in-
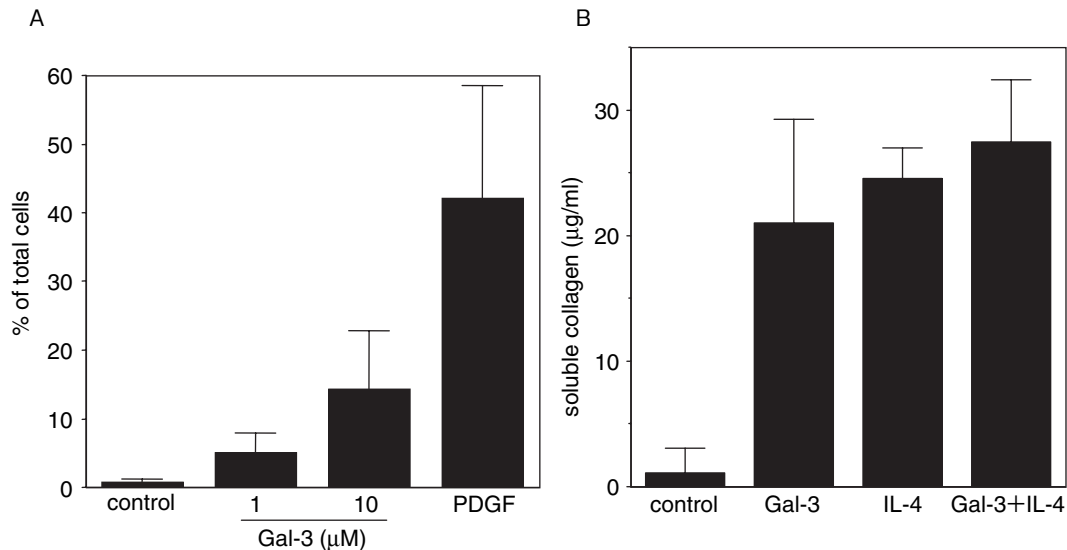
A

B

Fig. 3 (**A**) The effect of galectin-3 on fibroblast cell migration *in vitro*. Control vehicle, Galectin-3 (1 or 10 μM), or PDGF (10 ng/ml) were applied to the lower chambers, NIH-3T3 cells were applied on the membrane, and migration assay was performed as described in METHODS. Data are means ± SD of four individual experiments. (**B**) The effect of galectin-3 on collagen synthesis by fibroblasts. After NIH-3T3 cells were stimulated with 100 μM galectin-3, IL-4 (100 IU/ml), or both reagents for 48 hours, cell culture medium was harvested and soluble collagen was measured according to the manufacturer's instructions. Data are means ± SD of four individual experiments.

creased galectin-3 may participate in lung fibrosis by, at least partly, activation of resident macrophages to produce TNF-α and IL-8, thereby sustaining inflammation and fibrosis in the lung.

Galectin-3-induced migration and collagen production in fibroblasts. As galectin-3 shows chemotactic or chemokinetic activity for various types of cell,[28,35] we examined whether galectin-3 attracts fibroblasts to initiate lung fibrosis. We first confirmed that PDGF significantly increased NIH-3T3 fibroblast cell migration compared with control in a modified Micro Boyden chamber assay (vehicle only: $0.77 \pm 0.37\%$, 10 ng/ml PDGF: $40.1 \pm 13.9\%$; $p < 0.01$, $n = 4$ experiments). Using this assay, we showed that galectin-3 also significantly increased migration of NIH-3T3 at concentrations of 10 μM, and even 1 μM of the protein seemed to enhance cell migration, although it was not statistically significant [1 μM Galectin-3: $5.45 \pm 2.34\%$; 10 μM galectin-3: $14.2 \pm 6.89\%$; $p < 0.05$, $n = 4$] (Fig. 3A).

Sircol assay showed that galectin-3 induced collagen production by NIH-3T3 cells into the supernatant after 48 hours (control: 1.13 ± 1.95 mg/ml, 100 μM Galectin-3: 21.0 ± 8.3 mg/ml; $p < 0.01$, $n = 4$) (Fig. 3 B). The effect of galectin-3 was almost the same as that of 100 U/ml IL-4, a positive control of profibrotic cytokine. Combination of galectin-3 and IL-4 did not significantly exceed the effect of collagen synthesis by single stimulation, suggesting a common pathway of signal transduction, or its effect reached its maxi-

mum at that concentration. These results suggest that galectin-3 may directly participate in fibrogenesis by recruiting and stimulating the fibroblasts to synthesize collagen at the site.

DISCUSSION

In this report, we showed that galectin-3 was increased in the BALF from patients with fibrotic lung diseases including IPF and CVD-IP, compared with control BALF (Fig. 1A). The up-regulation of galectin-3 appears to be specific for these pulmonary fibrotic disorders, because patients with other interstitial lung diseases such as COP/BOOP, acute hypersensitive pneumonia, and *Pneumocystis jiroveci* infection did not show any significant increment of this protein in the BALF (Fig. 1A). Among various IIPs, we have analyzed BALF samples only from IPF and COP/BOOP but not from other entities. Thus, it is not yet determined whether galectin-3 is increased or not in other non-IPF IIPs. So far, we have only preliminary data that galectin-3 seemed to be slightly increased in BALF from (non-CVD-IP) NSIP patients, although it was not statistically significant compared with control [NSIP: 8.02 ± 3.77 ng/ml, ($n = 6$)]. On the other hand, it is well known that IPF is resistant to conventional immunosuppressive therapy,[1] but COP/BOOP is generally a steroid-responsive organizing pneumonia. Taken together, these results suggest that increased galectin-3 in the lung might be related to lung fibrosis

and disease obstinacy for immunosuppressive therapies. In addition, we showed that galectin-3 levels seemed to be lower in patients with CVD-IP receiving corticosteroid therapy, although it was not statistically significant. Thus, it may be useful to examine the galectin-3 level in BALF for not only diagnosis but also for assessing the response to medical treatment in cases of lung fibrosis.

The source of increased galectin-3 seemed to be, at least partially, derived from alveolar macrophages, which were shown to contain more galectin-3 inside the cells from IPF patients than in those from control subjects (Fig. 1B). Increased galectin-3 in the BALF of fibrotic lung disease is probably induced by proinflammatory cytokines, because its expression was induced *in vitro* in a monocyte/macrophage cell line U937 by stimulation with TNF-α (Fig. 1C), one of the key cytokines of lung fibrosis.[4-7]

We also propose that increased galectin-3 in BALF is related to the pathogenesis of lung fibrosis, because of its ability to induce TNF-α production by macrophages (Fig. 2A, B), suggesting a positive feedback mechanism between TNF-α and galectin-3 to sustain inflammation. It seemed curious that galectin-3 did not act additively with LPS for the secretion of cytokines unlikely to the mRNA induction pattern (Fig. 2A, B). This phenomenon may be explained by the fact that galectin-3 binds to LPS directly[46] to inhibit interaction of LPS with its cell surface receptors. Alternatively, it is possible that galectin-3 may inhibit the apoptotic pathway[33] by starvation in this assay contrary to LPS, which can induce apoptosis in macrophages.[48] Although we found no significant difference in numbers of cells within 24 hours by any stimulation, it is conceivable that galectin-3 might keep cell viability, resulting in continuous secretion of these cytokines. Further studies are required to determine the precise mechanism of this issue.

We also examined the correlation of BALF galectin-3 and serum KL-6, which is a frequently used marker to predict the outcome of IPF.[49] Both galectin-3 and KL-6 increased in 5 of all 7 patients examined, but only galectin-3 showed high levels in 2 patients. The latter cases consisted of IPF and CVD-IP with dermatomyositis (Galectin-3; 14.1 and 11.0 ng/ml, respectively), and showed radiologically obvious honeycombing and ground glass opacity, respectively. We also encountered a steroid-responsive patient with COP/BOOP with high KL-6 (1080 U/ml) and normal galectin-3 in BALF (2.61 ng/ml) before treatment. Although the number of cases is too small to draw a definite conclusion, BALF galectin-3 might be more specific than serum KL-6 for lung fibrosis.

Previous studies have demonstrated that profibrotic cytokines including TGF-β, IL-4, and IL-13 are also probably the key mediators as well as proinflammatory cytokines in the pathogenesis of pulmonary fibrosis,[8,9,13] suggesting that Th2 type immune responses also play central roles in the fibrotic process. In a mouse model, administration of IFN-γ, a Th1 type cytokine, ameliorated bleomycin-induced lung fibrosis via IP-10 induction and inhibition of TGF-β production.[40,41] Based on this concept, a clinical trial of IFN-γ1b therapy was conducted, and the therapy seemed to decrease the mortality of IPF patients.[42] Nevertheless, IFN-γ treatment did not improve physiological parameters after one-year therapy in a large clinical trial,[43] and did not suppress profibrotic molecular expression at 6 months.[44] Moreover, this therapy was reported to induce acute respiratory failure in several patients with advanced stage lung fibrosis.[45] One possible explanation for the discrepancy is that IFN-γ has dual opposing effects; one is downregulation of fibrosis as described above, and another is activation of the inflammatory pathway to promote fibrosis. As shown in this study, we speculate that IFN-γ-induced galectin-3 production from alveolar macrophages may enhance lung fibrosis by inducing TNF-α. Therefore, we propose that a combination of IFN-γ therapy with galectin-3 blockade may be a promising approach to prevent progression of lung fibrosis in IPF and CVD-IP.

We also propose one more possibility that galectin-3 is involved in lung fibrosis by inducing angiogenesis. The existence of extensive neovascularization in IPF was already reported by Turner-Warwick in 1963,[47] and a recent study revealed an imbalance in levels of CXC chemokines in IPF.[14] These chemokine family members containing the ELR (Glu-Leu-Arg) motif, including IL-8, are potent promoters of angiogenesis at physiologic concentrations of 1 to 100 nM.[14] In contrast, IFN-inducible members of the chemokine lacking the ELR motif, such as IP-10, are in general potent angiostatics at physiologic concentrations of 500 pM to 100 nM.[14] Indeed, IP-10 has been reported to attenuate lung fibrosis induced by bleomycin via inhibition of angiogenesis,[41] indicating the importance of chemokines and neovascularization in the disease. We found that galectin-3 stimulates macrophages to induce an angiogenic CXC chemokine, IL-8 (Fig. 2A, B). Its concentration in the supernatant of the *in vitro* experiments was around 100 pM (data not shown), suggesting that local concentration of the chemokine can reach 1 to 100 nM. Moreover, Raz and coworkers found that galectin-3 directly stimulated capillary tube formation of endothelial cells *in vitro* and induced angiogenesis *in vivo*.[28] Although we did not examine the effects of galectin-3 on the expression of other CXC chemokines such as IP-10, it is possible that this lectin may participate in vascular remodeling in IPF by acting directly on endothelial cells and indirectly through IL-8 production.

Finally, we found that galectin-3 induced migration and collagen synthesis of fibroblasts *in vitro*, suggesting direct effects of this protein on fibrogenesis (Fig.

3A, B). Our previous study showed that galectin-3 induced chemotactic activity and Ca^{2+} influx in monocytes/macrophages via its carbohydrate-binding activity.[27] Therefore, the phenomenon may have arisen by a similar mechanism in the case of fibroblasts, and these pathways are the subject of our on-going investigation. Taking together these results, this study is the first to propose that galectin-3, a galactose-binding animal lectin, plays an important role in the development of lung fibrosis by interacting with various cytokines and chemokines.

ACKNOWLEDGEMENTS

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