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Original Article

Advanced glycation end-products and receptor for advanced glycation end-products expression in patients with idiopathic pulmonary fibrosis and NSIP

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Abstract: Advanced glycation end products (AGEs) are associated with the pathogenesis of various diseases. AGEs induce excess accumulation of extracellular matrix and expression of profibrotic cytokines. In addition, studies on receptor for advanced glycation end products (RAGE) have shown that the ligand-RAGE interaction activates several intracellular signaling cascades associated with several fibrotic diseases. We investigated the expression of AGEs and RAGE in samples from patients with idiopathic pulmonary fibrosis (IPF) and non-specific interstitial pneumonia (NSIP). Lung tissues and plasma samples from patients with IPF (n=10), NSIP (n=10), and control subjects (n=10) were obtained. Expression of AGEs and RAGE was determined by immunofluorescence assay of lung tissue. Circulating AGEs were measured by Western blot and enzyme-linked immunosorbent assay. Lungs with IPF showed strong expression for both AGEs and RAGE compared to that in NSIP and controls. However, no difference in AGE or RAGE expression was observed in lungs with NSIP compared to that in the controls. Levels of circulating AGEs also increased significantly in lungs of patients with IPF compared to those with NSIP and normal control. Increased AGE-RAGE interaction may play an important role in the pathogenesis of IPF.

Keywords: Advanced glycation end products, Idiopathic pulmonary fibrosis, receptor for advanced glycation end product

Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic, progressive fibrosis of the lung interstitium without a definite cause [1]. It is characterized by progressive worsening of clinical symptoms and a poor prognosis. The molecular mechanisms of IPF are not fully understood [2].

Advanced glycation end products (AGEs), the irreversible products of nonenzymatic glycation of proteins, nucleic acids, and lipids, are overproduced in hyperglycemic or oxidative stress environments. AGEs have various structures such as N-ε-carboxymethylated lysine (CML), crosslinks, pentosidine, or pyrroline according to the precursor molecule. AGEs involve oxidative and non-oxidative molecular rearrangements and may be involved in several disorders [3]. Matsuse *et al*. reported the accumulation of AGE-modified proteins in alveolar macrophages of patients with IPF [4]. In addition, several investigators have reported that AGEs induce excessive deposition of extracellular matrix and enhance expression of profibrotic cytokines such as transforming growth factor-β (TGF-β) [5-7].

Receptor for advanced glycation end products (RAGE) is a single-chain transmembrane receptor found in various cell types. It recognizes a variety of ligands, including AGEs, amyloid β-peptides, high mobility group box-1 (HMGB-1), and S100/calgranulin [8]. The ligand-RAGE interaction activates several intracellular signaling cascades, such as the mitogen-activated protein kinase pathway, to produce reactive oxygen species, and nuclear factor-Kβ [8].

Table 1. Demographic data of patients enrolled this study

	Control	NSIP	IPF	*p*-value
Age, yrs	67.2±8.4	50.9±11.6	63.7±9.7	<0.05, Control, IPF vs NSIP
Gender, M:F	5:5	2:8	5:5	NS
Smoking, PGyrs	9.5±22.7	3.0±9.5	14.8±19.1	NS
Pulmonary function				
TLC, % pred.	-	83.1±24.4	83.4±24.3	NS
DLco, % pred	-	67.8±19.9	51.9±19.3	0.09
FVC, % pred	83.1±32.9	59.3±13.1	73.6±14.5	<0.05, Control vs NSIP
BALF analysis				
Neutrophils, %	-	37.6±21.5	30.5±21.4	NS
Lymphocytes, %	-	5.1±4.3	7.1±8.9	NS
Blood glucose, mg/dL	-	141.0±42.7	107.0±14.1	0.08
AGEs levels, μg/ml	7.5±5.9	11.4±5.4	17.1±8.7	<0.05, Control vs IPF
Death, n	-	0	4	
Median survival, m	-	102.6	26.6	<0.05

Data are shown as mean±SD. NS: non-significant; TLC: total lung capacity; DLco: diffusion capacity of carbon monoxide; FVC: forced vital capacity; BLAF: bronchoalveolar fluid; AGE: advanced glycation end products; NSIP: non-specific interstitial pneumonia; IPF: idiopathic pulmonary fibrosis; n: number; m: months.

Involvement of RAGE in renal fibrosis of diabetes and hepatic fibrosis has been demonstrated [8]. However, there is controversy regarding the role of RAGE in patients with IPF [9]. Some authors have reported that loss of RAGE contributes to IPF pathogenesis [10-12], whereas He *et al*. reported that RAGE, particularly the RAGE/HMGB-1 interaction, contributes to bleomycin-induced lung fibrosis [13]. Morbini *et al*. reported AGE and RAGE overexpression in bronchiolar epithelial cells, type II alveolar cells, and macrophages under pulmonary pathological conditions, including usual interstitial pneumonia [14].

We further investigated AGE and RAGE expression in lung tissues and the levels of circulating AGEs in patients with IPF and non-specific interstitial pneumonia (NSIP).

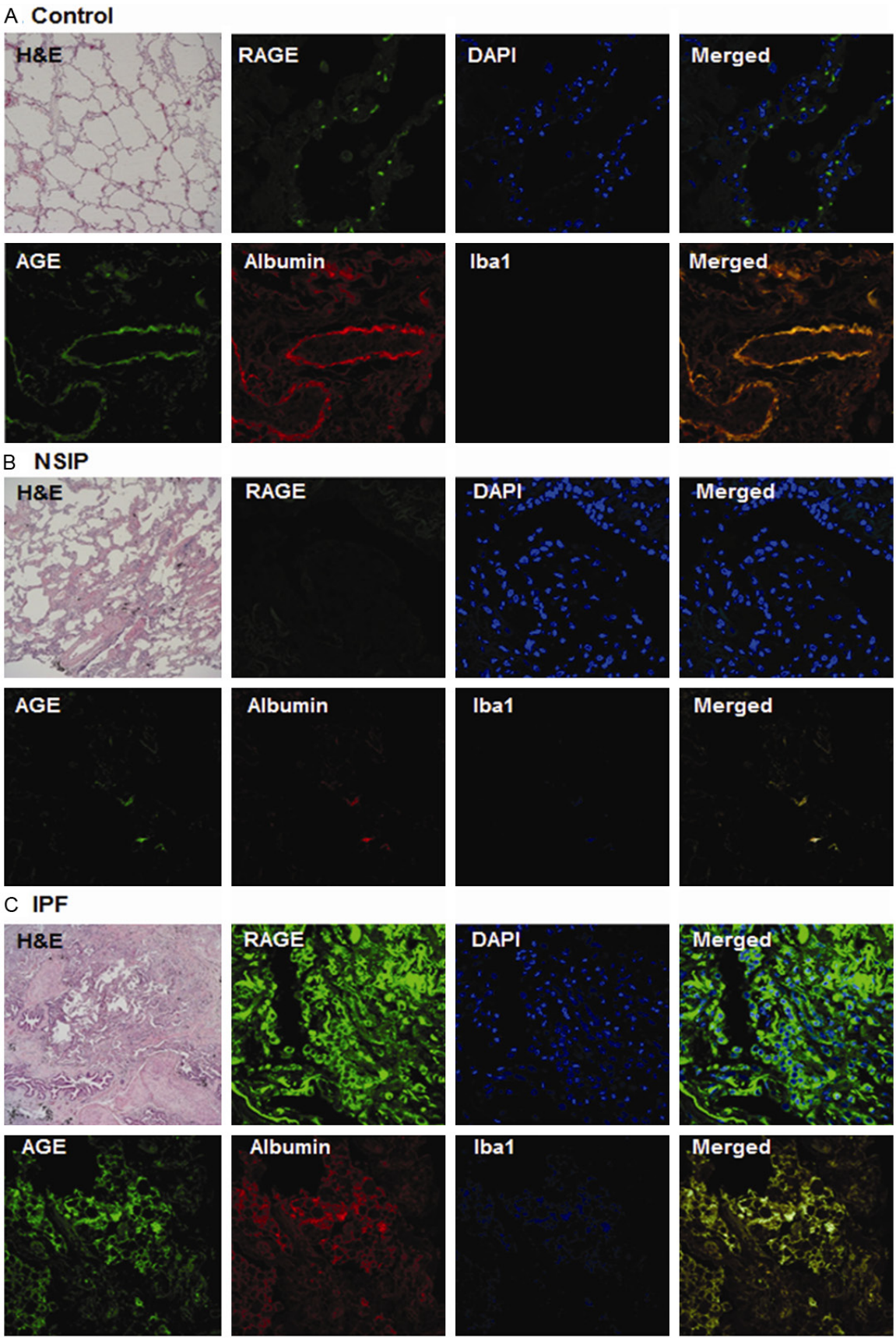
Material and methods

Sample preparation

Lung and plasma samples were obtained from 30 patients at Soonchunhyang University Bucheon Hospital. Ten samples were from patients with IPF, and ten were from patients with NSIP. Diagnoses were based on clinical, radiological, and histological findings. Ten control lung samples were obtained from patients with other pulmonary diseases who had undergone surgery. Medical history was reviewed in all patients. This study was approved by the Ethics Committee at Gachon University Gil Medical Center and Soonchunhyang University. Written consent was obtained from all patients prior to sample collection. All lung tissues were fixed overnight in 10% formalin, embedded in paraffin, and cut into sections. The sections were stained with hematoxylin and eosin, and subjected to immunofluorescence staining. Blood samples were collected in EDTA-containing tubes. Plasma was separated by centrifugation and stored at -70°C until use.

Immunofluorescence assay

The tissue sections were deparaffinized, rehydrolyzed, and blocked with normal serum for 1 h at room temperature, followed by a 1-h incubation with primary antibody at the appropriate dilution in antibody dilution buffer at room temperature, and then overnight at 4°C. After washing in PBS, the sections were incubated for 1 h at room temperature with fluorescent secondary antibody diluted in antibody dilution buffer. The slides were washed in PBS, mounted, and observed under a confocal microscope. Confocal microscopy (LSM710, Zeiss, Jena, Germany) was used to determine AGE and RAGE expression in lungs with IPF and NSIP and the control. Lung sections were stained to visualize immunofluorescent colocalization of AGE (anti-AGE antibody, Abcam, Cambridge, UK) with albumin (Abcam) and macrophages


A Control
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B NSIP
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AGE
Albumin
Iba1
Merged
C IPF
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RAGE
DAPI
Merged
AGE
Albumin
Iba1
Merged

Figure 1. Immunofluorescent staining for advanced glycation end-products (AGEs) and receptor for AGE (RAGE) in lung tissues of patients with non-specific interstitial pneumonia (NSIP) (B), idiopathic pulmonary fibrosis (IPF) (C), and the control (A) (×200). Enhanced expression of AGEs and RAGE was observed in the IPF lungs than NSIP and control. Most AGEs were found as glycated albumin (AGE-modified protein) in macrophages and alveolar epithelial lining cells.

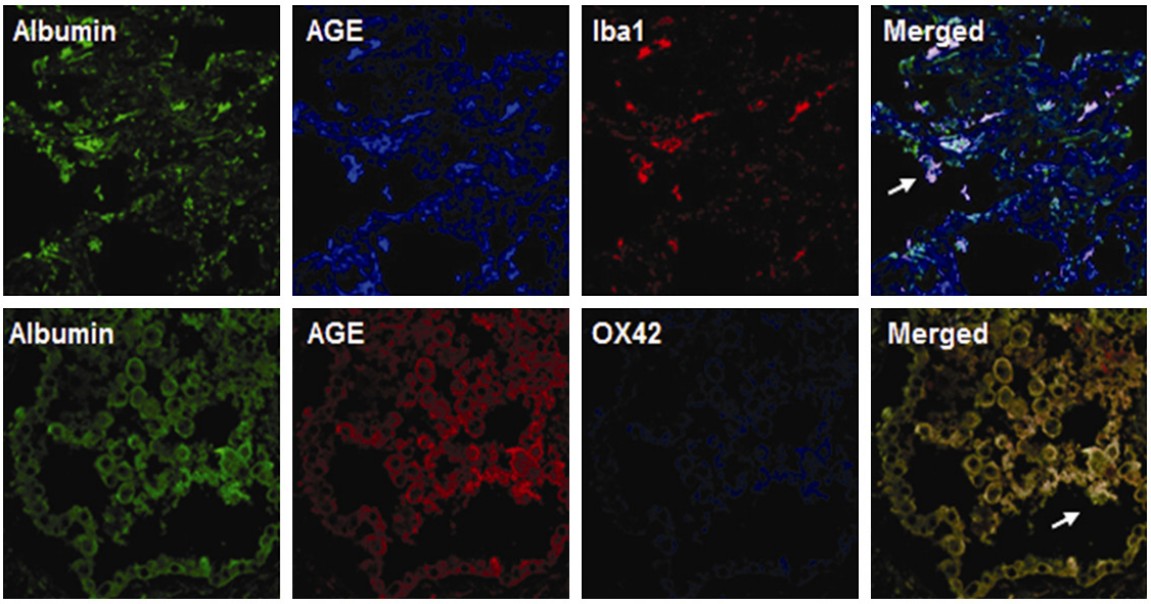

Figure 2. Localization of advanced glycation end-product (AGE) expression in lung tissues from patients with idiopathic pulmonary fibrosis (IPF). Immunofluorescent staining for macrophage-specific Iba1 and OX42 shown as a merge with AGE-modified albumin in lung tissue from patients with IPF.

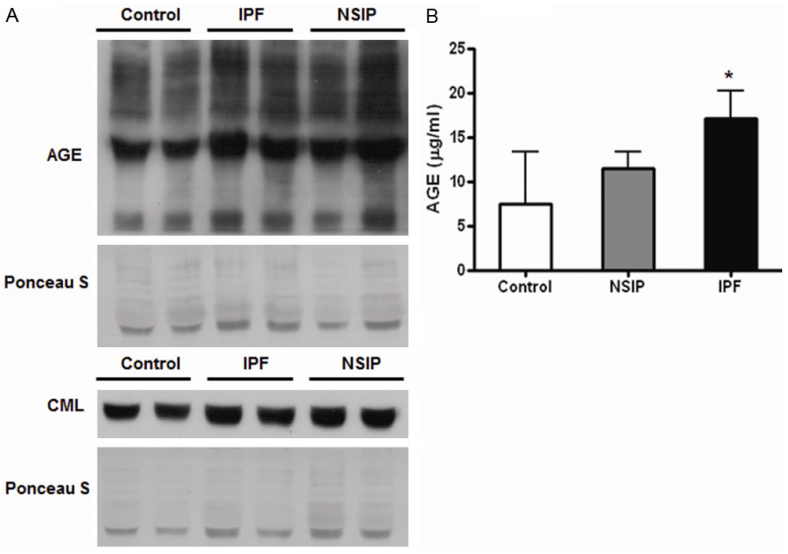

Figure 3. Circulating advanced glycation end-products (AGEs) in plasma from patients with idiopathic pulmonary fibrosis (IPF) and non-specific interstitial pneumonia (NSIP). Western blotting for AGEs and N-ε-carboxymethylated lysine (CML) (A), Enzyme-linked immunosorbent assay (ELISA) for AGEs (B). Western blot shows increased expression of circulating AGEs (AGEs and CML) in patients with IPF and NSIP, compared to that in the control. ELISA results show significantly higher levels of circulating AGEs in patients with IPF, compared to the NSIP and control. *$P<0.05$ compared to the control.

(Iba1 antibody, Abcam; OX42 antibody, Chemicon, Billerica, MA, USA). The anti RAGE antibody (Abcam) was used for RAGE, immunofluorescence staining. Counterstaining with 4'6-diamidino-2-phenyllindole (blue) was performed to identify nuclei.

Western blotting

After determining sample protein concentration by the Bradford assay, protein from each sample was mixed with sample buffer. Samples were separated on 10% sodium dodecyl sul-

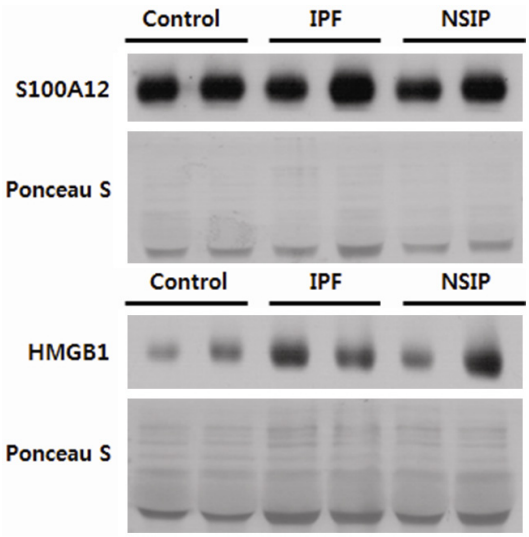

Figure 4. Western blotting for non-advanced glycation end-product (AGE) ligands for receptor for AGE (RAGE), S100A12, and HMGB1. Both S100A12 and HMGB1 showed increased expression in patients with idiopathic pulmonary fibrosis (IPF) and non-specific interstitial pneumonia (NSIP), compared to the control, no significant difference between IPF and NSIP.

fate-polyacrylamide gel electrophoresis and transferred to a PVDF membrane (Roche, Indianapolis, IN, USA). The immunoblots were sequentially incubated with primary antibody overnight at 4°C, followed by 1-h incubation with secondary antibody (AGE, Santa Cruz Biotechnology, Santa Cruz, CA, USA; CML, Amersham Biosciences, Little Chalfont, UK). The membranes were incubated with enhanced chemiluminescence detection reagents (WEST-ONE, Intron, Daejeon, Korea) and then stained with Ponceau-S as a loading control. The primary antibodies were the anti-AGE (Chemicon) and anti-CML (Abcam) antibodies.

Enzyme-linked immunosorbent assay (ELISA)

Protein concentration was quantified using the Bradford method. Plasma AGE levels were determined according to the manufacturer's instructions (Cell Biolabs Inc., San Diego, CA, USA). Samples were measured photometrically using an automated plate reader (PerkinElmer Inc., Waltham, MA, USA).

Statistical analysis

All values are expressed as means±standard deviation. A one-way analysis of variance was used to identify differences. Tukey's test was used to determine specific mean differences. All statistical analyses were performed using Graphpad Prism 4.0 (GraphPad, Inc., La Jolla, CA, USA). A $p<0.05$ was considered to indicate significance.

Results

Clinical characteristics

None of the patients had diabetes. Patients with NSIP were younger than control subjects and those with IPF (**Table 1**). Patients with IPF and NSIP had decreased pulmonary function compared to control subjects. Patients with IPF had high plasma AGE levels and a poor prognosis for survival. No correlation was observed between plasma AGE levels and clinical parameters, including survival (data not shown).

AGE and RAGE expression in lung tissues of patients with NSIP and IPF

Figure 1 shows the differences in AGE and RAGE expression in NSIP and IPF lung tissues. IPF lungs strongly expressed both AGE and RAGE compared to NSIP and control lungs. However, no difference in AGE or RAGE expression was observed in NSIP compared to control lungs. We performed staining with macrophage-specific Iba1 and OX42 to localize AGE expression in IPF lungs and observed a merged state with AGE-modified albumin (**Figure 2**). Immunofluorescence staining for both Iba1 and OX42 showed that macrophages contained AGE-modified albumin.

Circulating AGEs in patients with NSIP and IPF

Western blotting using NSIP and IPF patient plasma showed increased AGEs and CML protein (**Figure 3A**). In addition, levels of circulating AGE in the IPF samples were significantly higher than those in the NSIP and control (**Figure 3B**).

Circulating non-AGEs ligands for RAGE in patients with NSIP and IPF

Western blotting for non-AGE ligands of HMGB-1, and S100A12 indicated enhanced expression in plasma from patients with IPF and NSIP, compared to controls but no significant difference between IPF and NSIP (**Figure 4**).

Discussion

Increased AGE and RAGE expression was observed in lung tissues of patients with IPF,

compared to those with NSIP and control subjects. AGE-modified protein was localized primarily in macrophages in the IPF samples. In addition, the level of circulating AGEs showed a marked increase in patients with IPF compared to that in patients with NSIP and controls.

Strong evidence for the roles of AGEs in the pathogenesis of fibrosis has been reported. Huang *et al*. and Lee *et al*. reported that treatment with AGEs results in increased production of collagen and connective tissue growth factor (CTGF) in renal fibroblasts of rats [14, 15]. In addition, treatment with AGEs results in significantly increased accumulation of type IV collagen and fibronectin in renal glomeruli and induces marked expression of renal TGF-β1 and CTGF in rats [16]. These *in vitro* and *in vivo* results suggest that AGEs induces fibrosis. Significant evidence indicates an association between AGEs and pulmonary fibrosis. Matsuse *et al*. reported increased AGE expression in patients with IPF, particularly in macrophage and metastatic epithelium [4]. Chen *et al*. found that the accumulation of AGEs parallels the progression of bleomycin-induced pulmonary fibrosis and inhibits AGE production following treatment with the AGE inhibitor aminoguanidine, resulting in significant attenuation of bleomycin-induced pulmonary fibrosis [18]. Our data confirm that AGE expression is significantly increased in patients with IPF compared to those with NSIP and controls, even though functional vital capacity in patients with IPF was significantly greater than that in those with NSIP. This increased expression of AGE is estimated to be characteristic finding of the disease itself that may be independent of decreased lung function in IPF. Accumulation of these AGE-modified proteins occurred mainly in activated macrophages. These results are similar to previous reports [14, 17]. We also investigated the levels of circulating AGEs in plasma of patients with IPF and NSIP, as well as controls. Circulating AGE levels in patients with IPF were significantly higher than those in NSIP and controls. This is the first report of circulating AGE levels in patients with IPF. AGE formation is an important pathophysiological event in many diseases, not only diabetic complications but also age-related pathology. Increased circulating AGEs are correlated with the severity or complications of diabetes [19]. Patients with IPF clinically have a higher prevalence of diabetes, compared to that of healthy volunteers [20] and some antidiabetic agents attenuated lung fibrosis [21]. These results might be due to anti AGE effect for attenuating fibrosis. We confirmed that patients with IPF have increased AGE expression in lung tissue and high circulating levels of AGEs; thus, it is possible that increased AGE levels play an important role in IPF pathogenesis.

RAGE interacts and binds to several unrelated ligands, such as AGEs, amphoterin (*i.e*., HMGB1), S100/calgranulin, amyloid β-peptide, beta fibrils, and Mac-1. RAGE is normally found in low levels in most healthy tissues. However, pulmonary tissue shows a relatively high basal RAGE level, even under normal conditions [22]. High RAGE levels are present in bronchoalveolar lavage fluid from patients with acute lung injury/acute respiratory distress syndrome [23, 24]. In a recent study, bleomycin-induced lung fibrosis caused a loss of RAGE surface expression in lung tissues [10]. In addition, lack of RAGE in knockout mice leads to the development of spontaneous pulmonary fibrosis with age as well as more severe fibrosis induced by asbestos injury [11]. In contrast, He *et al*. reported that RAGE-knockout mice are almost entirely protected against the fibrotic effects of bleomycin [13]. Therefore, the function of RAGE in pulmonary fibrosis remains largely unknown [9]. We evaluated RAGE expression in patients with IPF and NSIP, and IPF lung tissues showed markedly increased RAGE expression. This result supports the suggestion of He *et al*. that RAGE or the RAGE/HMGB1 axis contributes to bleomycin-induced lung fibrosis [13]. We further evaluated other non-AGE ligands for RAGE expression in the plasma of patients with IPF and NSIP. Western blotting of plasma showed increased HMGB1 and S100A12 expression in patients with IPF or NSIP, compared to the controls but no significant difference in IPF and NSIP. Both AGE and non-AGE ligands for RAGE showed increased expression in the plasma of patients with IPF. In addition, RAGE expression in lung tissue of patients with IPF was higher than that of controls and patients with NSIP. These findings suggest a ligands (AGEs, non-AGE ligands)-RAGE interaction in the pathogenesis of IPF.

But, it is considered the limit of this study because we didn't measure for overall RAGEs such as circulating soluble RAGEs.

In summary, our study showed that a high level of circulating AGEs and increased expression of AGEs and RAGE in lung tissue of IPF patients, compared to those in NSIP and control. However, the NSIP patients did not show a difference in AGE or RAGE expression compared to that in the control. These finding was the major difference between IPF and NSIP. We can infer by these results that increased AGEs and non-AGE ligands-RAGE interaction may play a pathogenic role in the irreversible fibrosis of IPF. Further study of the roles of RAGE and the AGE-RAGE interaction will be more needed.

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Disclosure of conflict of interest

There is no conflict of interest in this study.

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