

# Immunohistochemical localisation of advanced glycation end products in pulmonary fibrosis

Takeshi Matsuse, Eijiro Ohga, Shinji Teramoto, Masashi Fukayama, Ryoji Nagai, Seikoh Horiuchi, Yasuyoshi Ouchi

Department of Geriatrics, Faculty of Medicine, University of Tokyo, Tokyo, Japan  
T Matsuse  
E Ohga  
S Teramoto  
Y Ouchi

Department of Pathology, Jichi Medical School, School of Medicine, Tochigi, Japan  
M Fukayama

The Second Department of Biochemistry, Kumamoto University School of Medicine, Kumamoto, Japan  
R Nagai  
S Horiuchi

Correspondence to: Dr Takeshi Matsuse, Department of Geriatrics, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo Bunkyo-ku, Tokyo 113, Japan.

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Table 1 Summary of 6D12 immunoreactivity of pulmonary tissue from cases of pulmonary fibrosis and cases with normal pulmonary parenchyma

Case	Age (years)	Macrophages*	Bronchiolar or metaplastic epithelium	Hyperplastic smooth muscle cells and/or interstitium
DAD 1	82	++	+	NE
DAD 2	75	+	++	NE
DAD 3	68	+	-	NE
IPF 1	78	++	+	±
IPF 2	69	++	+	+
IPF 3	74	++	-	-
IPF 4	82	++	±	+
IPF 5	74	++	+	±
IPF 6	73	++	±	±
IPF 7	53	++	++	+
Ctrl 1	62	++	-	-
Ctrl 2	37	-	-	-
Ctrl 3	71	-	-	-
Ctrl 4	30	+	-	-
Ctrl 5	62	-	-	-
Ctrl 6	61	-	-	-
Ctrl 7	48	-	-	-

Grading of immunoreactivity (see Methods): -, negative or very weak; ±, faintly positive; + positive; ++ strongly positive. Ctrl, control; DAD, diffuse alveolar damage; IPF, idiopathic pulmonary fibrosis; NE, not evaluated.

\*Tissue samples of cases with IPF showed increased expression of 6D12 immunoreactivity v control subjects (p < 0.05, Mann-Whitney rank test).

## Abstract

**Aim**—To investigate the presence and distribution of advanced glycation end products (AGE) in pulmonary fibrosis.

**Methods**—Lung tissue samples obtained from seven necropsy cases with idiopathic pulmonary fibrosis and seven with normal pulmonary parenchyma were examined immunohistochemically with a monoclonal antibody specific for AGE: 6D12. We also tested three cases with diffuse alveolar damage.

**Results**—All the specimens from cases with pulmonary fibrosis and diffuse alveolar damage showed strong AGE expression on macrophages. Lung specimens from normal parenchyma showed positive AGE immunoreactivity on macrophages from only two of seven cases.

**Conclusions**—These findings suggest that AGE modified proteins accumulate in alveolar macrophages in patients with diffuse alveolar damage and idiopathic pulmonary fibrosis.

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Keywords: advanced glycation end product; immunohistochemistry; pulmonary fibrosis

In the Maillard reaction, prolonged incubation of proteins with glucose leads to advanced glycation end products (AGE) through early stage intermediates such as Schiff base and Amadori rearrangement products.<sup>1,2</sup> Recent immunological studies have identified AGE modified

proteins in several human and animal tissues and suggest that AGE modification may be involved in the pathogenesis of several diseases including human diabetic complications and atherosclerosis.<sup>2-4</sup>

Pulmonary fibrosis is characterised by a complex process involving chronic inflammation, fibroblast proliferation, and abnormal deposition of interstitial collagen. The key cellular participants in the pathogenesis of pulmonary fibrosis have not been completely determined. However, lung macrophages are assumed to be one of the key cellular participants in these fibroproliferative processes.

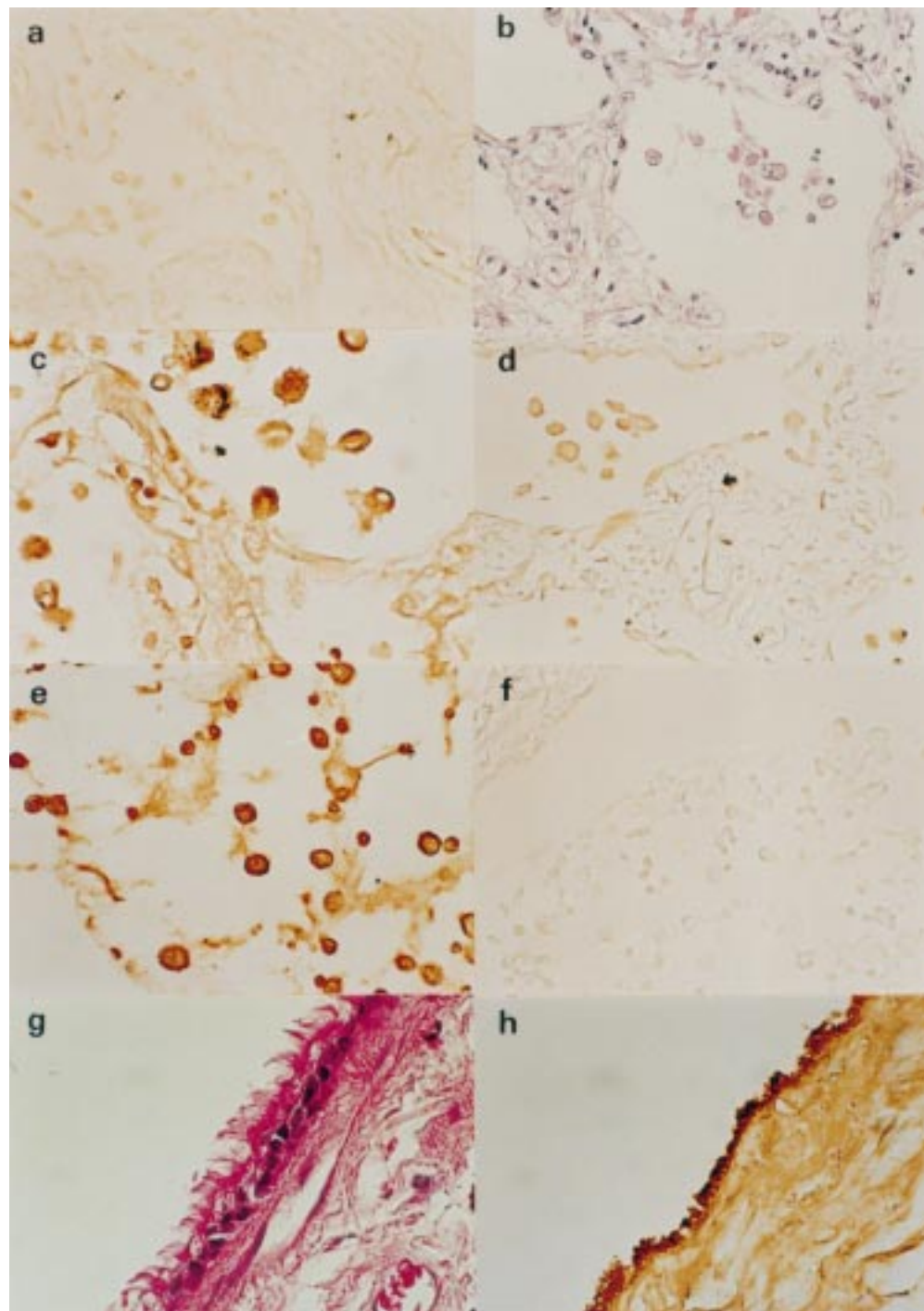
AGE modified proteins accumulate in aging extracellular matrix and it is thought that they are taken up by receptors or binding sites on macrophages. The receptor for advanced glycation end products (RAGE) has been supposed to mediate monocyte activation; it has been shown to stimulate monocytes/macrophages to synthesise and release inflammatory cytokines, in particular tumour necrosis factor (TNF) and interleukin-1 (IL-1).<sup>5</sup> In addition, AGE modified proteins induce monocytes/macrophages to generate growth factors—platelet derived growth factor (PDGF)<sup>6</sup> and insulin-like growth factor I (IGF-I)<sup>7</sup>—by the AGE receptor system. These cytokines and growth factors secreted by lung macrophages are thought to play cardinal roles in the pathogenesis of pulmonary fibrosis.

In this study, we examined the immunohistochemical localisation of AGE modified proteins in the remodelling lesions of pulmonary fibrosis, using a monoclonal antibody specific for AGE (6D12).<sup>8</sup> We showed accumulation of AGE modified proteins in lung macrophages in diffuse alveolar damage and remodelling lesions of pulmonary fibrosis, suggesting that chemical modification by AGE could be a biomarker of oxidative stress or might play some role in pulmonary fibrosis.

## Methods

### MONOCLONAL ANTI-AGE ANTIBODY

A monoclonal mouse anti-AGE antibody (6D12) was produced by immunisation of BALB/c mice with AGE bovine serum albumin (AGE-BSA) as described elsewhere.<sup>4,8</sup> Briefly, splenic lymphocytes from Balb/c mouse immunised with AGE-BSA were fused to myeloma P3U1 cells. The hybrid cells were screened to select cell lines positive for AGE-BSA but negative for BSA. One cell line, termed 6D12 cell, was selected and further purification by protein A affinity chromatography to IgG1 was



**Figure 1** Immunohistochemistry of lung specimens obtained from cases with normal parenchyma, diffuse alveolar damage, and idiopathic pulmonary fibrosis. (a) 6D12 immunoperoxidase staining of normal lung parenchyma showing no immunoreactivity on macrophages and alveolar epithelium ( $\times 233$ ). (b) Haematoxylin and eosin (H and E) staining of a lung specimen obtained from a case with diffuse alveolar damage showing interstitial oedema and alveolar macrophages ( $\times 233$ ). (c) 6D12 immunoperoxidase staining of the same specimen as in (b). Note that the 6D12 positive cells are macrophages ( $\times 467$ ). (d) Negative control for immunoperoxidase staining of the same lung specimen as in (c), in which primary antibody was replaced with non-specific mouse IgG1 ( $\times 233$ ). (e) 6D12 immunoperoxidase staining of a lung specimen obtained from a case with idiopathic pulmonary fibrosis ( $\times 467$ ). Note that the 6D12 positive cells are alveolar macrophages. (f) Negative control for immunoperoxidase staining of the same specimen as in (e), in which primary antibody was replaced with non-specific mouse IgG1 ( $\times 233$ ). (g) H and E staining of the same specimen as in (e), showing columnar cells (metaplastic epithelium) in the honeycomb lesion ( $\times 467$ ). (h) 6D12 immunoperoxidase staining of the same specimen as in (g), showing 6D12 positive metaplastic epithelium ( $\times 467$ ).

performed. 6D12 reacts not only with AGE proteins such as AGE-BSA, AGE human serum albumin, AGE human haemoglobin, and AGE collagen, but also with AGE preparation obtained either from lysine derivatives or monoaminocarboxylic acids, whereas it does

not react with unmodified counterparts.<sup>4</sup> Thus 6D12 reacts with the main AGE structure common to all AGE preparations.<sup>8</sup> A recent study showed that the epitope of 6D12 is an N-(carboxymethyl)lysine-protein (CML) adduct with a carbonyl group important for

immunological recognition of AGE modified proteins.<sup>9</sup>

#### CASES

Seventeen lung samples obtained from necropsies were analysed. Seven were of idiopathic pulmonary fibrosis and three of diffuse alveolar damage. These diagnoses were made on the basis of clinical course, chest x ray examinations, pulmonary function tests, and postmortem histopathology. Seven cases with normal parenchyma comprised the control group. Causes of death in the control group were as follows: two cases of renal cell carcinoma and one case each of acute myocardial infarction, malignant lymphoma, pancreatitis, purulent inflammation, and liver cirrhosis. Three cases from the control group had diabetic mellitus. The range of time from death to obtaining the specimens in each group was as follows: idiopathic pulmonary fibrosis, 2–5 hours; diffuse alveolar damage, 0.5–22.3 hours; normal control, 1.2–4.5 hours.

#### IMMUNOHISTOCHEMISTRY

Each specimen was fixed with buffered formalin and embedded in paraffin wax. Immunohistochemical detection for AGE was done by the immunoperoxidase technique. Briefly, the tissue sections were dewaxed and treated with 3% hydrogen peroxide in methanol to inhibit endogenous peroxidase. The sections were then preblocked and incubated with 1.2 µg/ml of a monoclonal mouse antibody for AGE (6D12) overnight, followed by a biotinylated antimouse IgG antibody (Nichirei Inc, Tokyo, Japan). After incubation with streptavidin conjugated with horseradish peroxidase, specifically bound antibodies were visualised using peroxidase substrate, 3,3'-diamino benzidine (DAB; Vector Laboratories Inc, Burlingame, California, USA). To compare the distribution and intensity of immunostaining, all samples were studied in an identical fashion using an identical concentration of antibody, identical batches of reagents, and identical development times. Immunohistochemical controls consisted of substitution of purified mouse IgG1 (Sigma, Missouri, USA) for the primary antibody.

#### EVALUATION OF IMMUNOSTAINING

The degree of immunostaining was evaluated by an arbitrary semiquantitative scale, as described previously<sup>10</sup>: for alveolar macrophages, - = negative or only a small subpopulation (less than 10 % of cells) faintly stained; + = positive, more than half of cells were stained; ++ = strongly positive, most cells (more than two thirds of cells) were stained. For bronchiolar epithelium, metaplastic epithelium, and smooth muscle cells, - = no staining or the same level of background staining; ± = faintly positive or only a small subpopulation was stained; + = positive, most cells were stained; ++ = strong positive means marked staining homogeneously.

Non-parametric comparison of the degree of immunoreactivity of alveolar macrophages between the control and idiopathic pulmonary

fibrosis groups was made by the Mann-Whitney U test. Probability (p) values less than 0.05 were taken as significant.

#### Results

Table 1 summarises the findings of the immunohistochemical studies. The lung specimens with normal parenchyma obtained from all but two cases without pulmonary diseases were negative for 6D12 immunohistochemistry. Only two cases with normal parenchyma showed positive immunoreactivity on macrophages (one case had diabetes mellitus and the other was not diabetic). Two of three cases with diabetes mellitus were negative for 6D12. No lung specimens with normal parenchyma expressed immunoreactivity on the bronchiolar epithelium.

In marked contrast, strong 6D12 immunoreactivity was observed on alveolar macrophages from all seven cases with idiopathic pulmonary fibrosis (table 1). The earliest lesion of pulmonary fibrosis detected histopathologically is a macrophage-rich fibrinous exudate within alveoli associated with epithelial injury, which is called diffuse alveolar damage. Strong 6D12 immunoreactivity was observed on alveolar macrophages from all three cases of diffuse alveolar damage. The bronchiolar epithelium from some cases with diffuse alveolar damage and the metaplastic columnar epithelium of pulmonary fibrosis also showed positive 6D12 staining on immunohistochemistry. Hyperplastic smooth muscle cells and interstitium were positive in some cases of pulmonary fibrosis.

Typical immunohistochemical findings from cases with diffuse alveolar damage and idiopathic pulmonary fibrosis are shown in fig 1. Positive immunoreactivity was observed on alveolar macrophages and on the metaplastic epithelium in the honeycomb lesions.

#### Discussion

Our study showed the presence of advanced glycation end products in all specimens from cases with pulmonary fibrosis and diffuse alveolar damage. However, the lung specimens from normal parenchyma showed positive AGE immunoreactivity on macrophages from only two of seven cases. Strong 6D12 immunoreactivity was observed on alveolar macrophages from all cases with pulmonary fibrosis. In marked contrast, most alveolar macrophages from control cases were negative for 6D12, which suggests that 6D12 immunoreactivity is not a marker for alveolar macrophages. Our findings also raise the interesting possibility that AGE modified proteins could be a biomarker of oxidant stress or might be involved in the pathogenesis of pulmonary fibrosis.

In the Maillard reaction, proteins react with glucose to form stable Amadori products through Schiff base adducts. Upon long term incubation, these early products are converted to AGE, which are characterised by a brown colour, autofluorescent properties, and inter- or intramolecular cross linking.<sup>1,2</sup> Immunohistochemical studies using anti-AGE antibodies including 6D12 have shown the presence of



AGE modified proteins in several tissues including atherosclerotic lesions of arterial walls,<sup>3 4 11</sup> human lens proteins,<sup>12</sup> and amyloid fibril deposits in patients with haemodialysis related amyloidosis.<sup>13 14</sup> The presence of AGE has also been demonstrated in lens, aorta, and kidney of streptozocin induced diabetic rats.<sup>15-17</sup> These findings from immunological studies with anti-AGE antibodies have suggested a potential involvement of AGE modification in disease processes such as diabetic complications and atherosclerosis.

Pulmonary fibrosis is characterised by the presence of chronic inflammation and increased deposition of collagen in lung parenchyma. The key cellular participants in the pathogenesis of pulmonary fibrosis have not been defined. However, lung macrophages are assumed to be one of the major participants in these fibroproliferative processes, because they secrete various inflammatory cytokines such as TNF and growth factors including platelet derived growth factor (PDGF), IGF-I, and granulocyte monocyte colony stimulating factor (GM-CSF).<sup>18-21</sup>

Macrophages play an essential role in eliminating AGE modified proteins. AGE modified proteins are biologically recognised by AGE binding proteins or AGE receptors of the cell surface membrane.<sup>22 23</sup> Recent experiments with Chinese hamster ovary cells overexpressing the macrophage scavenger receptor (MSR) or with peritoneal macrophages isolated from MSR-knockout mice have indicated that MSR plays a major role as the AGE receptor in the endocytic uptake of AGE modified proteins by macrophages.<sup>24 25</sup> The other AGE receptor—the receptor for AGE (RAGE)—has been reported to mediate macrophage activation and induce the production and secretion by macrophages of several cytokines such as TNF and IL-1<sup>7</sup> and growth factors such as PDGF<sup>9</sup> and IGF-1.<sup>7</sup> Moreover, it is suggested that the macrophage AGE receptor stimulates the cells to release GM-CSF, which induces macrophage growth in an autocrine or paracrine fashion.<sup>26</sup> These AGE induced cellular responses in macrophages raise the possibility that AGE modified proteins taken up by the AGE receptors of macrophages may play a role in the pathogenesis of pulmonary fibrosis.

Further, reactive oxygen intermediates are known to be implicated in the pathogenesis of pulmonary injury, diffuse alveolar damage, and pulmonary fibrosis. AGE modified proteins have been shown to generate reactive oxygen intermediates.<sup>27 28</sup> Reactive oxygen intermediates are also involved in the formation of AGE structures such as carboxymethyllysine (CML).<sup>29</sup> CML is thus also considered to be a potential biomarker of oxidative damage of tissue proteins in vivo.<sup>29 30</sup> The immunological demonstration of CML modified proteins in several animal and human tissues<sup>4 12-14 17</sup> indicates that reactive oxygen intermediates are generated in situ. Taken together, these results suggest that the interaction of AGE modified proteins with macrophages might induce oxidant stress, which could also contribute to the development of pulmonary fibrosis.

In addition, we found positive AGE staining on bronchiolar and metaplastic epithelium in cases of diffuse alveolar damage and idiopathic pulmonary fibrosis. No previous study has looked for AGE receptors on bronchial epithelium. Although we are not able to determine the significance of AGE in bronchiolar and metaplastic epithelium in this study, the presence of AGE modified protein in these epithelial cells in cases of diffuse alveolar damage and idiopathic pulmonary fibrosis suggests a potential role as a biomarker of oxidative damage to tissue proteins.

We found positive AGE staining on alveolar macrophages in normal parenchyma obtained from two cases, one of which had diabetes mellitus. Although positive AGE staining might be related to the presence of diabetes, macrophages in the other two cases of diabetes were found to be negative for AGE.

In conclusion, this study is the first to show the presence of AGE modified proteins on alveolar macrophages and bronchiolar epithelium in cases of diffuse alveolar damage and pulmonary fibrosis. The exact role of the AGE modified proteins that accumulate in the lung remain to be elucidated.

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