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Original Article

The Clinical Assessment of Protease-Activated Receptor-2 Expression in Inflammatory Cells from Peripheral Blood and Bronchoalveolar Lavage Fluid in Idiopathic Pulmonary Fibrosis

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Background: Idiopathic pulmonary fibrosis (IPF) is a lethal pulmonary fibrotic disease. In general, the exaggerated activation of the coagulation cascade has been observed during initiation or maintenance of the fibrotic disease. In our recent study, immunohistochemical expression of protease-activated receptor-2 (PAR-2), which plays a key role in coagulation cascade, was observed in surgical specimen of IPF patients, and associated with poor clinical outcome. The aim of this study was to evaluate the overexpression of PAR-2 in inflammatory cells from peripheral blood and bronchoalveolar lavage fluid in IPF patients.

Methods: From May 2011 to March 2012, IPF patients and controls were enrolled in Seoul National University Hospital. Peripheral blood and bronchoalveolar lavage fluid were collected for analysis of PAR-2 expression. Flow cytometry and reverse transcription polymerase chain reaction were used for PAR-2 receptor and mRNA assessment.

Results: Twelve IPF patients and 14 controls were included in this study. Among them, flow cytometry analysis was conducted from 26 peripheral blood (patient group, 11; control group, 13) and 7 bronchoalveolar lavage fluid (patient group, 5; control group, 2). The expression of PAR-2 receptor was not different between patient and control groups (p=0.074). Among all 24 population, PAR-2 mRNA assessment was performed in 19 persons (patient group, 10; control group, 9). The mRNA expression of PAR-2 was not significant different (p=0.633).

Conclusion: In IPF patients, PAR-2 receptor and mRNA expression were not different from control group.

Key Words: Receptor, PAR-2; Idiopathic Pulmonary Fibrosis; Bronchoalveolar Lavage

Introduction

Idiopathic pulmonary fibrosis (IPF) is an inflammatory fibrotic lung disease of unknown etiology. Unfortunately, IPF is a progressive and irreversible disorder. In general, median survival of IPF patients is 2.5 to 5 years with or without treatment[1,2]. The pathogenesis of IPF is poorly understood[3]. One of the major advances in the pathogenesis of IPF is the shift in current paradigms from inflammation to abnormal wound healing[4]. In the wound healing process, coagulation cascade is activated locally from tissue factor dependent extrinsic pathway[5]. Recent studies implicated protease-activated receptor-2 (PAR-2) in the fibrosis.

PAR-2 is a G-coupled 7-transmembrane receptor that is activated by tethered peptide ligand, which is exposed after enzymatic cleavage of the specific site in the extracellular N-terminal[6]. PAR-2 is expressed in various tissues and abundant in kidney, pancreas, and gastrointestinal tissue than in heart and lungs[7]. In each organ, PAR-2 expressions were mainly located in endothelial and epithelial cells[8]. Recent studies suggested association of PAR-2 activation with airway inflammation[9-11]

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and pulmonary fibrosis[12]. In addition, up-regulation of PAR-2 was observed in lung tissue of IPF patients and possible pathway of the development of pulmonary fibrosis[13]. Recently we conducted PAR-2 expression of surgical specimen from IPF patients, and we found the possibility of the association between PAR-2 expression and clinical outcome of IPF[14]. So the aim of this study was to evaluate the overexpression of PAR-2 in inflammatory cells from peripheral blood and bronchoalveolar lavage fluid in IPF patients.

Materials and Methods

1. Study population

We included the patients with IPF in Seoul National University between May 2011 and March 2012. The diagnosis of IPF was based on the established criteria (20) and clinical diagnosis was judged according to international guidelines. Peripheral blood and bronchoalveolar lavage fluid were collected after informed consent. The protocol was approved by the Institutional Review Board of the Seoul National University Hospital. All patients gave written informed consent. The study was conducted in accordance with the Declaration of Helsinki.

2. Mononuclear cell isolation

Blood (5 mL) was drawn into heparinized tubes and mononuclear cell isolation was performed within 6 hours from acquisition of specimen. Blood was diluted phosphate buffered saline with equal volume. The mixture was layered over 5 mL Ficoll-Paque-Plus solution (density 1.077/mL; Amersham Biosciences, Uppsala, Sweden). The layers of density gradient were separated after centrifugation (2,100 rpm for 20 minutes). Mononuclear cells were harvested with micropipette.

3. Flow cytometry

Mononuclear cells (1.0×10^6/mL) were fixed in 4% paraformaldehyde for 20 minutes at 4°C and then incubated 30 minutes room temperature with CD3 (BD Biosciences, San Jose, CA, USA), CD14 (BD Biosciences), CD20 (BD Biosciences), PAR-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Each isotype controls were used for calibration.

4. Reverse transcription polymerase chain reaction (RT-PCR) and PCR

The total RNA was extracted from the mononuclear cells using RNeasy Mini kit (Qiagen, Valencia, CA, USA), and PCR was followed using commercially available kit (Bioneer, Daejeon, Korea), according to manufacturere's instructions. Oligonucleotide primers (forward: 5'-GTT GAT GGC ACA TCC CAC GTC-3', reverse: 5'-GTA CAG GGC ATA GAC ATG GC-3') were designed for PAR-2. The PCR conditions were as follows; a pre-denaturating for 94°C for 1 minute, followed by 40 cycles fo denaturation at 94°C for 1 minute, annealing at 64°C for 1 minute, and extension at 72°C for 1 minute. PCR products were separated by electrophoresis through 1% agarose gel and the signal intensity was analyzed by ImageJ. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) controls were used to standardize the quantification of samples.

RT-PCR was conducted to examination the mRNA expression of PAR-2 and GAPDH.

5. Statistical analysis

All data were showed as mean±SD. Student t-test was used for comparison between two groups. SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis and $p<0.05$ was considered as statistically significant difference.

Results

1. Study population

Table 1 shows the clinical characteristics of IPF patients (n=12) and controls (n=14). Median age was 66 in IPF group and 63 control group. Pulmonary function test showed statistically significant different forced vital capacity (FVC) and forced expiratory volume in one second/FVC, because chronic obstructive pulmonary disease (COPD) patients (n=8) were included in control group.

Table 1. Baseline characteristics of included patients with idiopathic pulmonary fibrosis and controls

Variable	Patient (n=12)	Control (n=14)	p-value
Median age (range), yr	66 (43-80)	63 (37-75)	0.315
Male sex, n (%)	8 (66.7)	13 (92.9)	0.148
Pulmonary function test (n=22)			
FVC (% pred)	73	106	0.005
FEV_1 (% pred)	82	62	0.178
FEV_1/FVC	77.7	43.9	0.001
White blood cell count (n=24)	7,000	7,705	0.686
Neutrophil	3,938.5	3,892.8	0.862
Lymphocyte	1,931.8	2,462.7	0.083

FVC: forced vital capacity; FEV_1: forced expiratory volume in one second.

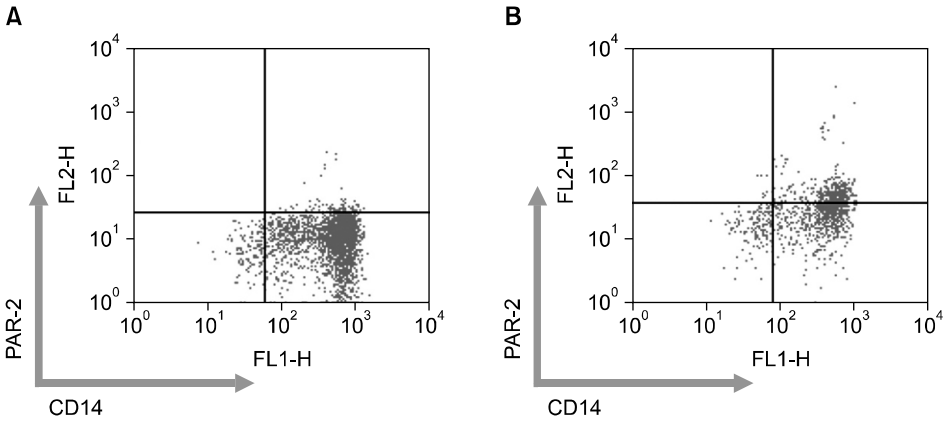

Figure 1. Distribution of protease-activated receptor-2 (PAR-2) and CD14 double positive cells in idiopathic pulmonary fibrosis patient (A) and control (B).

2. PAR-2 receptor expression in flow cytometry

We conducted flow cytometry analysis from 26 peripheral blood (patient group, 11; control group, 13) and 7 bronchoalveolar lavage (BAL) fluid (patient group, 5; control group, 2) (Figure 1). CD3, CD14, CD20, PAR-2 expression were analyzed. PAR-2 expression was not significant different between patient and control group in peripheral blood and BAL fluid (Figure 2). The proportion of PAR-2 and CD14 double positive among CD14 single positive was no different in both groups (p=0.247).

3. PAR-2 mRNA expression

Among 26 persons, PAR-2 mRNA assessment was performed in 19 persons (patient group, 10; control group, 9). mRNA expression of PAR-2 was not different between patient and control group (p=0.633) (Figure 3). Because there was paucity of cellularity in BAL fluid, only two cases (patient, 1; control, 1) were conducted RT-PCR for mRNA expression of PAR-2.

Discussion

The understanding of IPF pathogenesis has shifted from inflammatory response to aberrant wound healing of unknown etiology[4]. In other words, the pulmonary fibrosis is considered as the common end pathway of aberrant wound healing. Interestingly, during the fibrotic process, coagulation cascade activation was frequently observed not only IPF but also other inflammatory conditions such as systemic sclerosis and acute respiratory distress syndrome[15-17]. In acute lung injury animal model, uncontrolled activation of coagulation cascade and pulmonary fibrosis was also observed, and furthermore PAR-2 activated by Factor Xa was a sig-

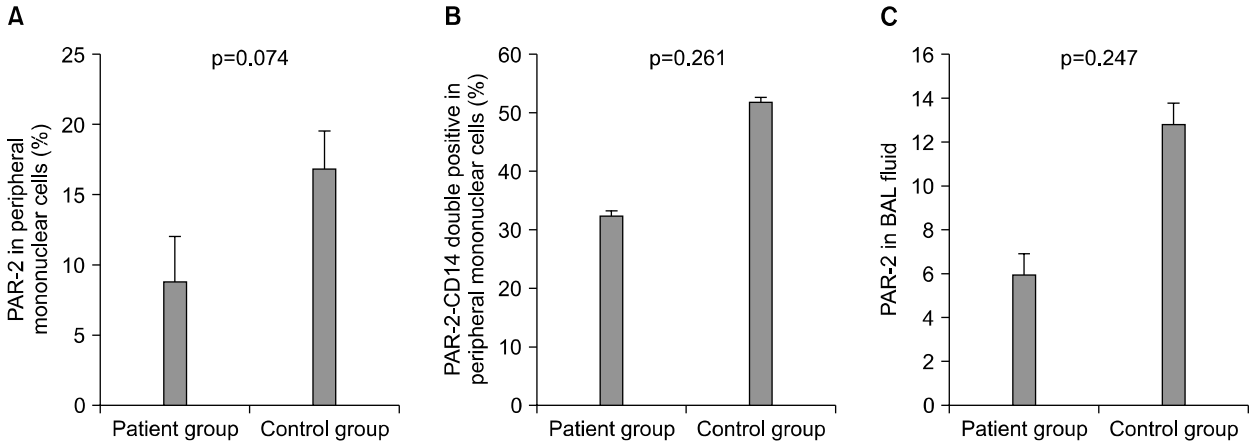

Figure 2. Comparison of protease-activated receptor-2 (PAR-2) expression in flow cytometry between patient and control group. PAR-2 expression was not significantly different in peripheral blood (A) and bronchoalveolar lavage fluid (C). The fraction of PAR-2 and CD14 double positive population in CD14 positive population was not significant different (B).

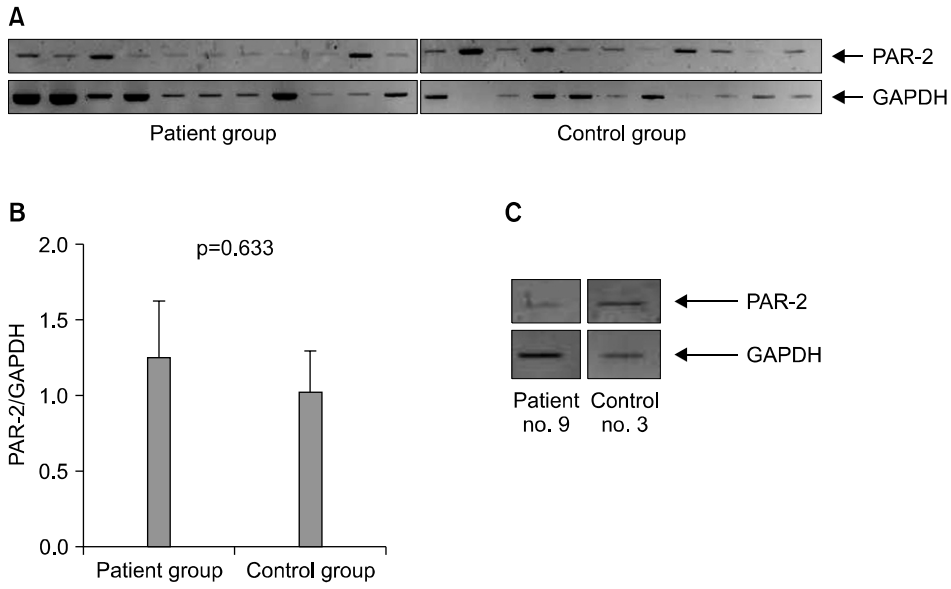

Figure 3. mRNA expression in peripheral mononuclear cells (A) and bronchoalveolar lavage fluid (C). The difference of mRNA expression in peripheral mononuclear cells was not significantly different (B, p=0.633). PAR-2: protease-activated receptor-2; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

nificant receptor for the development of pulmonary fibrosis[12]. These findings suggest the possible role of PAR-2 in the pathogenesis or progression of pulmonary fibrosis.

As described above, we reported the relationship between immunohistochemical expression of PAR-2 and worse clinical outcomes of IPF patients[14]. But this study did not show an increased PAR-2 receptor expression in BAL fluid and peripheral mononuclear cells with IPF patients. The relationship between activation of coagulation cascade and pathogenesis of IPF might be irrelevant. Moreover, warfarin did not show a beneficial effect in progressive IPF in recent study[18]. In addition, this study had some limitations. Most of all, study population was so small. We included only 12 IPF patients during 10 months. The second, control group was not homogeneous. Among 14 control group, 8 were COPD patients. COPD is well known systemic inflammatory disease and PAR-2 is associated with inflammation[19]. So it is explainable that PAR-2 receptor expression was increased in control group (Figure 3).

In conclusion, PAR-2 receptor and mRNA expression

was not increased in IPF patients compared to control group.

Acknowledgements

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