行政院國家科學委員會專題研究計畫　成果報告

原發性肺纖維化病人基因表現與多型性之分析

計畫類別：個別型計畫
計畫編號：NSC92-2314-B-002-304-
執行期間：92 年 08 月 01 日至 93 年 07 月 31 日
執行單位：國立臺灣大學醫學院檢驗醫學科

計畫主持人：李麗娜

報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 93 年 12 月 14 日

行政院國家科學委員會補助專題研究計畫 √ 成 果 報 告 □期中進度報告

（計畫名稱）

原發性肺纖維化病人基因表現與多型性之分析

計畫類別：√ 個別型計畫　　□ 整合型計畫
計畫編號：NSC 92-2314-B-002-304- －　　－　　－　　－　　－
執行期間：　2003 年 8 月 1 日至 2004 年 7 月 31 日

計畫主持人：李麗娜
共同主持人：
計畫參與人員：張秀靜

成果報告類型(依經費核定清單規定繳交)：√精簡報告　□完整報告

本成果報告包括以下應繳交之附件：
□赴國外出差或研習心得報告一份
□赴大陸地區出差或研習心得報告一份
□出席國際學術會議心得報告及發表之論文各一份
□國際合作研究計畫國外研究報告書一份

處理方式：除產學合作研究計畫、提升產業技術及人才培育研究計畫、列管計畫及下列情形者外，得立即公開查詢
□涉及專利或其他智慧財產權，□一年□二年後可公開查詢

執行單位：

中　華　民　國　93 年 12　月　13 日

中文摘要

原發性肺纖維化(IPF)是原因不明的間質性肺疾，其特徵是肺泡組織因細胞凋亡而破壞，纖維細胞增生，以及膠原蛋白之大量沉積.對原發性肺纖維化的研究常以 bleomycin 在小白鼠引發肺組織纖維化作為動物模式，Kaminski 等人利用 oligonucleotide microarrays 研究小白鼠之肺臟對於 bleomycin 所反應出之基因表現，因而發現有一組基因之表現與纖維化有關，其中表現最強的基因之一是 osteopontin.

Osteopotin 是一種含有 arginine-glycine-asparatic acid (RGD) 的蛋白質，很多細胞都會分泌 osteopontin，包括 osteoclasts，activated T cells，以及 activated macrophages. osteopontin 基因會進行 alternative RNA splicing，產生三種不同的 cDNA，這些 splice variants 的功能尚不清楚.

在本計劃中我們研究原發性肺纖維化病人，以及其他可能導致肺纖維化之疾病如急性間質性肺炎,肺泡蛋白沉著症之病人，其 osteopontin mRNA 之表現，以 RT-PCR 及 direct sequencing 方法，研究病人周邊血液單核血球所表現之 osteopontin mRNA. 我們發現, osteopontin mRNA 之一種 splice variant (缺乏 exon 5),可能與原發性肺纖維化之嚴重程度及預後有關，但與肺泡蛋白沉著症之嚴重程度及預後無關. 目前我們仍在繼續收集更多病例.

ABSTRACT

Idiopathic pulmonary fibrosis (IPF), is an interstitial lung disease of unknown cause, characterized by the loss of alveolar architecture through the apoptosis of epithelial and endothelial cells, proliferation of myofibroblasts , and extensive deposition of extracellular matrix proteins, especially collagens type I and III. The molecular basis of pulmonary fibrosis has been studied in a murine model. In mouse with pulmonary fibrosis induced by bleomycin, the expression of a large cluster of genes were augmented, and osteopontin was one of these most dramatically induced genes. Osteopontin is an arginine-glycine-asparatic acid (RGD)-containing protein secreted by a variety of cells, including osteoclasts, activated T cells, and activated macrophages. There is evidence of alternative RNA splicing of the osteopontin gene with three osteopontin cDNAs identified. The function of these splice variants is unknown.

In the present study we examined the mRNA expression of osteopontin gene in patients with idiopathic interstitial fibrosis IPF), acute interstitial pneumonitis (AIP) and idiopathic pulmonary alveolar proteinosis (PAP), by RT-PCR and direct sequencing of osteopontin mRNA transcripts. We found that the expression of a splice variant mRNA of osteopontin gene, which lacks exon 5, is associated with severity and poor prognosis of IPF, but not with prognosis of PAP. We are currently continuing to collect more cases of idiopathic pulmonary fibrosis and other interstitial lung disease which ends up with fibrosis, and examine the mRNA expression of osteopontin gene, including its variant forms caused by alternative splicing.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF), or usual interstitial pneumonitis (UIP), is an interstitial lung disease of unknown cause, characterized by the loss of alveolar architecture through the apoptosis of epithelial and endothelial cells, proliferation of myofibroblasts , and extensive deposition of extracellular matrix proteins, especially collagens type I and III (1,2). Despite of the identification of many of the extracellular mediators and genes which are associated with pulmonary fibrosis, the mechanism of this disease is little understood. Current therapies, aimed at inhibiting lung inflammation that often precedes fibrosis, are effective only in a minority of patients, and there are currently no proven therapies targeting the fibrotic process itself (1-3).

The molecular basis of pulmonary fibrosis has been studied in a murine model. Kaminski and colleagues (2) studied changes in gene expression of mouse with pulmonary fibrosis in response to bleomycin. They found a large cluster of genes that were associated with bleomycin-induced fibrosis, but not inflammation. Osteopontin was one of these most dramatically induced genes.

Osteopontin is an arginine-glycine-asparatic acid (RGD)-containing protein secreted by a variety of cells, including osteoclasts, activated T cells, and activated macrophages (4,5). The osteopontin gene is located on human chromosome 4q13 (6). There is evidence of alternative RNA splicing of the human osteopontin gene with three osteopontin cDNAs identified (7). The function of these splice variants is unknown.

In the present study we examined the mRNA expression of osteopontin gene in patients with idiopathic interstitial fibrosis IPF), acute interstitial pneumonitis (AIP) and idiopathic pulmonary alveolar proteinosis (PAP), by RT-PCR and direct sequencing of osteopontin mRNA transcripts. We found that the expression of a splice variant mRNA of osteopontin gene, which lacks exon 5, is associated with severity and poor prognosis of IPF, but not with prognosis of PAP.

MATERIALS AND METHODS

Patients

We collected peripheral blood monocytic cells (PBMCs) from: 2 patients with open-lung-biopsy proved IPF, one patient with systemic lupus erythematosus

associated AIP, and three with PAP.

Expression of osteopontin mRNA

PBMC are obtained by density gradient DNA Purification kit. Total cellular RNA is extracted using acid guanidinium thiocyanate method. The purified RNA is quantified by absorbance measurements at 260 nm, and its integrity is determined by electrophoresis through a 1% agarose-formaldehyde ethidium bromide gel followed by visualization of the 28S and 18S ribosomal RNA bands. One microgram of RNA from each sample is reverse transcribed using oligo(dT) as a primer and Moloney myeloblastic leukemia virus (MMLV) reverse transcriptase at 42^{o}C for 60 min. After terminating the reactions, the complimentary DNA samples are diluted in 10 mM tris (hydroxymethyl) aminomethane (Tris)-1 mM EDTA to a volume of 200 ul. Three-microliter aliquots of diluted cDNA are amplified in the presence of the osteoponin primers (8) listed below:

Sense:
CAT GAG AAT TGC AGT GAT TTG CTT TTG C (human osteopontin nt 101-128) (exon 2)

Anti-sense:
CAG TAC CCT GAT GCT ACA GAC GAG (human osteopontin nt 597-620)(exon 6)

The PCR protocol is: 40 cycles of 95^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C for 2 min, followed by a final extension at 72^{o}C. After PCR, 10 ul of the amplified product was electrophoressed on a 2.5% agarose gel and stained with ethidium bromide.

Sequencing of the amplified cDNA

The amplified DNA bands of osteopontin were cut from the agarose gel. The DNA extracted using a DNA extraction kit, and sequenced using the sense, anti-sense primers listed above and an automatic sequencing system.

RESULTS

mRNA expression of osteopontin gene

We found that by RT-PCR analysis, three cDNA fragments were amplified on agarose

gel electrophoresis. The shortest was about 520 base-pair (bp) in length; the middle one, about 560 bp; the longest one, about 600 bp.

Table. Osteopontin mRNA expression in six patients with interstitial lung disease

No.	Diagnosis	Diffusion Impairment	Outcome	Osteopontin mRNA		
				520 bp	560 bp	600 bp
1.	IPF*	mild	alive	-	+	-
2.	IPF	moderate	dead	+	+	+
3.	AIP**	mild	alive	+	+	-
4.	PAP***	moderate	alive	+	+	-
5.	PAP	severe	dead	+	+	-
6.	PAP	mild	alive	+	+	-

*IPF: idiopathic pulmonary fibrosis
**AIP: acute interstitial pneumonitis
***PAP: idiopathic pulmonary alveolar proteinosis

Sequencing analysis of osteopontin mRNA transcripts

Sequencing analysis of the three isoforms of osteopontin transcripts showed that the shortest isoform (520 bp) composed of exons 1, 2, 3, 4 and 6, and lacked exon 5 (42 bp long). The middle-length isoform (560 bp) contained exons 1,2,3,4,5 and 6. The longest isoform also contained exons 1 to 6. We havn't identified the sequential differences between the latter two isoforms.

DISCUSSION

From our preliminary data, milder form IPF was associated with the expression of the full length osteopontin mRNA transcript. The severer form, with diffusion impairment of greater severity, expressed both the full-length and the shorter mRNA isoform of osteopontin. AIP expressed both isoforms of osteopontin transcripts, but the shorter one was not associated with poor prognosis. As for PAP, all the three patients expressed both isoforms. The expression of osteopontin mRNA transcripts were not associated with outcome.

As we could find no previous data in the literature which can be compared with our own, the significance of the study results was undetermined. We are still collecting more cases of interstitial lung diseases, and sequencing the osteopontin transcripts.

REFERENCES

1. Strieter RM, Keane MP, Standiford TF, Kunkel SL. Cytokine biology and the pathogenesis of interstitial lung disease. In Interstitial Lung Disease, 3rd edition. M.I.Schwarz and J.T.E.King, editors. B.C.Decker, Inc., London. 181-205.
2. Kaminski N, Allard JD, Pittet JF, Zuo F, Griffiths MJ, Morris D, Huang X, Sheppard D, Heller RA. Global analysis of gene expression in pulmonary fibrosis reveals distinct programs regulating lung inflammation and fibrosis. Proc Natl Acad Sci 2000;97:1778-83.
3. Ryu JH, Colby TV, Hartman TE. Idiopathic pulmonary fibrosis: current concepts. Mayo Clin Proc 1998;73:1085-101.
4. Rodan G. Osteopontin overview. Ann NY Acad Sci 1995;760: 1-5.
5. Denhardt DT, Guo X. Osteopontin: a protein with diverse functions. FASEB J 1993; 7: 1475-1482.
6. Patarca R, Saavedra RA, Cantor H. Molecular and cellular basis of genetic resistance to bacterial infection: the role of the early T-lymphocyte activation-1/ osteopontin gene. Crit Rev Immunol 1993;13:225-46.
7. O'Regan A, Berman JS. Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. Int J Exp Pathol 2000;81:373-90.
8. Kiefer MC, Bauer DM, Barr PJ. The cDNA and derived amino acid sequence for human osteopontin. Nucleic Acids Res 1989;17:3306.