Acta Med Okayama 47 (2) 73-78 (1993)

Overexpression of Interleukin-2 Receptor α mRNA in Pulmonary Lymphocytes of Lung Cancer Patients Associated with Interstitial Pulmonary Shadow

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The activity of pulmonary lymphocytes was evaluated by the detection of interleukin-2 (IL-2) receptor α mRNA expression in lung cancer patients associated with diffuse interstitial shadow on roentgenograms of their lungs. Reverse transcription coupled with the polymerase chain reaction was used to detect mRNA expression. In 5 of 6 patients, IL-2Rα mRNA expression was increased in pulmonary lymphocytes compared with 4 normal controls. The expression in this mRNA in peripheral blood lymphocytes was almost undetectable in either normal controls or these patients. These results suggest that pulmonary lymphocytes in patients with lung cancer associated with diffuse interstitial shadows are activated and may promote the inflammatory process generating pulmonary fibrosis.

Key words : **pulmonary fibrosis, lung cancer, pulmonary lymphocytes, IL-2R α mRNA, RT-PCR**

It is well known that idiopathic interstitial pneumonia (IIP) is frequently associated with lung cancer (1), and pulmonary fibrosis is recognized as a precancerous lesion (2). It seems possible that continuing inflammatory fibrosis generates hyperplasia or metaplasia of the alveolar cells and respiratory bronchiolar epithelium (3) followed by carcinomatous change. Various cytokines (4) produced by activated macrophages, T lymphocytes, and B lymphocytes could play an important role in such processes. The interleukin-2 receptor α chain (IL-2R α) (5) is expressed by activated T cells and causes the clonal expansion of lymphocytes after IL-2 stimulation in inflammatory lesions. IL-2R α mRNA expression of pulmonary lymphocytes obtained by bronchoalveolar lavage (BAL) was examined to detect activated pulmonary lymphocytes in fibrotic lung lesions. Because BAL produces a small amount of clinical material, reverse transcription coupled with the polymerase chain reaction (RT-PCR) (6, 7) was applied to the detection of the target mRNA. In this study, IL-2 R α mRNA expression in pulmonary lymphocytes was greater in 5 of 6 patients with lung cancer associated with diffuse interstitial shadow than in 4 normal controls.

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Materials and Methods

Patients. Six patients with lung cancer associated with diffuse interstitial pulmonary shadow (IP + lung cancer) and 5 healthy individuals (normal controls) were studied. We analyzed both pulmonary and peripheral blood lymphocytes in 5 of 6 patients and in 4 of 5 normal controls. The patient profiles are presented in Table 1. The madian age was 66 and all the patients were men. The diagnosis of lung cancer was proven by biopsy in all cases, and the histologic diagnosis was adenocarcinoma in three patients and small cell carcionoma in the remaining three. The primary tumor was located peripherally in all 6 patients. The diffuse interstitial lung shadows around the lung cancers appeared as mostly reticulo-nodular shadows on roentgenograms of the chest.

Preparation of lung and blood mononuclear cells. BAL was performed under local anesthesia during diagnostic bronchoscopy for lung cancer patients and for the healthy normal individuals. A total of 200 ml of sterile 0.9 % saline (warmed to 37 ℃) was injected in 50 ml aliquots into the fibrotic lung parenchyma without cancer using a fiberoptic bronchoscope. Each aliquot was immediately aspirated and filtered through stainless steel mesh. BAL cells

Table 1 Patient characteristics

Patient number	Age (year)	Sex	Histlogical findings	Clinical stage	
1.	65	Male	Small cell carcinoma	T 2 N 2 M 0	III A
2.	71	Male	Adeno carcinoma	T 2 N 3 M 0	III B
3.	59	Male	Adeno carcinoma	T 2 N 0 M 0	I
4.	68	Male	Adeno cercinoma	T 2 N 2 M 1	IV
5.	62	Male	Small cell carcinoma	T 2 N 2 M 0	III A
6.	70	Male	Small cell carcinoma	T 1 N 2 M 0	III A

Patient numbers are correlated with Figs. 2 and 3.
Clinical stages are defined by TNM classifications.

were isolated by centrifugation at $100 \times g$ for 10 min at 20 ℃, washed with PBS, and then resuspended in RPMI 1640 medium (GIBCO, Gl and Island, NY) in plastic dishes. After 2 h of incubation at 37 ℃, non-adherent cells were recovered and used as lymphocyte-enriched preparations. Peripheral blood mononuclear cells were separated from heparinized venous blood by Ficoll/Hypaque density gradient centrifugation.

Reverse transcription of cytoplasmic RNA. We used the 10^5 cells of both pulmonary lymphocytes and peripheral blood lymphocytes (8). Cells were placed in a 100 μl microtube, diluted with PBS, and then pelleted at $500 \times g$ for 5 min . The cells were then resuspended in 20 μl of ice-cold lysis buffer containing 0.5 % NP-40, 10 mM Tris-HCl, 140 mM NaCl, and 1.5 mM $MgCl_2$. The cells were vortexed and the nuclei were pelleted by centrifugation at $12,000 \times g$ for 2 min. The postnuclear supernatant was transferred to another microtube and heated at 90 ℃ for 5 min. Then the aggregated material was removed by centrifugation and the supernatant was transferred to another tube. An aliquot (10 μl) of the above supernatant was mixed to a final volume of 20 μl with 50 mM KCl, 10 mM Tris · HCl (pH 8.3), 4 mM $MgCl_2$, 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 1 mM dCTP, 20 units of human placental RNase inhibitor (Takara Co., Kyoto, Japan), 2.5 μM random hexamer, and 7.5 units of RAV-2 reverse transcriptase (Takara). Then incubation was performed for 60 min at 42 ℃.

Polymerase chain reaction (PCR). PCR primers were synthesized by the phosphoramidite method (DNA synthesizer, Applied Biosystems). We used the primers (Clontech) derived from the 4th exon (sense primer 5′-GAATTTATCATTTCGTGGTGGGGCA-3′) and the 7th to 8th exons (antisense primer 5′-TCTTCTACTCTTCCTCTGTCTCCG-3′) of IL-2R α mRNA, so that the PCR product derived from the cDNA could easily be distinguished from that derived from genomic DNA. Reagent controls consisted of the reaction mixture above without reverse transcriptase. The target sequence defined by these primers included the 4th exon which is indispensable to the translation of functional IL-2R α. Reverse transcripition products were boiled for 5 min and cooled on ice. They were then diluted to obtain a final PCR mixture containing 50 mM KCl, 10 mM Tris · HCl (pH 8.0), 2.5 mM $MgCl_2$, all four $dNTP_S$ (each at 250 μM), 40 pmoles of each primers, and 3 units of Thermus aquaticus DNA polymerase (Perkin-Elmer/Cetus). Samples were subjected to 35 cycles of amplification which consisted of denaturation for 1 min at 95 ℃, primer annealing for 1 min at 37 ℃, and polymerization for 1.5 min at 72 ℃. A β-actin targeted gene sequence (9) served as the internal control, and was defined by the sense primer (5′-ATCATGTTTGAGACCTTCAA-3′) corresponding to the cDNA sequences 1854 to 1873 and the anti-sense primer (5′-CATCTCTTGCTCGAAGTCCA-3′) representing the cDNA sequences 2152 to 2171. The primer concentrations of β-actin were examined for 80 pmoles, 40 pmoles and 10 pmoles per reaction. The result of 80 pmoles per reaction showed excessive expressions and that of 10 pmoles showed low and unstable expressions, so 40 pmoles per reaction was apllied. PCR products were extracted by chloroform and directly applied to Southern blot analysis.

Southern blot analysis. PCR products (15 μl) were subjected to electrophoresis on 1.2 % Sea-Kem agarose gel, transferred to a nylon membrane (Gene Screen Plus, Dupont), and hybridized with^{32} P-labeled cDNA probe. Hybridization was performed in the 10 ml packed solution consisted of 50 % formamide, 1 % SDS and 10 % dextran for 24 h at 42℃ with constant agitation, then the membrane was washed in $2 \times$ SSC for 10 min at room temperature, followed by $2 \times$ SSC and 10 % SDS for 30 min at 60 ℃, and $0.1 \times$ SSC for 10 min at room temperature. It was then exposed to X-ray film for 48 h at −70 ℃ for autoradiography. The signal density of specific bands were measured by photodensitometry for the semiquantitation of mRNA expression.

Results

IL-2R α mRNA expression in MT-1 (10) cells was analyzed semiquantitatively by reverse transcriptase-PCR as the positive control. These are human T cell leukemia virus type- I infected cells that consititutively express IL-2R α. K 562 cells (11) (chronic myelogenous leukemia) were used as the negative control. IL-2R α mRNA expression by serially diluted MT-1 cells is shown in Fig. 1. The signal density of the IL-2R α mRNA bands shows a linear relationship to the number of cells. Under the same conditions, 6×10^4 cells of MT-1 were analyzed without applying reverse transcriptase, then no mRNA was detected indicating that cDNA did not contaminate the samples. For negative control, 6×10^4 cells of K 562 were analyzed in the same condition of positive control and IL-2R α mRNA expression was not detected.

The results of the semiquantitative analysis of IL-2R α mRNA expression by BAL lymphocytes are shown in Fig. 2. IL-2R α mRNA was almost undetectable in the BAL lymphocytes of the 4 healthy controls. BAL

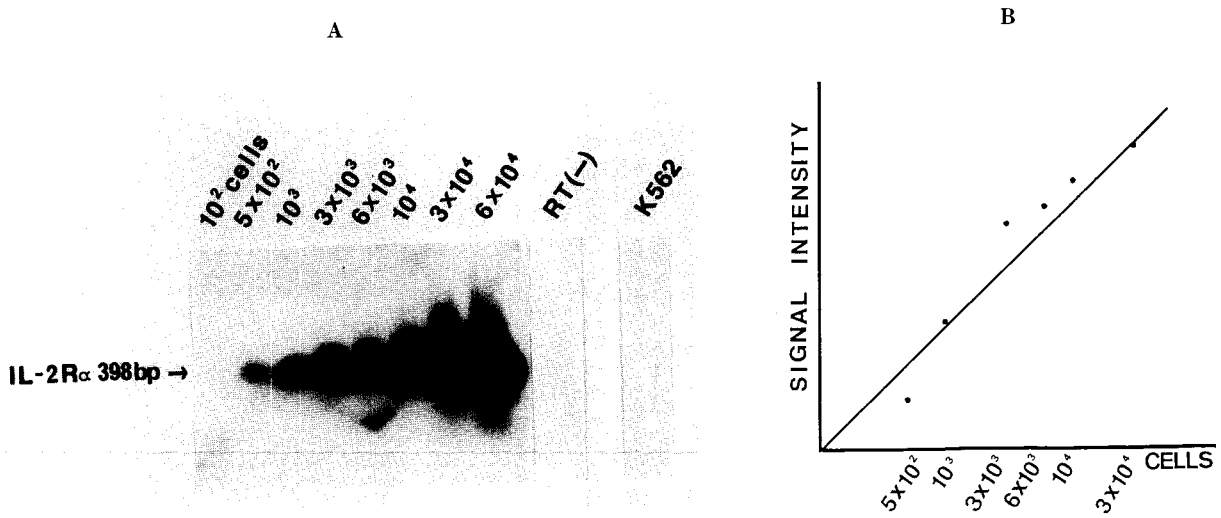

Fig. 1 IL-2R α mRNA expression in serial dilution of MT-1 cells for positive control and the results of K562 cells for negative control.
A. Southern blot analysis of PCR products. MT-1 cells for positive control were examined in serially diluted material from 10^2 cells to 6×10^4 cells. K562 cells for negative control were examined in 6×10^4 cells. RT(−): 6×10^4 cells of MT-1 were examined without reverse transcription.
B. Relative signal intensity were blotted on logarithmic scale of cell numbers.

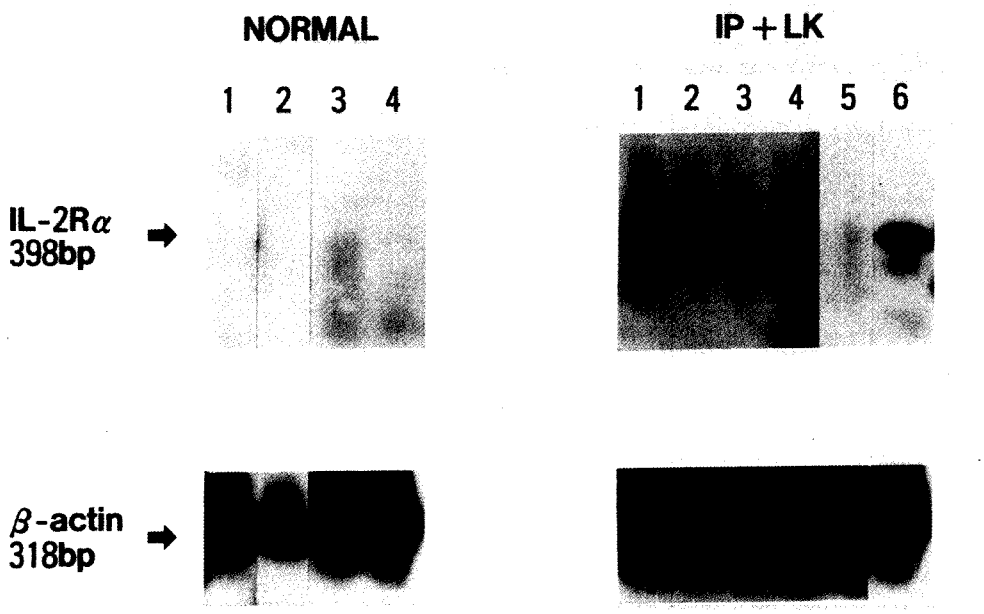

Fig. 2 IL-2R α mRNA expression in bronchoalveolar lavage lymphocytes. The number of lanes in IP + LK is corresponding to the number of patients listed in Table 1, and in NORMAL, is corresponding to the number of normal control shown in Fig. 3. All these results were obtained using 10^5 lymphocytes each. bp: base pairs. IP + LK: interstitial pulmonary shadow + lung cancer.

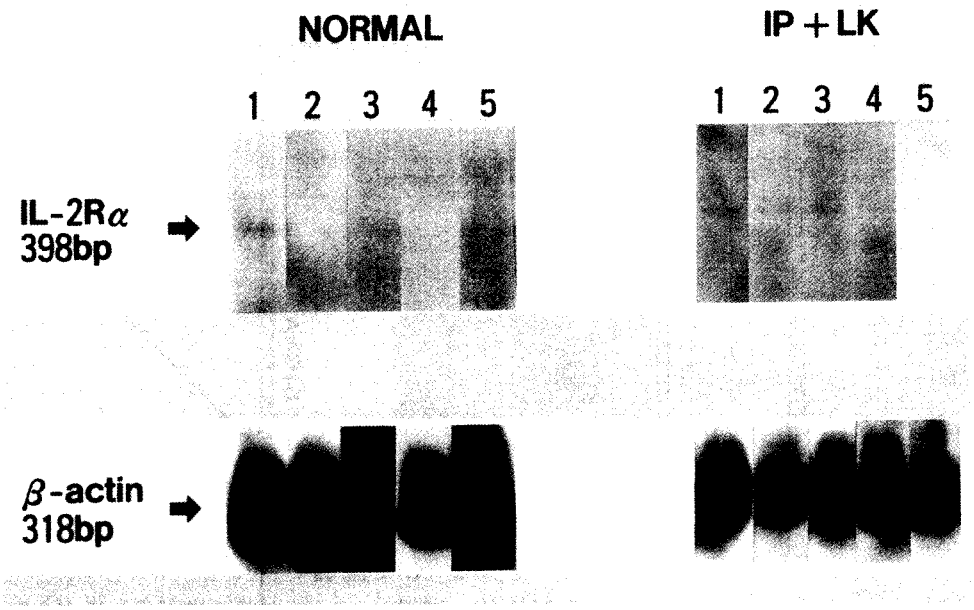

Fig. 3 IL-2 R α mRNA expression in peripheral blood lymphocytes. Lane numbers of the patients are correlated with Table 1 and Fig. 2. All these results were obtained using 10^5 lymphocytes each.

lymphocytes showed a high level of IL-2R α mRNA expression in 5 of 6 patients with IP + lung cancer. The results for peripheral blood lymphocytes are shown in Fig. 3. IL-2R α mRNA expression was very low or undetectable in peripheral blood lymphocytes in the five healthy controls, and also in the patients with IP + lung cancer. These results indicate that IL-2R α mRNA was overexpressed only by the pulmonary lymphocytes of patients with IP + lung cancer, while there was minimal or no IL-2R α mRNA expression by peripheral blood lymphocytes. The pulmonary lymphocytes of the patients with IP + lung cancer appear to be activated in the fibrotic regions around the tumor.

Discussion

In this study, the IL-2R α mRNA expression could be detected in pulmonary lymphocytes obtained by BAL using the method of reverse transcription coupled with the polymerase chain reaction (RT-PCR). Northern blot analysis has often been unsuccessful even if overexpressed lymphocytes existed in the sample, because the amount of lymphocytes harvested from lungs using ordinally BAL is very small (usually less than 10^6 cells). Fortunately, the RT-PCR method in extremely sensitive to detect the m RNA from very few samples as lymphocytes in BAL. But quantitative analysis by this method (12) has been considered difficult, because unhomogeneous or contaminated samples might give erroneous results due to its high sensitivity. Furthermore, reverse transcription and enzymatic amplification do not always work under the same conditions. The effectiveness of amplification was examined by detection of β-actin mRNA concomitantly, and the linear relationship between the amount of positive control cells and signal intensity was certified by serial dilution of MT-1 cells. Therefore, the semiquantitative analysis became possible.

Reactive fibrosis (13) as a result of active inflammation may be one of the causes of pulmonary fibrosis. However, in idiopathic interstitial pneumonia (13) both normal alveoli and fibrotic lesions exist at the same time (patchy distribution) and the fibrosis is chronic and progressive, so there must be an additional cause or contributing factor for such idiopathic pulmonary fibrosis. Various immunological abnormalities have been reported based on

the analyses of bronchoalveolar fluid of patients with idiopathic interstitial pneumonia. Some mediators or cytokines might play an important role in the pathogenesis of pulmonary fibrosis. For example (14), neutrophil chemotactic factor, eosinophil chemotactic factor, fibronectin, platelet derived growth factor (PDGF), IL-1 and insulin growth factor- I (IGF- I) are all produced by macrophages. Furthermore, it has been suggested that T lymphocytes and B lymphocytes as well as macrophages are activated. Crystal and Joachim (15, 16) have proposed that T lymphocyte alveolitis occurs in pulmonary sarcoidosis and hypersensitivity pneumonitis, while B lymphocyte alveolitis occurs in idiopathic interstitial pneumonia. Others (17, 18) have also reported that the activation of T cells plays an important role in the pathogenesis of chronic inflammation and pulmonary fibrosis.

The IL-2R α (5) is known to be expressed on activated T lymphocytes, and to play a major role in the lymphocyte proliferative response to IL-2 stimulation. In inflammatory lung lesions, activated T lymphocytes may undergo clonal expansion in response to autocrine and paracrine mechanism involving IL-2 and the IL-2R α. In addition, the IL-2R α is constitutively expressed by HTLV-I-infected cells. Kimura *et al.* (19) have recently suggested a close relationship between diffuse panbronchiolitis or idiopathic interstitial pneumonia and HTLV-I infection, and have proposed the clinicopathological entity of HTLV-I associated bronchiolo-alveolar disorder (HABA). Thus it may be possible that the overexpression of IL-2R α by pulmonary lymphocytes has a close relationship to the generation of pulmonary fibrosis. In addition, examination of soluble IL-2R α (20, 21) in the BAL fluid has shown increased levels in patients with idiopathic interstitial pneumonia.

We examined IL-2R α mRNA expression in the pulmonary lymphocytes of patients with lung cancer associated with diffuse pulmonary fibrosis. Lymphocytes obtained from fibrotic lesions, the possible source of the lung cancer, showed overexpression of IL-2R α mRNA in comparison with normal control lymphocytes. In healthy normal controls IL-2R α mRNA expression was very low or almost undetectable. In the peripheral blood lymphocytes of patients with IP + lung cancer, IL-2R α mRNA expression was also almost undetectable. These results suggest that the lymphocytes of IP + lung cancer patients were activated in the lungs only, so that the lymphocytes in the fibrotic lesions apparently play some role in generating the active inflammatory process. In the future it will be important to analyze expressions of the various cytokine mRNA related with pulmonary fibrosis and clarify the cytokine network in the pathogenesis of the disease.

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Received March 5, 1992; accepted November 4, 1992.