OK-432 Induces Production of Neutrophil Chemotactic Factors in Malignant Pleural Effusion

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We investigated the changes in cellular components and neutrophil chemotactic factors in pleural fluid from 19 lung cancer patients who received intrapleural injection of OK-432 to treat malignant pleurisy. Not only neutrophil chemotactic activity (NCA) but also neutrophil count and percentage were increased significantly at 6 hours after OK-432 injection. The neutrophil count was significantly correlated with NCA level. The levels of C5a and IL-8 in pleural fluid were increased significantly after OK-432 injection. The increased IL-8 level was associated with a increase of both NCA and neutrophil count. OK-432 treatment also induced a marked increase of IL-1β and IL-6 in pleural fluid. Thus, intrapleural injection of OK-432 induced production of neutrophil chemotactic factors (IL-8 and C5a) and cytokines (IL-1β and IL-6), which eventually attracted neutrophils into the pleural space. These observations suggest that neutrophil migration mediated by these factors and cytokines may contribute to the sclerosing effects of OK-432 treatment.

Key words: neutrophil chemotactic activity, IL-8, C5a, IL-1β, IL-6

Introduction

The pleura is often involved in the advanced stage of cancer, resulting in the development of pleural effusion (1). Biological response modifiers (BRM), anticancer agents, tetracyclines, and talc have been used to reduce the intrapleural accumulation of fluid (1–3). Among these agents, the streptococcal preparation, OK-432, has been most commonly used in Japan and is considered to be the most effective pleural sclerosing agent (4). OK-432 exerts an antitumor effect in experimental animals and humans by stimulating host immunity (5). Intraperitoneal or intrapleural administration of OK-432 in patients with malignant effusion has been shown to recruit many inflammatory cells, such as neutrophils, macrophages, and lymphocytes, to the site of effusion. Neutrophils are the first cells to migrate into the pleural or peritoneal cavity after the administration of OK-432 (6). In an attempt to clarify the mechanism of neutrophil accumulation, we investigated the changes in cellular components and the levels of neutrophil chemotactic factors in the pleural fluid before and after the intrapleural injection of OK-432 in 19 patients with malignant pleurisy. We found that OK-432 induced an increase of neutrophil chemotactic factors leading to the migration of neutrophils into the pleural cavity.

Materials and Methods

Subjects

We studied 19 patients with lung cancer, including 12 men and 7 women aged 40 to 77 years (median age: 66 years), all of whom had cytology-proven malignant pleural effusions. The histological diagnosis was adenocarcinoma in 12 patients, small cell carcinoma in 5 patients, and squamous cell carcinoma in 2 patients. After thoracocentesis was performed, 500–1,000 ml of pleural fluid was aspirated. Then the patients received a single intrapleural injection of 5 Klinische Einheit (KE) of OK-432 (Picibanil®, Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) in 50 ml of saline via the chest drainage tube. The chest tube was subsequently clamped for two hours and the patients rested in various positions to ensure wide intrapleural distribution of OK-432. Then the chest tube was unclamped and allowed to drain with continuous suction for a minimum of 48 hours. Pleural fluid was collected at 6, 24 and 48 hours after treatment by clamping the chest tube for two hours before each collection.
Preparation of pleural fluid

Pleural fluid was collected before and 6, 24 and 48 hours after OK-432 injection. After counting the number of cells, a cytocentrifuge preparation was made and stained with May-Giemsa solution for differential cell counting. The remaining fluid was centrifuged at 400xg for 15 minutes and the resulting supernatant was stored at below -80°C until use for measuring neutrophil chemotactic factors.

Measurement of neutrophil chemotactic activity (NCA)

Heparinized venous blood was obtained from normal volunteers and allowed to settle in a mixture of 6% dextran (Sigma Chemical Co., St. Louis, MO) for 30 minutes at room temperature. Then the buffy coat layer was placed on Histopaque (Sigma Chemical Co., St. Louis, MO) and centrifuged at 400xg for 30 minutes at 4°C. The resulting leukocyte-rich pellet was washed and contaminating erythrocytes were removed by hypotonic lysis. Neutrophils were resuspended in RPMI 1640 at a concentration of 1x10^6/ml. NCA was assayed using a modified Boyden chamber equipped with a Millipore filter having 5-μm pores (Millipore Corporation, Bedford, MA) (7). A pleural effusion sample (25 μl) was placed in the lower compartment of the chamber, while 200 μl of the neutrophil suspension was placed in the upper compartment. The chambers were incubated for 90 minutes at 37°C in humidified air. Chemotaxis was evaluated by counting the number of cells that migrated to 50 μm from the upper surface of the filter. NCA was expressed as the average number of migrating neutrophils in 5 high-power fields (hpf) at 400 times magnification.

Measurement of complement components

The level of complement component C5a in the pleural fluid was measured by radioimmunoassay (C5a des-Arg 125I Assay Kit, Amersham Japan, Tokyo, Japan). The cut-off value of the assay was set at 10 ng/ml according to the manufacturer's directions.

Measurement of cytokines

The level of interleukin-8 (IL-8) in pleural fluid and serum were measured by an ELISA (Quantikine Human IL-8 Immunoassay Kit, R&D Systems, Minneapolis, MN). The interleukin-1β (IL-1β) and interleukin-6 (IL-6) levels in pleural fluid were also measured by two different ELISA kits (IL-1β Immunoassay Kit, Medgenix Diagnostics, Fleurus, Belgium, and Predicta Interleukin-6 Kit, Genzyme, Cambridge, MA). Measurement of the serum IL-8 levels was performed in 10 lung cancer patients with pleural effusion and in 10 normal subjects.

Statistical analysis

Results were expressed as the mean ± standard deviation (SD). Statistical analysis was done by employing the Student’s t-test and correlation coefficients were determined by Pearson’s method.

Results

Changes of pleural fluid cellular components

All of the pleural effusions contained both malignant cells and numerous inflammatory cells. Mononuclear cells (MNC), consisting of lymphocytes and monocytes, predominated in the pleural fluid before OK-432 treatment. The average pleural cell count was 4.2 ± 2.9 x 10^3/μl before OK-432 treatment and reached a peak level of 24.2 ± 16.6 x 10^3/μl at 6 hours after treatment (p<0.01). Figure 1 shows the changes in the count and percentage of neutrophils and MNCs before and after OK-432 treatment. The absolute number of neutrophils in the pleural fluid increased significantly at 6 hours after treatment (p<0.01) and then decreased gradually. Similarly, the percentage of neutrophils also increased significantly from 4.4% to 86.1%...
(p<0.01). In contrast, the absolute number of MNCs in pleural fluid remained almost unchanged for up to 48 hours after OK-432 treatment.

**Changes of NCA**

The NCA level in pleural fluid was increased significantly at 6 hours after OK-432 treatment as compared to the pretreatment level (Fig. 1C) (p<0.05), but returned to the pretreatment level at 24 and 48 hours after treatment. NCA showed a significant correlation with the pleural fluid neutrophil count as demonstrated in Fig. 2A (r=0.86, p<0.001). In addition, the changes of NCA in the pleural fluid paralleled the changes of both the absolute neutrophil count and percentage.

**Changes of IL-8**

Before OK-432 treatment, the mean IL-8 level in pleural fluid samples from 10 lung cancer patients was 100-fold higher than that in their serum samples (1617 ± 1459 pg/ml vs 29.0 ± 24.1 pg/ml) (p<0.001). IL-8 was not detected in the serum obtained from 10 normal subjects. The IL-8 level in the pleural fluid was significantly increased at 6 hours after OK-432 treatment (p<0.01; Fig. 3A). IL-8 levels after treatment were significantly correlated with both NCA (r=0.85, p<0.001) and the neutrophil count (r=0.93, p<0.001), as shown in Fig. 2B and 2C, respectively.

**Changes of C5a**

Figure 3B shows the pleural fluid C5a levels before and 6 hours after OK-432 treatment. C5a was detected in 5 and 9 out of 12 patients before and after treatment, respectively. The mean C5a level increased from 6.8 ± 8.5 ng/ml to 62.1 ± 90.5 ng/ml after OK-432 treatment (p<0.025). The level of C5a in the pleural fluid was not correlated with either the IL-8 or NCA.
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level at 6 hours after treatment.

Changes of IL-1β and IL-6

IL-1β was not detected in the pleural fluid before treatment. After the injection of OK-432, the mean IL-1β level was 252 ± 339 pg/ml, which was significantly higher than before treatment (p<0.01; Fig. 4A). The pleural fluid IL-6 level also significantly increased, from 2.6 ± 2.3 ng/ml to 10.2 ± 2.7 ng/ml after OK-432 treatment (p<0.01; Fig. 4B).

Discussion

The treatment of malignant pleural effusion is one of the major problems in the management of patients with advanced cancer (1). Cell-mediated immunity is believed to play an important role in host defenses against cancer. Malignant pleural effusion is characterized by the accumulation of immune effector cells in the pleural space (5, 6). Accordingly, a number of agents that stimulate the immune system and may therefore induce pleural fibrosis have been used to treat malignant effusions. Although OK-432 is considered to be the agent of choice for treatment of malignant pleurisy in Japan (4, 8), its mechanism of action remains unclear. Intrapleural injection of OK-432 is reported to enhance local cell-mediated immunity in lung cancer patients with pleural effusions (5). In the present study, intrapleural injection of OK-432 significantly increased the number and percentage of neutrophils in the pleural fluid and the neutrophil count peaked 6 hours after OK-432 injection. The Boyden assay showed that NCA was not associated with OK-432, suggesting that neutrophil accumulation in the pleural fluid was induced by chemotactic factors or factors generated in the pleural cavity which promoted migration of cells along a chemotactic gradient to the site of inflammation. In the present study, the NCA level was increased significantly at 6 hours after OK-432 injection and the changes of NCA in the pleural fluid were correlated with changes in the absolute and relative neutrophil count. These observations suggest that neutrophil chemotactic factors may have been generated in the pleural fluid, causing the influx of neutrophils into the pleural space.

Several chemotactic factors, such as complement fragments, bacteria-derived peptides, lipid mediators, and cytokines, have chemotactic activity for leukocytes, particularly neutrophils (9). Fujimura and Torisu previously reported that C5a, a complement-derived chemotactic factor, was responsible for the major part of the chemotactic activity generated in malignant peritoneal effusions after the injection of OK-432 (10). In the present study, the pleural fluid levels of both C5a and IL-8 were increased after OK-432 injection. Moreover, changes of the pleural fluid IL-8 levels were correlated with changes of the neutrophil count and NCA. These findings indicate that in addition to C5a (11), IL-8 mediates neutrophil migration into the pleural space after OK-432 injection. IL-8 is an inflammatory cytokine and a chemotactic factor for neutrophils that is produced by pleural macrophages and other nonimmune cells, such as endothelial cells, fibroblasts, tumor cells, and mesothelial cells (12-14). It is presumed that the elevation of IL-8 levels after OK-432 treatment is due to the release from such cells, which are stimulated by either OK-432 or cytokines induced by OK-432 (12). IL-8 was already present in the pleural fluid before OK-432 injection and the IL-8 level was 100-fold higher than that in the serum of lung cancer patients with pleural effusion. The increased IL-8 levels in malignant pleural effusions may have resulted from production by mesothelial cells and tumor cells (13, 14), but the relative contribution of each cell type is unknown at present.

Several studies on neutrophil influx into the pleural space have shown that the major chemotactic factor for neutrophil depends on the type of inflammation (10, 11, 15–18). Thus, both C5a and IL-8 may contribute to neutrophil influx into the pleural space after OK-432 injection. Consistent with our results, the elevation of pleural fluid C5a and IL-8 levels is transient in models of pleurisy induced by sclerosing agents, and the levels of these chemotactic factors reach a peak at several hours after instillation (11, 17).

Migration of neutrophils from the vascular compartment to the site of inflammation is controlled by cell surface molecules on the migrating cells and on the endothelium (9). Cytokines like tumor necrosis factor (19), IL-1, and IL-6 induce the expression of selectins and ICAM-1 on the endothelium, allowing it to interact with migrating cells. Interactions with these molecules lead to neutrophil binding to the endothelium, and then neutrophil migration towards sites of inflammation is guided by chemotactic factors such as C5a and IL-8 (9).

Neutrophils induced by OK-432 have antitumor activity and may destroy tumor cells in the pleural space (15, 20). In addition, neutrophils release proteases and superoxide, which damage the pleural mesothelial cells and induce fibrosis (21, 22). Intrapleural injection of OK-432 induces marked fibrin production and this also promotes fibrosis (23).

In conclusion, intrapleural injection of OK-432 increased the level of chemotactic factors (C5a and IL-8) and inflammatory cytokines (IL-1β and IL-6) in the pleural fluid, and also altered the predominant cellular component from MNCs to neutrophils. These changes promoted antitumor activity and pleural fibrosis.

References

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