Effects of KP-496, a Novel Dual Antagonist for Cysteinyl Leukotriene Receptor 1 and Thromboxane A₂ Receptor, on Sephadex-Induced Airway Inflammation in Rats

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Bronchial asthma is characterized by chronic airway inflammation. Eosinophils are involved in airway inflammation and play crucial roles in asthma. Various types of mediators which recruit and activate eosinophils are involved in the development of airway inflammation.

The cysteinyl leukotrienes (cysLTs, including LTC₄, LTD₄, and LTE₄) which are lipid mediators generated from arachidonic acid are potent bronchoconstrictors.1,2 CysLT type 1 receptor (CysLT₁) antagonists including montelukast,3 pranlukast,4 and zaflurakast,4 exhibit beneficial effects on the clinical symptoms of asthma. In addition to bronchoconstriction, cysLTs are implicated in airway inflammation and induce eosinophil recruitment to the lung. Aerosolized cysLTs elicit recruitment of eosinophils into lung in guinea pigs, which is blocked by CysLT₁ antagonists.5,6 It has also been reported that LTD₄ challenge increased the percentage of eosinophils in sputum from asthmatics.7,8 CysLT₁ antagonist reduced sputum and mucosal eosinophils in asthmatics.8,9

In addition to CysLT₁ antagonists, the thromboxane (TX) A₂ receptor (TP) antagonist seratrodast10 is available in Japan. TXA₂ is a lipid mediator generated from arachidonic acid. TXA₂ is thought to be an important mediator in the development of asthma because of its potent bronchoconstrictive activity.11 Moreover, there is accumulating evidence to suggest that TXA₂ is involved in airway inflammation.12,13 In asthmatics, seratrodast significantly decreases the number of submucosal eosinophils and concentration of eosinophil cationic protein (ECP) in sputum.14,15

Although the mechanism by which cysLTs and TXA₂ induce recruitment of inflammatory cells is not fully clear, these findings suggest contributions of cysLTs and TXA₂ to the recruitment of eosinophils into lung in asthmatics. KP-496 is a novel dual antagonist for CysLT₁ receptor type 1 and TP receptors. The aim of this study was to evaluate the anti-inflammatory effects of KP-496 on Sephadex-induced airway inflammation. Sephadex suspension was intratracheally injected into rats. Amounts of regulated on activation, normal T cell expressed and secreted (RANTES) and eotaxin, and numbers of infiltrating cells in bronchoalveolar lavage fluid were measured 24 and 48 h after Sephadex injection, respectively. KP-496 (30, 100 μg/head) was intratracheally administered to rats 1 h before and 7 h after Sephadex injection. KP-496 and prednisolone (10 mg/kg, per os) exhibited significant inhibitory effects on infiltration of total cells and eosinophils into lung. Production of RANTES was significantly inhibited by KP-496 and prednisolone. Production of eotaxin was significantly inhibited by prednisolone. KP-496 also inhibited the production of eotaxin, though this effect was not significant. These results demonstrate that KP-496 exhibited the anti-inflammatory effects by inhibiting infiltration of inflammatory cells and productions of RANTES and eotaxin.

Key words dual antagonist; cysteinyl leukotriene; thromboxane A₂; airway inflammation; eosinophil

MATERIALS AND METHODS

Animals Male Sprague Dawley rats (six-week-old,
150—200 g) were purchased from Charles River Japan, Inc. (Yokohama, Japan). The handling and treatment of the animals were in accordance with the guidelines of the Japanese Association for Laboratory Animal Science (1987).

Drugs and Chemicals A dry powder formulation of KP-496 (KP-496DPI) containing 3 and 10% of KP-496 and vehicle for KP-496DPI were prepared by Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). Prednisolone (50 mg of water-soluble prednisolone) was purchased from Shionogi & Co., Ltd. (Osaka, Japan). Methylcellulose (MC) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Urethane was purchased from Sigma (St. Louis, MO, U.S.A.). Sephadex™ G75S Superfine was purchased from Amersham Biosciences (Uppsala, Sweden).

Drug Administration Vehicle and KP-496DPI were weighed into silicone tubes (1 mg per tube). A compressor (PARI GmbH, Starnberg, Germany) was connected to an air hose and a three-way stopcock. A silicone tube containing the test compound was connected to the other end of the three-way stopcock. A tracheal cannula for intratracheal administration was connected to the silicone tube. Animals were anesthetized with ether and the tracheal cannula was inserted into the trachea from the oral cavity noninvasively. It was confirmed that treatment with ether had no effect on inflammatory cell recruitment and expression of chemokines. The test compound was sprayed by rapidly and fully opening the three-way stopcock connected to the compressor and sending compressed air from the compressor. The amounts of KP-496 administered were 30 μg/head and 100 μg/head, respectively, when 3% and 10% of KP-496DPI were administered. The prednisolone was dissolved with 0.5% MC and administered 1 h before and 7 h after intratracheal administration of KP-496 treated with ether had no effect on inflammatory cell recruitment and expression of chemokines.

Measurement of Cells The animals were anesthetized with urethane (1 g/kg, i.p.) and sacrificed 24 h after Sephadex injection. BALF was recovered as described above. Supernatant of BALF was concentrated by ultrafiltration (1500 × g, 390 min, 4 °C) using the Centrifluor YM-3 (Billerica, MA, U.S.A.). Concentrated sample was dried using a centrifugal evaporator (300 min, 25 °C) and dissolved in PBS (−) to prepare 10-fold concentrated BALF. The amounts of RANTES and eotaxin were measured using ELISA Kit (Biosource Co., Ltd., Camarillo, CA, U.S.A. and R & D Systems, Minneapolis, MN, U.S.A., respectively).

Statistical Analysis The same experiment was conducted twice and the reproducibility of the results was confirmed. In this study, the values of 2nd experiment were expressed as the mean ± S.D. Statistical analysis was performed using one-way analysis of variance followed by Dunnett’s multiple range test and Student’s t-test, as appropriate. Values at p < 0.05 were considered significant.

RESULTS

Effects of KP-496 on Inflammatory Cell Recruitment Numbers of inflammatory cells in BALF were measured 48 h after intratracheal injection of Sephadex. Total cell number and numbers of all types of cells in the control group were significantly increased compared with the sham group (p<0.01, Table 1). Eosinophils, monocytes/macrophages, neutrophils, and lymphocytes in the control group were increased respectively 615-fold, 2.3-fold, 20.9-fold, and 9.9-fold compared with the sham group. The KP-496 (100 μg/head) group and prednisolone (10 mg/kg, p.o.) group exhibited significant inhibition of numbers of infiltrating total cells, eosinophils, monocytes/macrophages, and lymphocytes compared with the control group (Fig. 1, Table 1). Infiltration of all types of cells except neutrophils was decreased in the KP-496 (30 μg/head) group, though not to significant extents.

Effects of KP-496 on Amounts of RANTES and Eotaxin in BALF The amount of RANTES and eotaxin in

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose</th>
<th>Total cells</th>
<th>Eosinophils</th>
<th>Monocyte/macrophages</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
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<tbody>
<tr>
<td>Sham</td>
<td></td>
<td>5.34±1.55</td>
<td>0.01±0.01</td>
<td>5.18±1.52</td>
<td>0.09±0.05</td>
<td>0.07±0.04</td>
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<td>Control</td>
<td></td>
<td>20.48±12.04</td>
<td>6.15±4.69</td>
<td>11.76±6.15</td>
<td>1.88±1.47</td>
<td>0.69±0.59</td>
</tr>
<tr>
<td>Prednisolone</td>
<td></td>
<td>10.48±2.44</td>
<td>0.60±0.59</td>
<td>7.64±2.30</td>
<td>2.11±2.00</td>
<td>0.14±0.08</td>
</tr>
<tr>
<td>KP-496</td>
<td>10 mg/kg (p.o.)</td>
<td>17.12±4.05</td>
<td>4.29±1.24</td>
<td>9.62±1.94</td>
<td>2.75±2.29</td>
<td>0.46±0.33</td>
</tr>
<tr>
<td></td>
<td>30 μg/head (i.t.)</td>
<td>10.89±2.51</td>
<td>2.34±1.02</td>
<td>7.16±2.32</td>
<td>1.12±0.77</td>
<td>0.27±0.13</td>
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<tr>
<td></td>
<td>100 μg/head (i.t.)</td>
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Table 1. Effect of KP-496 on Infiltration of Inflammatory Cells into BALF in Sephadex-Induced Airway Inflammation Results are expressed as cell numbers (× 10^6) in BALF. Each value represents the mean ± S.D. of ten animals. *p<0.01 compared with sham (Student’s t-test), *p<0.05 and **p<0.01 compared with control (Dunnett’s multiple range test).
BALF was measured 24 h after intratracheal injection of Sephadex. The amount of RANTES in the control group increased significantly compared with the sham group (p<0.01, Fig. 2). Production of RANTES was significantly inhibited in the KP-496 (100 μg/head) group and prednisolone (10 mg/kg) group compared with the control group (p<0.05 in the KP-496 group and p<0.01 in the prednisolone group). The amount of RANTES also decreased in the KP-496 (30 μg/head) group, though not to a significant extent. The amount of eotaxin in the control group increased significantly compared to the sham group (p<0.05, Fig. 3).

**DISCUSSION**

In the present study, we investigated the effects of KP-496 on Sephadex-induced airway inflammation in rats. KP-496 inhibited the recruitment of inflammatory cells, especially eosinophils, into lung. KP-496 also reduced the amount of RANTES and eotaxin in lung.

Sephadex-induced airway inflammation is characterized by increase in number of eosinophils. The exact mechanism of airway inflammation caused by intratracheal instillation of Sephadex is not clear, but macrophage activation through phagocytosis, mast cell degranulation and T cell activation are thought to be involved in eosinophilic inflammation. The increase in eosinophil numbers is accompanied by increase in amounts of chemokines and Th2 cytokines in lung and AHR. Airway inflammation including eosinophil recruitment and AHR are characteristic features in asthmatics. Chemokines and Th2 cytokines play important role in the development of airway inflammation. It had been demonstrated that the degree of AHR is related to severity of asthma symptoms. Therefore, this model is thought to reflect important aspects of asthma and is useful and convenient for investigation of the anti-inflammatory effects of small compounds. In fact, many groups have investigated the anti-inflammatory effects of small compounds and steroids using this model.

The cysLTs are potent eosinophil chemotaxants and promote eosinophil survival. CysLTs also induce up-regulation of their own receptor, CysLT1, and CysLT1 antagonist inhibited the up-regulation of CysLT1 induced by cysLTs. The CysLT1 antagonistic activity of KP-496 thus contributes to the inhibition of production of eosinophils. In addition to cysLTs, RANTES and eotaxin are eosinophil activating and chemotaxant chemokines. In fact, numbers of cells expressing eotaxin and RANTES were sig-
nificantly increased, and enhanced expression of C–C chemokine receptor 3 (CCR3) was observed in asthmatics. Furthermore, there is a strong relationship between the expression of eotaxin and severity of asthma. It has been reported that cysLTs activate nuclear factor (NF)-κB and induce RANTES production from isolated lung mononuclear cells, which might be in turn cause recruitment of eosinophils into the lung. The CysLT₁ antagonists, montelukast and pranlukast, inhibited the activation of NF-κB. The CysLT₂, antagonistic activity of KP-496 should thus reduce the production of RANTES by inhibition of NF-κB activation and prevent the recruitment of eosinophils into lung.

Interleukin (IL)-5 also plays crucial role in eosinophil activation and survival. In the present study, we did not investigate the effect of KP-496 on IL-5 production. But, it has been demonstrated that CysLTs induced IL-5 production from inflammatory cells and CysLT antagonists inhibited IL-5 production. Additionally, some results suggested that IL-5 and eotaxin might promote the recruitment of eosinophils into lung cooperatively. Thus, it can be expected that CysLT₁ antagonistic activity of KP-496 might contribute to inhibit the recruitment of eosinophils through inhibiting IL-5 production and cooperative action of IL-5 with eotaxin.

It is not clear if TXA₂ has eosinophil chemoattractant activity like cysLTs. However, some groups have reported the involvement of TXA₂ in airway inflammation. Hoshino et al. reported that administration of seratrodast to asthmatics reduced the number of activated eosinophils in bronchial mucosa and the production of the C–C chemokines RANTES and eotaxin. Although the mechanisms by which TXA₂ induced production of these chemokines are unclear in detail, it has been reported that TXA₂ analogue induced the production of RANTES. Thus, C–C chemokines might be produced in part by direct stimulation via TP, and antagonism by seratrodast and KP-496 of TP might contribute to the inhibition of their production.

These findings and those of previous reports suggest that KP-496 exhibit the anti-inflammatory effects by inhibiting infiltration of inflammatory cells into lung and productions of RANTES and eotaxin. In addition to these results, KP-496 exhibited anti-inflammatory effects on a guinea pig chronic airway inflammation model (manuscript in preparation).

According to the GINA guideline, inhaled corticosteroids (ICS) are the first choice for control of asthma. However, it is known that ICS do not inhibit the production of cysLTs. It has also been reported that CysLT₁ antagonists and a TP antagonist exhibit additive effects with ICS. Moreover, there is some evidence that inhibition of multi-mediators, including KP-496, provides more potent effects on airway obstruction than inhibition of single mediators. It can thus be expected that KP-496 will provide new therapeutic approach and choice such as combination of ICS and KP-496 to asthmatics.

REFERENCES

35) Ying S., Robinson D. S., Meng Q., Rottman J., Kennedy R., Ringler D.