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Original Article

Protective Effect of Leukotriene Receptor Antagonist Montelukast on Smoking-Induced Lung Injury in Wistar Rats

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Increased activation of alveolar macrophage, neutrophil and mast cell has been proven in cigarette smoking (CS)-related lung disorders (CSLD). An increased production of cysteinyl-leukotrienes (LTs), which are mediators secreted from the mentioned cells, in response to CS has been shown in humans. The protective effect of LT1 receptor-1 antagonist (LTR-1AT) on CSLD is, however, not known. In this study we aimed to determine whether there is any protective effect of a LTR-1AT, montelukast (MK), on CSLD in Wistar rats. Nine controls and twenty-three smoke-exposed rats were enrolled into this study. Controls were exposed to non-filtered air, and the smoke-exposed rats were exposed to CS for 6 h/day, 6 days/week for three weeks. The CS-exposed rats were also treated with 0.1 mg/kg/day of MK or saline. Morphometric criteria for lung injury were determined as the mean linear intercept of alveolar septa (L_m), the volume density of alveolar septa (V_{vspt}) and the density of the alveolar surface area per unit volume of lung parenchyma ($S_{va.pa}$). Lung mast cells (LMC), which are a major source of LTs, were also counted. Results showed that L_m of the control group was significantly lower and V_{vspt}, $S_{va.pa}$ of the controls were significantly higher compared to those of the CS-exposed groups. Animals treated with MK had significant protection against CSLD. L_m was significantly higher and V_{vspt}, $S_{va.pa}$ were lower in the saline group than in the MK-treated group. The number of LMC in the CS-exposed groups was also significantly higher than that in the control group. Based on these results, one can suggest that some part of the pathogenesis of CSLD may be related to an enhanced LTs synthesis and LTR-1AT. Therefore, montelukast may protect against active or passive smoking-induced lung injury and related disorders.

Key words: cigarette smoking, cysteinyl-leukotrine, lung, montelukast, rats

Exposure to cigarette smoking (CS) is a significant risk factor for chronic obstructive lung disease (COLD) in adulthood and plays an important role in the development of wheeze and asthma in childhood [1, 2]. Although the link between CS and smoking-related lung disease has been poorly understood, recent studies have suggested that inflammatory mechanisms may be responsible [3, 4]. As in patients with COLD and asthma, increased numbers of neutrophils, macrophages, mast cell *etc* have been found in the bronchioli of smokers [4, 5]. Moreover, the mucosa of the bronchioli in COLD and asthma patients are characterized by inflammatory cell infiltration and increased synthesis of inflammatory mediators from these cells, such as leukotrienes [1, 6]. Cysteinyl-leukotrienes (LTs), including LTC4, LTD4 and LTE4, play many roles in smoking-related lung

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diseases [7, 8]. Fauler *et al*. showed that CS stimulates and enhances LTs production in humans, and they claimed that some of the adverse effects of CS exposure on pulmonary structures might be related to an enhanced LTs synthesis [7]. LTs are synthesized from the 5-lypoxygenase pathway of arachidonic acid metabolism by 5-lypoxygenase (5-LO) [8–11]. Lung mast cells (LMC) are an important source of LTs in pulmonary parenchyma [11]. Current data suggest that the biological effects of LTs appear via a single receptor for all the LTs, LTs receptor-1 [11]. Increased numbers of mast cell, neutrophils and T-lymphocytes and an enhanced activation of alveolar macrophages, which are the major cellular sources of LTs, have been shown in active smokers. These cells may also be responsible for CSLD [4, 7, 8, 11, 12]. Therefore, active or passive CS-induced LTs secretion from inflammatory cells in lung parenchyma may be responsible for CSLD and airway hyperreactivity. However, it is not known whether inhibition of the effects of LTs by LTs receptor-1 antagonist (LTR-1AT) protects against active or passive CSLD. Accordingly, we designed the present study to clarify whether montelukast, which is a potent LTR-1AT, could protect against CS-induced lung injury in CS-exposed Wistar albino rats.

Materials and Methods

Animals. Thirty-two 210 ± 25 gram, 12- to 17-week-old female Wistar rats were used in this study. None of them had any sign of infection, respiratory disorders or pneumonia. After a 2-week quarantine and acclimatization period, twenty-three animals were exposed to mainstream CS for 6 h/day, 6 days/week for 3 weeks. Nine rats were used as the controls, and they were exposed to non-filtered room air. CS-exposed animals and controls were fed a pelleted ration and provided water ad libitum. The signed study protocol was approved by the Celal Bayar University Ethics Committee and carried out in accordance with our institutional Policies and Guidelines for the Care and Use of Laboratory Animals.

Study Design and Drug Treatment. The CS-exposed rats were divided into 2 groups. Twelve CS-exposed animals were treated with montelukast (Singulair, Merck-Sharp Dohme Co., WA, USA), an oral and potent LTR-1AT, at a dosage of 0.1 mg/kg/day by oral route for 3 weeks. The remaining 11 CS-exposed rats were treated with the same volume of oral saline during the same period. None of the control animals received any medication. At the end of the study period, all animals were sacrificed and their lung tissues were processed for histologic and morphometric examination to reveal CS-induced lung injury and LMC counts.

Cigarette Smoking Exposure. Passive CS exposure was performed with non-filtrated smoke fume using a previously described method [13]. One 50-cm^3 puff per minute from a non-filtered, low-quality cigarettes was generated by a smoking device delivered to a 75 × 75 × 50-cm whole-body exposure chamber constructed of polyethylene material and joined to the smoking device. Controls were sham-exposed to non-filtered room air.

Lung Tissue Preparation. CS-exposed and control animals were sacrificed at the end of the third week. Their lungs were immediately extracted by thoracoabdominal incision and separated at the level of the trachea. All lungs were perfused, via the trachea, with Cornoy fixative and 10% formaldehyde overnight. After the fixation period, nonpulmonary tissues were dissected from the pulmonary structure and embedded in paraffin. The left lungs were systematically sectioned in a dorsoventral-transverse direction at 3-mm intervals, with the first slice randomly positioned within 3 mm of tissue as described previously [14]. Blocks were sliced into 5-μm thick sections using a microtome. Formalin-fixed preparations were stained with haematoxylin-eosin, and Cornoy-fixed preparations were stained with toluidine blue.

Camera-Adapted Computerized Microscopy. Histopathologic and morphometric examinations of lungs were performed on haematoxylin-eosin-stained lung sections by using a digital computerized system adapted to light microscopy. In this system, fields were examined at a magnification of 10x for the eyepiece and a 40- or 100-objective lens on a digital camera (Samsung SAG 410PA, Seoul, Korea) interfaced with a light microscope (Olympus BX40F-3, Tokyo, Japan). Using these instruments, we obtained digitized images of the chosen lung fields and transported them to the computer. Digitized images were saved using Microsoft image software (WinTV 4.7.6255, Hauppage Computer Works, NY, USA). The objective of the light microscope was randomly positioned within the extreme dorsomedial region of a section, and the area was marked as number 1, 2, *etc*. This processing was made in a zigzag pattern (first laterally, then ventrally, then medially) repeatedly, and the objective was moved across the entire section. Every

third field was selected and was changed to provide a magnification of 200x prior to creation of a software image. All morphometric measurements to demonstrate CS-induced changes in parenchyma were performed by the image software using saved digitized images.

Morphometric Measurements. A computer-generated quadrangle grid containing 100 spaced points and sine-wave straight lines on a perpendicular and dorsoventral axis was placed on the projections. The length of a straight line was measured, by using the total augmentation ratio, as 18 μ in real-time. The accepted criteria that demonstrated CS effects on pulmonary parenchyma were alveolar airspace enlargement and tissue loss, as described previously [14, 15]. The most commonly used morphometric indicator of airspace enlargement is the mean linear intercept of alveolar septa (L_m, in μm). Thus, L_m was measured as described by Dunnill *et al* [16]. Also, the most commonly used criteria for tissue loss are the measurement of volume density of alveolar septa (V_{vspt}, %) and the density of the alveolar surface area per unit volume of lung parenchyma ($S_{va.pa}$, mm^{-1}) calculated by using L_m as described previously by March *et al* [14].

Lung Mast Cell Counting. Since the most important source of secreted LTs from lung parenchyma is mast cells, we counted LMC in all sections of toluidine blue-stained samples. Enumeration of LMC was performed with 10x eyepiece and a 40x objective lenses. All the LMC in a single section were counted and categorized as "intact" (identifiable by their methachromatic granules) or "degranulated" (partially degranulated LMC having visible extracellular granules) cells as previously reported [17]. A total LMC count was obtained by using a magnification ratio and an identically magnified micrometer scale image as an index of magnification. Then, the mean total LMC count was calculated and expressed as per mm^2. In addition, the degranulation ratio of LMC, as an activation criteria of mast cells, was accounted as a percent (%).

Statistics. Data including L_m, V_{spt}, $S_{va.pa}$ and LMC count were expressed as means $\pm$ SDs. Morphometric parameters and LMC count of all groups were analyzed by two-way analysis of variance (ANOVA) for significant differences among groups. After analyzing by ANOVA, we made comparisons between the groups of treated and untreated CS-exposed rats and the control rats using Tukey-multiple comparisons test as a post hoc test. Statistical significance was accepted as a P value of less than 0.05.

Results

Histopathology of Lung Sections. None of the animals had any life-threatening events during the study, and body weights of members of all groups at the end of study were not significantly different from weights at the beginning of the study. In the general viewing by light microscopy, there were no significant pathological changes in the pulmonary structure of the control rats (Fig. 1a). The lungs from CS-exposed untreated rats had diffuse and nonuniform alveolar airspace enlargement, decreasing of alveolar septal thickening, irregular widening of alveolar ducts, increased visceral pleural thickening and multifocally distributed intraalveolar or parenchymal bleeding (Fig 1b). Most of the alveoli were distorted and had prominent alveolar septal attenuation (Fig. 1b). However, the mentioned parenchymal changes in rats treated by MK were not as severe as those in the untreated CS-exposed group (Fig. 1c).

Morphometric Results of Lung Sections. A two-way ANOVA (3 groups) of morphometric measurements showed significant airspace enlargement and differences by means of the increased L_m and decreased V_{vspt} and $S_{va.pa}$ of both CS-exposed animals and control subjects ($F_{2,29} = 16.988$, $P < 0.05$). Of particular interest, the mean L_m of both groups of CS-exposed subjects was significantly higher than that in the control animals ($P < 0.05$). Also, the density and tissue loss of alveolar septa of both CS-exposed groups were significant as shown by the decreases in V_{vspt} and $S_{va.pa}$ ($P < 0.05$). When we compared the results of treated and untreated groups, we found the airspace enlargement and tissue loss of untreated animals were significantly worse than that in treated rats. That is to say, mean L_m of untreated rats was significantly higher and mean V_{vspt}, $S_{va.pa}$ of same group was significantly lower than that of the MK-treated animals. All measurements including L_m, V_{vspt} and $S_{va.pa}$ of all groups are listed in Table 1. These results indicated that exposure to CS led to the thinning of alveolar septa, increased alveolar airspace and tissue loss and that treatment with MK protected against those changes.

Lung Mast Cell Counts. There was a significant difference in the mean total LMC counts of both CS-exposed groups and the controls ($F_{2,29} = 14.947$, $P < 0.05$) (Table 2). Also, there was a significant

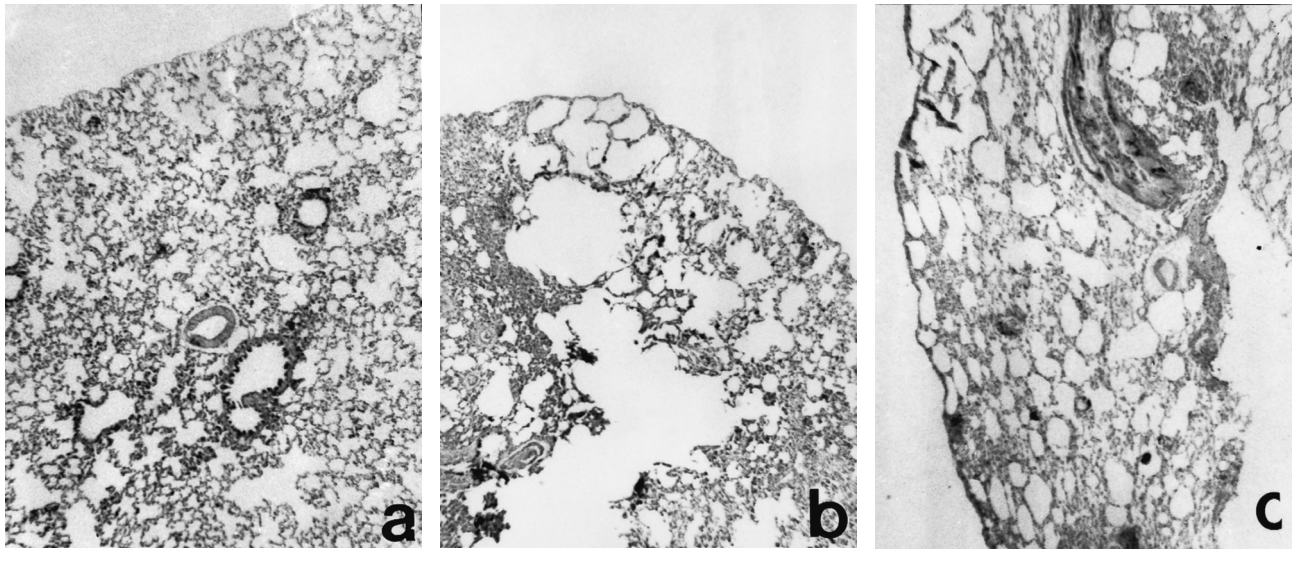

Fig. 1 Histopathology of lungs and mast cells. (a) In control rats, there was no sign of pulmonary inflammation or degeneration in histopathology of the lungs (haemotoxylin-eosin, × 40). (b) In cigarette smoking-exposed untreated rats, most of the alveoli were distorted, with alveolar septal attenuation, airspace enlargement and tissue loss (haemotoxylin-eosin, × 40). (c) Alveolar distortion, septal attenuation and tissue loss in rats treated with MK were not as severe as that in members of the untreated CS-exposed group (haemotoxylin-eosin, × 40).

Table 1 Morphometric measurements of lungs of CS-exposed and control groups

Parameters	Untreated CS-Exposed Group (n = 12)	Treated CS-Exposed Group (n = 11)	Control Group (n = 9)	P*, ϕ
L_m (mm)[1]	83.8 ± 27.2	61.8 ± 21.6	28.3 ± 9.6	< 0.05
V_{vspt} (%)[2]	18.2 ± 4.1	22.1 ± 3.9	29.1 ± 5.7	< 0.05
$S_{va,pa}$ (mm^{-1})[3]	0.13 ± 0.05	0.18 ± 0.04	2.11 ± 0.06	< 0.05

[1], Linear intercept of alveolar septa; [2], volume density of alveolar septa; [3], density of the alveolar surface area per unit volume of lung parenchyma; *, P value is significant if less than 0.05. ϕ, The differences between the same parameters of each group by post hoc test (Tukey-Multiple Comparisons Test) were significant.

difference in the proportion of degranulated LMC among the groups ($F_{2,29} = 19.668$, $P < 0.05$). That is, both CS-exposed groups had higher LMC and proportion of degranulated LMC than that in controls (Table 2). However, there was no significant difference between the mean total LMC and the degranulated LMC ratio of untreated and treated CS-exposed groups ($P > 0.05$) (Table 2). We observed an increased LMC density in the subpleural area of lungs of the CS-exposed rats; however, there was no significant heterogeneity or increased number of LMC in the control group. An "intact" and "degranulated" LMC are shown in Fig. 1.

Discussion

This study provides *in vivo* evidence of the protective effect of the LTR-1AT montelukast on CS-induced injury within the lungs of CS-exposed rats. We demonstrate that MK treatment was associated with fewer alveolar airspace enlargements and less tissue loss, which are the accepted criteria showing CS-induced lung injury. In addition, although the differences were not statistically significant, we found that MK-treated rats had fewer LMC (which is the most important source of LTs in the lungs) and lower activation than that in subjects treated with saline.

Table 2 Lung mast cell counts and their degranulation ratio in study and control lungs

Parameters	Untreated CS-Exposed Group (n = 12)	Treated CS-Exposed Group (n = 11)	Control Group (n = 9)	$P^{*,\S}$
LMC[1] count (mm^3)	$20.1 \pm 3.4^{\#}$	$17.2 \pm 3.8^{\#}$	$7.3 \pm 2.1^{\P}$	< 0.05
Degranulated LMC (%)	$23.2 \pm 6.3^{\#}$	$20.7 \pm 4.9^{\#}$	$9.1 \pm 2.9^{\P}$	< 0.05

*P value is significant if less than 0.05; [1], lung mast cell. §, the differences between # symbols were not significant and between # and ¶ were significant.

Leukotrienes are derived from arachidonic acid of membrane lipids by 5-LO and play many roles in the pathogenesis of asthma and other pulmonary diseases by their bronchoconstrictor and inflammatory effects [11]. They increase vascular permeability by the exudation of plasma into the airway wall and lumen, which are important pathological features of COLD and bronchial asthma [11, 18]. Leikauf *et al*. showed that LTs [exhibit] potent harmful effects on pulmonary epithelial cells, and McAlexander *et al*. showed that LTs exacerbate the sensitizing effects of epithelial and alveolar wall denudation by stimulating local afferent nerves to release tachykinines, increasing plasma exudation and vascular permeability [19, 20]. Moreover, several studies suggested that LTs are potent chemoattractants for inflammatory cells to lung parenchyma. Laitinen *et al*. demonstrated that LTs are capable of recruiting granulocytes, particularly eosinophils, into the lamina propria [21]. Also, Calhoun *et al*. and Diamant *et al*. showed that LTs led to granulocyte and basophil accumulation in the lung [22, 23]. Nakamura *et al*. believe that LTs cause T- and B-lymphocytes infiltration into the pulmonary parenchyma [24]. CS exposure is a major risk factor for 2 different disorders of 2 different age groups, and both disorders may be called CSLD. The first is the occurrence of wheeze and airway hyperreactivity or asthma related to passive CS exposure in childhood, and the second is COLD related to active CS in adulthood. Despite our understanding of the immunopathogenesis and cellular mechanisms of asthma and wheeze as inflammatory disease, the cellular and molecular mechanisms of COLD have been neglected until recently. Although these types of inflammation differ markedly; both diseases are characterized by chronic inflammation of the airways and pulmonary parenchyma of the lungs [25]. Both disorders have identical inflammatory cell infiltration such as increased mast cells, neutrophils, eosinophils, lymphocytes and macrophages [25]. While bronchial asthma and recurrent wheeze are characterized by an increase in CD4 + Th2 lymphocytes, mast cells, neutrophils and activated eosinophils in bronchial biopsies, the pathology of COLD has been shown as an increase in the proportion of mast cells, neutrophils, CD8-T-lymphocytes and macrophages in the lung parenchyma [25, 26]. All these inflammatory cells and bronchial epithelial cells are the main source of LTs in lungs [8, 9, 11]. Also, increased mast cells, neutrophils, eosinophils, lymphocytes and macrophages are clearly evident and activation has been proven in the bronchioli and pulmonary parenchyma in active and passive smokers [4, 5]. An important observation reported by Fauler and Frolich demonstrated that CS exposure causes a dose-related increase in LTs production in smokers [7]. The authors claimed that some of the adverse effects of CS exposure in the CS-induced lung injury might be related to enhanced LTs synthesis [7]. Our results support this observation, as evidenced by the fact that LTR-1AT treatment with MK was associated with significantly diminished CS-induced lung injury demonstrated by morphometric measurements. This evidence is also supported by previous studies using anti-inflammatory treatment with LTR-1AT, which inhibited the accumulation of mast cells/basophils, eosinophils, basophils, T- and B-lymphocytes and neutrophils into the lung parenchyma. Moreover, the increased plasma exudation and pulmonary epithelial denudation, which are the main causes of pathological changes in CS exposure-induced lung injury have been proven [20, 23, 24].

Lung mast cells are mononuclear cells and are almost exclusively localized to lung tissues [27]. The capacity of LMC to release plenty of powerful inflammatory mediators makes this cell a unique member of the lung's immune response network [27]. These mediators are usually released within minutes after activation. These inflammatory mediators or cytokines and chemical sub-

stances, such as LTB4, PgD2, TxA2, PAF, IL-8, IL-5, TNF-α, TGF-β, tyriptase, elastase and β-glucronidase, have clearly harmful and degenerative effects on lung parenchyma [28]. One group of these inflammatory mediators is LTs, which are classified as nonpreformed or newly synthesized mediators. LMC is a major pulmonary cellular source of LTs [11, 27]. The effects of LTs on CS-induced lung injury were discussed above. Several studies reported that, indeed, the number of mast cells in the lungs of smokers was significantly increased compared to that of non-smokers. Experimental studies showed an increase of mast cell density and degranulation rate in CS-exposed animals [4, 17, 29]. Knowing this causes us to hypothesize that CS may increase the number and the activation of LMC, which may contribute to CS-induced lung injury by increasing production of LTs in the lung parenchyma. Our results showed that CS-exposed rats had significantly increased LMC count and activation compared to control subjects. Although there was no significant correlation between LMC count and degranulation ratio as an activation criteria of mast cells in MK-treated and untreated groups, we demonstrated that CS-induced lung injury was clearly diminished in MK-treated rats by blocking the activity of LTs using the LTR-1AT montelukast. Calhoun and coworkers reported that LTR-1AT inhibited the rise in basophils, which resemble mast cells, in asthmatic patients [23]. In our study, despite the fact that LMC count and degranulation ratio of MK-treated rats were lower than that in untreated CS-exposed rats, the difference was not statistically significant. Therefore, we cannot claim with certainty that LTR-1AT inhibits the rises in number and activation of LMC due to CS exposure.

We showed that treatment with MK, a potent LTR-1AT, is protective against pathological changes such as alveolar airspace enlargement and tissue loss secondary to CS exposure. It is well known that LTR-1AT has anti-inflammatory effects during both early and late phases of inflammation [12]. Therefore, we suggest that LTR-1AT may have a protective effect against smoking-induced acute lung injury and late-phase chronic degeneration due to CS-induced chronic persistent inflammatory processes in the lungs. Further studies are needed to clarify the mechanism of the effect of leukotrienes receptor antagonists.

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