

S. Hashimoto
Y. Gon
Y. Asai
Y. Asai
T. Machino
I. Jibiki
I. Takeshita
H. Anazawa
T. Horie

p38 MAP kinase regulates RANTES production by TNF- α -stimulated human pulmonary vascular endothelial cells

Authors' affiliations:

S. Hashimoto, Y. Gon, Y. Asai, Y. Asai,
T. Machino, I. Jibiki, I. Takeshita, H. Anazawa,
T. Horie, First Department of Internal Medicine,
Nihon University School of Medicine, Tokyo,
Japan

Correspondence to:

Dr Shu Hashimoto
First Department of Internal Medicine
Nihon University School of Medicine
30-1 Oyaguchikamimachi
Itabashi-Ku
Tokyo 173-8610
Japan

Key words: p38 MAP kinase; pulmonary vascular endothelium; RANTES.

Background: RANTES plays an important role in the production of allergic inflammation of the airway through its chemotactic activity for eosinophils. However, the intracellular signal regulating RANTES expression in human pulmonary vascular endothelial cells has not been determined. In the present study, therefore, we examined the role of p38 mitogen-activated protein (MAP) kinase in RANTES production by tumor necrosis factor (TNF)- α -stimulated pulmonary vascular endothelial cells in order to clarify the signal transduction pathway regulating RANTES production by pulmonary vascular endothelial cells.

Methods: We examined p38 MAP kinase activation, and the effect of SB 203580, as the specific inhibitor for p38 MAP kinase, on p38 MAP kinase activity and RANTES production by TNF- α -stimulated human pulmonary vascular endothelial cells.

Results: The results showed that TNF- α induced RANTES production and p38 MAP kinase activity in human pulmonary vascular endothelial cells. Abrogation of p38 MAP kinase activity by SB 203580 repressed TNF- α -induced p38 MAP kinase activity and RANTES production.

Conclusions: These results indicate that p38 MAP kinase plays an important role in the TNF- α -activated signaling pathway which regulates RANTES production by human pulmonary vascular endothelial cells.

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Bronchial asthma is characterized by an episode of reversible airway obstruction, airway hyperresponsiveness to exogenous and endogenous stimuli, and eosinophilic inflammation in the airway (1). Various cells and their products may contribute to the feature of allergic inflammation (2, 3). Of these cells, eosinophils and their products have been

demonstrated to play an important role in the production of allergic inflammation (4). In the allergic inflammatory responses in the lung, circulating eosinophils accumulate in the pulmonary capillaries and migrate through the vascular endothelium to the submucosa of the asthmatic airway. RANTES, which displays chemotactic activity for eosinophils, participates in this process through the recruitment of eosinophils (5–7). Therefore, it has been suggested that RANTES produced by vascular endothelial cells, as well as other types of cells such as airway epithelial cells and T cells, plays a role in facilitating eosinophil migration from the circulation. Vascular endothelial cells are well known to express a variety of cytokines (8–10). Recently, RANTES has been shown to be expressed in vascular endothelial cells (8–10). Therefore, it is important to clarify the mechanism of the production of RANTES by vascular endothelial cells.

p38 mitogen-activated protein (MAP) kinase, which belongs to the MAP kinase superfamily, is activated in various cells by proinflammatory cytokines, environmental stresses, DNA-damaging agents, and hematopoietic growth factor (11–21); however, little is known about the role of p38 MAP kinase in RANTES production by human pulmonary vascular endothelial cells.

In the present study, we examined the role of p38 MAP kinase in the induction of RANTES production by human pulmonary vascular endothelial cells in order to clarify the signal transduction pathway in RANTES production by these cells.

Material and methods

Reagents

Human recombinant TNF- α was kindly provided by Dainippon Pharmaceutical Co. Ltd (Osaka, Japan). The pyridinyl imidazole SB 203580, the specific inhibitor for p38 MAP kinase activity (22) (kindly provided by SmithKline Beecham), was dissolved in dimethyl sulfoxide.

Cell culture

Normal human pulmonary artery endothelial cells (HPAEC) were obtained from Clonetics (San Diego, CA, USA). The cells (1×10^4 cells/ml) were placed onto 24-well, flat-bottom, tissue-culture plates (Corning, Corning, NY, USA) for determination of cytokine production and tissue-culture dishes (Falcon 1007, Oxnard, CA) for Western blot analysis with vascular endothelial growth medium (EGM-2;

Clonetics) containing 0.2% fetal bovine serum (FBS), gentamycin-amphotericin B, epidermal growth factor (EGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), ascorbic acid, heparin, and hydrocortisone. The cells were grown until they were subconfluent, and the medium was changed. In order to examine TNF- α -induced RANTES production and the effect of SB 203580 on it, the cells that had been preincubated with EGM-2 medium without FBS, EGF, FGF, IGF, VEGF, ascorbic acid, and hydrocortisone (growth-factor-free medium) for 16 h were preincubated with growth-factor-free medium containing or not SB 203580 for 1 h. Thereafter, the cells were stimulated with TNF- α and cultured in growth-factor-free medium for the desired times at 37°C in humidified 5% CO₂ atmosphere. At the end of culture periods, the culture supernatants were harvested and centrifuged, and the supernatants were collected, passed through a Millipore filter and stored at –80°C until assay. For examination of p38 MAP kinase activity and the effect of SB 203580 on p38 MAP kinase activity in TNF- α -stimulated cells, the cells that had been preincubated with growth-factor-free medium for 16 h were preincubated with growth-factor-free medium containing or not SB 203580 for 1 h. Thereafter, the cells were stimulated with TNF- α and cultured in growth-factor-free medium for 10 min.

Measurement of RANTES

The concentration of RANTES in the culture supernatants from HPAEC was measured by commercially available ELISA kits (Amersham International, Aylesbury, UK). ELISA was performed according to the manufacturer's instructions. All samples were assayed in duplicate.

p38 MAP kinase assay

The activity of p38 MAP kinase was analyzed by commercially available kits (p38 MAP Kinase Assay Kit, New England Biolabs, Inc.). The kit employs two different antibodies, anti-p38 MAP kinase antibody, which is specific for p38 MAP kinase and does not cross-react with ERK1/2 or JNK, and anti-phospho-specific ATF-2 antibody to detect p38 MAP kinase-induced phosphorylation of ATF-2. p38 MAP kinase activity was analyzed according to the manufacturer's instructions, as described previously (18). Briefly, the cell lysate containing 200 μ g of protein was incubated with anti-p38 MAP kinase antibody to immunoprecipitate selectively p38 MAP kinase from the cell lysates,

and the immunoprecipitates were incubated with ATF-2 fusion protein in the presence of ATP, a process which allowed immunoprecipitated active p38 MAP kinase to phosphorylate its substrate, ATF-2. The samples were separated by a 15% SDS gel, transferred to membranes, and blotted with anti-phospho-specific ATF-2 antibody.

Statistical analysis

Statistical significance was analyzed by analysis of variance (ANOVA). A *P* value less than 0.05 was considered significant.

Results

TNF- α induces RANTES production

Firstly, we examined a dose- and time-dependent induction of RANTES production by HPAEC. The concentrations of RANTES in the culture supernatants from TNF- α -stimulated culture increased in a dose- and time-dependent manner (Figs. 1 and 2).

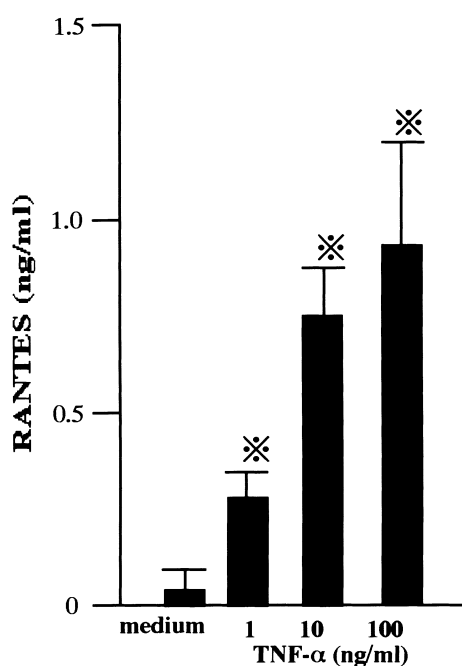


Figure 1. Dose-dependent production of RANTES by TNF- α -stimulated human pulmonary vascular endothelial cells. HPAEC were cultured with either medium or various concentrations of TNF- α . Concentrations of RANTES in culture supernatants were determined at 48 h after cultivation, as described in Material and methods. Results are expressed as mean $\pm$ SD in five different experiments. **P* < 0.01 compared with RANTES concentrations in HPAEC cultured with medium only.

TNF- α activates p38 MAP kinase, and SB 203580 inhibits p38 MAP kinase activity

TNF- α induced p38 MAP kinase activity, as demonstrated by the increased phosphorylation of its substrate, ATF-2. Pretreatment of the cells with SB 203580 inhibited the phosphorylation of ATF-2 in TNF- α -stimulated cells (Fig. 3).

SB 203580 inhibits TNF- α -induced RANTES production

TNF- α induced RANTES production and p38 MAP kinase activity, and SB 203580 inhibited p38 MAP kinase activity in TNF- α -stimulated cells. To test the possible involvement of p38 MAP kinase in TNF- α -induced RANTES production, we examined the effect of SB 203580 on TNF- α -induced RANTES production. As shown in Fig. 4, the concentrations of RANTES in the culture supernatants from the cells cultured with TNF- α in the presence of SB 203580 (5 and 10 μ M) were significantly lower than those from the cells cultured with TNF- α in the absence of SB 203580, indicating that SB 203580 inhibited TNF- α -induced RANTES production. The total number of cells and cell viability at the end of the culture period of each experiment, as determined by trypan blue exclusion assay, did not differ with culture conditions. Figs. 1, 2, and 4 suggest that TNF- α -induced RANTES production, and the inhibition by SB 203580 of RANTES production did not result from cell cytotoxicity.

Discussion

In the present study, we examined the signal transduction pathway in TNF- α -induced RANTES production by human pulmonary vascular endothelial cells. The results showed that TNF- α induced RANTES production and p38 MAP kinase activity in human pulmonary vascular endothelial cells. Abrogation of p38 MAP kinase activity by SB 203580 repressed RANTES production and p38 MAP kinase activity in TNF- α -stimulated human pulmonary vascular endothelial cells. These results indicate that p38 MAP kinase plays an important role in the TNF- α -activated signaling pathway which regulates RANTES production by human pulmonary vascular endothelial cells.

In the present study, TNF- α was used as the inducer of RANTES production by human pulmonary vascular endothelial cells, since TNF- α has been shown to be involved in the production of allergic inflammation and can stimulate vascular endothelial cells to induce cytokine expression (8–10, 23–27).

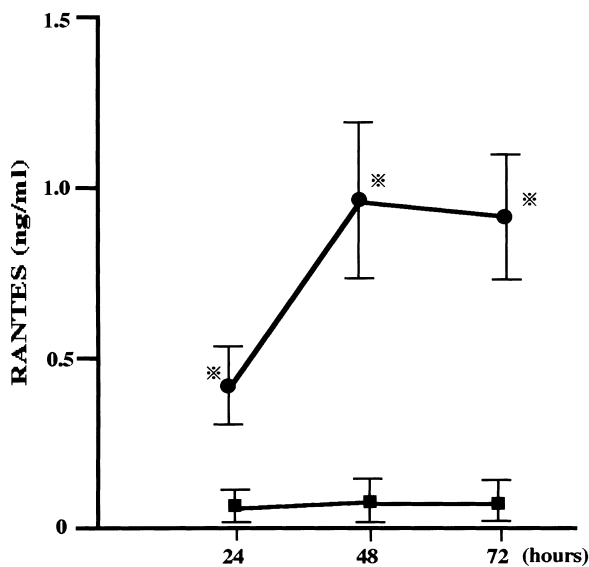


Figure 2. Time-dependent production of RANTES by TNF- α -stimulated human pulmonary vascular endothelial cells. HPAEC were cultured with either medium (■) or 100 ng/ml of TNF- α (●). Concentrations of RANTES in culture supernatants were determined at 24, 48, and 72 h after cultivation, as described in Material and methods. Results are expressed as mean $\pm$ SD in five different experiments. * $P < 0.01$ compared with RANTES concentrations in HPAEC cultured with medium only.

Bronchial asthma is regarded as eosinophilic inflammation of the airway (1–4). Eosinophils and their products have been shown to play an important role in the production of allergic inflammation. The mechanism in the recruitment of eosinophils into the sites of allergic inflammation has been investigated, and various cytokines and chemical mediators that display chemotactic activity for eosinophils have been shown to participate in the recruitment of eosinophils into the sites of allergic inflammation (5–7).

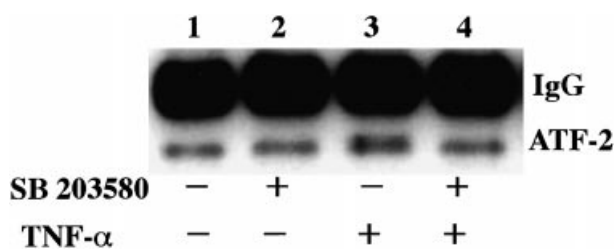


Figure 3. TNF- α activated p38 MAP kinase and SB 203580 inhibited p38 MAP kinase activity. HPAEC that had been preincubated with or without SB 203580 (10 μ M) for 1 h were stimulated with TNF- α (100 ng/ml) and cultured for 10 min. p38 MAP kinase activity was analyzed as described in Material and methods. Cells were cultured with medium (lane 1), SB 203580 (lane 2), TNF- α (lane 3), and TNF- α and SB 203580 (lane 4). Three identical experiments independently performed gave similar results. SB 203580 was dissolved in dimethyl sulfoxide. Amount of 10 μ M of SB 203580 contained 0.01% of dimethyl sulfoxide; 0.01% of dimethyl sulfoxide had no effect on p38 MAP kinase activity in TNF- α -stimulated cells.

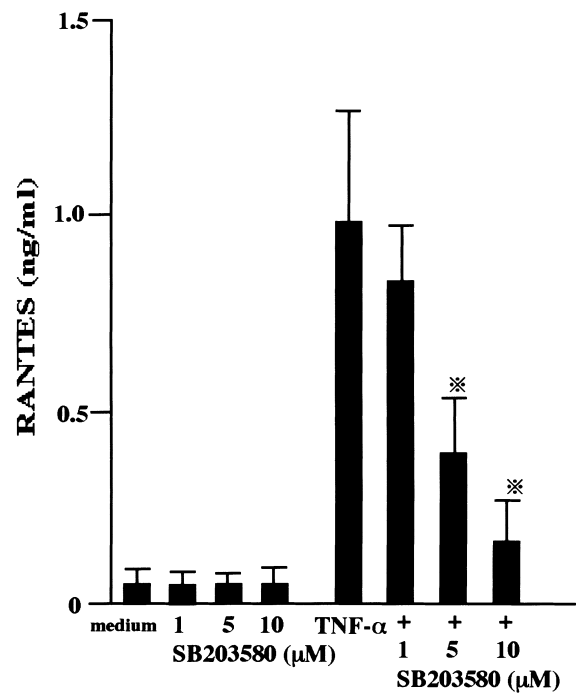


Figure 4. SB 203580 inhibited TNF- α -induced RANTES production by human pulmonary vascular endothelial cells. HPAEC that had been preincubated with or without various concentrations of SB 203580 for 1 h were cultured with either medium or 100 ng/ml of TNF- α , and concentrations of RANTES in culture supernatants were determined at 48 h after cultivation, as described in Material and methods. SB 203580 was dissolved in dimethyl sulfoxide; 1, 5, and 10 μ M of SB 203580 contained 0.001%, 0.005%, and 0.01% of dimethyl sulfoxide, respectively. These concentrations of dimethyl sulfoxide had no effect on TNF- α -induced RANTES production. Results are expressed as mean $\pm$ SD in five different experiments. * $P < 0.01$ compared with RANTES concentrations in HPAEC cultured without SB 203580.

The traffic of eosinophils to the sites of allergic inflammation is presumed to be regulated at the several steps. A primary step in the infiltration of eosinophils into the submucosa of the asthmatic airway is the adherence of eosinophils to vascular endothelial cells (23). At this step, therefore, cytokines produced by pulmonary vascular endothelial cells, as well as the interaction of adhesion molecules on eosinophils and their counterreceptors on pulmonary vascular endothelial cells, are thought to play an important role in tethering eosinophils to vascular endothelial cells. Recently, RANTES has been shown to play an important role in the production of allergic inflammation through chemotactic activity for eosinophils (5–7). From this evidence, we attempted to clarify the mechanism of the production of RANTES by pulmonary vascular endothelial cells. Our results showed that pulmonary vascular endothelial cells produced RANTES and that abrogation of p38 MAP kinase activity by SB 203580, as the specific inhibitor for p38 MAP kinase activity, repressed RANTES production,

indicating that p38 MAP kinase plays an important role in RANTES production.

SB 203580, the specific inhibitor of p38 MAP kinase activity, was originally discovered as an inhibitor of LPS-induced production of IL-1 and TNF- α in human monocytic cell lines, THP-1 (21). The specific inhibitor for p38 MAP kinase has been shown to be useful in the treatment of inflammatory diseases (28, 29). In the present study, we showed that SB 203580 inhibited RANTES production by human pulmonary vascular endothelial cells. Therefore, the

specific inhibitor for p38 MAP kinase activity, SB 203580, may have a potential effect on controlling the allergic inflammation of bronchial asthma through inhibiting RANTES production.

From the data presented here, we conclude that TNF- α induces RANTES production by human pulmonary vascular endothelial cells through the p38 MAP kinase-dependent pathway. However, the precise role of p38 MAP kinase in the production of airway inflammation of bronchial asthma remains to be clarified.

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