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Suppressive Activity of Quercetin on the Production of Eosinophil Chemoattractants from Eosinophils *In Vitro*

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Abstract. *Quercetin, a flavonoid found in a wide variety of plants, has been studied for possible health benefits, and it has been found to have potent anti-oxidant, anti-viral and anticancer effects. Although quercetin is also reported to act as an antihistamine and an anti-inflammatory through the suppression of mast cell activation, the influence of quercetin on eosinophil activation is not fully understood. The present study, therefore, was undertaken to examine the influence of quercetin on eosinophil activation, especially chemokine production by using an in vitro cell culture technique. Eosinophils (5×10^5 cells/ml) obtained from Mesocestoides corti-infected mice were stimulated with 200 ng/ml stem cell factor in the presence of different concentrations of quercetin for 24 h. Chemokine, eotaxin, regulated on activation normal T cell expressed and secreted and macrophage inflammatory protein-1β, levels in culture supernatants were examined by enzyme-linked immunosorbent assay (ELISA). We also examined the influence of quercetin on chemokine mRNA expression and transcription factor, nuclear factor kappa B and activator protein 1, activation by real-time reverse transcription polymerase chain reaction and ELISA, respectively. Treatment of eosinophils with quercetin at more than 4.5 μM caused a significant decrease in chemokine levels in culture supernatants. Quercetin also suppressed transcription factor activation in 4 h-cultured cells and mRNA expression of chemokine in 12 h-cultured cells, which were increased by stem cell factor stimulation. These results may suggest that quercetin inhibits eosinophil activation, especially chemokine production, and results in inhibition of the development of eosinophilic inflammatory responses.*

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Airway mucosal eosinophilia is a prominent feature of allergic airway diseases such as asthma and rhinitis (1). Eosinophils are believed to play an essential role as final effector cells in the development and maintenance of allergic disease through the secretion of cationic proteins, lipid mediators, cytokines, and chemokines that can directly cause tissue damage and lead to the exacerbation of inflammatory responses (2). This was confirmed by ultrastructural studies, which showed extensive de-granulation of eosinophils in airway tissues during active diseases (3, 4). Accordingly, immunohistochemical analysis revealed high levels of extracellular deposition of granule proteins in the diseased tissues (5). These reports suggest that the manipulation of eosinophil functions, such as activation and mediator release, may be a good therapeutic aim in the treatment and prevention of allergic diseases.

Quercetin is a common chemical pigment in the rind and bark of a wide variety of plants (6). It is one of the main flavonoids in the diet, being found in apple skin, onions, and red wine, among others (7). For many years, quercetin has been studied for its possible health benefits, and it was revealed that it exerts anti-viral activity through the inhibition of reverse transcriptase, which is essential for replication of HIV and retroviruses (8-10). It is also reported that quercetin acts as scavenger of free radicals and attenuates oxidative stress responses (11). In regard to allergic inflammatory responses, quercetin is reported to inhibit the production of both inflammatory cytokines and chemical mediators from mast cells after immunological stimulation *in vitro,* which are responsible for the development of allergic inflammatory diseases (12, 13). Furthermore, oral administration of quercetin attenuated clinical symptoms such as bronchial hyper-reactivity to specific allergen challenge in murine and guinea pig models of asthma (14, 15). Although these reports strongly suggest that quercetin will be a good candidate as a dietary supplement for prevention of the development of allergic diseases, the mode of action of quercetin in allergic immune responses is not well understood.

Histological investigation of allergic diseases has shown the presence of activated inflammatory cells, such as type-2 helper

(Th2) T-cells, basophils and eosinophils (16). Out of these, eosinophils are well accepted as being key cells and play essential roles in the development and maintenance of allergic diseases through the secretion of a number of harmful mediators, such as eosinophil cationic protein and major basic protein, which are responsible for the development of airway reactivity, destruction of epithelium and other inflammatory changes that underlie allergic diseases (17-19). Eosinophils are also reported to be able to secret inflammatory cytokines and chemokines such as interleukin (IL)5, and macrophage inflammatory protein-1beta (MIP1β), which are implicated in recruitment and activation of inflammatory cells, especially eosinophils, at the site of allergic inflammatory reactions (19). In this regard, several experiments have been performed to examine the influence of quercetin on eosinophil functions, which revealed the inhibitory effects of quercetin on eosinophil activation such as migration and de-granulation after immunological stimulation *in vitro* and *in vivo* (15, 20), but the pharmacological actions of quercetin on eosinophil activation have not been sufficiently investigated. In the present study, therefore, we examined the influence of quercetin on eosinophil activation by using an *in vitro* cell culture technique.

Materials and Methods

Agent. Quercetin was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA) as preservative-free pure powder. This was firstly dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM, diluted with RPMI-1640 medium (Sigma-Aldrich Co., Ltd.) supplemented with 10% fetal calf serum (RPMI-FCS; Nippon Bio-Supply Center, Tokyo, Japan) at a concentration of 1.0 mM. BAY11-7085 (BAY), a nuclear factor kappa B (NFκB) inhibitor, and SP600125 (SP), an activator protein1(AP1) inhibitor, were purchased from Sigma-Aldrich Co., Ltd. and dissolved in RPMI-FCS at a concentration of 1.0 mM. These chemicals were then sterilized by passing through 0.2 μm filters, and stored at 4˚C until used.

Eosinophil preparation and cell culture. Specific pathogen-free male BALB/c mice (five weeks of age) purchased from Charles River Japan Inc. (Atsugi, Japan) were infected intraperitoneally with 500 *Mesocestoide corti* larvae kindly donated by Dr. A. Niwa (Kinki University, Osaka, Japan). These mice were maintained in our animal facilities under controlled environment (25±3˚C, 55±5% humidity and a 12 hour light/dark cycle). Mouse peritoneal exudate eosinophils were obtained from five individual mice 21 days after infection as previously described (21). Briefly, BALB/c mice were killed under ether anesthesia and peritoneal exudate cells were obtained by washing mouse peritoneal cavity with 10 ml sterile phosphate-buffered saline (PBS). The cells were washed three times with PBS and incubated in plastic tissue culture plates to remove plastic-adherent cells in a humidified atmosphere with 5% CO_2 at 37˚C. After 2 h, non-adherent cells were collected and suspended in RPMI-FCS at a density of 5×10^5 cells/ml and used as eosinophils. The purity of eosinophils was >95% as judged by Giemsa staining. Eosinophils (1.0 ml) were treated in triplicate with various doses of quercetin for 1 hour and stimulated with 200.0 ng/ml stem cell factor (SCF) obtained from R&D Corp. (Minneapolis, MN, USA) for 24 h (20) in a final volume of 2.0 ml. The culture supernatants were collected after pelleting cells by centrifugation at 2000 ×*g* for 15 min at 25˚C and stored at −40˚C until used. In examining the influence of quercetin on transcription factor activation and chemokine mRNA expression, cells were cultured in a similar manner for 4 hours and 12 h, respectively (22). All animal experimental procedures were approved by the Animal Care and Use Committee of Showa University(approval number 54011) and were carried out in accordance with the guidelines of the Physiological Society of Japan.

Assay for eosinophil chemoattractants. Levels of eosinophil chemoattractants, eotaxin, regulated on activation normal T cell expressed and secreted (RANTES) and macrophage inflammatory protein, (MIP1)β, in culture supernatants were examined by commercially available mouse enzyme-linked immunosorbent assay (ELISA) test kits (R & D Corp.). The ELISA was carried out in duplicate according to the manufacturer's recommendation. The minimum detectable levels by these ELISA kits were 3.0 pg/ml for eotaxin, 2.0 pg/ml for RANTES and 3.0 pg/ml for MIP1β.

Assay for transcription factor activation. NF-κB (p65) activity in cultured-eosinophils was analyzed by commercially available NF-κB ELISA test kits (Active Motif, Co., Ltd., Carlsbad, CA, USA) that contained sufficient reagents and monoclonal antibody against p65, according to the manufacturer's recommendations. In brief, nuclear extract (5.0 mg of protein) from eosinophils was introduced into each well of 96-well microplates pre-coated with oligonucleotide containing NFκB consensus site (5'-GGGACTTTCC-3') in a volume of 20.0 μl in duplicate, followed by incubation for 1 h at 25˚C. After washing three times, 100 μl of monoclonal antibody against p65 was added to the appropriate wells and microplates were incubated for a further 1 h at 25˚C. Anti-IgE horseradish peroxidase (HRP) conjugate in a volume of 100 μl was then added and microplates were incubated for a further hour at 25˚C. The absorbance at 450 nm was measured after the addition of TMB solution. AP1 (JUN B) activity was also measured with commercially available AP1 (JUN B) ELISA test kits (Active Motif, Co., Ltd.) in a similar manner.

Assay for mRNA expression. Poly A^+ mRNA was separated from cultured cells with oligo (dT)-coated magnetic micro beads (Milteny Biotec, Bergisch Gladbach, Germany). The first-strand cDNA was synthesized from 1.0 μg of PolyA^+ mRNA using a Superscript cDNA synthesis kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was then carried out using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR mixture consisted of 2.0 μl of sample cDNA solution (100 ng/μl), 25.0 μl of SYBR-Green Mastermix (Applied Biosystems), 0.3 μl of both sense and antisense primers, and distilled water to give a final volume of 50.0 μl. The reaction was conducted as follows: 4 min at 94˚C, followed by 40 cycles of 15 s at 95˚C and 60 s at 60˚C. β-actinwas amplified as an internal control. mRNA levels were calculated by using the comparative parameter threshold cycle and normalized to β-actin. The nucleotide sequences of the primers were as follows: for β-actin: 5'-GTGGGCCG- CTCTAGGCACCAA-3 (sense) and 5'-CTCTTTGATGTCACGCACGATTTC-3' (antisense); for RANTES: 5'-CATCCTCACTGCAG- CCGCC-3' (sense) and 5'-CCAAGCTG GCTAGGACTAGAG-3 (antisense); for eotaxin: 5'-AGCTCC-ACAGCGCTTC TATT-3' (sense) and 5'-GGTGCATCTGTTGTTGG

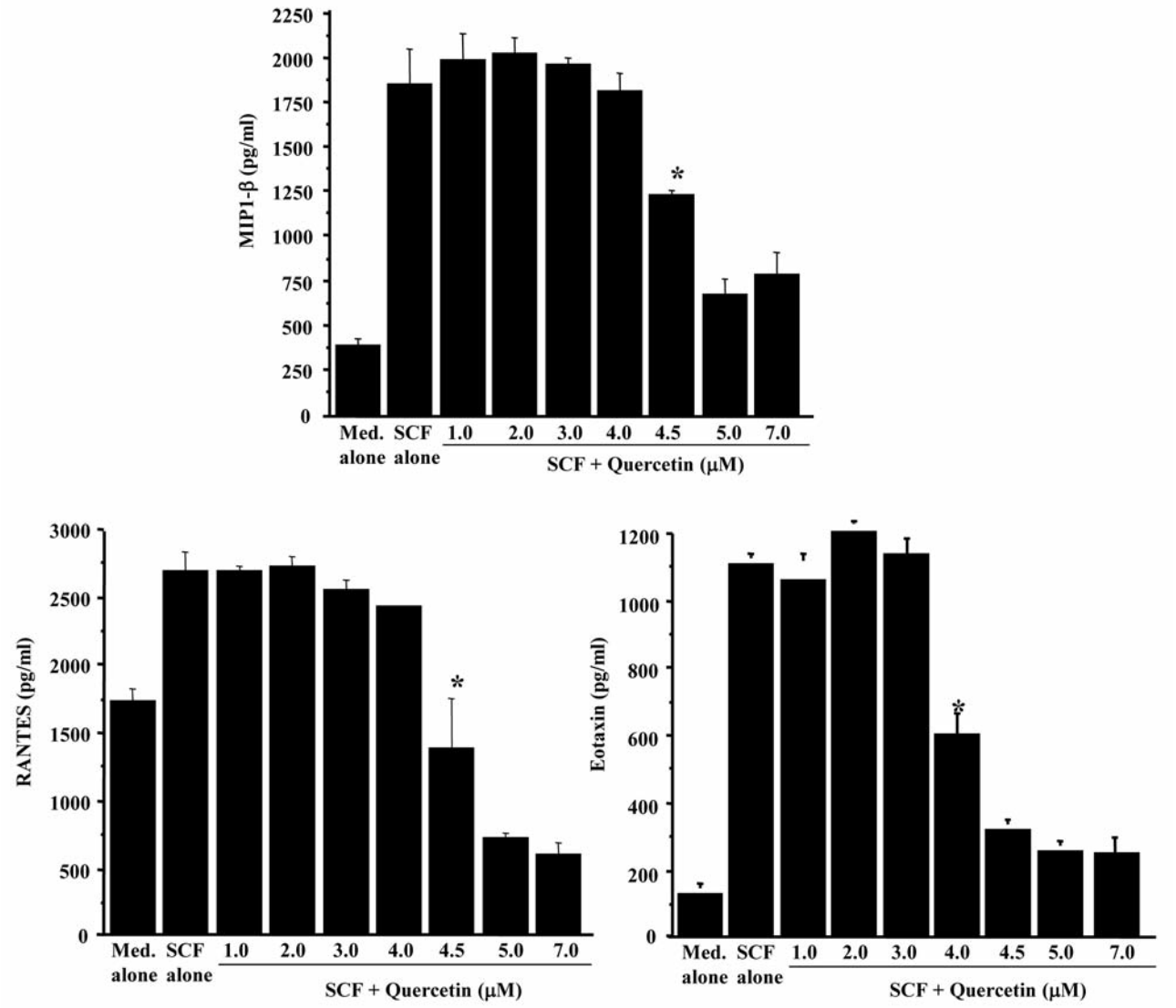

Figure 1. *Influence of quercetin on eosinophil chemoattractant production by eosinophils after stem cell factor (SCF) stimulation in vitro. Eosinophils obtained from five individual mice infected with Mesocestoides corti (500 larvae/mouse) were stimulated with 200 ng/ml SCF in the presence of different concentrations of quercetin for 24 h. Factor levels in culture supernatants were examined by enzyme-linked immunosorbent assay. The data are expressed as the mean pg/ml±standard errors of the means. Med. alone: Medium alone. *p<0.05 vs. SCF alone.*

TGATT-3' (antisense) (23); for MIP1β: 5'-ATGAAG CTCTGC GTGTCTGC-3' (sense) and 5'-TGTCTGCCTCTT TTGGTCAG-3' (antisense) (24).

Statistical analysis. All data are expressed as the means±SE of five individual mice. All results were analyzed with analysis of variance (ANOVA) followed by Bonferroni correction. A value of $p<0.05$ was considered significant.

Results

Effect of quercetin on the production of eosinophil chemoattractants. The first set of experiments was carried out to examine the influence of quercetin on the production of eosinophil chemoattractants, eotaxin, RANTES and MIP1β, after SCF stimulation. Eosinophils (5×10^5 cells/ml) were stimulated with 200 ng/ml SCF in the presence of different concentrations of quercetin. After 24 h, the chemoattractant levels in the culture supernatants were examined by ELISA. As shown in Figure 1, quercetin dose-dependently suppressed the ability of eosinophils to produce eotaxin, RANTES and MIP1β under SCF stimulation. The minimum concentration that caused significant suppression of the chemoattractant production was 4.5 μM.

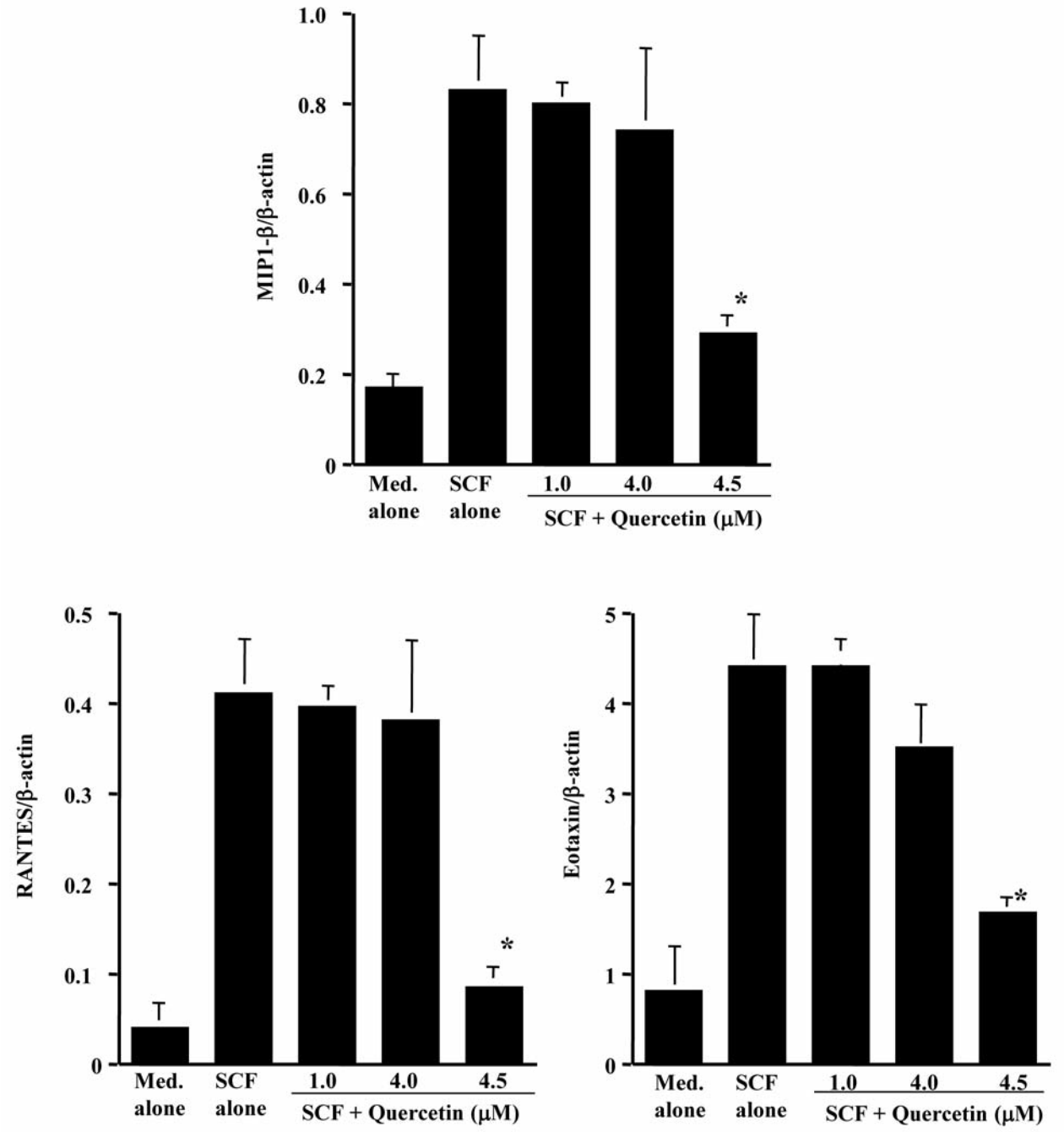

Figure 2. *Influence of quercetin on mRNA expression for eosinophil chemoattractants in eosinophils after stem cell factor (SCF) stimulation in vitro. Eosinophils obtained from five individual mice infected with Mesocestoides corti (500 larvae/mouse) were stimulated with 200 ng/ml SCF in the presence of different concentrations of quercetin for 12 h. mRNA expression was examined by real-time reverse transcription polymerase chain reaction. The data are expressed as the mean±standard errors of the means relative to β-actin expression. Med. alone: Medium alone. *p<0.05 vs. SCF alone.*

Influence of quercetin on the expression of mRNA for eosinophil chemoattractants. The second set of experiments was designed to examine the influence of quercetin on mRNA expression. Eosinophils were stimulated with 200 ng/ml SCF in the presence of various concentrations of quercetin. After 12 h, mRNA expression was examined by real-time RT-PCR. As shown in Figure 2, addition of quercetin at 4.5 μM, but not 1.0 and 4.0 μM, inhibited mRNA expression of eotaxin, RANTES and MIP1β, which were increased by SCF stimulation.

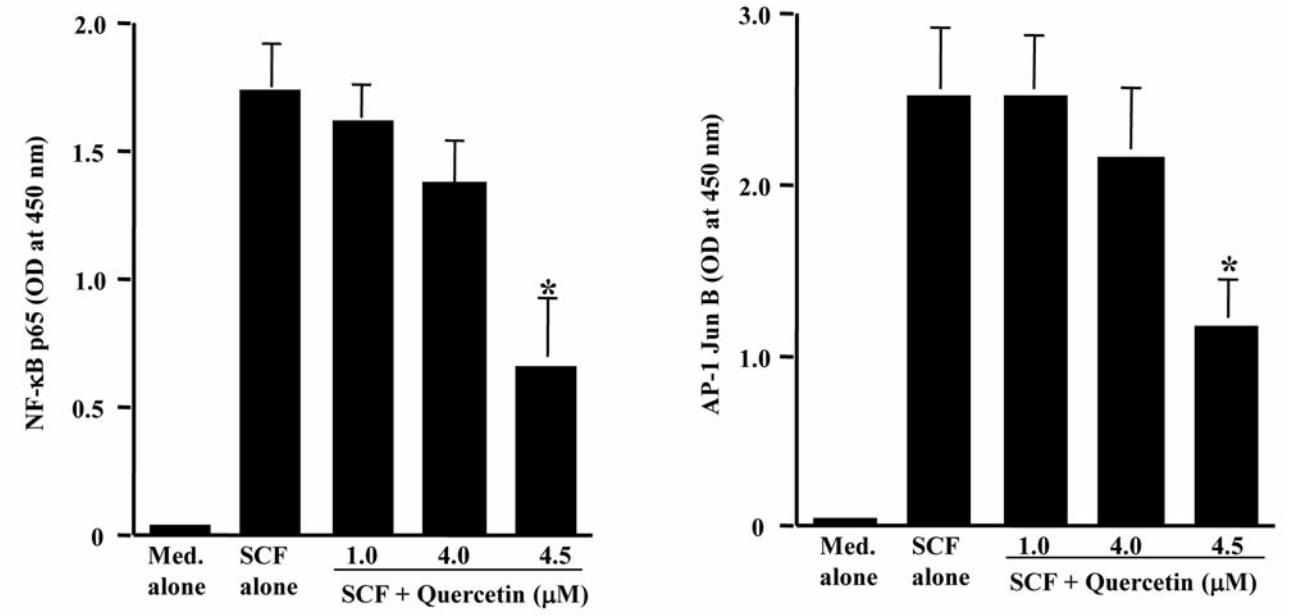

Figure 3. *Influence of quercetin on transcription factor activation in eosinophils after stem cell factor (SCF) stimulation in vitro. Eosinophils obtained from five individual mice infected with Mesocestoides corti (500 larvae/mouse) were stimulated with 200 ng/ml SCF in the presence of different concentrations of quercetin for 4 h. Transcription factor activation was examined by enzyme-linked immunosorbent assay. The data are expressed as the mean optical density at 450 nm±standard errors of the means. Med. alone: Medium alone. *p<0.05 vs. SCF alone.*

Influence of quercetin on transcription factor activation. The third set of experiments was carried-out to examine the influence of quercetin on the activation of transcription factor NFκB (p65) and AP1 (JUN B). Eosinophils were stimulated 200 ng/ml SCF in the presence of quercetin for 4 hours. Transcription factor activation was assayed by ELISA. As shown in Figure 3, treatment of cells with quercetin at 4.5 μM caused significant suppression of NFκB (p65) activation: the optical density observed in experimental cultures was significantly lower than that in cells cultured with SCF-alone. The data in Figure 3 also show the suppressive action of quercetin on AP1 (JUN B) activation, which was increased by SCF stimulation.

Influence of the inhibition of transcription factor activation on the production of eosinophil chemoattractants from eosinophils. The final set of experiments was carried-out to examine the influence of suppression of transcription factor activation on eosinophil chemoattractant production from eosinophils in response to SCF stimulation. Eosinophils (5×10^5 cells/ml) were stimulated with 200 ng/ml SCF in the presence of either BAY or SP. After 24 h, eotaxin and RANTES levels in culture supernatants were examined by ELISA. As shown in Figure 4 (upper panels), treatment of eosinophils with SP at more than 5 μM caused significant suppression of both eotaxin and RANTES production from eosinophils after SCF stimulation. The data in Figure 4 (lower panels) also showed the suppressive effects of BAY on the production of eotaxin and RANTES from eosinophils in response to SCF stimulation. The minimum concentration that caused significant suppression was 5 μM (Figure 4, lower panels).

Discussion

Quercetin is an important member of a large group of plant compounds, so-called flavonoids, and is one of the most abundant flavonoid in our diets (7, 11). For many years, much effort was made to elucidate the possible health benefits of quercetin and revealed that it has antioxidant, anticarcinogenic, and cardioprotective effects (6-11). Quercetin is also reported to exert anti-allergic activity through the inhibition of histamine, a chemical that causes allergic reactions, released from allergic inflammatory cells, such as mast cells (12-15). On the other hand, there is established concept that eosinophils are essential for the development of allergic immune responses and associated with disease severity (1-5), suggesting that eosinophils may be important targets for the treatment and management of allergic diseases. However, the influence of quercetin on eosinophil functions is not fully understood. The present study, therefore, was undertaken to examine the influence of quercetin on eosinophil functions, especially chemokine production after immunological stimulation *in vitro*. The data

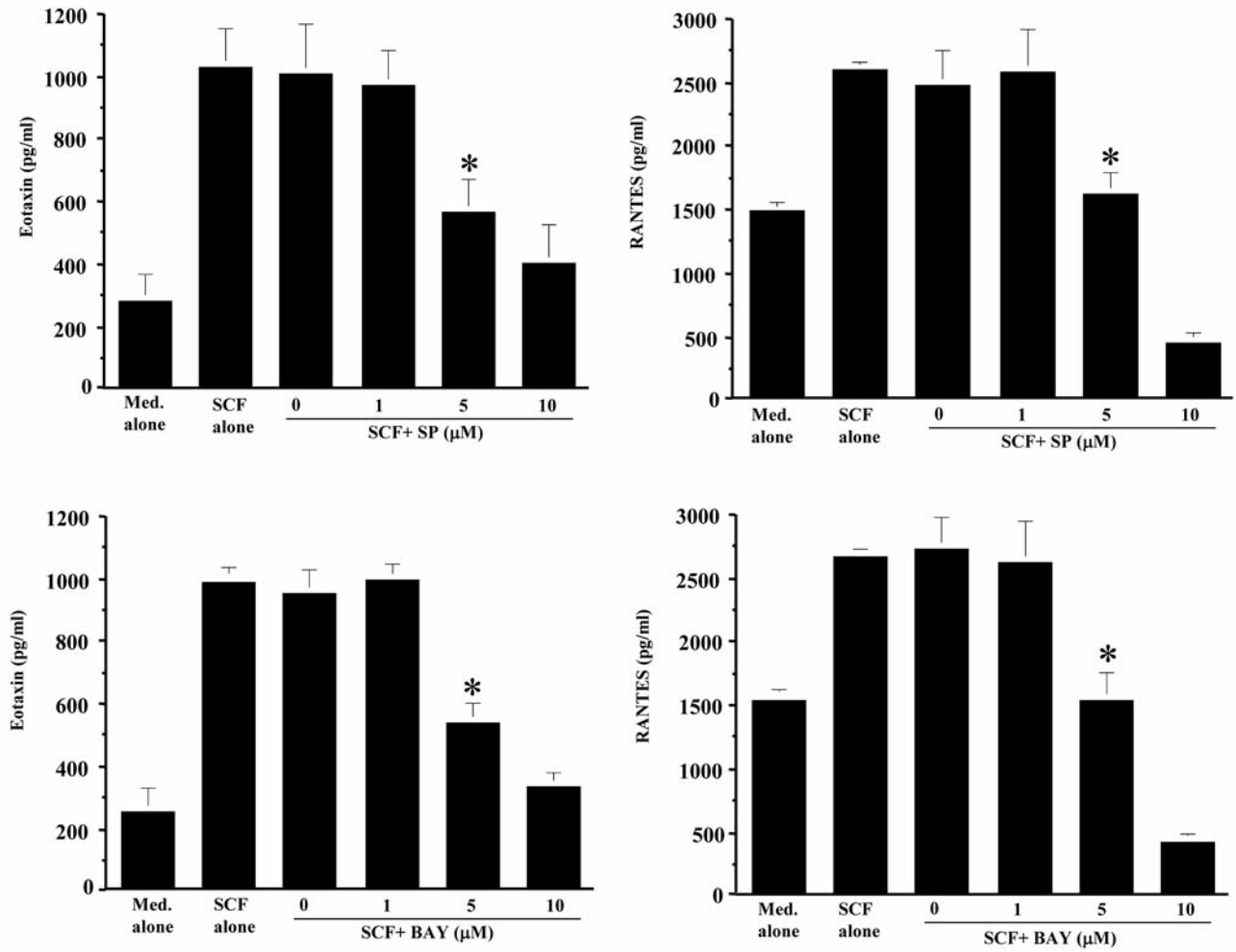

Figure 4. *Influence of transcription factor inhibitor on eosinophil chemoattractant production by eosinophils after stem cell factor (SCF) stimulation in vitro. Eosinophils obtained from five individual mice infected with Mesocestoides corti (500 larvae/mouse) were stimulated with 200 ng/ml SCF in the presence of different concentrations of either BAY11-7085 (BAY), a nuclear factor kappa B inhibitor, or SP600125 (SP), an activator protein-1 inhibitor, for 24 h. Eotaxin and regulated on activation normal T-cell expressed and secreted (RANTES) levels in culture supernatants were examined by enzyme-linked immunosorbent assay. The data are expressed as the mean pg/ml±standard errors of the means. Med. alone: Medium alone. *p<0.05 vs. SCF alone.*

obtained clearly show that quercetin at more than 4.5 μM inhibits the ability of eosinophils to produce chemokines, RANTES, eotaxin and MIP1β after stimulation with SCF that plays a significant role in eosinophil-associated inflammatory responses (25, 26).

Allergic inflammatory responses are characterized by structural abnormalities, so-called tissue remodeling, associated with intense infiltration of eosinophils and macrophages (17-19). Tissue eosinophilia is due to a combination of specific and coordinated cellular processes appearing at different stages of eosinophil extravasation including adhesion, chemotaxis, and activation (19). In addition to IL5, RANTES and eotaxin are responsible for eosinophil attraction, and imply their participation in the specific recruitment of eosinophils to the site of allergic inflammation (16, 17, 19). Furthermore, these two chemokines stimulate basophils to secrete histamine, and also cause eosinophil de-granulation and secretion of various granule proteins such as eosinophil major basic protein and eosinophil cationic protein (17, 19, 27). MIP1β is a member of the CC subfamily of chemokines, which induce the migration and recruitment of monocytes and T-cells to the sites of inflammation (28). MIP1β has also been reported to enhance macrophage effector functions by inducing the production of nitric oxide, which is the most important final effector molecule in inflammatory diseases (29). After oral administration of 64 mg quercetin to humans, plasma levels of quercetin gradually increased and peaked at 650 nM, with an elimination half-life of quercetin of 17 to 24 h (30). Although there is no standard

recommended dosage of quercetin, a dose of 1,200 mg or 1,500 mg per day is commonly used (31). Assuming first-order kinetics, a 1,200 mg dose could lead to a plasma concentration of up to 12 μM (30), which is much higher than that inducing suppressive effects of eosinophil activation *in vitro*. Judging from these reports, the present results showing the suppressive activity of quercetin on chemokine production from eosinophils provide possible mechanisms that could explain the favorable effects of quercetin on eosinophil-mediated allergic inflammatory diseases.

SCF is well accepted as playing essential roles in the hematopoiesis during embryonic development (26). SCF exerts its biological effect through a specific interaction with the cell surface receptor c-KIT, which is a member of the receptor tyrosine kinase family (26). SCF and c-KIT complex has been reported to cause activation of several types of transcription factor, including NF-κB and AP1 (32), which are responsible for the production of chemokines from mast cells and eosinphils (22), suggesting that quercetin inhibits transcription factor activation and results in suppression of chemokine production by eosinophils. Therefore, we then examined the possible suppressive mechanisms of quercetin on chemokine production by eosinophils *in vitro*. The present data clearly showed that quercetin at more than 4.5 μM suppressed the activation of transcription factors NF-κB and AP1, which are essential factors for chemokine production by eosinophils after immunological stimulation (22) and results in decrease in factor levels in culture supernatants. Quercetin is reported to inhibit the increase in intracellular Ca^{2+} levels induced by compound 48/80 in human mast cells line *in vitro* (20), which are essential for transcription factor activation. It is also reported that quercetin inhibits the activation of tyrosine kinases, which are essential for the production of eosinophil chemoattractants, including IL5 (22). From these reports, there is another possibility that quercetin inhibits the activation of tyrosine kinases in eosinophils after SCF stimulation through the suppression of Ca^{2+} in cytosol and results in the inhibition of chemokine production by eosinophils *in vitro*. Further experiments are required to clarify this point.

In conclusion, the present results strongly suggest that some of the therapeutic effects of quercetin on allergic diseases depend on its ability to inhibit eosinophil activation.

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