

Original article

Soluble vascular cell adhesion molecule-1 induces human eosinophil migration

Background: Tissue eosinophilia is one of the hallmarks of allergic diseases and Th2-type immune responses including asthma. Adhesion molecules are known to play an important role in the accumulation of eosinophils in allergic inflammatory foci, and they contribute to eosinophil activation. Elevated levels of the soluble forms of adhesion molecules in the body fluid of asthmatic patients have been observed, although their pathophysiological significance remains to be fully elucidated.

Methods: Peripheral blood eosinophils were purified, and the effect of soluble vascular cell adhesion molecule-1 (sVCAM-1) on eosinophil migration was investigated using *in vitro* systems.

Results: We found that sVCAM-1 (1 to 10 µg/ml) induced eosinophil chemotaxis, rather than chemokinesis, in a concentration-dependent fashion. In addition, sVCAM-1 induced cell shape change and actin polymerization, which are necessary for cell movement. Manipulations with very late antigen (VLA)-4-neutralizing antibody and signal inhibitors indicated that the sVCAM-1-induced chemotaxis was mediated through ligand-dependent activation of tyrosine kinase Src, p38 mitogen-activated protein kinase (MAPK), and extracellular signal-regulated kinase (ERK) MAPK. Rapid phosphorylation of these signaling molecules was observed using a bead-based multiplex assay.

Conclusion: Our results raise the possibility of sVCAM-1 in the fluid phase as a significant contributor to the heightened eosinophilic inflammatory response.

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Tissue eosinophilia is one of the hallmarks of allergic diseases and T-helper-2 (Th2)-type immune responses. Eosinophils contribute to tissue injury, vascular leakage, mucus secretion, and tissue remodeling by releasing cytotoxic granule proteins, reactive oxygen species (ROS), and lipid mediators (1).

Adhesion molecules are known to play an important role in the accumulating of leukocytes in the inflamed airway. Adherence of eosinophils to vascular endothelial cells via adhesion molecules is considered to be an essential and initial event in eosinophilic allergic inflammation. Vascular cell adhesion molecule-1 (VCAM-1) contributes to the firm adhesion of eosinophils to vascular endothelial cells through a counter receptor for VCAM-1, very late antigen-4 (VLA-4, a heterodimer of $\alpha 4$ and $\beta 1$ subunits) (2). VLA-4 is believed to be involved in the selective accumulation of eosinophils because it is

expressed by eosinophils but not by neutrophils (3). In addition, it is of interest to evaluate not only eosinophil accumulation but also varying eosinophil activation status. Accumulating evidence indicates that adhesion molecules augment the release of granule proteins (4, 5), increase production of ROS (6), and prolong eosinophil survival (7, 8).

Adhesion molecules, including VCAM-1, undergo proteolytic cleavage, which releases the soluble ectodomain from the cell surface. Soluble forms of adhesion molecules are increased in the sputum (9), serum (10, 11), and bronchoalveolar lavage fluid (BALF) (12) of asthmatic patients. Elevated levels of soluble adhesion molecules reflect the activity of asthma; therefore, these molecules can be useful inflammatory markers (10, 11). Yet relatively little is known about the pathophysiological function(s) of soluble adhesion molecules in allergic inflammation. We hypothesized that soluble VCAM-1 (sVCAM-1) could play a role in human eosinophil accumulation in inflammatory foci, particularly in chemotaxis. Our results indicate that sVCAM-1 induces eosinophil chemotaxis via VLA-4, which is regulated by multiple signaling.

Abbreviations: BALF, bronchoalveolar lavage fluid; MAPK, mitogen-activated protein kinase; MFI, mean fluorescence intensity; PGD₂, prostaglandin D₂; VCAM-1, vascular cell adhesion molecule-1; sVCAM-1, soluble VCAM-1; VLA-4, very late antigen-4.

Materials and methods

Isolation of eosinophils

Peripheral venous blood sample was obtained from the subjects with mild eosinophilia. Informed consent was obtained from each subject, and the study protocols were approved by the Ethics Committee of Akita University School of Medicine. Eosinophils were isolated by sedimentation with 6% dextran followed by centrifugation on 1.088 Percoll (Pharmacia, Uppsala, Sweden) density gradients (13). The cells were further purified by negative selection using anti-CD16 immunomagnetic beads and a MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). The eosinophils (>98% purity) were then suspended in Hank's balanced salt solution (HBSS) with 1% fetal calf serum (FCS).

Chemotaxis assay

Chemotaxis of eosinophils was conducted in duplicate using 5- μ m-pore polycarbonate, polyvinylpyrrolidone-free membranes in Boyden chambers. Recombinant sVCAM-1 (R&D Systems, Minneapolis, MN, USA) was diluted in HBSS with 0.1% bovine serum albumin (BSA) and placed in the lower wells (100 μ l) at the indicated concentration. Aliquots of 100 μ l of the cell suspension at 0.5×10^6 cells/ml were placed in the upper chambers. Checkerboard analysis was also performed by varying the concentration of sVCAM-1 in both upper and lower wells. The loaded chambers were incubated at 37°C in humidified air with 5% CO₂ for 1 h. Then the membrane was removed, followed by fixation and staining for 3 min in May-Grünwald solution. The cells that migrated and adhered to the lower surface of the membrane were counted from 10 fields by light microscopy. The role of intracellular signaling pathways was investigated by pretreatment of eosinophils with cytochalasin D (1 μ M) (Biomol, Plymouth Meeting, PA, USA) for 20 min, anti-VLA-4 blocking antibody (5 μ g/ml) (R&D Systems) for 60 min, Src inhibitor PP2 (20 μ M) (Biomol) for 30 min, p38 mitogen-activated protein kinase (MAPK) inhibitor SB-202190 (1 μ M) (CalBiochem, La Jolla, CA, USA) for 30 min, or MEK inhibitor PD-98059 (10 μ M) (Cell Signaling Technology, Inc., Beverly, MA, USA) for 30 min at 37°C before the chemotaxis assay.

Measurement of changes in eosinophil shape using cytometry

Eosinophil shape change was assayed as previously described (13), modified from the method of Sabroe et al. (14). Purified eosinophils were washed in buffer (10 mM PBS containing Ca²⁺ and Mg²⁺, 10 mM HEPES, 10 mM glucose, and 0.1% BSA, pH 7.4) and pre-incubated for 10 min at 37°C. Stimulation of eosinophils by 100 nM prostaglandin D₂ (PGD₂) (Sigma-Aldrich, St Louis, MO, USA) for positive control, or sVCAM-1, resulted in changes in cell shape that were measured as changes in their ability to scatter light when illuminated in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). The data are displayed as mean fluorescence intensity (MFI) increase in forward scatter (FSC) compared with samples treated with vehicle alone.

Fluorescent staining of F-actin

The content of microfilamentous actin (F-actin) was analyzed by confocal microscopy with NBD-phalloidin (Molecular Probes, Eugene, OR, USA) staining. Purified eosinophils were incubated with PGD₂ (100 nM) for positive control, or sVCAM-1 (10 μ g/ml)

for 10 min at 37°C. The cells were then mounted, fixed with 4% formaldehyde in PBS for 10 min, permeabilized in PBS for 30 min, blocked with 2% BSA in PBS for 20 min, and rinsed with PBS. The fixed cells were incubated with 3.3×10^7 M NBD-phalloidin for 20 min in the dark. The cells were washed three times with PBS and observed by a confocal laser-scanning microscope system (LSM510; Carl Zeiss Co., Ltd, Jena, Germany).

Phosphoprotein assay

A panel of phosphoproteins was measured in duplicate using a bead-based multiplex assay (Bio-Plex phosphoprotein assay; Bio-Rad, Hercules, CA, USA) (15, 16), according to the manufacturer's instructions. Briefly, purified eosinophils (0.5×10^6 cells) were incubated with and without sVCAM-1 (10 μ g/ml) in human serum albumin-coated tubes for the indicated time intervals. After incubation, the cells were rinsed with ice-cold Cell Wash Buffer and pelleted by centrifugation. The cell lysate was collected and the protein concentration was calculated with a DC (detergent compatible) protein assay (Bio-Rad). The Bio-Plex assay was customized to detect and quantify phosphoproteins of Src, p38 MAPK, and ERK MAPK. The prepared first antibody with coupled beads was captured under 96-well plates, and the samples were incubated overnight at room temperature. Samples were incubated with biotin-labeled detection antibodies followed by further incubation with the fluorescence-labeled avidin reporter. The level of phosphoproteins bound to the beads was indicated by the intensity of the reporter signal. The signal was measured with Bio-Plex Manager software (Bio-Rad) interfaced with a Bio-Plex Reader (Bio-Rad).

Statistical analysis

Data are presented as mean $\pm$ SEM. Comparisons of two groups of data were performed using the Mann-Whitney *U*-test. One-way ANOVA with repeated measures was used for comparison of more than two variables. When the initial *P*-value was less than 0.05, Tukey's multiple comparison test was used to determine the significance between groups. Significance was established at the *P* < 0.05 level.

Results

Eosinophil migration was induced by sVCAM-1

Eosinophils were isolated by CD16-negative selection, and migration was investigated by using Boyden chambers stimulated with sVCAM-1. To rule out the possibility that recombinant soluble adhesion molecules absorbed to the filters in the migration assay, we first compared two filters, one coated with BSA overnight and one without coating. No significant differences were observed between the two filters at the time point studied (data not shown), so we performed migration assay with filters without BSA coating. sVCAM-1-stimulated eosinophil migration was significantly increased compared with the control ($304.6 \pm 50.9\%$ vs control, when stimulated at 10 μ g/ml, *P* < 0.01) in a concentration-dependent fashion (Fig. 1A). To distinguish between concentration-dependent cell migration (chemotaxis) and random migration (chemokinesis), eosinophils were

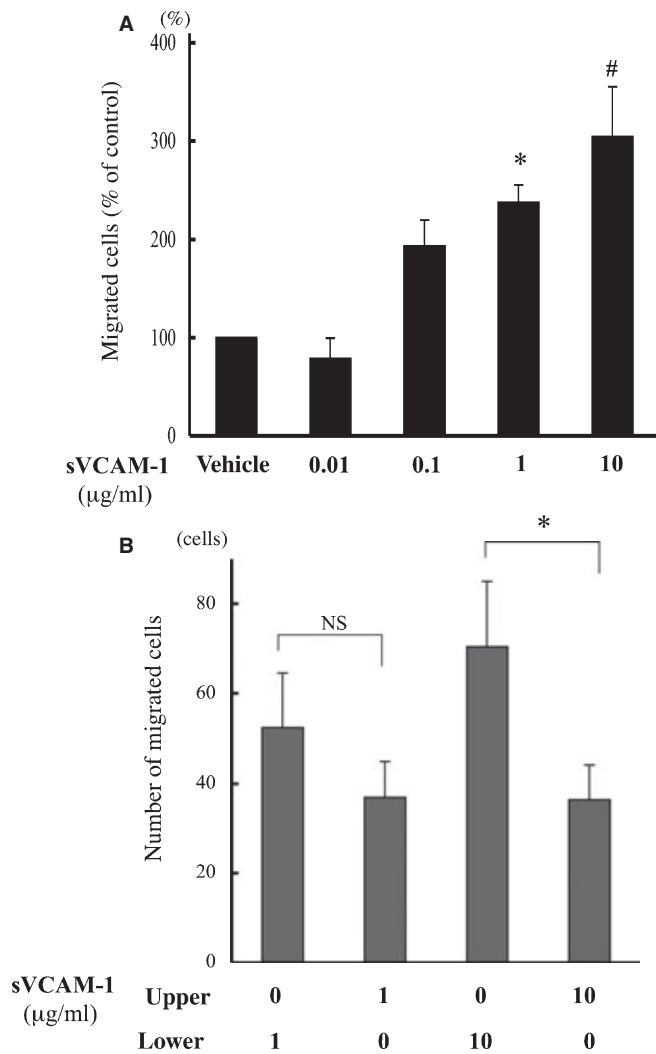


Figure 1. Effect of sVCAM-1 on eosinophil chemotaxis. (A) sVCAM-1-directed migration assays were performed using Boyden chambers. Purified eosinophils were placed in the upper wells, and chemotactic responses to varying concentrations of sVCAM-1 in the lower wells were studied. The loaded chambers were incubated at 37°C for 1 h. Data are expressed as the mean of four to five experiments $\pm$ SEM. # $P < 0.01$, * $P < 0.05$ vs unstimulated control. (B) Ability of sVCAM-1 to induce chemokinesis and chemotaxis. Cells were stimulated with sVCAM-1 in the upper or lower chemotaxis chamber as indicated. Results are expressed as the mean number of migrated cells ($n = 4$). * $P < 0.05$. NS, nonsignificant.

stimulated by varying the concentrations of sVCAM-1 in the upper and lower chambers, which revealed that this effect on eosinophils was chemotactic rather than chemokinetic (Fig. 1B).

sVCAM-1 causes eosinophil shape change and cytoskeletal rearrangement

Eosinophil shape change is consistent with a relationship between chemoattractant-induced responses and the

earliest phases of leukocyte recruitment from the microcirculation (14). Because shape change must occur before or during its movement, we next examined eosinophil shape change using a modified autofluorescence/FSC assay, which allows quantitative measurement of chemoattractant-induced cell shape change by flow cytometry. PGD₂, a potent eosinophil chemoattractive mediator, was used for positive control (17). Stimulation of eosinophils by sVCAM-1 and PGD₂ for 30 min results in the changes in cell shape that are measured as changes in their ability to scatter light when illuminated in a flow cytometer (Fig. 2A). A significant increase in MFI (60.4 ± 7.6) was observed at a concentration of 10 µg/ml (Fig. 2B), with the maximum effect at 30 to 120 min (Fig. 2C).

Actin rearrangement presumably governs the physical process of chemotaxis by directly regulating changes in cell shape and motility (18). Using a Boyden chamber system, we confirmed the actin reorganization-dependent migration by cytochalasin D that disrupts actin microfilaments. sVCAM-1 (10 µg/ml)-directed migration was completely inhibited when eosinophils were pretreated with 1 µM cytochalasin D (97.3% inhibition, $n = 3$). To assess the cytoskeletal rearrangement at shape change and chemotaxis, we visualized F-actin content using confocal microscopy. Compared with eosinophils with no treatment, stimulation with sVCAM-1 and PGD₂ promoted actin polymerization and distribution into bundles (Fig. 3).

Mechanisms of sVCAM-1-induced eosinophil chemotaxis

To further explore the mechanisms of the chemotactic activity of sVCAM-1, we examined the effect of receptor-blocking antibody and pharmacological inhibitors for several signaling pathways (Fig. 4). Pretreatment of cells with anti-VLA-4-neutralizing antibody, but not IgG1 control antibody (data not shown), completely reversed the chemotactic activity of sVCAM-1, which indicated that the activity is mediated through activation of VLA-4. Blockade of the Src tyrosine kinase pathway with the pharmacological inhibitor PP2 strongly reduced the effect of sVCAM-1. Although there was no statistical significance, the response of PP2-treated eosinophils was lower than that of unstimulated control (85.0% inhibition), suggesting the important role of the Src pathway in the chemokinesis of eosinophils. In addition, we also examined the MAPK pathways. Pretreatment with p38 MAPK inhibitor SB-202190, MEK (an upstream activator of ERK) inhibitor PD-98059 reversed the ability of sVCAM-1. We observed no obvious cytotoxic effects of inhibitors because the number of trypan blue-stained cells was insignificant. Finally, to confirm the activation of these signaling pathways, we performed phosphorylation assay using a bead-based multiplex assay. Increased phosphorylation of Src, p38 MAPK, and

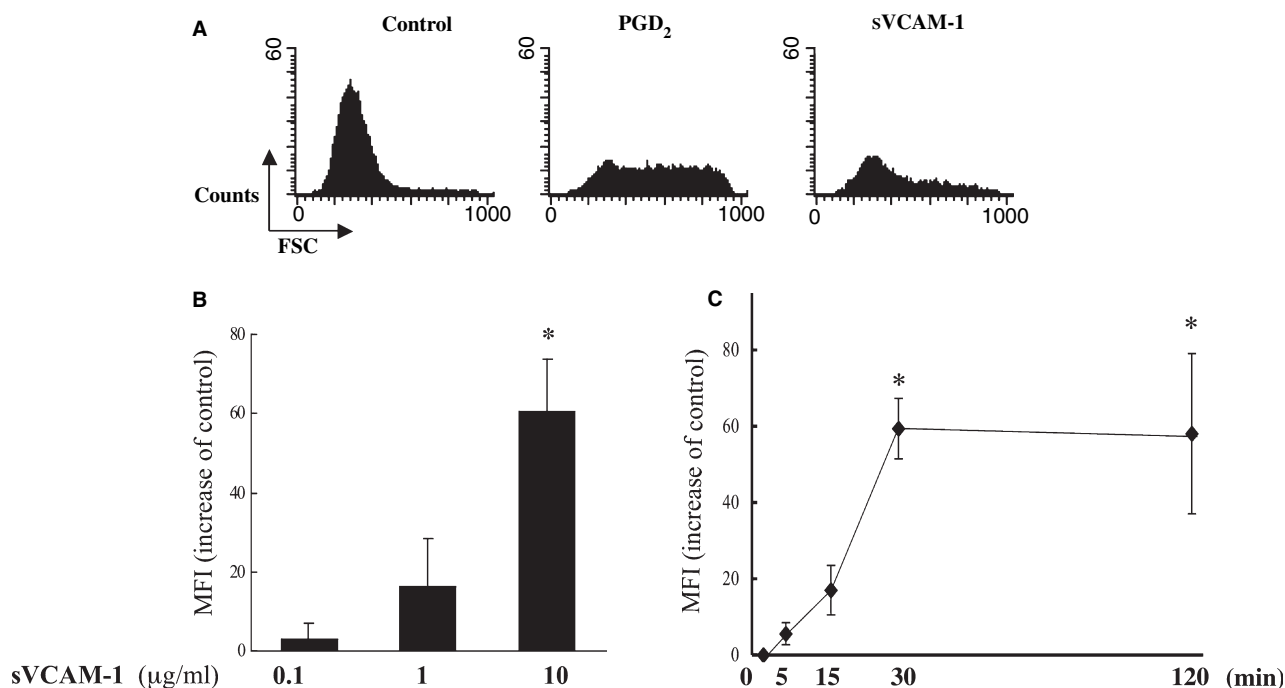


Figure 2. Effect of sVCAM-1 on eosinophil shape change. (A) Stimulation of purified eosinophils with 100 nM PGD₂ or 10 µg/ml sVCAM-1 for 30 min, followed by fixation with formaldehyde. The shape change assays were performed using flow cytometry. PGD₂ and sVCAM-1 increased fluorescence intensity in FSC, reflecting the cell shape change. (B) Concentration-dependent response of sVCAM-1 on eosinophil shape. Cells were stimulated with the indicated concentration of sVCAM-1 for 30 min, after which assays were performed. (C) Time course of shape change caused by stimulation with 10 µg/ml sVCAM-1. Data are expressed as the mean of three experiments $\pm$ SEM (B and C). * $P < 0.05$ vs unstimulated control.

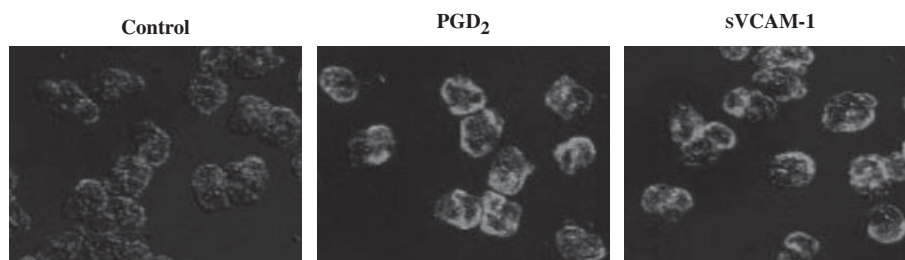


Figure 3. Effect of sVCAM-1 on actin polymerization of eosinophils. Purified eosinophils were incubated with sVCAM-1 for 10 min, followed by fixation with formaldehyde and staining of F-actin with NBD-phalloidin. F-actin content was visualized by confocal microscopy ($\times 200$) in the same setting. The results are representative of experiments with similar results using eosinophils from three different donors.

ERK MAPK was observed by sVCAM-1 treatment, which peaked at 1 min (Fig. 5).

Discussion

Soluble forms of adhesion molecules are released from either passive shedding or active proteolytic cleavage from the cell surface (19). Indeed, previous studies have shown evidence of active cleavage; stimulation of endothelial cells with cytokines and phorbol 12-myristate 13-acetate (PMA), a phorbol ester, induced surface

expression and rapid cleavage of adhesion molecules (20, 21). However, the pathophysiological significance of soluble adhesion molecules remains to be fully elucidated. To make progress in understanding the mechanisms of allergic inflammation and the biological functions of soluble adhesion molecules, we investigated the role of sVCAM-1 in eosinophil function. In the present study, we report the novel chemotactic activity of sVCAM-1 on human eosinophils. In addition, sVCAM-1 rapidly induces eosinophil shape change and actin polymerization, which are necessary for cell movement. To our knowledge, this is the first report that directly shows the

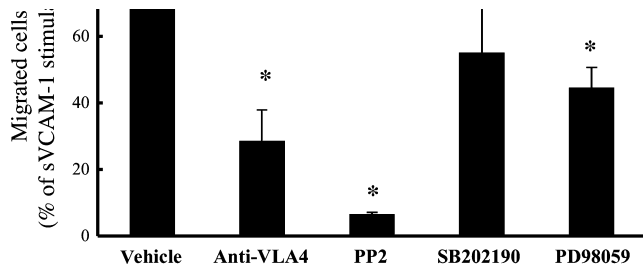


Figure 4. Effect of various inhibitors on eosinophil chemotaxis toward sVCAM-1 (10 μ g/ml). Purified eosinophils were mixed with anti-VLA-4-blocking antibody (5 μ g/ml) for 60 min, Src inhibitor PP2 (20 μ M) for 30 min, p38 MAPK inhibitor SB-202190 (1 μ M) for 30 min, or MEK inhibitor PD-98059 (10 μ M) for 30 min at 37°C before chemotaxis assay. Chemotactic response to sVCAM-1 alone was considered as 100%. Data are expressed as the mean of three to five experiments $\pm$ SEM. * P < 0.05 vs sVCAM-1 alone.

effect of soluble adhesion molecules on human eosinophil chemotaxis. Our study adds additional information about the potential role of sVCAM-1 in allergic inflammatory responses.

Multiple lines of evidence have shown the production of sVCAM-1 in the circulation and inflammatory sites of asthmatics. The plasma level of sVCAM-1 in asthmatic patients (753 ± 60.8 ng/ml) is higher than that of healthy controls (580 ± 70.7 ng/ml) (10). Koizumi et al. also reported that serum sVCAM-1 was elevated in asthmatics but not in patients with pneumonia, and circulating sVCAM-1 was higher during asthma attacks than in asymptomatic periods (22). After segmental antigen challenge, the concentration of sVCAM-1 in BALF from allergic asthmatics showed an increase (1.79 ± 0.31 to 139.39 ± 68.58 ng/ml), and this was not observed in normal controls (12). This large increase even in BALF was diluted by the lavage procedure, indicating that sVCAM-1 could play a role in eosinophil influx at an allergic inflammatory site.

Prior to eosinophil migration to the extravascular tissue, cell adhesion molecules mediate the rolling and the firm adhesion of these cells. During these processes, signals of adhesion molecules, especially VLA-4 ($\alpha 4\beta 1$ integrin), are known to play important roles in the pathogenesis of airway inflammation. In this context, targeting VLA-4 is an attractive therapeutic modality for the treatment of asthma. To date, several antagonists or neutralizing antibodies against VLA-4 have been developed, which ameliorate various aspects of asthmatic responses such as eosinophil infiltration and hyperresponsiveness in animal models of asthma (23–26). In our

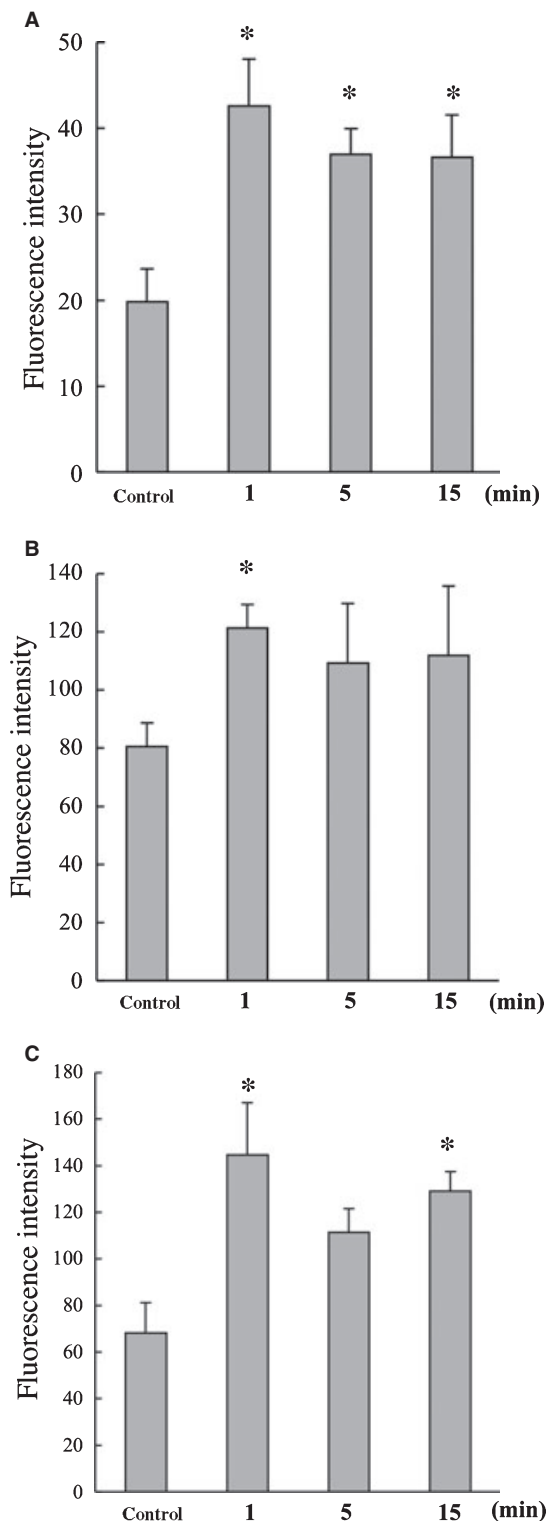


Figure 5. The fluorescence intensity of phosphoproteins in the eosinophils was counted by bead-based multiplex assay. Purified eosinophils (0.5×10^6 cells) were incubated with sVCAM-1 (10 μ g/ml) for the indicated time intervals. (A) Phospho-Src; (B) phospho-p38 MAPK; (C) phospho-ERK MAPK. Data are expressed as the mean of three experiments $\pm$ SEM. * P < 0.05 vs unstimulated control.

study, a neutralizing antibody of VLA-4 completely inhibited the chemotactic activity of sVCAM-1. Taken together, the attenuated allergic airway responses caused by VLA-4 blockade might be partially attributed to the inhibition of chemotactic activity of sVCAM-1 on eosinophils.

Outside-in signaling induced by integrin is mediated through two mechanisms: generation of biochemical signals within the cells as a result of ligand occupation and receptor clustering of the integrin receptor on the cell surface (27). Our observation revealed that Src and MAPK are involved in signal transduction of sVCAM-1-induced eosinophil chemotaxis. In other words, the binding of sVCAM-1 in the fluid phase, which induced only VLA-4 receptor occupancy, can generate these signals in eosinophils and induce the subsequent cytoskeletal reorganization necessary for cell motility. As chemokine-induced eosinophil migration is also regulated by multiple complementary signaling pathways of phosphoinositide 3 kinase (PI3K) (28), Src (29), p38, and ERK MAPK (30), it is suggested that VLA-4 engagement activated the required signals for cell migration.

In line with our findings, sVCAM-1-induced cellular chemotaxis was observed in other types of cells, such as T cells and smooth muscle cells (31, 32). These chemotactic responses were reported to be inhibited by

pharmacological Rho inhibitors. Previously, we showed that the p21 G protein Rho is upstream of ERK and regulates eotaxin-induced chemotaxis in eosinophils (30). Hence, the Rho-ERK pathway is suggested to be involved in sVCAM-1-induced eosinophil chemotaxis, although further studies are needed to confirm this.

In summary, we found that the chemotactic activity of soluble VCAM-1 was mediated through rapid phosphorylation of Src and MAPK. This evidence raises the possibility of soluble forms of adhesion molecules as a significant contributor to sustainment of the heightened eosinophilic inflammatory response, and it supports a therapeutic strategy that interferes with VCAM-1/VLA-4 interaction in asthma and other allergic inflammatory diseases.

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