An antioxidant modulates expression of receptor activator of NF- κ B in asthma

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Abbreviations: BAL, bronchoalveolar lavage; EBD, Evans blue dye; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; ODF, osteoclast differentiation factor; OPGL, osteoprotegerin ligand; OTC, L-2-oxothiazolidine-4-carboxylic acid; OVA, ovalbumin; RANK, receptor activator of NF- κ B; RANKL, RANK ligand; ROS, reactive oxygen species; TRANCE, TNF-related activation-induced cytokine

Abstract

Oxidative stress plays critical roles in airway inflammation that is usually accompanied by increased vascular permeability and plasma exudation. VEGF increases vascular permeability and leads to airway inflammation. In addition, VEGF has been shown to enhance receptor activator of NF- κ B (RANK) expression in endothelial cells. An aim of the study was to determine the potential role of antioxidant in the regulation of RANK expression in murine model of asthma. We have used a C57BL/6 mouse model of allergic asthma to evaluate the effect of L-2-oxothiazolidine-4-carboxylic acid (OTC), a prodrug of cysteine, which acts as an antioxidant, and VEGF receptor inhibitor on RANK mRNA expression. The mice develop the following pathophysiological features of asthma in the lungs: increased expression of RANK mRNA, increased number of inflammatory cells of the airways, increased vascular permeability, and increased levels of VEGF. Administration of OTC and VEGF receptor inhibitor markedly reduced plasma ex-

travasation and VEGF levels in allergen-induced asthmatic lungs. We also showed that the increased RANK mRNA expression at 72 h after ovalbumin inhalation were reduced by the administration of OTC or VEGF receptor inhibitor. The results indicate that OTC and VEGF receptor inhibitor which inhibit up-regulation of VEGF expression modulate RANK expression that may be in association with the regulation of vascular permeability, and suggest that VEGF may regulate the RANK expression. These findings provide a crucial molecular mechanism for the potential use of antioxidants to prevent and/or treat asthma and other airway inflammatory disorders.

Keywords: capillary permeability; eosinophilia; oxidative stress; signal transduction; vascular endothelial growth factor

Introduction

Bronchial asthma is a chronic inflammatory disease of the airways characterized by airway eosinophilia, goblet cell hyperplasia with mucus hypersecretion, and hyperresponsiveness to inhaled allergens and nonspecific stimuli, which usually induce increased vascular permeability, resulting in plasma exudation (Kay, 1991; Bousquet *et al.*, 2000). Oxidative stress is caused by a large variety of free oxygen radicals known as reactive oxygen species (ROS). ROS play a crucial role in the pathogenesis of airway inflammation (Rahman *et al.*, 1996; Dworski, 2000; MacNee, 2001). ROS can lead to endothelial barrier dysfunction with subsequent increased permeability to fluids, macromolecules, and inflammatory cells (Henricks and Nijkamp, 2001). The inflammatory cells recruited to the asthmatic airways have a capability of producing ROS. Evidence for increase oxidative stress in asthma is further provided by the finding of defective endogenous antioxidant capacity in asthmatic patients (Dworski, 2000). L-2-Oxothiazolidine-4-carboxylic acid (OTC), a prodrug of cysteine, works as an antioxidant by increasing intracellular glutathione level (Asti *et al.*, 1995; Blesa *et al.*, 2002). OTC raises the plasma concentrations of cysteine, which is the rate-limiting amino acid for intracellular GSH synthesis (Porta *et al.*, 1991; Vita *et al.*, 1998; Oiry *et al.*, 1999). GSH is synthesized from cysteine and is a vital intra- and extracellular

protective antioxidant against oxidative stress (Lu, 1999). Several studies have also demonstrated that OTC replenishes cellular GSH stores when GSH is depleted (Hazelton *et al.*, 1986; Moslen *et al.*, 1989). Recently, we have demonstrated that OTC which can reduce the increased ROS generation is able to reduce inflammation and hyperreactivity in animal models of asthma (Lee *et al.*, 2004c).

VEGF is an endothelial cell-specific mitogenic peptide and plays a key role in vasculogenesis and angiogenesis (Dvorak *et al.*, 1995). VEGF also increases vascular permeability so that plasma proteins can leak into the extravascular space, which leads to edema and profound alterations in the extracellular matrix. VEGF is one of the major determinants of asthma and thus the inhibition of VEGF receptor may be a good therapeutic strategy (Lee and Lee, 2001; Lee *et al.*, 2002; Lee *et al.*, 2004a; Lee *et al.*, 2004b). ROS are shown to induce VEGF expression *in vitro* and *in vivo* (Kuroki *et al.*, 1996).

TNF-related activation-induced cytokine [TRANCE, also known as receptor activator of NF- κ B ligand (RANKL), osteoprotegerin ligand (OPGL), and osteoclast differentiation factor (ODF)] is a novel member of the TNF family ligands that regulates immune responses and bone remodeling (Anderson, 1997; Green and Flavell, 1999; Kong *et al.*, 1999; Kong *et al.*, 2000). TRANCE expressed on activated T cells promotes the survival of dendritic cells and modulates T helper cell responses to viral infections (Wong *et al.*, 1997a; Bachmann *et al.*, 1999). TRANCE is also highly expressed on osteoblasts and plays a key role in osteoclast differentiation from hematopoietic precursors and calcium metabolism (Hsu *et al.*, 1999; Li *et al.*, 2000). These effects of TRANCE are exerted by its binding to the transmembrane receptor activator of NF- κ B (RANK). RANK is detected on mature dendritic cells, chondrocytes, osteoclast precursors, and mature osteoclasts (Wong *et al.*, 1997a; Lacey *et al.*, 1998) and induces activation of extracellular signal-regulated kinase (ERK) and JNK, antiapoptotic serine/threonine kinase Akt/protein kinase B, and NF- κ B in dendritic cells and osteoclasts (Wong *et al.*, 1997b; Wong *et al.*, 1998; Wong *et al.*, 1999). TRANCE is shown to promote angiogenesis *in vivo*, and the binding of TRANCE to receptor activator of nuclear factor- κ B expressed on endothelial cells directly stimulates proliferation, migration, and tube formation of endothelial cells *in vitro* (Kim *et al.*, 2002), and VEGF enhances RANK expression in endothelial cells through Flk-1/KDR-protein kinase C-ERK signaling pathway (Min *et al.*, 2003). Thus, these observations suggest that the TRANCE-RANK system is important for the processes of increases

vascular permeability of VEGF.

In this study, we investigate the potential role of VEGF in the regulation of the TRANCE receptor RANK expression. We here show that OTC and VEGF receptor inhibitor, which inhibit up-regulation of VEGF expression, reduce the increased RANK mRNA expression after ovalbumin (OVA) inhalation in lung tissues of a murine model of asthma.

Materials and Methods

Animals and experimental protocol

Female C57BL/6 mice, 8-10 wk of age and free of murine specific pathogens, obtained from the Korean Research Institute of Chemistry Technology (Daejeon, Korea) housed throughout the experiments in a laminar flow cabinet and maintained on standard laboratory chow ad libitum. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Mice were sensitized on d 1 and 14 by i.p. injection of 20 μ g OVA (Sigma-Aldrich, St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce Chemical Co., Rockford, IL) in a total volume of 200 μ l, as previously described (Kwak *et al.*, 2003). On d 21, 22, and 23 after the initial sensitization, the mice were challenged for 30 min with an aerosol of 3% (wt/vol) OVA in saline (or with saline as a control) using an ultrasonic nebulizer (NE-U12, Omron, Japan). Bronchoalveolar lavage (BAL) was performed. At the time of lavage, the mice (6 mice in each group) were sacrificed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg/kg of body wt, administered i.p.). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed 0.9% NaCl solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and then kept at 4°C. A part of each pool was then centrifuged and the supernatants were kept at -70°C until use. Total cell numbers were counted with a hemocytometer. Smears of BAL cells were prepared with a cytopsin (Thermo Electron, Waltham, MA). The smears were stained with Diff-Quik solution (Dade Diagnostics of P. R. Inc., Aguada, Puerto Rico) in order to examine the cell differentials. Two independent, blinded investigators counted the cells using a microscope. Approximately 400 cells were counted in each of four different random locations. Interinvestigator variation was <5%. The mean number from the two investigators was used to estimate the cell differentials.

Administration of OTC or VEGF receptor inhibitor

OTC solution (80 or 160 mg/kg body wt/d, Sigma-Aldrich) was freshly prepared as described elsewhere (Han *et al.*, 2002) and administered i.p. four times at 24 h intervals on d 21-24, beginning 1 h before the first challenge. An inhibitor of VEGF receptor tyrosine kinase, SU5614 {Flk-1; IC_{50} =1.2 μ M, 5-chloro-3-[(3,5-dimethylpyrrol-2-yl)methylene]-2-indolinone, Calbiochem-Novabiochem, San Diego, CA}, was used to inhibit VEGF activity. SU5614 (2.5 mg/kg body wt/d) was dissolved in dimethyl sulfoxide, and administered i.p. three times at 24 h intervals, beginning 1 h after the last airway challenge with OVA.

Measurement of plasma exudation

To assess lung permeability, Evans blue dye (EBD) was dissolved in 0.9% saline at a final concentration of 5 mg/ml. Animals were weighed and injected with 20 mg/kg EBD in the tail vein. After 30 min, the animals were killed and their chests were opened. Normal saline containing 5 mM EDTA was perfused through the aorta until all venous fluid returning to the opened right atrium was clear. The lungs were removed and weighed wet. EBD was extracted in 2 ml formamide kept in a water bath at 60°C for 3 h and the absorption of light at 620 nm was measured in a spectrophotometer (Spectra Max Plus Microplate Spectrophotometer, Sunnyvale, CA). The dye extracted was quantified by interpolation against a standard curve of dye concentration in the range of 0.01-10 μ g/ml and is expressed as ng of dye/mg of wet lung.

Histology and immunocytochemistry

At 72 h after the last challenge, lungs were removed from the mice after sacrifice. Before the lungs were removed, the lungs and trachea were filled intratracheally with a fixative (0.8% formalin, 4% acetic acid) using a ligature around the trachea. Lung tissues were fixed with 10% (vol/vol) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 4 μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI). Inflammation score was graded by three independent blinded investigators. The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3, as described elsewhere (Kwak *et al.*, 2003). A value of 0 was adjudged when no inflammation was detectable, a

value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by thin layer (one to five cells) of inflammatory cells, and a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells. For immunocytochemistry of VEGF, the cytocentrifuge preparations of BAL cells were incubated sequentially in accordance with the R. T. U. Vectastain Universal Quick kit instruction from Vector Laboratories (Burlingame, CA). Briefly, the slides were incubated in Endo/Blocker (Biomed, Foster City, CA) for 5 min and in pepsin solution for 4 min at 40°C. The slides were incubated in normal horse serum for 15 min at room temperature. The slides were then probed with an affinity-purified rabbit polyclonal VEGF IgG (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, and were incubated with prediluted biotinylated pan-specific IgG for 10 min. The slides were incubated in streptavidin/peroxidase complex reagent for 5 min, and then in 3-amino-9-ethylcarbazole substrate kit for 12 min. For the control, BAL cells prepared from mice were treated without the primary Ab under the same conditions. After immunostaining, the slides were counterstained for 1 min with Gill's hematoxylin in 20% ethylene glycol and then mounted with Aqueous Mounting Medium (InnoGenex, San Ramon, CA) and photomicrographed (Vanox T; Olympus, Tokyo, Japan).

Measurements of VEGF in BAL fluids

Levels of VEGF were quantified by an enzyme immunoassay according to the manufacturer's protocol (R&D Systems Inc., Minneapolis, MN). The minimum detectable level of mouse VEGF is less than 3.0 pg/ml.

Western blot analysis

Lung tissues were homogenized in the presence of protease inhibitors and protein concentrations were determined using the Bradford reagent (Bio-Rad, Hercules, CA), as described previously (Kwak *et al.*, 2003). Samples (30 μ g of protein per lane) were loaded on a 12% SDS-PAGE gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by the wet transfer method (250 mA, 90 min). Nonspecific sites were blocked with 5% non-fat dry milk in TBST buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20) for 1 h, and the blots were then incubated with an anti-VEGF Ab (Santa Cruz Biotechnology), anti-Akt Ab (Cell Signaling Technology, Beverly, MA), or anti-phosphorylated Akt

(p-Akt) Ab (Cell Signaling Technology) overnight at 4°C. Anti-rabbit horseradish peroxidase conjugated-IgG was used to detect binding of Abs. The membranes were stripped and reblotted with anti-actin Ab (Sigma-Aldrich) to verify equal loading of protein in each lane. The binding of the specific Abs was visualized by exposing to photographic film after treating with enhanced chemiluminescence system reagents (Amersham Pharmacia Biotech).

RNA isolation and RT-PCR

Total RNA from lung tissues was isolated using a rapid extraction method (TRI-Reagent) as previously described (Chomczynski and Sacchi, 1987). RNA was quantified by measuring absorption at 260 nm and was stored at -80°C until use. Total RNA (4 µg) was reverse-transcribed to cDNA in a buffer containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 5 mM MgCl₂, 10 mM DTT; 0.5 µg random hexanucleotide primers, 2.5 mM dNTP, 40 U RNase inhibitor, and 50 U/µl SuperScript II RT (Invitrogen, Carlsbad, CA), in a final volume of 20 µl. This mixture was incubated for 50 min at 42°C and was finally digested with 2 U/µl *E. coli* RNase H for 20 min at 37°C. The first-strand cDNAs were used for PCR amplification of RANK or the housekeeping gene, GAPDH. PCR amplification was performed by mixing 3 µl of the RT reaction mixture with 47 µl of buffer containing 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI) and 30 pmol of specific primer pairs for mouse cDNA of RANK or GAPDH, designed from published mouse gene sequences. The primers used were as follows: RANK (predicted length 458 bp) sense: 5'-CCA TCA TCT TCG GCG TTT ACT-3', antisense: 5'-ACT GTC GTT CTC CCC CAC TT-3' and GAPDH (predicted length 609 bp) sense: 5'-GCC ATC AAC GAC CCC TTC ATT GAC-3', antisense: 5'-ACG GAA GGC CAT GCC AGT GAG CTT-3'. PCR reactions were performed in a thermocycler (GeneAmp® PCR System 2400, Foster City, CA) using the following reaction conditions; after an initial incubation for 2 min at 95°C, samples were subjected to 35 cycles of 1 min at 94°C, 2 min at 54°C (GAPDH) or 59°C (RANK) and 1 min at 72°C. A final extension step at 72°C for 10 min was performed. The RT-PCR products were electrophoretically fractionated on 2% agarose gels stained with ethidium bromide. DNA bands were visualized under UV light.

Quantitative real-time RT-PCR

Quantitative RT-PCR analysis was performed using the LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany). The sequences of primers used were as follows: RANK sense: 5'-CCA TCA TCT TCG GCG TTT ACT-3',

antisense: 5'-ACT GTC GTT CTC CCC CAC TT-3' and β -actin sense: 5'-CAG ATC ATG TTT GAG ACC TTC-3' and antisense: 5'-ACT TCA TGA TGG AAT TGA ATG-3'. Calculation of the relative mRNA levels of each sample was performed according to the manufacturer's protocol. The data have been normalized to the expression of β -actin.

Densitometric analysis and statistics

All immunoreactive and phosphorylation signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad). Data were expressed as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA followed by the Fisher's test. Significant differences between groups were determined using the unpaired Student's *t* test. The significance of correlations was evaluated by determining Pearson rank correlation coefficients. Statistical significance was set at $P < 0.05$.

Figure 1. RANK mRNA in lung tissues of OVA-sensitized and -challenged mice. (A) Representative RT-PCR analysis of RANK. Sampling was performed in lung tissues from sensitized mice challenged with ovalbumin or saline. (B) Quantitative analysis of RANK mRNA expression by means of real-time PCR. Data represent mean $\pm$ SEM from 6 mice per group. 6, 12, 24, 48, and 72 h are time periods after the last challenge. Control, no treatment; Pre, 1 h before the first challenge; * $P < 0.05$ versus Pre; * $P < 0.05$ versus saline inhalation.

Results

RANK mRNA expression increased in OVA-induced asthma

Real-time RT-PCR analysis revealed that RANK mRNA expression had increased approximately 2.1-, 3.1-, 3.7-, 4.8-, and 4.9-fold at 6, 12, 24, 48, and 72 h after OVA inhalation, respectively, compared with the expression in before OVA inhalation or in the control group (Figure 1B). In contrast, no significant changes in the RANK mRNA expression were observed after saline inhalation (Figure 1A and B).

Figure 2. Effect of OTC and VEGF receptor inhibitor on RANK mRNA expression in lung tissues of OVA-sensitized and -challenged mice. (A) Representative RT-PCR analysis of RANK. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered SU5614 (OVA + SU5614), OVA-inhaled mice administered OTC 80 mg/kg (OVA + OTC 80), and OVA-inhaled mice administered OTC 160 mg/kg (OVA + OTC 160). (B) Quantitative analysis of RANK mRNA expression by means of real-time PCR. Data represent mean $\pm$ SEM from 6 mice per group. # P < 0.05 versus SAL + SAL; * P < 0.05 versus OVA + SAL.

Effect of OTC and VEGF receptor inhibitor on RANK mRNA expression in lung tissues of OVA-sensitized and -challenged mice

RT-PCR and real-time RT-PCR analyses showed that RANK mRNA expression in lung tissues was increased at 72 h after OVA inhalation compared with the levels after saline inhalation (Figure 2A and B). The increased RANK mRNA expression at 72 h after OVA inhalation were reduced by the administration of OTC or SU5614.

VEGF protein levels increased in OVA-induced asthma

Western blot analysis revealed that VEGF protein levels in lung tissues were increased at 6, 12, 24, 48, and 72 h after challenge with OVA compared with the levels in before OVA inhalation or in the control group (Figure 3A and B). In contrast, no significant changes in the VEGF protein level were observed after saline inhalation. Consistent with these results, enzyme immunoassays revealed that VEGF levels in BAL fluids were significantly increased at 6, 12, 24, 48, and 72 h after OVA inhalation compared with levels in before OVA inhalation or in the control group (Figure 3C). On the other hand, no significant changes in VEGF levels were observed after saline inhalation.

Effect of OTC and VEGF receptor inhibitor on VEGF protein levels in lung tissues and in BAL fluids of OVA-sensitized and -challenged mice

Western blot analysis showed that levels of VEGF in lung tissues were increased at 72 h after OVA inhalation compared with the levels in the control mice (Figure 3D and E). The increased VEGF levels in lung tissues at 72 h after OVA inhalation were decreased by the administration of OTC or SU5614. Consistent with the results obtained from the Western blot analysis, enzyme immunoassay revealed that OTC or SU5614 reduced the increased levels of VEGF in the BAL fluids at 72 h after OVA inhalation (Figure 3F).

Localization of immunoreactive VEGF in BAL fluids of OVA-induced asthma

Immunocytologic analysis of BAL fluids showed the localization of immunoreactive VEGF in the precipitated cells from the OVA-sensitized and -challenged mice (Figure 4B). Immunoreactive VEGF was markedly reduced in BAL cells from control mice (Figure 4A) and from OVA-sensitized and -challenged mice treated with OTC (Figure 4C and D).

Figure 3. Effect of OTC and VEGF receptor inhibitor on VEGF protein expression in lung tissues and in BAL fluids of OVA-sensitized and -challenged mice. (A) Western blot analyses of VEGF protein. Sampling was performed in lung tissues from sensitized mice challenged with OVA or saline. The Western blot was probed with an anti-VEGF antibody and reprobed with an anti-actin antibody to verify equal loading of protein in each lane. (B) Densitometric analyses are presented as the relative ratio of VEGF to actin. The relative ratio of VEGF in the lung tissues of control mice is arbitrarily presented as 1. (C) Enzyme immunoassay of VEGF in BAL fluids. Data represent mean $\pm$ SEM from 6 mice per group. 6, 12, 24, 48, and 72 h are time periods after the last challenge. Control, no treatment; Pre, 1 h before the first challenge. $^{\#}P < 0.05$ versus Pre; $^{*}P < 0.05$ versus saline inhalation. (D) Western blotting of VEGF. The protein expression of VEGF was measured at 72 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered SU5614 (OVA + SU5614), OVA-inhaled mice administered OTC 80 mg/kg (OVA + OTC 80), and OVA-inhaled mice administered OTC 160 mg/kg (OVA + OTC 160). (E) Densitometric analyses are presented as the relative ratio of VEGF to actin. The relative ratio of VEGF in the lung tissues of SAL + SAL is arbitrarily presented as 1. (F) Enzyme immunoassay of VEGF. Data represent mean $\pm$ SEM from 6 mice per group. $^{\#}P < 0.05$ versus SAL + SAL; $^{*}P < 0.05$ versus OVA + SAL.

OTC and VEGF receptor inhibitor reduced plasma extravasation in OVA-sensitized and -challenged mice

The EBD assay showed that plasma extravasation was significantly increased at 72 h after the last

challenge (Figure 5). The increase in plasma extravasation at 72 h after OVA inhalation was significantly reduced by the administration of OTC or SU5614. These results suggest that the treatment of

Figure 4. Localization of immunoreactive VEGF in BAL fluids of OVA-sensitized and -challenged mice. Sampling was performed 72 h after the last challenge in BAL fluids from sensitized mice challenged with saline (A) sensitized mice challenged with OVA (B) OVA-inhaled mice administered OTC 80 mg/kg (C) and OVA-inhaled mice administered OTC 160 mg/kg (D). Representative light microscopy shows VEGF-positive cells in the BAL fluids; the brown color indicates VEGF-positive cells. Bars indicate 50 μ m.

OTC decreases the vascular permeability by inhibiting up-regulation of VEGF expression.

Effect of OTC and VEGF receptor inhibitor on cellular changes in BAL fluids

Numbers of total cells, eosinophils, lymphocytes, and neutrophils in BAL fluids were increased significantly at 72 h after OVA inhalation compared with the numbers after saline inhalation (Figure 6A). The increased numbers of these cells were significantly reduced by the administration of OTC or SU5614.

Effect of OTC and VEGF receptor inhibitor on pathological changes of OVA-induced asthma

Histological analyses revealed typical pathologic features of asthma in the OVA-exposed mice. Numerous inflammatory cells including eosinophils infiltrated around the bronchioles (Figure 6C) as compared to the control (Figure 6B). Mice treated with SU5614 (Figure 6D) or OTC (Figure 6E)

showed marked reductions in the infiltration of inflammatory cells in the peribronchiolar and perivascular regions. The scores of peribronchial, perivascular, and total lung inflammation were increased significantly at 72 h after OVA inhalation compared with the scores after saline inhalation (Figure 6F). The increased peribronchial, perivascular, and total lung inflammation was significantly reduced by the administration of OTC or SU5614. These results suggest that OTC and VEGF receptor inhibitor attenuate antigen-induced inflammation in the lungs, including the influx of eosinophils.

Relationship between numbers of BAL fluids cells and vascular permeability in OVA-induced asthma

There were significant correlations between numbers of total cells, eosinophils, lymphocytes, or neutrophils in BAL fluids and vascular permeability index in OVA-sensitized and -challenged mice. (Figure 7A-D). These results suggest that there is a close relationship between the inflammatory cells and vascular permeability.

Figure 5. Effect of OTC and VEGF receptor inhibitor on plasma exudation. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered SU5614 (OVA + SU5614), OVA-inhaled mice administered OTC 80 mg/kg (OVA + OTC 80), and OVA-inhaled mice administered OTC 160 mg/kg (OVA + OTC 160). Data represent mean $\pm$ SEM from 6 mice per group. # $P < 0.05$ versus SAL + SAL; * $P < 0.05$ versus OVA + SAL.

Determination of Akt phosphorylation

We used Western blotting to determine the activation of Akt which is a downstream signaling mediator of RANK. Levels of p-Akt protein in the lung tissues were increased at 72 h after OVA inhalation compared with the levels in the control mice (Figure 8A and B). However, no significant changes in Akt protein levels were observed in any of the groups tested. The increased p-Akt but not Akt protein levels in lung tissues at 72 h after OVA inhalation were significantly reduced by the administration of OTC or SU5614.

Discussion

Bronchial asthma is characterized by inflammation of the airways which is usually accompanied by increased vascular permeability, resulting in plasma exudation. Oxidative stress has been shown to play critical roles in airway inflammation, including vascular leakage (Rahman *et al.*, 1996; Dworski, 2000; MacNee, 2001). VEGF increases vascular permeability and leads to airway inflammation (Thurston *et al.*, 2000; Lee *et al.*, 2002; Lee *et al.*, 2004a; Lee *et al.*, 2004b). Recently, it has been reported that VEGF enhances RANK expression in endothelial cells (Min *et al.*, 2003). In this study, we have used

OTC, which is a precursor of GSH biosynthesis and thus functioning as an antioxidant, and VEGF receptor inhibitor, SU5614, to examine roles of VEGF in the regulation of the TRANCE receptor RANK expression in OVA-induced asthma murine model. The results have revealed that RANK mRNA expression, VEGF expression, and HIF-1 α protein level as well as vascular permeability are increased in induction of asthma. The increased expression of RANK mRNA expression is significantly reduced by administration of OTC or SU5614. These observations suggest that OTC or VEGF receptor inhibitor, which inhibits up-regulation of VEGF expression, modulate RANK expression and are in association with the regulation of vascular permeability and that VEGF may regulate the RANK expression.

Cysteine derivatives, N-acetylcysteine and carbocysteine, have been used to block inflammation and hyperreactivity in animal models of asthma (Asti *et al.*, 1995; Blesa *et al.*, 2002). Previous studies have demonstrated that OTC is more effective than N-acetylcysteine in replenishing intracellular GSH stores (Williamson and Meister, 1981; Mesina *et al.*, 1989) and blocks airway hyperresponsiveness and inflammation in an asthmatic animal model (Lee *et al.*, 2004c). Consistent with previous observations, administration of OTC decreases airway inflammation. Our results also indicate that plasma extravasation caused by increased vascular permeability is elevated after inducing asthma and that administration of OTC significantly reduces the increased plasma extravasation. VEGF plays a critical role in asthma (Hoshino *et al.*, 2001; Lee and Lee, 2001; Lee *et al.*, 2004a). One of the major roles of VEGF in asthma appears to be the enhancement of vascular permeability. The mechanism of VEGF-mediated induction of the vascular permeability seems to be the enhanced functional activity of vesicular-vacuolar organelles (Dvorak *et al.*, 1995). VEGF can be produced by a wide variety of cells including macrophages, neutrophils, eosinophils, and lymphocytes (Horiuchi and Weller, 1997; Taichman *et al.*, 1997; Hoshino *et al.*, 2001; Lee *et al.*, 2002; Lee *et al.*, 2004a; Lee *et al.*, 2004b). Several studies have shown that overproduction of VEGF causes an increase in vascular permeability, which results in leakage of plasma proteins, including inflammatory mediators and cells, into the extravascular space and allowing the migration of inflammatory cells into the airways (Thurston *et al.*, 2000; Lee *et al.*, 2002; Lee *et al.*, 2004b). Consistent with these observations, we have found that VEGF expression is up-regulated in OVA-induced asthma. Administration of the antioxidant, OTC, reduces the increased VEGF expression. In addition, we have found that VEGF receptor inhibitor, SU5614, inhibits the in-

Figure 6. Effect of OTC and VEGF receptor inhibitor on total and differential cellular components of BAL and on pathologic changes in lung tissues of OVA-sensitized and -challenged mice. (A) The numbers of each cellular component of BAL from saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered SU5614 (OVA + SU5614), OVA-inhaled mice administered OTC 80 mg/kg (OVA + OTC 80), and OVA-inhaled mice administered OTC 160 mg/kg (OVA + OTC 160). (B-E) Representative hematoxylin and eosin-stained sections of the lungs. Sampling was performed at 72 h after the last challenge in saline-inhaled mice administered saline (B) OVA-inhaled mice administered saline (C) OVA-inhaled mice administered SU5614 (D) OVA-inhaled mice administered OTC 80 mg/kg (E). Bars indicate scale of 50 μ m. (F) Peribronchial and perivascular lung inflammation were measured at 72 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered SU5614 (OVA + SU5614), OVA-inhaled mice administered OTC 80 mg/kg (OVA + OTC 80), and OVA-inhaled mice administered OTC 160 mg/kg (OVA + OTC 160). Total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. Data represent mean $\pm$ SEM from 6 mice per group. [#] P < 0.05 versus SAL+SAL; ^{*} P < 0.05 versus OVA + SAL.

creased vascular permeability, the airway inflammation, and the increased VEGF expression and that there are significant correlations between numbers of total cells, eosinophils, lymphocytes, or neutrophils in BAL fluids and vascular permeability in OVA-sensitized and -challenged mice. These results indicate that oxidative stress is associated with the regulation of VEGF expression and that treatment of the antioxidant may decrease the vascular permeability by inhibiting up-regulation of VEGF expression which leads to airway inflammation in asthma.

RANK is known to be an essential signaling

receptor for TRANCE, which regulates the development of osteoclasts and lymph nodes and also influences the development of the B-cell lineage and dendritic cell function *in vivo* (Dougall *et al.*, 1999). TRANCE-RANK engagement plays important roles in mammary gland development and angiogenesis (Wong *et al.*, 1999; Fata *et al.*, 2000). Thus, RANK is thought to control a variety of biological phenomena in mammalian cells. RANK mRNA is ubiquitously expressed in mammalian tissues, and the expression of RANK protein is also observed in several cell types such as myeloid-derived dendritic cells, activated T cells, osteoclast progenitors, mammary

Figure 7. Relationship between total cells, eosinophils, neutrophils, and lymphocytes in BAL fluids and vascular permeability in OVA-sensitized and -challenged mice.

epithelial cells, and endothelial cells (Dougall *et al.*, 1999). Although TGF- β and IL-4 have been shown to up-regulate RANK expression in peripheral blood T lymphocytes (Anderson *et al.*, 1997) and the murine monocytic cell line, RAW 264.7 (Yan *et al.*, 2001), the regulatory mechanism of RANK expression has not been well elucidated. Min *et al.* have demonstrated that VEGF positively regulates RANK expression in endothelial cells (Min *et al.*, 2003). Our results have revealed that RANK mRNA expression is increased after OVA inhalation and that the increased RANK mRNA expression is reduced by the administration of SU5614 and OTC. Previous studies have shown that TRANCE activates a serine/threonine kinase Akt/protein kinase B through a signaling complex in osteoclasts, dendritic cells, and endothelial cells (Wong *et al.*, 1999; Kim *et al.*, 2002). Activation of the receptor downstream signaling mediator, Akt, is also observed in our murine

model of asthma. We demonstrate that the increased p-Akt but not Akt protein levels in lung tissues after OVA inhalation are significantly reduced by the administration of the VEGF receptor inhibitor and OTC. Taken together, these findings suggest that OTC which inhibits ROS generation modulates VEGF expression and that VEGF activates Akt which is a downstream signaling mediator of RANK. Thus, these findings provide an important molecular mechanism for the potential use of antioxidants to prevent and/or treat asthma and other airway inflammatory disorders.

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Figure 8. Effect of OTC and VEGF receptor inhibitor on p-Akt and Akt protein expression in lung tissues of OVA-sensitized and -challenged mice. (A) Western blotting of p-Akt and Akt. p-Akt and Akt protein expression were measured at 72 h after the last challenge in saline-inhaled mice administered saline (SAL + SAL), OVA-inhaled mice administered saline (OVA + SAL), OVA-inhaled mice administered drug vehicle (OVA + VEH), OVA-inhaled mice administered OTC 80 mg/kg (OVA + OTC 80), OVA-inhaled mice administered OTC 160 mg/kg (OVA + OTC 160), OVA-inhaled mice administered SU5614 (OVA + SU5614). (B) Densitometric analyses are presented as the relative ratio of p-Akt to Akt. The relative ratio of p-Akt in the lung tissues of SAL+SAL is arbitrarily presented as 1. Data represent mean $\pm$ SEM from 6 mice per group. * $P < 0.05$ versus SAL + SAL; # $P < 0.05$ versus OVA + SAL.

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