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Contribution of Vascular Endothelial Growth Factor to Airway Hyperresponsiveness and Inflammation in a Murine Model of Toluene Diisocyanate-Induced Asthma¹

Yong C. Lee,^{3*} Yong-Geun Kwak,[†] and Chang H. Song[‡]

Isocyanate chemicals, including toluene diisocyanate (TDI), are currently the most common causes of occupational asthma. Although considerable controversy remains regarding its pathogenesis, TDI-induced asthma is characterized by hyperresponsiveness and inflammation of the airways. One of the histological hallmarks of inflammation is angiogenesis, but the possible role of vascular endothelial growth factor (VEGF), a potent angiogenic cytokine, in TDI-induced asthma is unknown. We developed a murine model to investigate TDI-induced asthma by performing two courses of sensitization with 3% TDI and one challenge with 1% TDI using ultrasonic nebulization to examine the potential involvement of VEGF in that disease. These mice develop the following typical pathophysiological features: airway hyperresponsiveness, airway inflammation, and increased VEGF levels in the airway. Administration of VEGFR inhibitors reduced all these pathophysiological symptoms. These results suggest that VEGF is one of the major determinants of TDI-induced asthma and that the inhibition of VEGF may be a good therapeutic strategy. *The Journal of Immunology*, 2002, 168: 3595–3600.

Toluene diisocyanate (TDI)³ is a leading cause of occupational asthma in Korea (1) as well as in North America (2), as it is widely used in industry and is a potent sensitizer. However, the pathogenesis of TDI-induced asthma is controversial. An increase in TDI-specific IgE Abs has been reported in only 20% of subjects with TDI-induced asthma (3). An IgG-mediated reaction is not likely to be the only mechanism responsible for the action of TDI. TDI-induced asthma is characterized by hyperresponsiveness and inflammation of the airways (4). Angiogenesis, the process of growth and proliferation of new blood vessels, is one of the histological hallmarks of inflammation (5). Although angiogenesis has not been studied in asthma, an increase in vessel area has been reported (6).

Vascular endothelial growth factor (VEGF; also known as vascular permeability factor) is one of the most potent proangiogenic cytokines, and it plays a central role in mediating the process of angiogenesis. 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 (7). However, little is known about the possible pathophysiological role of VEGF in TDI-induced asthma.

In the present study, we developed a murine model to investigate TDI-induced asthma to examine the possible involvement of VEGF in the pathogenesis of that disease. We found that VEGF is one of the major determinants of TDI-induced asthma. An additional aim of the present study was to evaluate the effect of VEGFR inhibitors on hyperresponsiveness and inflammation of the airways induced by TDI in this murine model.

Materials and Methods

Animals and experimental protocol

Female BALB/c mice, free of murine specific pathogens, were obtained from the Korean Research Institute of Chemistry Technology (Daejeon, Korea), and were 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 (Chonju, South Korea). Mice, 10–12 wk of age, were sensitized by intranasal administration of 20 μ l of 3% TDI dissolved in ethyl acetate plus olive oil (1/4 dilution) under light anesthesia (sodium pentobarbitone, 30 mg/kg i.p.) once daily for 5 consecutive days according to the method of Scheerens et al. (8), with some modifications (Fig. 1). Animals were kept in a supine position for 10 min after each sensitization. After 3 wk, these animals were further sensitized with the same reagent for 5 consecutive days. On day 7 after the second course of sensitization (day 38), mice were individually placed in a horizontal cylindrical chamber and challenged via the airways with 1% TDI dissolved in ethyl acetate plus olive oil (1/4 dilution) by ultrasonic nebulization (NE-U12; Omron, Tokyo, Japan) (Fig. 1). As a control, mice were sensitized and challenged using the same protocol but using only the solvent, ethyl acetate plus olive oil (1/4).

Bronchoalveolar lavage (BAL) was performed at 0, 6, 24, 48, and 72 h after the challenge. At the time of lavage, the mice were sacrificed with an overdose of sodium pentobarbitone (pentobarbital sodium, 100 mg/kg of body weight). The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter was 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. 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 cytospin. The smears were stained with Diff-Quik solution (Dade Diagnostics, Aguada, Puerto Rico) to examine the cell differentials and were used for immunocytochemistry of VEGF. Two independent, blinded investigators counted the cells using a microscope. Approximately 400 cells were counted in each of four different

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³ Abbreviations used in this paper: TDI, toluene diisocyanate; Penh, enhanced pause; EBD, Evans blue dye; BAL, bronchoalveolar lavage; VEGF, vascular endothelial growth factor; VVO, vesicular-vacuolar organelle; EO, ethyl acetate plus olive oil (vehicle control).

random locations. Interinvestigator variation was <5%. The mean number from the two investigators was used to estimate the cell differentials.

Administration of VEGFR inhibitor

Inhibitors of VEGFR tyrosine kinase, SU5614 (Flk-1; $IC_{50} = 1.2 \mu\text{M}$; 5-chloro-3-[(3,5-dimethylpyrrol-2-yl)methylene]-2-indolinone; Calbiochem, San Diego, CA) and SU1498 ((E)-3-(3,5-diisopropyl-4-hydroxyphenyl)-2-[(3-phenyl-*n*-propyl)amino-carbonyl]acrylonitrile; Calbiochem) were used to inhibit VEGF activity. SU5614 (2.5 mg/kg of body weight per day) or SU1498 (9 mg/kg of body weight per day) were dissolved in DMSO and administered i.p. three times at 24-h intervals, beginning 1 h after the TDI challenge (Fig. 1).

Determination of airway responsiveness to methacholine

Airway responsiveness was measured in mice 3 days after the TDI challenge in an unrestrained conscious state, according to the method of Hamelmann et al. (9). Mice were placed in a barometric plethysmographic chamber (All Medicus, Seoul, Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine in increasing concentrations (2.5–50 mg/ml) were nebulized through an inlet of the main chamber for 3 min. Readings were taken and averaged for 3 min after each of the nebulizations, which were administered at 5-min intervals. Enhanced pause (Penh), calculated as (expiratory time/relaxation time 1) \times (peak expiratory flow/peak inspiratory flow), according to the manufacturer's protocol, is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. Penh was used as a measure of airway responsiveness to methacholine. Results are expressed as the percentage increase of Penh following challenge with each concentration of methacholine, where the baseline Penh (after saline challenge) is expressed as 100%.

Measurement of plasma exudation

To further 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 ethylenediaminetetraacetic acid 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 (Spectrace Max Plus Microplate Spectrometer; Sunnyvale, CA). The dye extracted was quantified by interpolation against a standard curve of dye concentration in the range of 0.01–10 $\mu\text{g/ml}$ and is expressed as nanograms of dye per milligram of wet lung. This technique of EBD has been shown to correlate well with the extravasation of radiolabeled albumin (10, 11).

Histology, immunohistochemistry, and immunocytochemistry

At 72 h after challenge lungs were removed from mice. 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% (v/v) neutral buffered formalin. The specimens were dehydrated and embedded in paraffin. For histological examination, 4- μm sections of fixed and embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with H&E (Richard-Allan Scientific, Kalamazoo, MI). For immunohistochemistry and immunocytochemistry of VEGF, the deparaffinized 4- μm sections or the cytocentrifuge preparations of BAL cells were incubated sequentially in accordance with the instructions of the R.T.U. Vectastain Universal Quick kit from Vector Laboratories (Burlingame, CA). Briefly, the slides were incubated in Endo/Blocker (Biomedica, 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. Controls consisted of sections of lung tissue or BAL cells from mice incubated without the primary Ab. 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 (VENOX-T; Olympus, Tokyo, Japan).

Measurements of VEGF in BAL fluids

Levels of VEGF were quantitated by enzyme immunoassays according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). The minimum detectable level of mouse VEGF is <3 pg/ml. Levels of VEGF were also measured by Western blot analysis. BAL fluids were obtained from the tracheas of TDI-challenged mice with 0.8 ml saline solution and were centrifuged at 4000 $\times g$ for 1 min. Each supernatant was recovered and the remaining cell pellet was resuspended in PBS for cytospin. Each BAL fluid supernatant was quantified using the Bradford reagent (Bio-Rad, Hercules, CA) and 3 μg of BAL protein was loaded on a 12% SDS-PAGE gel and separated at 120 V for 90 min. After electrophoresis, 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% nonfat milk in TBST buffer (25 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20) for 2 h and the anti-mouse VEGF Ab (Santa Cruz Biotechnology), diluted 1/500, was then incubated for 2 h in TBST buffer at room temperature. Anti-mouse HRP-conjugated IgG was coupled to the anti-mouse VEGF Ab and specific binding was visualized using ECL system reagents (Amersham Pharmacia Biotech) and exposed to photographic film.

Statistical analysis

Data are expressed as means \pm SD. 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. Correlations were calculated using Spearman's rank test. Statistical significance was set at $p < 0.05$.

Results

Cellular changes in BAL fluids after TDI inhalation

Total cell numbers in BAL fluids were significantly greater from 6 to 72 h after TDI inhalation compared with cell numbers before the inhalation or those in the control group (Fig. 2). At 6 h after TDI inhalation, neutrophils were increased in the BAL fluid and the increased number of neutrophils continued up to 72 h after TDI inhalation. The numbers of lymphocytes and eosinophils were also increased significantly from 6 to 72 h after TDI inhalation. In contrast, no significant changes in cellular components were observed in BAL fluids in the control group.

VEGF levels in BAL fluids were increased in TDI-induced asthma

Enzyme immunoassay revealed that VEGF levels in the BAL fluids were significantly increased at 6, 24, 48, and 72 h after TDI inhalation compared with levels before inhalation or in the control group. In contrast, no significant changes in VEGF levels were

FIGURE 1. Schematic diagram of the experimental protocol. Mice were sensitized twice by two courses of intranasal administration of 3% TDI once a day for 5 consecutive days with a 3-wk interval. Seven days later, mice were challenged via the airways with 1% TDI for 10 min by ultrasonic nebulization. In the case of treatment with SU5614 or SU1498, it was administered i.p. three times at 24-h intervals, beginning 1 h after the TDI challenge.

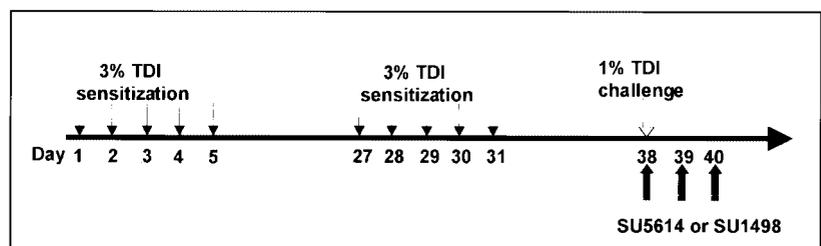
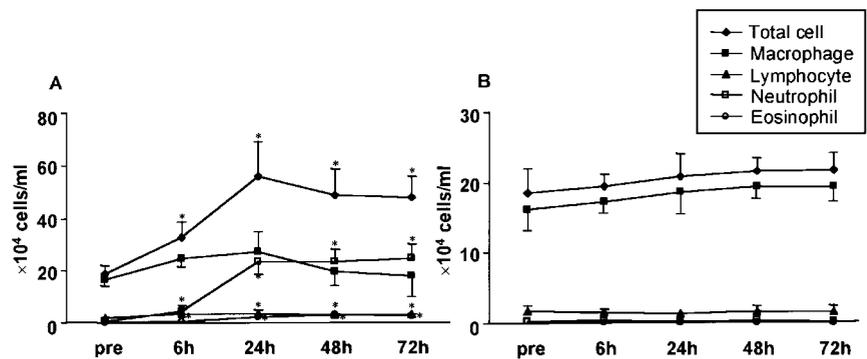


FIGURE 2. Effect of TDI challenge on total and differential cellular components in BAL fluids. In BAL fluids from TDI-sensitized and -challenged mice (A) or from vehicle control mice (B), the numbers of cells were counted. *, Significantly different from preinhalation and vehicle control mice ($p < 0.05$). Shown is the average number of cells in each sample \pm SD ($n = 6$ mice per group).



observed after inhalation of the vehicle control (Fig. 3A). Consistent with the results obtained from the enzyme immunoassays, Western blot analysis revealed that VEGF protein levels were increased after challenge with TDI (Fig. 3B). Neutrophils and eosinophils were counted in the BAL fluids at the same time points, and there were significant correlations between the level of VEGF and the number of neutrophils ($r = 0.695$ and $p = 0.000$) or eosinophils ($r = 0.635$ and $p = 0.000$) (data not shown).

VEGFR inhibitors reduced plasma extravasation in TDI-sensitized and -challenged mice

The EBD assay revealed that plasma extravasation was significantly increased at 72 h after TDI inhalation (Fig. 4). However, the administration of the VEGFR inhibitors, SU5614 or SU1498, significantly reduced the increase in plasma extravasation at 72 h after TDI inhalation.

VEGFR inhibitors decreased VEGF levels in BAL fluids of TDI-sensitized and -challenged mice

Administration of the VEGFR inhibitors, SU5614 or SU1498, dramatically reduced the levels of VEGF in BAL fluids 72 h after TDI

inhalation (Fig. 5A). Consistent with the results obtained from the enzyme immunoassays, Western blot analysis revealed that SU5614 or SU1498 reduced the levels of VEGF in the BAL fluid 72 h after TDI inhalation (Fig. 5B).

Effect of VEGFR inhibitors on cytopathological changes of TDI-induced asthma

The administration of SU5614 or SU1498 significantly abrogated the increase in total cells, neutrophils, lymphocytes, and eosinophils elicited in the airway lumen 3 days after TDI inhalation compared with controls (Fig. 6).

Histologic analyses revealed typical pathologic findings of asthma in the TDI-exposed mice. Numerous inflammatory cells infiltrated around the bronchioles, the airway epithelium was thickened, and mucus and debris had accumulated in the lumen of bronchioles (Fig. 7D). SU5614-treated mice showed marked reductions in the thickening of the airway epithelium, the infiltration of inflammatory cells in the peribronchial region, and the number of inflammatory cells and the amount of debris in the airway lumen (Fig. 7G).

Immunohistochemical analyses showed the localization of immunoreactive VEGF in inflammatory cells around the bronchioles of mice with TDI-induced asthma (Fig. 7E). In control mice and in TDI-sensitized and -challenged mice treated with SU5614, almost no VEGF-positive cells were detected (Fig. 7, B and H).

Immunocytologic analysis of BAL fluids showed localization of immunoreactive VEGF in the precipitated cells, including cells that resembled macrophages, neutrophils, and eosinophils, from

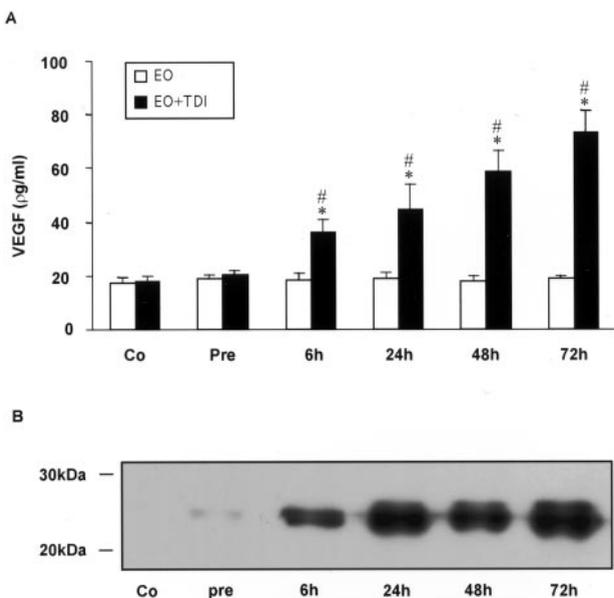


FIGURE 3. Effect of TDI challenge on VEGF secretion in BAL fluids. Mice were treated as described in Fig. 1. A, Enzyme immunoassay of VEGF. B, Western blotting. EO, Vehicle control; Co, no treatment; Pre, before the challenge; 6, 24, 46, and 72 h, after challenge. Data represent the mean \pm SD from six independent experiments. *, $p < 0.05$ vs Pre; #, $p < 0.05$ vs EO.

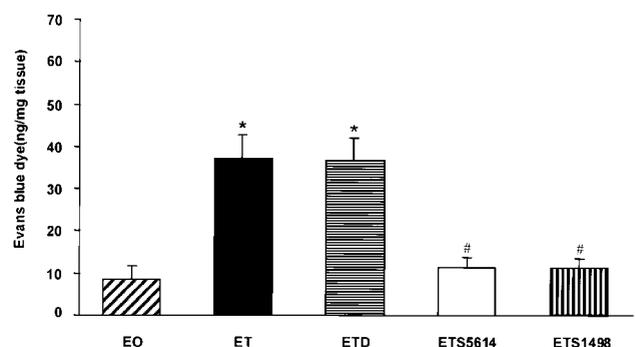


FIGURE 4. Effect of SU5614 or SU1498 on plasma exudation in the TDI-sensitized and -challenged mice. Mice were treated as described in Fig. 1 and sampling was performed at 72 h after treatment with vehicle only (EO), EO plus TDI (ET), EO plus TDI plus DMSO (ETD), EO plus TDI plus SU5614 (ETS5614), or EO plus TDI plus SU1498 (ETS1498). Bars represent the mean \pm SD from six independent experiments. *, $p < 0.05$ vs EO; #, $p < 0.05$ vs EO plus TDI.

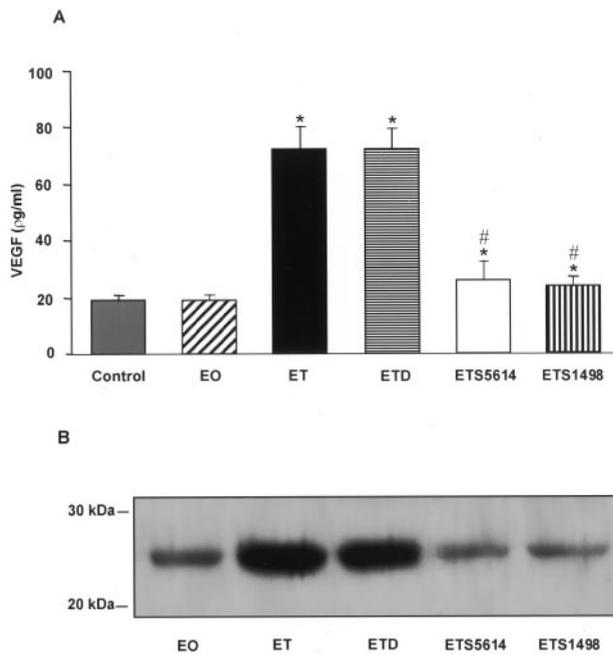


FIGURE 5. Effect of SU5614 or SU1498 on VEGF secretion in BAL fluids of TDI-sensitized and -challenged mice. *A*, Enzyme immunoassay of VEGF. *B*, Western blotting. Mice were treated as described in Fig. 1 and sampling was performed at 72 h after treatment with vehicle only (EO), EO plus TDI (ET), EO plus TDI plus DMSO (ETD), EO plus TDI plus SU5614 (ETS5614), or EO plus TDI plus SU1498 (ETS1498). Data represent the mean \pm SD from six independent experiments. *, $p < 0.05$ vs EO; #, $p < 0.05$ vs EO plus TDI.

the TDI-induced mice (Fig. 7F). However, immunoreactive VEGF was markedly reduced in BAL cells from control mice and from TDI-sensitized and -challenged mice treated with SU5614 (Fig. 7, C and D). These results suggest that the migration of inflammatory cells, especially neutrophils and eosinophils, into the bronchial lumens of TDI-sensitized and -challenged mice was strongly inhibited by SU5614.

VEGFR inhibitors reduced TDI-induced airway hyperresponsiveness

Airway responsiveness was assessed as a percentage of increase of Penh in response to increasing doses of methacholine. In TDI-sensitized and -challenged mice, the dose response curve of the percentage of Penh shifted to the left side compared with control mice (Fig. 8). In addition, the percentage of Penh produced by

methacholine (from 2.5 to 50 mg/ml) increased significantly in the TDI-sensitized and -challenged mice compared with controls. Sensitized mice treated with SU5614 or SU1498 showed a dose response curve of percentage of Penh that shifted to the right in a dose-dependent manner compared with untreated mice. Thus, these results indicate that SU5614 or SU1498 treatment reduces TDI-induced airway hyperresponsiveness.

Discussion

To our knowledge, this report is the first to describe the overexpression of VEGF in a murine model of TDI-induced asthma. Accordingly, administration of VEGFR inhibitors, SU5614 or SU1498, almost completely prevented the pathophysiological symptoms of occupational asthma.

TDI, a low-molecular weight compound widely used in the production of polyurethane foams, automobile paints, varnishes, and related products, is a leading cause of occupational asthma (1, 2). Although considerable controversy remains regarding its pathogenesis, TDI-induced asthma is characterized by airway hyperresponsiveness to TDI. In addition, an important pathophysiological feature of TDI-induced asthma is airway inflammation. One of the histological hallmarks of inflammation is angiogenesis, the growth and proliferation of new blood vessels (5). Angiogenesis is important in a variety of pathophysiologic processes, such as wound repair, tumor growth, and rheumatoid arthritis (12–14). Although little is known about the role of angiogenesis in asthma, more vessels are found in asthmatic airways than in those of normal controls (6). The formation of new blood vessels may play an important role both in the development of bronchial asthma and in the pathophysiologic repair process. In addition, the increased vascularity of the bronchial mucosa in asthmatic subjects is closely related to the expression of VEGF and its receptors, flt-1 and flk-1, which may then contribute to the pathogenesis of asthma (15). Recently, we reported that overproduction of VEGF is associated with airway inflammation during acute asthma (16).

VEGF is an endothelial cell-specific mitogen that has been shown to play a key role in vasculogenesis and angiogenesis. VEGF was purified on the basis of its ability to induce transient vascular leakage and endothelial migration. Studies indicate that VEGF binds to high-affinity cell surface receptors, flt-1 and KDR/flk-1, which are predominantly expressed in endothelial cells. VEGF expression patterns coincide spatially and temporally with blood vessel growth under physiological and pathological conditions. Furthermore, VEGF enhances microvascular permeability with a potency some 50,000 times greater than histamine, and it behaves as a vasodilator substance (17). VEGF can be produced by a wide variety of human cells including vascular smooth muscle

FIGURE 6. Effect of SU5614 or SU1498 on total and differential cellular components of BAL fluids in the TDI-sensitized and -challenged mice. Mice were treated as described in Fig. 1 and sampling was performed at 72 h after treatment with vehicle only (EO), EO plus TDI, EO plus TDI plus DMSO, EO plus TDI plus SU5614, or EO plus TDI plus SU1498. The numbers of each cellular component were counted. Bars represent the mean \pm SD from six independent experiments. *, $p < 0.05$ vs EO; #, $p < 0.05$ vs EO plus TDI.

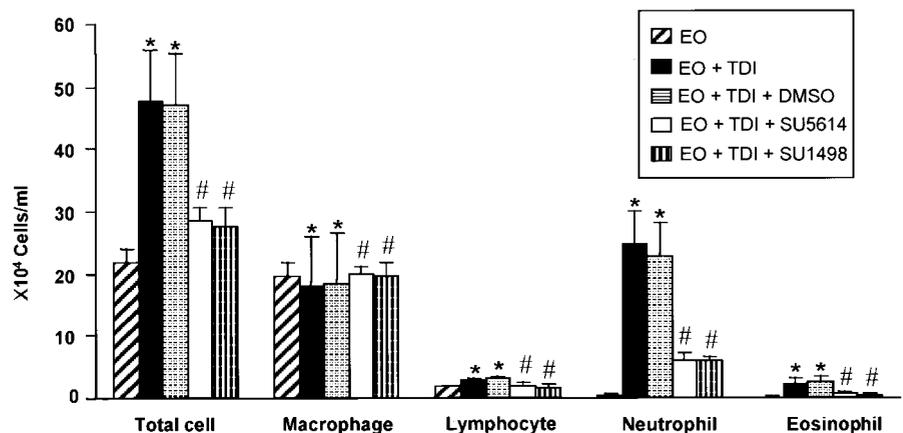
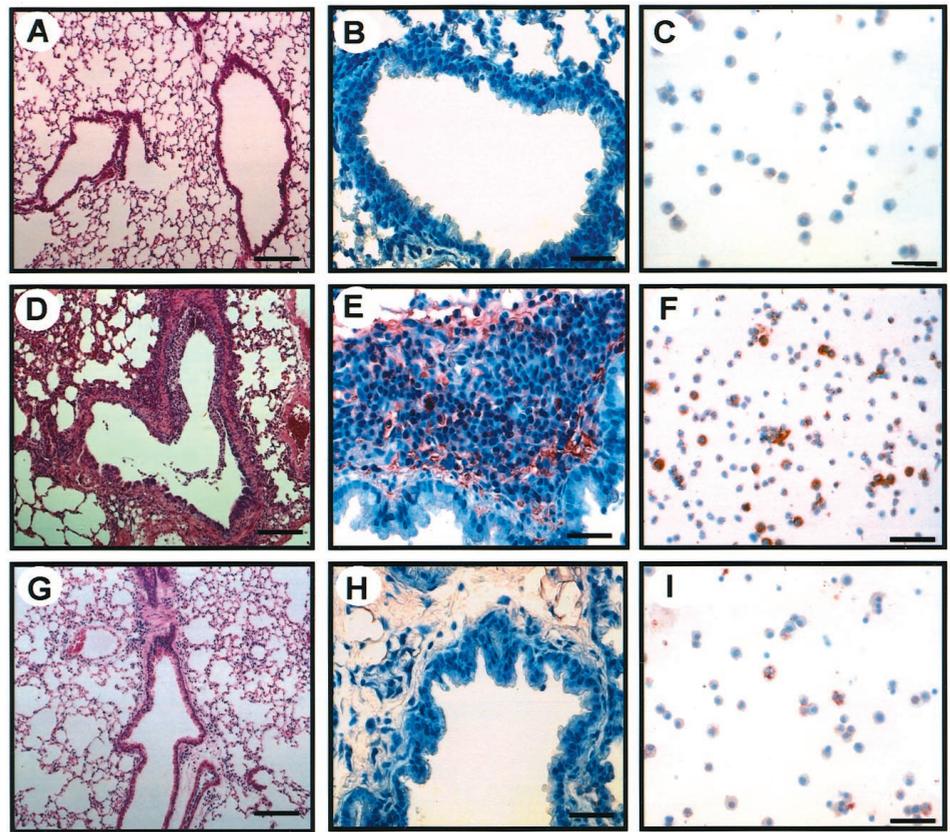


FIGURE 7. Effect of TDI challenge and SU5614 treatment on VEGF expression in lung tissues and BAL fluids of TDI-sensitized and -challenged mice. Mice were treated as described in Fig. 1 and sampling was performed at 72 h after treatment with vehicle only (A–C), vehicle plus TDI (D–F), or vehicle plus TDI plus SU5614 (G–I). A, D, and G, Representative H&E-stained sections of the lungs. B, E, and H, Representative light microscopy of VEGF-positive cells in the bronchioles; the dark brown color indicates VEGF-positive cells. C, F, and I, Representative light microscopy of VEGF-positive cells in the BAL fluids; the brownish pink color indicates VEGF-positive cells. Bars indicate scale of 10 (B, E, and H), 50 (C, F, and I), or 100 (A, D, and G) μ m.



cells, tumor cells, keratinocytes, fibroblasts, and epithelial and mesangial cells (7). In addition, VEGF may also be released by macrophages, neutrophils, and eosinophils (15, 18, 19). In this

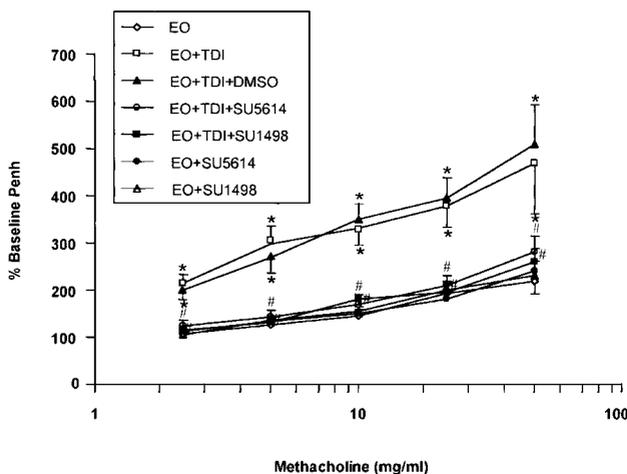


FIGURE 8. Effect of TDI challenge and SU5614 or SU1498 on airway responsiveness in TDI-sensitized and -challenged mice. Mice were treated as described in Fig. 1 and airway responsiveness was measured at 72 h after treatment with vehicle only (EO), EO plus TDI, EO plus TDI plus DMSO, EO plus TDI plus SU5614, EO plus TDI plus SU1498, EO plus SU5614, or EO plus SU1498. Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. Mice were placed into the main chamber and were nebulized first with saline, then with increasing doses (2.5–50 mg/ml) of methacholine for 3 min for each nebulization. Readings of breathing parameters were taken for 3 min after each nebulization during which Penh values were determined. Data represent the mean \pm SD from six independent experiments. *, $p < 0.05$ vs EO; #, $p < 0.05$ vs TDI inhaled mice.

study, our data suggest that macrophages, neutrophils, and eosinophils are the major cellular sources of VEGF in the airways.

It has long been appreciated that VEGF increases vascular permeability so that plasma proteins can leak into the extravascular space, which leads to edema and profound alterations in the extracellular matrix. Inflammation of the asthmatic airway mucosa is usually accompanied by increased vascular permeability and plasma exudation. VEGF apparently increases microvascular permeability by enhancing the functional activity of vesicular-vacuolar organelles (VVOs) so that plasma proteins can leak into the extravascular space (7). VVOs provide the major route of extravasation of macromolecules at sites of increased vascular permeability induced by VEGF. Dvorak et al. (20) reported the presence of extensive numbers of VVOs in the venular endothelium of an animal model of allergic inflammatory disease. In the airways, the plasma exudate may readily pass the inflamed mucosa and reach the airway lumen through leaky epithelium. Plasma protein leakage induces a thickened, engorged, and edematous airway wall, resulting in the airway lumen narrowing that is observed in asthma exacerbations. In addition, plasma may also traverse the epithelium and collect in the airway lumen. Plasma exudation may compromise epithelial integrity (21) and its presence in the lumen may slow ciliary function and thus reduce mucus clearance (22). Plasma proteins may also promote the formation of viscid luminal plugs of exudate mixed with mucus and inflammatory and epithelial cells. Van de Graaf et al. (23) reported that exudation of plasma proteins into the airways correlates with bronchial hyperreactivity. Hoshino et al. (15) showed that VEGF expression correlates with airway caliber and airway hyperresponsiveness. Together, these effects contribute to airflow obstruction and bronchial hyperresponsiveness. Therefore, VEGF plays a crucial role in the obstruction of the airway lumen and in the induction of airway hyperresponsiveness in TDI-induced asthma. Treatment with

SU5614 or SU1498 decreases the bronchial hyperresponsiveness and the plasma exudation by inhibiting the overexpression of VEGF.

Based on the marked increase of VEGF in the airway, we administered the VEGFR inhibitors, SU5614 or SU1498, to examine their potential therapeutic effects. The VEGFR inhibitors were dramatically effective at reversing all pathophysiological symptoms examined. One likely mechanism for the effectiveness of SU5614 or SU1498 is that the VEGF can rapidly increase vascular permeability so that plasma proteins, including inflammatory mediators and cells, can leak into the extravascular space to allow the migration of inflammatory cells, including neutrophils and eosinophils, into the airways. Our data demonstrate that the VEGF-induced plasma leakage can be blocked by acute administration of SU5614 or SU1498. In this study, the administration of SU5614 or SU1498 not only inhibits VEGF activity in the bronchial lumen but also inhibits the migration of inflammatory cells through the endothelial basement membrane. Without these inflammatory cells, the other pathological changes do not occur. Our results strongly indicate that the VEGFR inhibitors are potentially powerful therapeutic agents for TDI-induced asthma.

References

1. Park, H. S., and D. H. Nahm. 1996. Isocyanate-induced occupational asthma: challenge and immunologic studies. *J. Korean Med. Sci.* 11:314.
2. Chan-Yeung, M. 1990. Occupational asthma. *Chest* 98:S148.
3. Baur, X., M. Dewair, and G. Fruhmann. 1984. Detection of immunologically sensitized isocyanate workers by RAST and intracutaneous skin tests. *J. Allergy Clin. Immunol.* 73:610.
4. Mapp, C. E., P. Boschetto, E. Zocca, G. F. Milani, F. Pivrotto, V. Tegazzin, and L. M. Fabbri. 1987. Pathogenesis of late asthmatic reactions induced by exposure to isocyanates. *Bull. Eur. Physiopathol. Respir.* 23:583.
5. Cotran, R., V. Kumor, and S. Robin. 1989. Inflammation and repair. In *Robbins Pathologic Basis of Disease*. R. Cotran, V. Kumor, and S. Robin, eds. Saunders, Philadelphia, p. 39.
6. Li, X., and J. W. Wilson. 1997. Increased vascularity of the bronchial mucosa in mild asthma. *Am. J. Respir. Crit. Care Med.* 156:229.
7. Dvorak, H. F., L. F. Brown, M. Detmar, and A. M. Dvorak. 1995. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am. J. Pathol.* 146:1029.
8. Scheerens, H., T. L. Buckley, T. L. Muis, J. Garssen, J. Dormans, F. P. Nijkamp, and H. Van Loveren. 1999. Long-term topical exposure to toluene diisocyanate in mice leads to antibody production and in vivo airway hyperresponsiveness three hours after intranasal challenge. *Am. J. Respir. Crit. Care Med.* 159:1074.
9. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156:766.
10. Kidney, J. C., J. O. Lotvall, Y. Lei, K. F. Chung, and P. J. Barnes. 1996. The effect of inhaled K^+ channel openers on bronchoconstriction and airway microvascular leakage in anaesthetised guinea pigs. *Eur. J. Pharmacol.* 296:81.
11. Rogers, D., F. P. Boschetto, and P. J. Barnes. 1989. Plasma exudation: correlation between Evans blue dye and radiolabeled albumin in guinea pig airways in vivo. *J. Pharmacol. Methods* 21:309.
12. Folkman, J., and M. Klagsburn. 1987. Angiogenic factors. *Science* 235:442.
13. Klagsbrun, M., and P. A. D'Amore. 1991. Regulators of angiogenesis. *Annu. Rev. Physiol.* 53:217.
14. Folkman, J., and Y. Shing. 1992. Angiogenesis. *J. Biol. Chem.* 267:10931.
15. Hoshino, M., Y. Nakamura, and Q. A. Hamid. 2001. Gene expression of vascular endothelial growth factor and its receptors and angiogenesis in bronchial asthma. *J. Allergy Clin. Immunol.* 107:1034.
16. Lee, Y. C., and H. K. Lee. 2001. Vascular endothelial growth factor in patients with acute asthma. *J. Allergy Clin. Immunol.* 107:1106.
17. Senger, D. R., S. J. Galli, A. M. Dvorak, V. S. Harver, and H. E. Dvorak. 1983. Tumors cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219:983.
18. Horiuchi, H., and P. F. Veller. 1997. Expression of vascular endothelial growth factor by human eosinophils: upregulation by granulocyte macrophage colony-stimulating factor and interleukin-5. *Am. J. Respir. Cell Mol. Biol.* 17:70.
19. Taichman, N. S., S. Young, A. T. Cruchley, P. Taylor, and E. Paleolog. 1997. Human neutrophils secrete vascular endothelial growth factor. *J. Leukocyte Biol.* 62:397.
20. Dvorak, A. M., R. I. Tepper, P. F. Weller, E. S. Morgan, P. Estrella, R. A. Monahan-Earley, and S. J. Galli. 1994. Piecemeal degranulation of mast cells in the inflammatory eyelid lesions of interleukin-4 transgenic mice: evidence of mast cell histamine release in vivo by diamine oxidase-gold enzyme-affinity ultrastructural cytochemistry. *Blood* 12:3600.
21. Persson, C. G. 1996. Epithelial cells: barrier functions and shedding restitution mechanisms. *Am. J. Respir. Crit. Care Med.* 153:S9.
22. Foster, W. M., E. G. Langenback, and E. H. Bergofsky. 1982. Lung mucociliary function in man: interdependence of bronchial and tracheal mucus transport velocities with lung clearance in bronchial asthma and healthy subjects. *Ann. Occup. Hyg.* 26:227.
23. Van de Graaf, E. A., T. A. Out, C. M. Roos, and H. M. Jansen. 1991. Respiratory membrane permeability and bronchial hyperreactivity in patients with stable asthma: effects of therapy with inhaled steroids. *Am. Rev. Respir. Dis.* 143:362.