Neovastat(Æ-941) inhibits the airway inflammation and hyperresponsiveness in a murine model of asthma

Sook-Young Lee[1], Soon-Young Paik[2,*] and Su-Mi Chung[3,*]

*[1]Department of Internal Medicine, [2]Department of Microbiology,
[3]Department of Radiation Oncology, College of Medicine, The Catholic University of Korea, Seoul, 137-701, Republic of Korea*

Matrix metalloproteinase (MMP)-9 plays an important role in the pathogenesis of bronchial asthma. Neovastat, having significant antitumor and antimetastatic properties, is classified as a naturally occurring multifunctional antiangiogenic agent. We evaluated the therapeutic effect of Neovastat on airway inflammation in a mouse model of asthma. BALB/c mice were immunized subcutaneously with ovalbumin (OVA) on days 0, 7, 14, and 21 and challenged with inhaled OVA on days 26, 29, and 31. Neovastat was administrated by gavage (5 mg/kg body weight) three times with 12 h intervals, beginning 30 min before OVA inhalation. On day 32, mice were challenged with inhaled methacholine, and enhanced pause (Penh) was measured as an index of airway hyperresponsiveness. The severity of airway inflammation was determined by differential cell count of bronchoalveolar lavage (BAL) fluid. The MMP-9 concentration in BAL fluid samples was measured by ELISA, and MMP-9 activity was measured by zymography. The untreated asthma group showed an increased inflammatory cell count in BAL fluid and Penh value compared with the normal control group. Mice treated with Neovastat had significantly reduced Penh values and inflammatory cell counts in BAL fluid compared with untreated asthmatic mice. Furthermore, mice treated with Neovastat showed significantly reduced MMP-9 concentrations and activity in BAL fluid. These results demonstrate that Neovastat might have new therapeutic potential for airway asthmatic inflammation.

Keywords: airway resistance, asthma, inflammation, matrix metalloproteinase-9, neovastat

Airway inflammation and hyperresponsiveness (AHR) are fundamental features of bronchial asthma (Jonathan and Lee, 1992). Airway inflammation in asthmatics is characterized by an accumulation of activated inflammatory cells, including eosinophils, lymphocytes, and mast cells/basophils in airway lumens and walls. Their accumulation in the airway necessitates their travel from the peripheral circulation, through the vascular endothelium, through the peribronchial areas, and ultimately, to the bronchial and bronchiolar spaces. This process involves a complex interplay of a series of molecules including proteolytic enzymes, adhesion molecules and chemokines (Lukacs, 2000).

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidases capable of proteolytically degrading many of the components of the extracellular matrix (Nagase, 1997). Of the MMP family, MMP-9 (gelatinase B) is one of the major proteinases involved in bronchial asthma. MMP-9 has been reported

to play a crucial role in the infiltration of airway inflammatory cells and the induction of AHR (Kelly and Jarjour, 2003). MMP-9 cleaves type IV collagen, which is an important constituent of the basement membrane. Moreover, MMP-9 was shown to play a crucial role in the transmigration of lymphocytes (Leppert *et al.*, 1995), neutrophils (Delcaux *et al.*, 1996), and eosinophils (Okada *et al.*, 1997) through basement membrane components in *in vitro* systems. MMP-9 is inhibited by tissue inhibitors of metalloproteinase (TIMP)-1 that form a 1:1 complex with MMP-9.

Neovastat is a naturally occurring inhibitor of angiogenesis derived from marine cartilage that contains a high concentration of biologically relevant molecules (Gingras *et al.*, 2001), and it shows significant antitumor and antimetastatic properties in animal models (Castronovo *et al.*, 1999). Neovastat has reached Phase III clinical trial evaluation for the treatment of malignant disease (Falardeau *et al.*, 2001). At the molecular level, the antiangiogenic activity of Neovastat correlates with the inhibition of metalloproteinases (MMP-2, 9, 12) (Gingras *et al.*, 2001). In addition, Neovastat interferes with several steps associated with the development of angiogenesis through its ability to induce endothelial cell apoptosis (Boivin *et al.*,

---

* To whom correspondence should be addressed.
(Tel) 82-2-590-1217; (Fax) 82-2-535-6473
(E-mail) paik@catholic.ac.kr
(Tel) 82-2-590-1567; (Fax) 82-2-3476-1365
(E-mail) sumic@catholic.ac.kr

2002) and vascular endothelial growth factor-mediated signaling pathways (Beliveau *et al.*, 2002).

We hypothesized that Neovastat inhibits the airway inflammation and AHR in bronchial asthma. To examine this hypothesis, we studied the effects of Neovastat on allergic airway inflammation and AHR using a murine model of allergic asthma. To further explore the mechanism, we also assessed the effects of Neovastat on MMP-9 TIMP-1 levels.

Materials and Methods

Animals

Specific pathogen free BALB/c mice (female) were used when they reached 8-10 weeks of age. Mice were housed in environmentally controlled specific-pathogen free conditions for 1 week prior to the study, and for the duration of experiments. All animal experimental protocols were approved by the Catholic University of Korea, Animal Subjects Committee. The mice were divided into 3 different groups of animal with 10-15 mice, and asthma was induced in 2 groups.

Sensitization and antigen challenge protocol

Mice were sensitized by a subcutaneous injection of 25 μg of ovalbumin (OVA, grade V; Sigma, USA) emulsified in 1 mg of aluminum hydroxide (Aldrich, USA) in a total volume of 200 μl of phosphate buffered saline (PBS) on days 0, 7, 14 and 21. Intranasal OVA challenges (20 ng/50 μl in PBS) were administered on days 26, 29 and 31 under isoflurane (Vedco, USA) anesthesia. Age- and sex-matched control mice were not sensitized and challenged.

Administration of Neovastat

Neovastat was administrated by gavage three times with 12 h intervals, beginning 30 min before OVA inhalation.

Measurement of airway hyperresponsiveness

Airway responsiveness was assessed 24 h after the final OVA inhalation by using a single-chamber whole-body plethysmograph obtained from Allmedicus (Korea), as described previously (Cho *et al.*, 2004). In this system, an unrestrained, spontaneously breathing mouse is placed into the main chamber of the plethysmograph, and pressure differences between this chamber and a reference chamber are recorded. In the plethysmograph, mice were exposed for 3 min to nebulized PBS and subsequently to increasing concentrations of nebulized methacholine (Mch; Sigma, USA) in PBS by using an aerosonic ultrasonic nebulizer (DeVilbiss). After each nebulization, recordings were taken for 3 min. The Penh values measured during each 3-min sequence were averaged and expressed as the percentage of baseline Penh values following PBS exposure for each Mch concentration (3-50 mg/ml).

Bronchoalveolar larvage

Mice were killed by CO_2 asphyxiation after measuring airway responsiveness. The trachea was exposed and cannulated with silicone tubing attached to a 23-guage needle on an 800 μl tuberculin syringe. After instillation of 1 ml of sterile PBS through the trachea into the lung, bronchoalveolar larvage fluid (BALF) was withdrawn. BALF total white blood cell counts were performed using a hemacytometer. The BALF was cytospun (3 min at 1000 rpm) onto microscope slides and stained with Wright-Giemsa. The percentages of BALF macrophages, eosinophils, lymphocytes and neutrophils were obtained by counting 400 leukocytes on randomly selected portions of the slide by light microscopy. Supernatants were stored at -70°C.

ELISA

The MMP-9 and TIMP-1 concentrations in the BAL fluid were measured with an ELISA-kit (R&D system Inc, USA). The protocol was followed according to the manufacturer's instructions.

Zymography

Gelatin zymography was performed as described previously (Yang *et al.*, 2002).

Data analysis

Results from each groups were compared by ANOVA with the nonparametric Kruskal-Wallis test, followed by posttesting with Dunns multiple comparison of means. A statistical software package (In Stat, USA) was used for the analysis. A P value less than 0.05 was considered statistically significant. All results are given as means ±SEM.

Results

Effect on airway inflammation

The numbers of total cells, macrophages, lymphocytes, and eosinophils in BAL fluids were significantly increased in the untreated asthma group compared with those in the normal control group. The administration of Neovastat significantly reduced the increase in total cells, macrophages, lymphocytes, and eosinophils elicited from the asthmatic airway lumens (P<0.05). These results indicate that the migration of inflammatory cells into the bronchial lumens was strongly inhibited by Neovastat (Fig. 1).

Effect on airway hyperresponsiveness

AHR was assessed as a percent increase of enhanced pause (Penh) in response to increasing the dose of methacholine (Mch). The Penh values are expressed as the percentage of baseline Penh values following PBS exposure for each Mch concentration (3-50 mg/ml). The percent Penh increased significantly by 25 and 50 mg/ml Mch in

the untreated asthma group (453±21% and 557±12%, respectively) compared with that in the normal control group. Mice treated with Neovastat showed a decreased Penh value after receiving 25 mg/ml Mch compared with that in the untreated asthma group (453±21 % vs 254±10 %, respectively, P<0.05). This trend was also observed with 50 mg/ml Mch (557±12% vs 345±14 %, respectively, P<0.05) (Fig. 2).

Effect on MMP-9 and the TIMP-1 expression.

To investigate potential immunomodulatory mechanisms by which Neovastat inhibited airway inflammation, MMP-9 and TIMP-1 concentrations in BAL fluid were determined by ELISA (Fig. 3). MMP-9 protein levels in BAL fluids were significantly increased in the untreated asthma

group compared with those in the normal control group (2.93±0.79 ng/ml vs 0.24±0.03 ng/ml, respectively, P< 0.05). The administration of Neovastat significantly reduced the increase of MMP-9 protein in the BAL fluid compared with that in the untreated asthma group (0.99 ±0.16 ng/ml vs 2.93±0.79 ng/ml, respectively, P<0.05). The OVA challenge also significantly increased the TIMP-1 level in untreated mice compared with that in normal control mice (822.5±190.5 pg/ml vs 40.9±8.89 pg/ml, respectively, P< 0.05). However, there was no significant decrease in mice treated with Neovastat compared with that in untreated asthma mice (813.6±179.7 pg/ml vs 822.5±190.5 pg/ml, respectively, P >0.05). The BAL fluid
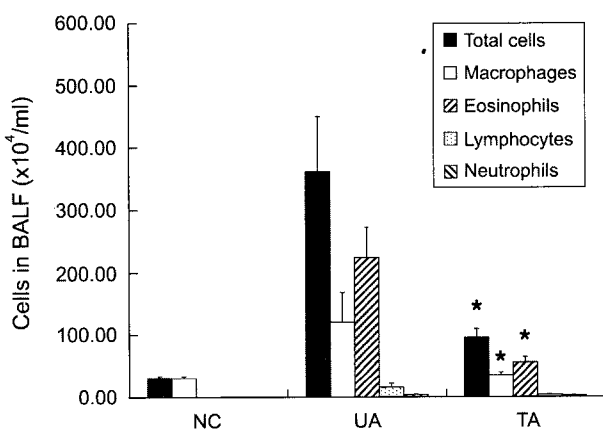
Fig. 1. Effect of Neovastat treatment on airway inflammation. Differential cell counts in bronchoalveolar lavage (BAL). Mice were sacrificed 24 h after the final ovalbumin (OVA) challenge, and BAL cells were isolated. NC, normal control mice; UA, untreated asthmatic mice; TA, asthmatic mice treated with Neovastat. Values are expressed as mean±SEM, n=10-15/group in three separated experiments, and *, p<0.05 in comparison with the untreated asthmatic group.
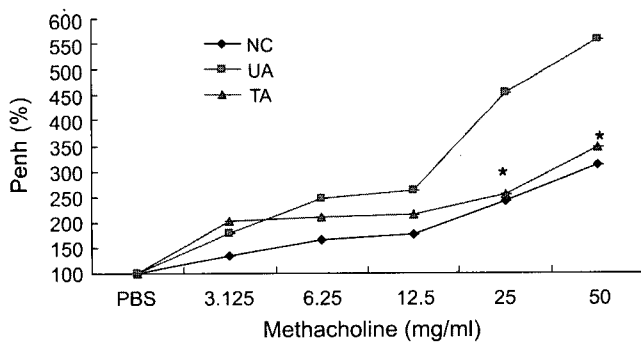
Fig. 2. Effect of Neovastat treatment on airway hyperresponsiveness (AHR) to inhaled methacholine (Mch). AHR was measured 24 h after the final ovalbumin (OVA) challenge using an Allmedicus system in which mice were exposed to increasing concentrations of methacholine (3-50 mg/ml). NC, normal control mice; UA, untreated asthmatic mice; TA, asthmatic mice treated with Neovastat. Values are expressed as mean±SEM, n=10-15/group in three separated experiments, and *, p<0.05 in comparison with the untreated asthmatic group.
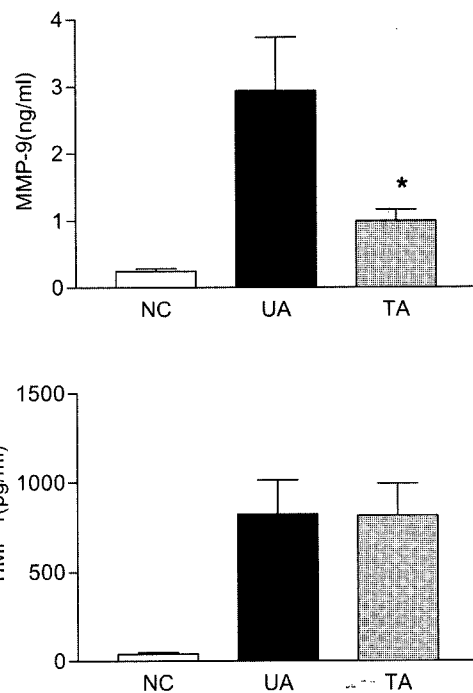
Fig. 3. Effect of Neovastat on MMP-9 and TIMP-1 levels in bronchoalveolar lavage (BAL) fluid. Mice were sacrificed 24 h after the final obalbumin (OVA) challenge, BAL fluids were separated, then MMP-9 and TIMP-1 levels were measured with ELISA. NC, normal control mice; UA, untreated asthmatic mice; TA, asthmatic mice treated with Neovastat. Values are expressed as mean±SEM, n=10-15/group in three separated experiments, and *, p<0.05 in comparison with the untreated asthmatic group.

◄ Pro MMP-8 (92 kDa)
◄ Active MMP-9 (82 kDa)

Fig. 4. Analysis of bronchoalveolar lavage fluids by gelatin zymography. Mice were sacrificed 24 h after the final obalbumin (OVA) challenge, BAL fluids were separated, then MMP-9 activity was measured by gelatin zymography. PC, positive control; NC, normal control mice; UA, untreated asthmatic mice; TA, asthmatic mice treated with Neovastat.

Fig. 5. Effect of Neovastat on the MMP-9 expression in a mouse model of OVA-induced asthma. A, normal control mice; B, untreated asthmatic mice; C, asthmatic mice treated with Neovastat. The dark-brown color indicates immunoreactive inflammatory cells (x200).

was also evaluated for MMP-9 activity by zymography. In most mice, two gelatinolytic bands were observed with MWs of 92 and 82 kd, corresponding to the pro (latent) and active forms (Fig. 4). The pro and active forms of MMP-9 in mice treated with Neovastat were both lower than the amounts in the untreated asthma group.

The expression of MMP-9 was assessed by immunostaining (Fig. 5). Immunohistochemical analysis of lung tissues from the untreated asthma group revealed immunoreactive MMP-9 on inflammatory cells and the extracellular matrix around each bronchus, and on inflammatory cells and debris in the airway lumens. Immunoreactive MMP-9 was markedly reduced in Neovastat treated mice.

Discussion

The present study shows that Neovastat, an antiangiogenic drug used in the field of cancer treatment, effectively suppressed airway inflammation and AHR in a mouse model of asthma. These effects of Neovastat were associated with a decrease in MMP-9 activity. These results suggest that Neovastat may be used as adjuvant therapy for patients with bronchial asthma.

The up-regulation of MMP-9 release observed in this study is consistent with the previous reports that showed the MMP-9 content in BAL fluids from untreated asthmatics was increased compared with that from control subjects including steroid-treated asthmatics (Mautino et al., 1997), and an antigen challenge increased the enzyme activity of MMP-9 in BAL fluids from asthmatics (Warner et al., 1997). MMPs are clearly elevated during asthma exacerbations, and from genetic linkage analysis it would appear that at least one MMP, ADAM33, is closely associated with AHR (Van Eerdewegh et al., 2002).

The inhibition of MMP-9 may be a good therapeutic strategy in bronchial asthma. Previously, several studies reported that inhibitors of MMP-9 decreased the airway inflammation and AHR in animal asthma models (Kumagai et al., 1999; Lee et al., 2001; Lee et al., 2003). However, the inhibitors used in these studies were chemically synthesized substances that have limitations for clinical application. Neovastat is a naturally occurring inhibitor of angiogenesis and orally bioavailable. This study showed

that Neovastat inhibits the airway inflammation and AHR. Inflammation is an important component of the development and progression of asthma. Thus, anti-inflammatory interventions are therapeutically effective and may prevent disease progression. Inhaled corticosteroids are presently a first-line anti-inflammatory drug for chronic asthma. However, corticosteroids, particularly at high doses, have significant and severe adverse effects. Furthermore, a subgroup of patients with severe asthma exhibited a poor response to treatment with corticosteroids; indeed, they might have been corticosteroids resistant (Adcock, 1996). The current study suggests that Neovastat may be a novel therapeutic modality for the treatment of asthma.

To investigate potential immunomodulatory mechanisms by which Neovastat inhibits airway inflammation, the MMP-9 level and activity in the BAL fluid were determined. The administration of Neovastat significantly reduced an increase in MMP-9 protein in the BAL fluid compared with that in the untreated asthma group. The mechanism by which MMPs contributes to asthma pathogenesis remains somewhat unclear. MMPs are important for proteolysis and processing of both cell surface and secreted factors, which alter the biological activities of these substrates. MMPs may contribute to inflammatory cell migration by releasing chemokines from extracellular matrix stores and degrading the extracellular matrix into unique chemotatic fragments. Another particular function most likely to be affected by MMPs is the recruitment of cells across endothelial basement membranes. MMPs can degrade components of the vascular endothelium basement membrane, facilitating extravasation of inflammatory cells into the surrounding tissues. In the processes of cellular infiltration from circulation to inflammatory sites, MMP-9 could be involved in the migration of inflammatory cells through endothelial and epithelial basement membranes (Nagase, 1997; Sternlicht and Werb, 2001).

MMP-9 activity can be inhibited by another family of proteins, the TIMPs, consisting of the related proteins TIMP-1 (Stricklin and Welgus, 1983) and TIMP-2 (Stetler-Stevenson et al., 1989). The TIMP-1 expression is highly regulated and has been found in the same tissue and cells that express MMPs. Thus the regulation of MMP and

TIMP activities in tissues is complex and occurs at many levels. In this study, we observed that MMP-9 and TIMP-1 levels were significantly increased in asthmatic mice. Neovastat inhibited MMP-9 production without altering the biosynthesis of TIMP-1.

The airways of bronchial asthmatics show tissue remodeling including subepithelial basement thickening, angiogenesis, and hypertrophy/hyperplasia of airway smooth muscles. This airway remodeling has been speculated to be irreversible airway obstruction and one of the factors that make the treatment of patients with asthma difficult. In particular, angiogenesis is an essential component of tissue growth and remodeling. An early study on the pathology of asthma showed edematous bronchial mucosa with dilated and congested blood vessels in patients with fatal disease (Dunnill, 1960). A more recent study found an increase in the total number of vessels and in vascular area in patients with mild asthma when compared with control subjects (Li and Wilson, 1997). Further investigation is necessary to characterize the effect of Neovastat on airway angiogenesis in bronchial asthma.

In summary, the present study addressed the effect of Neovastat on airway inflammation and AHR in a mouse asthma model. We demonstrated that Neovastat was effective in inhibiting both airway inflammation and AHR. Furthermore, Neovastat inhibited the MMP-9 expression. These results imply that Neovastat, in addition to its known antiangiogenic effects in malignancy, has anti-asthma effects. Neovastat may be a new therapeutic modality for the treatment of asthma.

Acknowledgment

References

Adcock, I.M. 1996. Steroid resistance in asthma. Molecular mechanisms. *Am. J. Respir. Crit. Care Med.* 154, S58-61.

Béliveau, R., D. Gingras, E.A. Kruger, S. Lamy, P. Sirois, B. Simard, M.G. Sirois, L. Tranqui, F. Baffert, E. Beaulieu, V. Dimitriadou, M.C. Pepin, F. Courjal, I. Ricard, P. Poyet, P. Falardeau, W.D. Figg, and E. Dupont. 2002. The antiangiogenic agent neovastat (AE-941) inhibits vascular endothelial growth factor-mediated biological effects. *Clin. Cancer Res.* 8, 1242-1250.

Boivin, D., S. Gendron, Beaulieu, D. Gingras, and R. Béliveau. 2002. The Antiangiogenic Agent Neovastat (Æ-941) Induces Endothelial Cell Apoptosis. *Molecular Cancer Therapeutics.* 1, 795-802.

Castronovo, V., V. Dimitriadou, P. Savard, M. Rivière, and E. Dupont. 1999. Cartilage as a source of natural inhibitors of angiogenesis. Antiangiogenic Agents in Cancer Therapy, p. 175-83. Humana Press, Totowa, New Jersey.

Cho, J.Y., M. Miller, K.J. Baek, G. W. Han, J. Nayar, M. Rodriguez, S.Y. Lee, K. McElwain, S. McElwain, E. Raz, and D.H.

Broide. 2004. Immunostimulatory DNA Inhibits Transforming Growth Factor-β Expression and Airway Remodeling. *Am. J. Respir. Cell Mol. Biol.* 30, 651-661.

Delcaux, C., C. Delacourt, M.P. d'Ortho, V. Boyer, C. Lafuma, and A. Harf. 1996. Role of gelatinase B and elastase in human polymorphonuclear neutrohil migration across basement membrane. *Am. J. Respir. Cell Mol. Biol.* 14, 288-295.

Dunnill, M.S. 1960. The pathology of asthma with special reference to changes in the bronchial mucosa. *J. Clin. Patho.* 13, 27-33.

Falardeau, P., P. Champagne, P. Poyet, C. Hariton, and E, Dupont. 2001. Æ-941 (Neovastat), a naturally occurring multifunctional anti-angiogenic product in Phase clinical trials. *Semin. Oncol.* 28, 620-625.

Gingras, D., A. Renaud, N. Mousseau, E. Beaulieu, Z. Kachra, and R. Béliveau. 2001. Matrix proteinase Inhibition by Æ-941, a multifunctional antiangiogenic compound. *Anticancer Res.* 21, 145-155.

Gingras, D., G. Batist, and R. Béliveau. 2001. Æ-941 (Neovastat®): A novel multifunctional antiangiogenic compound. *Expert Rev. Anticancer Ther.* 1, 341-347.

Jonathan, P.A. and T.H. Lee. 1992. The pathobiology of bronchial asthma. *Adv. Immunol.* 51, 323-329

Kelly, E.A. and N.N. Jarjour. 2003. Role of matrix metalloproteinases in asthma. *Current Opinion in Pulmonary Medicine.* 9, 28-33.

Kumagai, K., I. Ohno, S. Okada, Y. Ohkawara, S.K. uzuki, T. Shinya, H. Nagase, K. Iwata, and S.K. hirato. 1999. Inhibition of matrix metalloproteinases prevents allergen-induced airway inflammation in a murine model of asthma. *J. Immunol.* 162, 4212-4219.

Lee, Y.C., C.H. Song, H.B. Lee, J.L. Oh, Y.K. Rhee, H.S. Park, and G.Y. Koh. 2001. A murine model of toluene diisocyanate-induced asthma can be treated with matrix metalloproteinase inhibitor. *J. Allergy Clin. Immunol.* 108, 1021-1026.

Lee, K.S., S.M. Jin, H.J. Kim, and Y.C. Lee. 2003. Matrix metalloproteinase inhibitor regulates inflammatory cell migration by reducing ICAM-1 and VCAM-1 expression in a murine model of toluene diisocyanate-induced asthma. *J. Allergy Clin. Immunol.* 111, 1278-1284.

Leppert, D., E. Waubant, R. Galardy, N.W. Bunnett, and S.L. Hauser. 1995. T cell gelatinases mediate basement membrane transmigration in vitro. *J. Immunol.* 154, 4379-4389.

Li, X. and J.W. Wilson. 1997. Increased vascularity of the bronchial mucosa in mild asthma. *Am. J. Respir. Crit. Card. Med.* 156, 229-233.

Lukacs, N.W. 2000. Migration of helper T-lymphocyte subsets into inflamed tissues. *J. Allergy Clin. Immunol.* 106, 264-269.

Mautino, G., N. Oliver, P. chanez, J. Bousquet, and F. Capony. 1997. Increased release of matrix metalloproteinase-9 in bronchoalveolar lavage fluid and by alveolar macrophgages of asthmatics. *Am. J. Respir. Cell Mol. Biol.* 17, 583-591.

Nagase, H. 1997. Activation mechanisms of matrix metalloproteinases. *Biol. Chem.* 378, 151-160.

Okada, S., H. Kita, T.J. George, G.J. Gleich, and K.M. Leiferman. 1997. Migration of eosinophil through basement membrane components in vitro:role of matrix metalloproteinase-9. *Am. J. Respir. Cell Mol. Biol.* 17, 519-528.

Stetler-Stevenson, W.G., H.C. Krutzsch, and L.A. Liotta. 1989. Tissue inhibitor of metalloproteinase (TIMP-2): A new member of the metalloproteinase family. *J. Biol Chem.* 264, 17374-17378

Sternlicht, M.D. and Z. Werb. 2001. How Matrix Metalloprotein-ases Regulate Cell Behavior. *Ann. Rev. Cell Dev. Biol.* 17, 463-516.

Stricklin, G. and H. Welgus. 1983. Human skin fibroblasts collage-nase inhibitor : purification and biochemical characterization. *J. Biol. Chem.* 258, 12252-12258

Yang, H., S. Han, H. Kim, Y.M. Choi, K.J. Hwang, H.C. Kwon, S.K. Kim, and D.J. Cho. 2002. Expression of integrines, cyclooxygenases and matrix metalloproteinases in three-dimensional human endometrial cell culture system. *Exp. Mol. Med.* 34, 75-82.

Van Eerdewegh, P., R.D. Little, and J. Dupuis. 2002. Association of the ADAM33 gene with asthma and bronchial hyperrespon-siveness. *Nature* 418, 426-430.

Warner, J.A., P. Julius, W. Luttmann, and C. Kroegel. 1997. Matrix metalloproteinases in bronchoalveolar lavage fluid following antigen challenge. *Int. Arch. Allergy Immunol.* 113, 318-320.