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A pivotal role of cytosolic phospholipase A_2 in bleomycin-induced pulmonary fibrosis

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Pulmonary fibrosis is an interstitial disorder of the lung parenchyma whose mechanism is poorly understood. Potential mechanisms include the infiltration of inflammatory cells to the lungs and the generation of pro-inflammatory mediators. In particular, idiopathic pulmonary fibrosis is a progressive and fatal form of the disorder characterized by alveolar inflammation, fibroblast proliferation and collagen deposition. Here, we investigated the role of cytosolic phospholipase A_2 ($cPLA_2$) in pulmonary fibrosis using $cPLA_2$-null mutant mice, as $cPLA_2$ is a key enzyme in the generation of pro-inflammatory eicosanoids. Disruption of the gene encoding $cPLA_2$ (*Pla2g4a*) attenuated IPF and inflammation induced by bleomycin administration. Bleomycin-induced overproduction of thromboxanes and leukotrienes in lung was significantly reduced in $cPLA_2$-null mice. Our data suggest that $cPLA_2$ has an important role in the pathogenesis of pulmonary fibrosis. The inhibition of $cPLA_2$-initiated pathways might provide a novel therapeutic approach to pulmonary fibrosis, for which no pharmaceutical agents are currently available.

Pulmonary fibrosis comprises a group of interstitial disorders of the lung parenchyma[1–5]. Idiopathic pulmonary fibrosis (IPF) is the most common form of the disorder and is characterized by progressive development of alveolar inflammation, fibroblast proliferation and collagen deposition. IPF is clinically manifested as increased lung elasticity, restrictive ventilation impairment and respiratory failure[1]. Although IPF is a progressive and fatal disease, no useful drugs are currently available for its treatment. Current medical intervention for IPF is either lung transplantation or supportive treatment, such as oxygen therapy. Although the molecular mechanisms underlying development of IPF remains to be elucidated, potential mechanisms might be the infiltration of inflammatory cells to the lungs, and the generation of pro-inflammatory and proliferative mediators, leading to fibroblast proliferation and accumulation of extracellular collagen[1,2].

Platelet-activating factor (PAF) and metabolites of arachidonic acid might be involved in pathogenesis of pulmonary fibrosis, although little attention has been paid to these mediators. PAF is a pro-inflammatory phospholipid mediator that has various biological effects including cell proliferation and the production of cytokines and eicosanoids via activation of the G protein–coupled PAF receptor (PAFR)[6–17]. Thromboxanes (Txs) and leukotrienes (LTs) are potent mediators generated from arachidonic acid by cyclooxygenase and 5-lipoxygenase, respectively. TxA_2 may induce lung inflammation, whereas LTB_4 is a potent neutrophil chemoattractant. Cysteinyl LTs (LTC_4, LTD_4 and LTE_4) are among the most important mediators in inflammatory lung diseases. A crucial enzyme for generation of these pro-inflammatory mediators, including eicosanoids and PAF, is phospholipase A_2. In particular, cytosolic phospholipase A_2 ($cPLA_2$) is a key player, as it is activated by submicromolar concentration of Ca^{++} and by phosphorylation by mitogen-activated protein kinases[18–22].

Our aim was to elucidate the role of $cPLA_2$ in the experimental model of pulmonary fibrosis induced by bleomycin. To perform this study, we used mutant mice lacking the gene encoding $cPLA_2$ (ref. 23; also known as *Pla2g4a*) and their wild-type littermates as controls.

Pulmonary fibrosis induced by bleomycin administration

Fig. 1 demonstrates the lung elasticity in bleomycin and saline-treated groups (n = 4–6). In the bleomycin-treated wild-type group, the value of lung elastance (EL) was significantly greater than those in the other groups, whereas there was a no difference in EL values among bleomycin-treated $cPLA_2$-null group and saline-treated groups. In terms of alterations in lung parenchyma, physiological data suggest that bleomycin-induced responses in $cPLA_2$-null mice were significantly reduced compared with their wild-type controls.

We assessed collagen content of the lungs by measuring hydroxyproline-content values. Values in bleomycin-treated wild-type group were significantly greater than those in bleomycin-treated $cPLA_2$-null group (83.6 ± 4.4 and 56.0 ± 3.0 μg per left lung; n = 6, respectively; $P < 0.05$), indicating that bleomycin-induced collagen synthesis in $cPLA_2$-null mice was significantly attenuated compared with bleomycin-treated wild-type group. There was no difference in hydroxyproline content among bleomycin-treated $cPLA_2$-null group and saline-treated groups.

Histopathological assessment of pulmonary fibrosis

Fig. 2 shows lung histology 14 days after bleomycin or saline

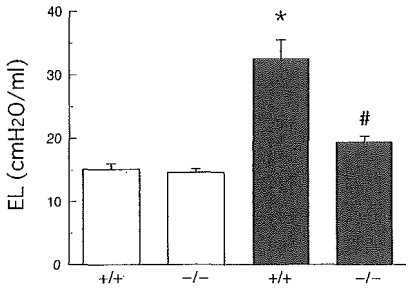

Fig. 1 Physiological roles of $cPLA_2$ in IPF induced by bleomycin treatment. Responses in EL 14 d after the administration of bleomycin (■) or saline (□) in the wild-type (+/+) or $cPLA_2$-null (−/−)mice (n = 4–6). *, $P < 0.001$ versus saline-treated; #, $P < 0.001$ versus bleomycin-treated wild-type group.

administration. Bleomycin treatment elicited alveolar thickening and subpleural fibrous foci in wild-type mice. In $cPLA_2$-null mice, histological changes including alveolar thickening and fibrosis were minimal. We performed further histopathological evaluation of pulmonary fibrosis using an established scoring method[3]. There was a significant difference in the scores of fibrotic lesions between bleomycin-treated wild-type and $cPLA_2$-null mice (Fig. 3).

Bleomycin-induced inflammation

In the wild-type group, bleomycin administration increased protein content and the numbers of macrophages, polymorphonuclear leukocytes (PMNs) and lymphocytes in bronchoalveolar lavage fluid (BALF), indicating bleomycin-induced protein leakage and leukocyte infiltration (Table 1 and Fig. 4). The protein leakage was significantly attenuated in $cPLA_2$-null mice, which had lesser macrophage, PMN and lymphocyte sequestration compared with the control mice (Fig. 4).

Thromboxane and leukotriene content

To assess the biosynthesis of $cPLA_2$ products, we performed BALF assays of TxA_2 (measured as TxB_2), LTB_4 and $LTC_4/D_4/E_4$. Table 1 summarizes the results of BALF TxB_2, LTB_4 and $LTC_4/D_4/E_4$ assays in each experimental group. Each eicosanoid level in bleomycin-treated wild-type mice was significantly greater than that in any other group, whereas any eicosanoid measured in similarly treated $cPLA_2$-null mice was reduced to the same level as the saline-treated groups. Bleomycin administration increased TxB_2, LTB_4 and $LTC_4/D_4/E_4$ levels in BALF, whereas the levels of these eicosanoids were significantly reduced in $cPLA_2$-null mice.

Relative contribution of downstream $cPLA_2$ mediator, PAF

To assess the relative contribution of downstream mediators of $cPLA_2$, we examined the role of PAF in bleomycin-induced pulmonary fibrosis using mutant mice lacking the gene encoding the PAF receptor (*Pafr*$^{-/-}$ mice)[17] and their wild-type littermates as controls. The disruption of *Pafr* reduced the score of fibrotic lesions, although there was a marked difference between bleomycin-treated *Pafr*$^{-/-}$ mice and saline-treated groups (Table 2). However, there was no significant difference in either EL or hydroxyproline content between bleomycin-treated wild-type and *Pafr*$^{-/-}$ mice.

Discussion

Our results suggest that $cPLA_2$ has a pivotal role in the pathogenesis of pulmonary fibrosis. Disruption of the gene encoding $cPLA_2$ significantly attenuated pulmonary fibrosis and inflammation induced by bleomycin. These observations indicate that $cPLA_2$ may be involved in pulmonary fibrosis by mediating overproduction of inflammatory mediators including Txs and LTs.

The fibrotic lung disorders such as IPF are characterized by chronic lung parenchymal injury and inflammation, that is, alveolitis[1,2]. The inflammation of pulmonary fibrosis is associated with infiltration of leukocytes including macrophages, lymphocytes and neutrophils. It is thought that these inflammatory cells might generate and release chemical mediators, leading to proliferation of mesenchymal cells and deposition of extracellular matrix protein. This pathologic process is associated with clinical findings including reduced vital capacity and impaired gas exchange[1]. In investigating the pathogenesis of pulmonary fibrosis, the efficacy of the pharmacological approach is limited as the progression of fibrotic disorders is a chronic and continuous process. We therefore used a mutant mouse model to examine whether $cPLA_2$ would mediate development of IPF.

In bleomycin-treated wild-type mice, we observed increases in EL, pulmonary fibrosis, protein leakage and infiltration of inflammatory cells including macrophages, lymphocytes and

Table 1 Physiological and biochemical data after saline or bleomycin treatment

Mouse type/ Treatment	EL (cmH_2O/ml)	BALF total protein (mg)	BALF total cell counts ($\times 10^4$)	BALF total TxB_2 (ng)	BALF total LTB_4 (ng)	BALF total $LTC_4/D_4/E_4$ (ng)
Wild type Saline	14.5 ± 0.2	0.122 ± 0.006	9.9 ± 0.8	0.011 ± 0.011	0.000 ± 0.000	1.103 ± 0.144
$cPLA_2$-null Saline	15.4 ± 0.8	0.129 ± 0.011	8.5 ± 0.8	0.000 ± 0.000	0.000 ± 0.000	0.281 ± 0.169
Wild type Bleomycin	33.0 ± 3.0*	0.703 ± 0.142†	63.1 ± 6.1*	0.077 ± 0.018*	0.026 ± 0.005*	23.400 ± 5.201*
$cPLA_2$-null Bleomycin	20.0 ± 1.1#	0.224 ± 0.073#	23.4 ± 1.3#	0.015 ± 0.009#	0.000 ± 0.000#	1.838 ± 0.643#

Data collected 7 d after treatment. †, $P < 0.05$; *, $P < 0.01$ versus saline-treated groups; #, $P < 0.01$ versus bleomycin-treated wild-type group (n = 4–7).

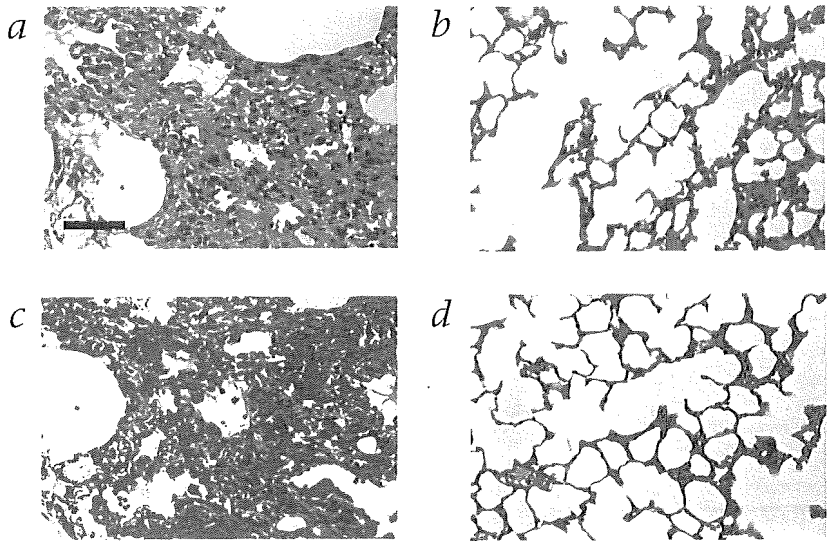

Fig. 2 Photomicrograph of lung tissues from wild-type and $cPLA_2$-null mice 14 d after bleomycin administration. ***a–d***, In wild-type mice, there was marked accumulation of neutrophils and collagen (*a* and *c*), while there were little histological changes in $cPLA_2$-null (*b* and *d*). *a* and *b*, H&E stain; *c* and *d*, Masson's trichrome stain. Scale bar, 50 μm.

neutrophils. Consistently, increases in Txs and LTs were found in the BAL fluid of bleomycin-treated wild-type mice. These observations were markedly attenuated by $cPLA_2$ gene disruption, suggesting that $cPLA_2$ could mediate the process of lung fibrotic disorder via the production of pro-inflammatory mediators. Moreover, $cPLA_2$ products are related to infiltration of various leukocytes, which may lead to the fibrotic disorder. Indeed, increases in leukocytes, especially PMNs, are found in BAL fluid of patients with IPF (ref. 1). It has been reported that lung homogenates from IPF patients contain much higher levels of LTB_4 and LTC_4 compared with non-fibrotic controls[2]. LTB_4 is an important mediator of neutrophil-mediated inflammation including lung injury[24–26]. It is possible that not only PMN, but also macrophages and lymphocytes, may be necessary to induce the bleomycin-induced lung inflammation. The $cPLA_2$-initiated pathways may mediate both infiltration and activation of these leukocytes triggered by bleomycin, resulting in pulmonary fibrosis. Our results suggest that $cPLA_2$ has a dominant role in leukocyte sequestration, and potential interaction between mediators such as PAF and eicosanoids may further upregulate the development of fibrotic process.

To investigate the relative contribution of downstream pathways of $cPLA_2$ mediators, we investigated the role of PAF in bleomycin-induced fibrosis using mutant mice established in our laboratory[17]. We observed that the disruption of the PAF receptor modestly attenuated the histopathological changes induced by bleomycin, although we found no significant differences in physiological data or collagen content between bleomycin-treated mutant mice and controls. These findings suggest that PAF may be potentially involved in the development of bleomycin-induced pulmonary fibrosis. However, the relative contribution of PAF is only partial and limited when compared with the crucial role of $cPLA_2$. We therefore suggest that other downstream mediators of $cPLA_2$, that is, eicosanoids produced by cyclooxygenase and/or lipoxygenase, may also contribute to the development of bleomycin-induced pulmonary fibrosis.

Here we measured TxB_2, LTB_4 and cysteinyl LTs (LTC_4, LTD_4 and LTE_4) in BALF to confirm the generation of $cPLA_2$ products. We observed significant increases in each eicosanoid in the model of bleomycin-induced pulmonary fibrosis, but the $LTC_4/D_4/E_4$ levels were much higher than the other eicosanoids. As there is abundant cysteinyl LT receptors in the lung[27], the coupling of these cysteinyl LTs and receptors may induce inflammatory process including increased permeability and leukocyte infiltration in the lung[28]. Our findings suggest that the cysteinyl LTs might have an important role in the development of pulmonary fibrosis.

Recent studies have shown that $cPLA_2$ may be involved in the pathogenesis of various inflammatory diseases including bronchial asthma and adult respiratory distress syndrome (ARDS)[23,29]. Although there are extreme differences in clinical features between ARDS and IPF, both diseases are fatal disorders with no useful drug therapies. Whereas no effective $cPLA_2$ inhibitors are available, $cPLA_2$ might be a potential target to intervene development of pulmonary fibrosis as well as acute lung injury.

Bleomycin is an antibiotic agent with antitumor activity and commonly used to treat various types of tumors[30]. It has been reported that bleomycin is an essential component of combination chemotherapy in patients with germ-cell tumors[31]. The most important and severe side effects of bleomycin are pulmonary injuries including bleomycin-induced pulmonary fibrosis[30]. In terms of both clinical and pathological features, bleomycin-induced pulmonary fibrosis resembles IPF. Histologically, bleomycin-induced pulmonary fibrosis is associated with infiltration of inflammatory cells into alveoli, focal collagen depositions and fibrotic lesions[30]. Recent studies have shown that bleomycin stimulates cytokine secretion by alveolar macrophage or fibroblast proliferation in human cells[32,33]. However, the exact mechanism underlying the development of bleomycin-induced pulmonary fibrosis also remains to be elucidated.

Notably, although disruption of the gene encoding $cPLA_2$ attenuated bleomycin-induced lung injury, it did not com-

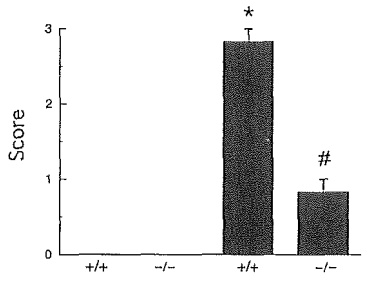

Fig. 3 Histopathological roles of $cPLA_2$ in pulmonary fibrosis induced by bleomycin treatment. Using a scoring method[3], quantitative assessment was performed 7 d after the administration of bleomycin (■) or saline (□) in the wild-type (+/+) or $cPLA_2$-null (−/−) mice ($n = 4–6$). *, $P < 0.001$ versus saline-treated; #, $P < 0.001$ versus bleomycin-treated wild-type group.

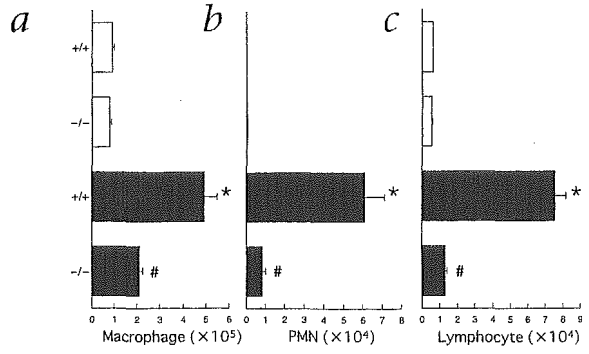

Fig. 4 Roles of $cPLA_2$ in leukocyte infiltration associated with pulmonary fibrosis induced by bleomycin treatment. ***a–c***, The numbers of macrophages (*a*), PMNs (*b*) and lymphocytes (*c*) in BALF are shown. BAL was performed 7 d after the administration of bleomycin (■) or saline (□) in the wild-type (+/+) or $cPLA_2$-null (–/–) mice (n = 4–7). *, $P < 0.01$ versus saline-treated; #, $P < 0.01$ versus bleomycin-treated wild-type group.

pletely abolish the pathology. Indeed, we found a significant difference in histopathologic scores of fibrosis between bleomycin-treated $cPLA_2$-null mice and saline-treated controls. This observation indicates that factors other than $cPLA_2$ should also be involved in the process of pulmonary fibrosis. It has been postulated that oxygen radicals, adhesion molecules, plasminogen-activator inhibitor and cytokines are also involved in this mechanism[1–5]. Because no pharmacological agents are available to treat pulmonary fibrosis and increase survival rates, these factors are also potential targets to develop agents. Our data suggest that the intervention of $cPLA_2$ may also be a promising tool to improve management of pulmonary fibrosis.

In summary, the disruption of $cPLA_2$ significantly attenuated lung inflammation and fibrosis induced by bleomycin treatment. $cPLA_2$ pathways might be involved in the pathogenesis of pulmonary fibrosis caused by bleomycin, and inhibition of these pathways may provide a novel and potential therapeutic approach to pulmonary fibrosis.

Methods

Mice. $cPLA_2$-null mice were established by gene targeting[23]. Mice heterozygous for $cPLA_2$ mutant allele with the genetic background of the C57BL/6J × 129/Ola hybrid were mated. *Pafr*$^{-/-}$ mice were also established as reported[17,34,35]. Offspring were genotyped at 4 wk of age. For genotyping, genomic DNAs were isolated from biopsied tail and subjected to PCR amplification[23]. The animals were maintained on a light/dark cycle with light from 7:00 to 20:00 at 25 °C. Mice were fed with a standard laboratory diet and water *ad libitum*. Mutant homozygous mice and their littermate homozygous controls were used in this study. All animal experiments were approved by the University of Tokyo Ethics Committee for Animal Experiments.

Table 2 Roles of PAF in bleomycin-induced pulmonary fibrosis

Mouse type/ Treatment	EL (cm H_2O/ml)	Hydroxyproline value (μg/left lung)	Score of fibrotic lesions
Wild type			
Saline	14.8 ± 1.1	43.2 ± 3.3	0.0 ± 0.0
Pafr$^{-/-}$			
Saline	14.1 ± 1.1	43.0 ± 3.7	0.0 ± 0.0
Wild type			
Bleomycin	32.5 ± 2.2*	82.0 ± 7.2†	2.8 ± 0.2*
Pafr$^{-/-}$			
Bleomycin	26.9 ± 2.3†	59.8 ± 12.1	2.0 ± 0.0*#

†, $P < 0.05$; *, $P < 0.001$ versus saline-treated groups; #, $P < 0.05$ versus bleomycin-treated wild-type group (n = 4–5).

Experimental pulmonary fibrosis induced by bleomycin administration. Mice were anesthetized with ketamine and tracheostomized. Then, 1.5 ml/kg solution containing bleomycin (5 mg/kg, Nippon Kayaku, Tokyo, Japan) was intratracheally administered. Control animals were treated with saline instead of bleomycin in the same manner. These procedures were performed in a sterile environment. To assess the development of pulmonary fibrosis physiologically, EL (a reciprocal of lung compliance) was measured 7 or 14 d after bleomycin or saline administration as described[36–40]. Briefly, we measured the tracheal pressure (*Ptr*), flow and volume (*V*). EL and lung resistance (RL, data not shown) were calculated by adjusting the equation of motion: $Ptr = EL(V) + RL(dV/dt) + K$, where K was a constant. Values in EL reflect lung parenchymal alterations and stiffening of the lungs.

Assessment of collagen synthesis. To assess collagen synthesis, a hydroxyproline assay was performed. Mice were anesthetized and the lungs were removed 14 d after bleomycin or saline treatment. The left lung was then excised and hydroxyproline content was measured as reported[3].

Histopathological assessment of pulmonary fibrosis. The right lung of each animal was excised 14 d after bleomycin or saline treatment. Following fixation, the tissue blocks obtained from midsagittal slices of the lungs were embedded in paraffin. The slices were stained with H&E or with Masson's trichrome stain. Histopathological evaluation of pulmonary fibrosis was performed using a scoring method as described[3]. Briefly, the scores of fibrotic lesions were defined as follows: 0, absence of lesion; 1, occasional small localized subpleural foci; 2, thickening of interalveolar septa and subpleural foci; 3, thickened continuous subpleural fibrosis and interalveolar septa.

Bronchoalveolar lavage fluid. 7 d after bleomycin or saline treatment, bronchoalveolar lavage (BAL) was performed (1 ml PBS × 5). In each animal, 90% (4.5 ml) of the total injected volume was consistently recovered. After BAL fluid was centrifuged at 450*g* for 10 min, the total and differential cell counts of the BAL fluid were determined from the cell fraction. The supernatant was used for protein determination by Lowry's method using BSA as a standard.

Thromboxane and leukotriene assay. TxA_2 (measured as TxB_2), LTB_4 and $LTC_4/D_4/E_4$ in the BAL fluid were determined by using enzymeimmunoassay (EIA) kits (Amersham Pharmacia Biotech, Piscataway, New Jersey). The detection limits of the EIA assays for TxB_2, LTB_4 and $LTC_4/D_4/E_4$ were 3.6, 6 and 10 pg/ml, respectively.

Data analysis. Comparisons of data among each experimental group were carried out with analysis of variance (Scheffé test). Data are expressed as means ± s.e. P values less than 0.05 were considered significant.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Airway Hyperresponsiveness in Transgenic Mice Overexpressing Platelet Activating Factor Receptor Is Mediated by an Atropine-Sensitive Pathway

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Platelet activating factor (PAF) is a potent mediator potentially involved in the pathogenesis of inflammatory disorders, including bronchial asthma. Recently, transgenic mice overexpressing the PAF receptor (PAFR) gene have been established, and exhibit bronchial hyperresponsiveness, one of the cardinal features of asthma. To elucidate the molecular and pathophysiologic mechanisms underlying PAF-associated bronchial hyperreactivity, we studied airway responsiveness to methacholine (MCh) and serotonin (5-hydroxytryptamine; 5-HT) in PAFR-transgenic mice. In addition, we examined the role of the muscarinic receptor in PAF-induced responses and the binding activities of the muscarinic receptor. The PAFR-transgenic mice exhibited hyperresponsiveness to MCh and PAF; however, no significant differences in 5-HT responsiveness were observed between the control and PAFR-transgenic mice. The administration of atropine significantly blocked PAF-induced responses in PAFR-transgenic mice. There were no differences between the two phenotypes in the binding activities of muscarinic receptor. Morphometric analyses demonstrated that PAFR overexpression did not affect airway structure. These findings suggest that the muscarinic pathway may have a key role in airway hyperresponsiveness associated with PAFR gene overexpression. More generally, PAFR-transgenic mice may provide appropriate models for study of the molecular mechanisms underlying PAF-associated diseases.

Keywords: platelet activating factor; asthma; airway hyperresponsiveness; transgenic mouse

Platelet activating factor (PAF) is a proinflammatory phospholipid mediator that has various potent properties (1–4). PAF mediates its biologic effects via activation of a G-protein–coupled, seven transmembrane receptor (1, 3–5). Complementary DNAs (cDNAs) and genes for the PAF receptor (PAFR) have been cloned from various species, including guinea pigs and humans (5–11). To examine the pathophysiologic role of PAF *in vivo*, we have established transgenic mice ubiquitously overexpressing PAF receptor (12). Recently, we also established a mutant mouse lacking PAFR, and demonstrated that PAF could be involved in anaphylactic responses (13).

It has been indicated that PAF plays a substantial role in the pathogenesis of bronchial asthma (14, 15). Moreover, it has been demonstrated that the level of expression of PAFR messenger RNA (mRNA) in the lung is increased in humans with asthma (16). In asthmatic children, deficiency of plasma PAF acetylhydrolase is associated with respiratory dysfunction (17, 18), and in a mouse model of asthma, recombinant PAF acetylhydrolase inhibits airway inflammation and hyperreactivity (19). Bronchial hyperreactivity, which is a major characteristic of asthma, is augmented by the administration of exogenous PAF (20). However, the exact pathophysiologic roles of PAF in the pathogenesis of bronchial asthma remain to be elucidated.

In the current study, we investigated the pathophysiologic mechanisms underlying PAF-associated airway hyperresponsiveness (AHR). We studied the airway responsiveness of PAFR-transgenic mice to methacholine (MCh) and serotonin (5-hydroxytryptamine; 5-HT). We then examined the involvement of the muscarinic pathway in PAF-induced responses, and performed binding assays for muscarinic receptor. We further assessed airway structure, including smooth muscle, using morphometry.

METHODS

Mice

PAFR-transgenic mice were established as previously described (12). Mutant mice and their littermate controls were used in this study.

Animal Preparation

Animals were anesthetized with pentobarbital sodium (25 mg/kg, intraperitoneally) and ketamine hydrochloride (25 mg/kg, intraperitoneally) in combination, and were mechanically ventilated with tidal volumes of 10 ml/kg, respiratory frequencies of 2.5 Hz and a positive end-expiratory pressure of 2 cm H_2O. The thorax was widely opened by means of a midline sternotomy, but the vagus nerve was not sectioned. Lung resistance (R_L) and elastance (E_L) were measured as previously described (21–23).

Airway Responsiveness to 5-HT or MCh

Following baseline measurement, each dose of 5-HT aerosol (0.15 to 10 mg/ml) or MCh aerosol (0.63 to 80 mg/ml) was administered for 1 min in a dose–response manner. As previously reported (22, 23), airway responsiveness to 5-HT or MCh was assessed from the concentration of agonists required to increase R_L to 200% of baseline values (EC_{200} R_L).

Airway Responsiveness to PAF

Following baseline measurement, each dose of PAF (5 to 20 μg/kg) was administered intravenously and measurements were made.

Effects of Atropine on PAF-Induced Responses

Two minutes before receiving the intravenous bolus of 10 μg/kg PAF, mice were intraperitoneally pretreated with either saline or 10 μmol/kg atropine sulfate. After baseline measurements, PAF was administered via the jugular vein and measurements were made.

Binding Assays for Muscarinic Receptor

From each mouse preparation, five or six whole lungs were obtained and homogenized together in a ×10 volume of binding buffer (25 mM 4-(2-hydroxyethyl)-1-piperazine-*N*′-2-ethanesulfonic acid [pH 7.4], 0.25 M sucrose, 10 mM $MgCl_2$) containing a proteinase inhibitor cocktail (Complete; Roche, Mannheim, Germany) and 20 μM amidino phenyl methyl sulfonyl fluoride (Sigma, St. Louis, MO). After centrifugation (800 × *g*

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for 20 min at 4° C), the supernatants were further centrifuged at 123,000 × *g* for 1 h. The pellets were mashed once and recentrifuged at 140,000 × *g* for 1 h. The final pellets were resuspended in the same buffer. The binding assays were done in duplicate in the absence or presence of 50 nM PAF. The reaction mixture consisted of 150 μl of the binding buffer containing [*N*-methyl-^{3}H]scopolamine (2.51 TBq/mmol), a nonselective muscarinic antagonist, at increasing concentrations of 0.0625 to 2 nM, and 50 μl of the membrane protein (95 μg). The mixtures were incubated in a 96-well microplate at 25° C for 1.5 h, followed by filtration and washing as described previously (24). The radioactivities of dried filters were determined as reported (24). Nonspecific binding was defined as the binding measured in the presence of 20 μM methacholine.

Binding Assays for PAFR

The lung membrane was obtained as described previously. Binding assays for PAFR were performed in the same manner as previously described, with [^{3}H]-WEB2086, a PAFR-selective antagonist, at increasing concentrations at 3.13 to 100 nM (703 GBq/mmol). Nonspecific binding was defined as the binding in the presence of 50 μM cold WEB2086.

Morphometric Study

In four animals from each group, we quantitated airway smooth muscle and lamina propria, using morphometric techniques as previously reported (25, 26).

Data Analysis

Comparisons of physiologic and morphometric data among the experimental groups were done through analysis of variance (Scheffe's test) or Student's *t* test. Data are expressed as mean ± SE. Values of $p < 0.05$ were taken as significant.

RESULTS

Airway Responsiveness to 5-HT or MCh Administration

The control and PAFR-transgenic mice showed no significant differences in baseline EL and RL. Figure 1 shows 5-HT dose–response curves for EL in the two groups. There was no difference in elastic responses to 5-HT. MCh dose–response curves for EL are shown in Figure 2. Responses in the PAFR-transgenic mice were significantly greater than in the control group at MCh doses of > 2.5 mg/ml.

Responses for RL are summarized in Figure 3. As shown, there were no differences between the two groups in 5-HT responsiveness. However, airway responsiveness to MCh in PAFR-transgenic mice was significantly greater than in control mice ($logEC_{200}$ RL = 0.950 ± 0.076 versus 1.462 ± 0.039, $p < 0.001$).

Airway Responsiveness to PAF

Following the administration of 20 μg/kg PAF to five PAFR-transgenic mice, two animals died immediately from cardiac arrest, whereas no animals died in the other groups. Responses for EL and RL are shown in Figure 4. After administration of each dose of PAF (5 to 20 μg/kg), significant differences in pulmonary responses were observed between the control and PAFR-transgenic mice, and the effects of PAF were enhanced at higher doses in the PAFR-transgenic group.

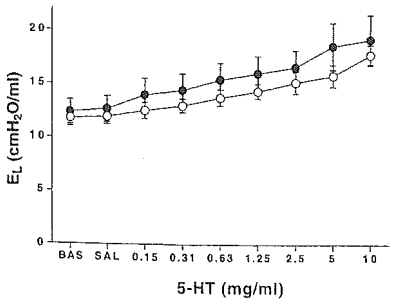

Figure 1. Serotonin (5-HT) dose–response curves for EL in control and PAFR-transgenic mice (n = 4 for each group). BAS = baseline; SAL = saline. -○- Control; -●- Transgenic.

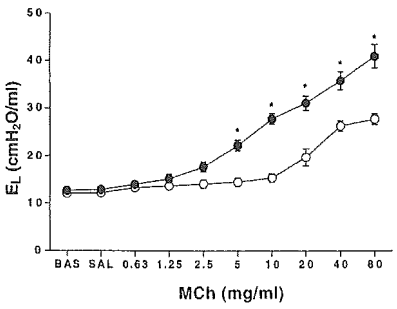

Figure 2. MCh dose–response curves for EL in control and PAFR-transgenic mice (n = 7 for each group). *p < 0.01 compared with control mice. BAS = baseline; SAL = saline. -○- Control; -●- Transgenic.

Effects of Atropine on PAF-Induced Pulmonary Responses

Effects of atropine on PAF-induced pulmonary responses are demonstrated in Figure 5. As shown, the control mice exhibited no responses to 10 μg/kg PAF after either saline or atropine pretreatment. In the PAFR-transgenic mice pretreated with saline, PAF administration induced increases in RL and EL. Pretreatment with atropine significantly inhibited PAF-induced responses in the PAFR-transgenic mice.

Binding Assays for Muscarinic Receptor and PAFR

Results of binding assays for muscarinic receptor are summarized in Table 1. In either the absence or presence of PAF, there were no differences between the control and PAFR-transgenic mice in dissociation constant (*K*d) or binding maximum (Bmax) values for [*N*-methyl-^{3}H]scopolamine, a nonselective muscarinic antagonist, suggesting that the binding activities for muscarinic receptors in the PAFR-transgenic lungs were similar to those in the controls.

As shown in Figure 6, the binding activities for PAFR in the PAFR-transgenic lungs were markedly greater than in the control lungs.

Morphometric Study

Table 2 summarizes the morphometric data for airway size and roundness. There were no significant differences between the PAFR and control mice in number of airways, airway size, or airway roundness, indicating that there were no significant biases between the experimental groups in terms of airway selection.

As shown in Figure 7, there were no significant differences between the PAFR and control groups in thickness of the lamina propria or airway smooth muscle. In addition, no significant difference in inner wall area was observed between the

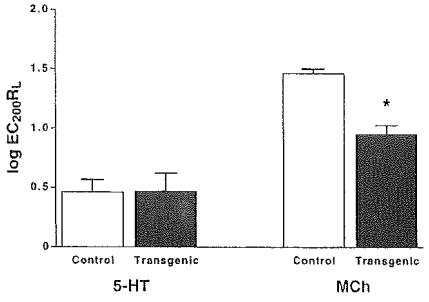

Figure 3. Airway responsiveness expressed as the concentration of agonists required to double lung resistance (EC_{200} RL). *p < 0.001 compared with control mice. 5-HT = serotonin; MCh = methacholine.

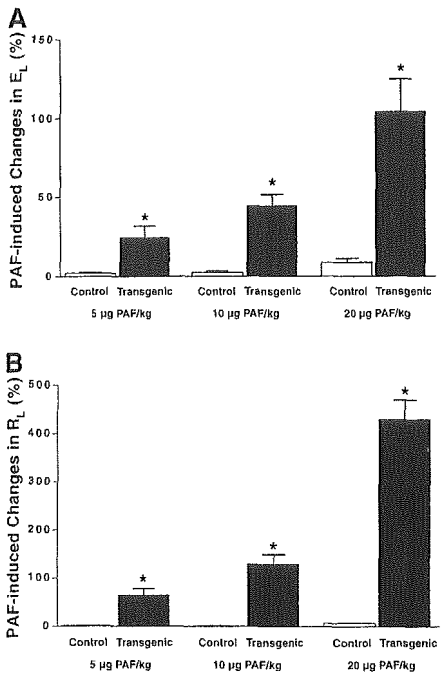

Figure 4. PAF-induced responses in EL (*A*) and RL (*B*) in control and PAFR-transgenic mice (n = 3 or 4 for each group). *p < 0.001 compared with control mice.

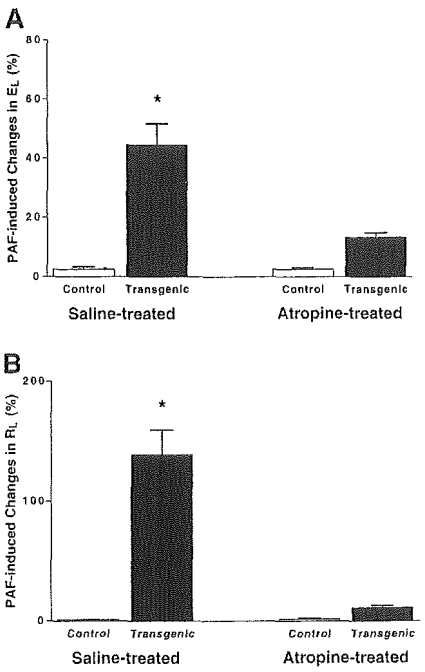

Figure 5. Effects of atropine on PAF-induced responses in EL (*A*) and RL (*B*) (n = 4 for each group). *p < 0.001 compared with other groups.

control and PAFR-transgenic mice (0.224 ± 0.018 and 0.215 ± 0.015, respectively), suggesting that the airway structure of PAFR-transgenic mice is not altered as compared with that of control mice.

DISCUSSION

The results of the experiments reported here show that airway responsiveness to MCh and PAF in PAFR-transgenic mice was significantly greater than in control mice. Blockade of the muscarinic pathway with atropine inhibited PAF-induced responses in the PAFR-transgenic mice, whereas the overexpression of PAFR did not change the binding activity of muscarinic receptor. Similarly, airway responsiveness to 5-HT was unaffected by overexpression of the PAFR gene, and morphometric analyses showed that airway structure, including airway smooth muscle, was not altered by overexpression of the PAFR gene. These findings suggest that the muscarinic pathway may have a key role in AHR associated with overexpression of the PAFR gene. Thus, expression of the PAFR gene is involved in airway responsiveness to MCh in mice through a functional but not a structural mechanism.

Before the results of the study are discussed, the issue of anesthesia warrants consideration. In the study, it was necessary to reduce the vagal function of the animals used, since effects of the experimental procedures on vagal activity might have affected the results and interpretation of data. We therefore chose to use anesthesia with pentobarbital sodium and ketamine hydrochloride in combination with one another. It has been demonstrated that both pentobarbiturate and ketamine have inhibitory effects on vagal pathways (27–29). Although the vagus nerve was not surgically sectioned, we believe that vagal function was significantly attenuated with the anesthesia applied in this study and that comparisons between the control and PAFR-transgenic mice are meaningful.

The airway responsiveness to MCh of the PAFR-transgenic mice was markedly greater than that of control mice, suggesting that the transgenic mice have an asthmalike phenotype. There was also a marked difference in MCh-induced changes in lung elasticity between the asthmalike and normal phenotypes (Figure 2). It has been reported that changes in EL reflect lung parenchymal alterations and stiffening of the lungs induced by various contractile stimuli (30), although the contraction of conducting airways can also cause changes in EL (31). By comparison, increases in RL represent decreases in airway luminal cross-sectional area (30). In the present study, enhanced responses in both EL and RL to MCh administration were observed in the PAFR-transgenic mice, suggesting that overexpression of the PAFR gene elicits increased responses to MCh in both the lung parenchyma and airways.

It has been postulated that PAF may be related to AHR in various species, including human (14, 15). The administration of exogenous PAF increases airway responsiveness in humans (20), whereas a specific PAFR antagonist (Y-24180) reduces AHR to MCh in asthmatic patients (32). In their recent study, Henderson and colleagues (19) found that increasing plasma levels of PAF acetylhydrolase through its administration was effective in blocking late-phase pulmonary inflammation in a murine model of asthma. However, the exact mechanism for the involvement of PAF in AHR remains to be clarified. In the current study, the molecular and pathophysiologic mechanisms underlying AHR were examined with PAFR-transgenic mice overexpressing the PAFR gene.

One possible mechanism for the involvement of PAF in AHR is that PAF and PAFR gene expression affect airway structure, and especially the lamina propria and airway smooth muscle. Airway remodeling, including thickening of airway smooth muscle, is a feature in asthmatic subjects and could be

TABLE 1. RESULTS OF BINDING ASSAYS FOR MUSCARINIC RECEPTOR

	Kd (*nM*)	Bmax (*fmol/mg protein*)
Control membrane + vehicle*	0.51 ± 0.04	109.2 ± 16.3
Control membrane + PAF*	0.55 ± 0.02	122.2 ± 21.2
PAFR-transgenic membrane + vehicle*	0.56 ± 0.06	130.3 ± 2.3
PAFR-transgenic membrane + PAF*	0.54 ± 0.06	127.4 ± 0.3

Definition of abbreviations: Bmax = binding maximum; Kd = dissociation constant for PAF from PAF receptor; PAF = platelet activating factor; PAFR = platelet activating factor receptor.

* n = 3 for each group.

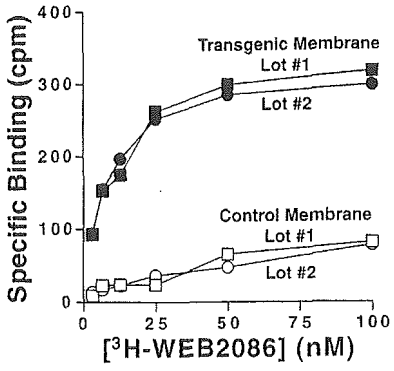

Figure 6. Binding assays for PAFR in control and PAFR-transgenic mouse lung membranes, showing isoforms for binding of [^{3}H]-labelled WEB2086 to membrane fractions. Because of low specific binding to control membranes, Scatchard analysis was not performed. Results shown are means from duplicate wells. Two independent membrane fractions (Lots #1 and #2), from whole lungs for each genotype, were used.

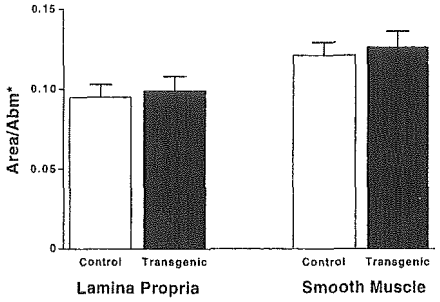

Figure 7. Thickness of lamina propria and airway smooth muscle in control and PAFR-transgenic mice. Area/Abm* = area normalized with ideally relaxed airway size.

involved in bronchial hyperresponsiveness (33, 34). Lambert and Paré have shown that a marked increase in airway responsiveness is theoretically induced by thickening of the airway wall, including its smooth muscle layer (33). In the current study, however, no significant difference in thickness of either the lamina propria or airway smooth muscle was observed in the control as opposed to the PAFR-transgenic mice. These results suggest that overexpression of the PAFR gene has little effect on airway remodeling in mice. Whereas PAF may have proliferative effects on various cells (35), it seems that in the mouse model used in our study, the effect of PAF on airway smooth muscle proliferation is not remarkable. In this model, AHR elicited by overexpression of the PAFR gene may be associated with airway dysfunction, but not with airway remodeling.

It has been shown that in PAFR-transgenic mice, a high level of transgenic mRNA exists in the trachea (12), and one could assume from this that airway smooth-muscle cells overexpressing PAFR may play a role in PAF-induced bronchopulmonary responses. Consistently, the binding activities for PAFR in the PAFR-transgenic mouse lungs in our study were remarkably augmented in comparison with those of the controls. After each dose of PAF given *in vivo*, marked pulmonary responses were observed in the PAFR-transgenic mice as compared with the controls. Notably, the effects of PAF were more remarkable at the higher doses in the PAFR-transgenic mice, indicating that PAF *per se* has a significant role in the AHR of PAFR-transgenic mice to PAF. On the other hand, blockade of the muscarinic pathway completely ablated PAF-induced responses in the PAFR-transgenic mice. This indicates that PAF-induced bronchoconstriction is mediated by an atropine-sensitive pathway. Stimler-Gerard (36) has postulated that PAF may stimulate neural elements proximal to the end plate to release constrictive neurotransmitters including acetylcholine, which might explain the mechanism for the findings in our study.

TABLE 2. MORPHOMETRIC DATA FOR AIRWAYS

	Control Mice	PAFR-Transgenic Mice
Total number of airways	40	40
Number of airways obtained from individual animals	10.0 ± 0.7	10.0 ± 0.4
Pbm, mm	0.780 ± 0.039	0.772 ± 0.040
D_2/D_1	0.831 ± 0.015	0.826 ± 0.015

Definition of abbreviations: D_2/D_1 = index of airway roundness (airways with a D_1/D_2 ratio > 0.6 were analyzed); PAFR = platelet activating factor receptor; Pbm = length of basement membrane.

The results of the binding assays indicate that PAFR overexpression *per se* has little effect on the binding activity of muscarinic receptors in either the absence or presence of PAF. The interaction between PAF and muscarinic receptors may therefore instead occur through an indirect pathway. It has been reported that PAF may affect the biologic actions of other potent mediators, such as thromboxanes and leukotrienes, which are involved in AHR (37). Potentially, overexpression of the PAFR gene may modulate the production levels of these potent mediators.

Airway responsiveness to 5-HT was not affected by overexpression of the PAFR gene. This observation suggests that PAF and the PAFR gene may be specifically related to bronchial responsiveness to MCh, but not to 5-HT. Recently, it was demonstrated that AHR induced by 5-HT and acetylcholine is inherited independently in mice, and that murine nonspecific AHR is determined by multiple genes (38). The results of the present study indicate that mutation of the PAFR gene affects muscarinic receptor-specific responsiveness, but not general bronchial responsiveness.

Genetic features are potentially associated with the etiology of asthma. On the basis of the inheritance pattern of bronchial asthma a number of genes could have substantial roles in its pathogenesis (39). Murine models of asthma have recently been used to investigate individual genes associated with AHR (40). Since PAF may be one of the potent mediators involved in bronchial asthma (14, 15, 20), genes regulating the function and metabolism of PAF could be targets of study in the pathogenesis of asthma. These genes consist of the PAFR gene (11, 41) and genes encoding PAF-metabolic enzymes, including PAF acetylhydrolases (42, 43). The PAFR-transgenic mice used in the present study may contribute to study of the genetic roles of PAF in bronchial asthma.

PAF has pleiotropic and pathophysiologic effects on various cells and organs (1–4). It exerts its actions at concentrations as low as 10^{-12} M in some cells, and almost always at levels of at least 10^{-9} M as an intercellular messenger (2). PAF activates phospholipase A_2 (PLA_2), protein kinase C, protein tyrosine phosphorylation, and gene expression (1, 2, 44). It has been shown that PAFR knockout mice exhibit markedly reduced anaphylactic responses as compared with wild-type mice, suggesting that PAF plays an important role in the development of anaphylactic shock (13). On the other hand, PAFR-transgenic mice show an increased lethality to bacterial lipopolysaccharide endotoxin (12). Melanogenesis and melanocytic proliferation were observed in the skin of PAFR-transgenic mice, and melanoma was occasionally seen in aged PAFR-transgenic mice (12). Recent studies done with genetically-engineered mice have shown that cytosolic PLA_2 ($cPLA_2$) is essential in the production of PAF (24, 45), indicat-

ing that both $cPLA_2$ and PAF are key mediators in the development of inflammatory disorders. For example, both $cPLA_2$ and PAF play significant roles in the molecular mechanism underlying acute lung injury (46–48). The PAFR-transgenic mice used in our study may further provide novel insights for study of the pathophysiologic roles of PAF and PAFR *in vivo*.

In summary, the PAFR-transgenic mice in our study exhibited hyperresponsiveness to MCh and PAF, but not to 5-HT. The muscarinic pathway may have a key role in PAF-induced responses in these PAFR-transgenic mice. Meanwhile, binding activities for muscarinic receptor were not altered by the overexpression of PAFR. We observed no differences in airway structure between the control and PAFR-transgenic mice to suggest that PAFR gene overexpression would be involved in MCh airway responsiveness by acting as a functional mediator. The PAFR-transgenic mice may provide appropriate models for studying molecular and pathophysiologic mechanisms underlying diseases related to PAF metabolism.

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Resistance to Neointimal Hyperplasia and Fatty Streak Formation in Mice With Adrenomedullin Overexpression

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Objective—Several in vitro studies have implicated that adrenomedullin (AM) plays an important role in the pathogenesis of vascular injury and fatty streak formation. To test this possibility in vivo, we evaluated 2 experimental models using transgenic mice overexpressing AM in a vessel-selective manner (AMTg mice).

Methods and Results—Placement of a periarterial cuff on femoral arteries resulted in neointimal formation at 2 to 4 weeks to a lesser extent in AMTg mice than in their wild-type littermates (at 28 days, intima/media area ratio 0.45±0.14 versus 1.31±0.41, respectively; $P<0.001$). This vasculoprotective effect observed in AMTg mice was inhibited by N^{ω}-nitro-L-arginine methyl ester. We further examined the effect of AM on hypercholesterolemia-induced fatty streak formation by crossing AMTg mice with apolipoprotein E knockout mice (ApoEKO mice). The extent of the formation of fatty streak lesions was significantly less in ApoEKO/AMTg mice than in ApoEKO mice (percent lesion area 12.0±3.9% versus 15.8±2.8%, respectively; $P<0.05$). Moreover, endothelium-dependent vasodilatation as indicative of NO production was superior in AMTg/ApoEKO mice compared with ApoEKO mice.

Conclusions—Taken together, our data demonstrated that AM possesses a vasculoprotective effect in vivo, which is at least partially mediated by NO. (***Arterioscler Thromb Vasc Biol.* 2002;22:1310-1315.**)

Key Words: adrenomedullin ■ transgenic mice ■ neointimal hyperplasia ■ fatty streak ■ apolipoprotein E knockout mice

Many vasoactive factors derived from endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are known to regulate regional vascular tone as well as cellular proliferation and migration, thereby affecting vascular structure. Dysregulated production of these factors is assumed to make a contribution to pathological states, such as hypertension, atherosclerosis, and postangioplasty restenosis.

Adrenomedullin (AM), originally identified as a potent vasodilatory peptide produced by human pheochromocytoma,[1] has been shown to be released from ECs[2] and, to a lesser extent, VSMCs.[3] In addition to its direct[4] and NO-mediated[5] vasodilatory activities, in vitro studies have demonstrated its direct inhibitory effect on the migration[6] and proliferation[7] of VSMCs and its antiapoptotic effect on ECs[8,9] via NO. Therefore, it is suggested that AM has a compensatory effect on deteriorating physiological status and a protective effect against vascular injury as a possible counterpart against vasoconstrictive and mitogenic peptides, including angiotensin II and endothelin-1. However, direct evidence for such in vivo roles has still not been well documented.

Recently, we established transgenic mice overexpressing AM (AMTg mice) in a vessel-selective manner driven by the murine preproendothelin-1 promoter.[10] AMTg mice exhibited an increase in AM levels by 2- to 4-fold in the plasma and major organs and by 2- to 8-fold in the aorta by radioimmunoassay. Immunohistochemistry detected intense AM signals in both ECs and VSMCs in the aorta and major arteries as well as in arterioles in vascular-rich organs, including the heart, kidney, and lung. Concerning hemodynamics, chronic AM overexpression in the vessel wall resulted in decreased blood pressure through, at least in part, the stimulation of NO production. We postulated that these mice could also be useful for analyzing the role of AM in the pathophysiological process associated with vascular injury and atherosclerosis. In the present study, we compared the formation of injury-induced neointimal hyperplasia and hypercholesterolemia-

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induced fatty streak formation between mice with and without AM overproduction to clarify whether AM could be involved in the protective mechanisms in the vasculature.

Methods

The experiments were performed in accordance with the Declaration of Helsinki and the University of Tokyo Institutional Guidelines for Animal Experiments. The mice were housed in an animal room with a 12-hour light-dark cycle and a temperature of 22°C. The mice were given standard chow and water ad libitum, unless otherwise indicated.

Animals

The AMTg mouse line has already been established as previously described.[10] We crossed AMTg mice with apoE knockout (ApoEKO) mice.[11] To obtain AMTg/ApoEKO mice, homozygous AMTg mice were bred with homozygous ApoEKO mice. The resultant offspring, which overexpressed AM and were obligatorily heterozygous for the mutant apoE locus, were further bred with each other to obtain homozygous ApoEKO mice with or without the AM transgene. The parental ApoEKO mice were F2 hybrids between 129/Sv and C57BL/6, and the parental AM transgenic mice originated from hybrids from the B6C3F1 and C3H strains, which were backcrossed for at least 5 generations into the original background of the ApoEKO mice.

Hemodynamics

We evaluated blood pressure with a programmable sphygmomanometer (BP98 Softron) by use of the tail-cuff method.[12] All measurements were repeated 10 times for each animal, and the average was obtained. To evaluate the effect of blood pressure reduction, several mice were treated with hydralazine at a dose of 5 mg/kg per day in their drinking water.[13]

Vascular Injury by Cuff Placement

We used 10- to 12-week-old AMTg and wild-type mice from the same genetic background. The cuff placement surgery was performed according to a method described previously.[14,15] In brief, the mice were anesthetized with ketamine (70 mg/kg) and xylazine (4 mg/kg) by intraperitoneal injection. The left femoral artery was isolated from the surrounding tissues. A polyethylene tube (PE-50, 2 mm long, inner diameter 0.56 mm, outer diameter 0.965 mm; Becton-Dickinson) was cut longitudinally, loosely placed around the artery, and closed with a suture. After the experimental period, the mice were killed, and arterial tissues were fixed in 10% formalin and embedded in paraffin. The middle segment of the artery was cut into 5 subserial cross sections at intervals of 200 μm. The sections were stained by elastica van Gieson or hematoxylin and eosin staining. The areas of the neointima, media, and adventitia were measured by using image-analyzing software (NIH image). The average of 5 sections was taken as a representative value for each animal. To evaluate DNA synthesis, bromodeoxyuridine (BrdU, Sigma Chemical Co) was injected at doses of 100 mg/kg SC and 30 mg/kg IP 18 hours before euthanasia and then at a dose of 30 mg/kg IP 12 hours before euthanasia.[16] Immunohistochemistry using anti-BrdU antibody in serial sections was performed (BrdU Staining Kit, Zymed Laboratories), and the BrdU index (the ratio of BrdU-positive nuclei versus total nuclei) was calculated.

To evaluate the chronic inhibition of NO synthase, N^{ω}-nitro-L-arginine methyl ester (L-NAME) was dissolved in the drinking water at a concentration of 200 mg/L[17] for 28 days. Moreover, several mice were treated with the AM receptor antagonist CGRP(8-37) to evaluate the effect of AM antagonism. Alzet micro-osmotic pumps (model 1002, Alza) were implanted intraperitoneally at the time of cuff placement. The pumps delivered vehicle (saline) or CGRP(8-37) (20 mg/kg per day, Peptide Institute)[18] continuously for 28 days at a rate of 0.125 μL/h.

Evaluation of Fatty Streak Formation

Two diets were used: (1) a normal chow diet (MF diet from Oriental Yeast Co) that contained 5.6% (wt/wt) fat with 0.09% (wt/wt) cholesterol, and (2) an atherogenic diet, which consisted of the MF diet containing 0.15% (wt/wt) cholesterol and 15% (wt/wt) butter.[19] Eight-week-old ApoEKO/AMTg and ApoEKO mice were fed the atherogenic diet for 2 months and euthanized by an overdose of anesthetic, and the extent of fatty streak formation was evaluated by 2 methods: (1) the en face surface lesion area[20] and (2) the cross-sectional lesion area of aortic origin.[21] In brief, for evaluation of the entire aorta, the aorta from the aortic sinus to the iliac bifurcation was dissected, and lipid-rich atheroma were visualized by staining with Sudan IV. As cross-sectional lesions of the aortic sinuses, 4 serial sections at intervals of 60 μm were prepared, stained with oil red O, and counterstained with hematoxylin.

Vascular Reactivity to Endothelium-Dependent and -Independent Vasodilators

Vascular reactivity to endothelium-dependent and -independent vasodilators was tested by using the descending thoracic aorta to evaluate endothelial function as previously described.[22] The aortic rings were suspended under 1.0 g of tension and preconstricted with phenylephrine (5×10^{-8} mol/L). Acetylcholine (ACh) (10^{-9} to 10^{-5} mol/L) and sodium nitroprusside (SNP, 10^{-8} to 10^{-5} mol/L) were added cumulatively to the organ bath. For ACh-induced vasodilatation after blockade of NO production, L-NAME (10^{-5} mol/L) was administered to evaluate endogenous NO production. Data are expressed as the percentage relaxation of phenylephrine-induced preconstriction.

Statistical Analysis

Quantitative values are expressed as the mean±SD. Comparisons of means were made by using the Student t test for unpaired values; when >2 means were compared, an ANOVA with repeated measurements was used. If a significant F value was found, the Scheffé post hoc test for multiple comparisons was used to identify any differences among groups. A value of $P<0.05$ was considered significant, unless otherwise indicated. In aortic ring experiments, the negative logarithm of the concentration of ACh or SNP that produced half the putative maximal response was referred to as ED_{50}. Maximum vasorelaxation was shown as E_{max} (a percentage).

Results

Neointimal Formation Induced by Cuff Placement

We compared neointimal formation induced by cuff placement between AMTg and wild-type mice. In contralateral sham-operated arteries, there was no neointima in either the wild-type or AMTg mice (Figure 1A). On the other hand, cuff placement resulted in neointimal hyperplasia, which grew for up to 28 days. Intimal hyperplasia was significantly smaller in AMTg mice than in wild-type mice at 14 days and also at 28 days after surgery (Figure 1A). Twenty-eight days after surgery, the ratio of the neointima area to the intimal/medial area was 16 390±1890 μm^2/1.31±0.41 in wild-type mice (n=10) and 5830±1620 μm^2/0.45±0.14 in AMTg mice (n=10, $P<0.001$; Figure 1B).

For DNA synthesis of VSMCs, we examined BrdU uptake 3 and 7 days after injury. BrdU uptake in subendothelial layers mainly composed of VSMCs was significantly higher in the wild-type mice (n=7) than in the AMTg mice (n=7). BrdU incorporation was relatively high within 1 week after injury and almost completely diminished ≥14 days after injury in AMTg and wild-type mice. These data indicate that AM overexpression inhibited the proliferation of VSMCs (Figure 2A).

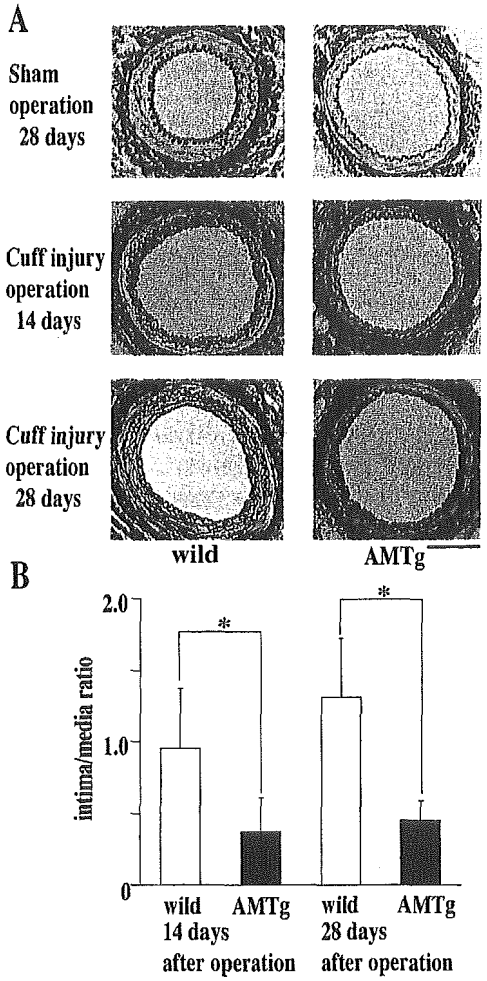

Figure 1. A, Photomicrographs showing representative cross sections of the femoral arteries 14 and 28 days after cuff placement. Sections of 5-μm thickness were stained by elastica van Gieson staining. B, Morphometric analysis of the cuffed femoral arteries. Cross-sectional areas of media and intima were evaluated at 14 days and 28 days after cuff placement, and the intima/media ratio was calculated from the area of intima divided by the area of media. *$P<0.001$.

Inflammatory cell infiltration around the vessels was prominently observed, especially within 1 week after injury. The number of inflammatory cells was not different between AMTg and wild-type mice at 3 and 7 days after injury, indicating that the inflammatory process at the adventitia may not be affected by AM overexpression (Figure 2B).

Effect of L-NAME, Hydralazine, and CGRP(8-37) on Neointimal Hyperplasia

The systolic blood pressure measured by the tail-cuff method was significantly lower in the AMTg mice than in the wild-type mice (101.2±9.3 versus 115.3±13.2 mm Hg, respectively; $P<0.05$). When L-NAME was chronically administered, the systolic blood pressure was significantly elevated, and the difference between the 2 groups diminished (136.5±7.3 versus 141.2±11.4 mm Hg for AMTg versus wild-type mice, respectively; P=NS). For the vascular injury, the vasculoprotective effect in AMTg mice was diminished, and there was no difference in the neointimal response

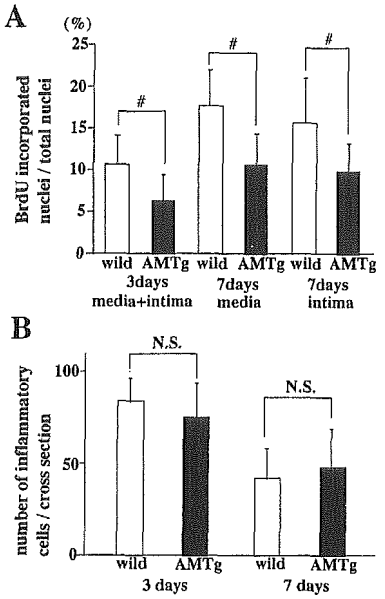

Figure 2. A, BrdU uptake in the media and neointima of the cuffed femoral arteries. The BrdU index (the number of BrdU positive nuclei/number of total nuclei) was analyzed 3 and 7 days after cuff placement. B, Number of inflammatory cells invading around injured arteries. The average of 5 sections was taken as the value for each animal. Error bars indicate SD. #$P<0.05$. NS indicates not significant.

between the AMTg and wild-type littermates (Figure 3A), suggesting that the vasculoprotective action of AM is mediated via an NO-dependent mechanism. Next, to confirm that the vasculoprotective effect of AM is independent of its hypotensive effect, we evaluated the neointimal responses between wild-type mice with and without hydralazine administration. The systolic blood pressure of wild-type mice treated with hydralazine was 98.3±8.5 mm Hg, which was almost equivalent to that of AMTg mice. We evaluated the neointimal formation 28 days after injury in the wild-type mice treated with hydralazine, finding that the intima/media ratio was 1.25±0.50, which was not significantly different from that of wild-type mice without hydralazine treatment (1.31±0.41, P=NS; Figure 3B). Therefore, the protective effect of AM was independent of the pressure reduction. Moreover, to test the effect of AM antagonism, we treated wild-type mice and AMTg mice with CGRP(8-37) or vehicle. This demonstrated that CGRP(8-37) infusion partially inhibited the beneficial effect of AM overexpression compared with vehicle administration and did not significantly affect neointimal formation in the wild-type mice (Figure 3C).

Fatty Streak Formation in ApoEKO and AMTg/ApoEKO Mice

Next, we examined the protective effect of AM on fatty streak formation by breeding AMTg with ApoEKO mice. In AMTg/ApoEKO mice, AM transgene expression was detected in the aorta, heart, lung, and kidney, as evaluated by reverse transcription–polymerase chain reaction and Northern blot analysis (data not shown), and plasma AM levels evaluated by radioimmunoassay[10] were significantly higher in AMTg/ApoEKO mice than in ApoEKO mice

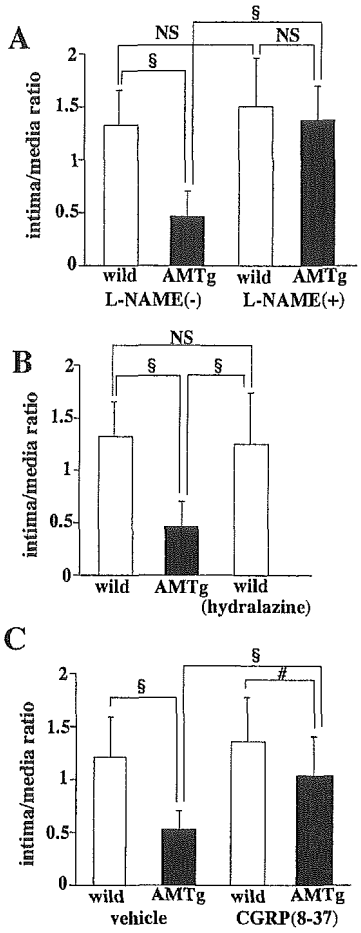

Figure 3. Effects of several drug interventions on neointimal formation. A, L-NAME. B, Hydralazine. C, CGRP(8-37). #$P<0.05$ and §$P<0.01$.

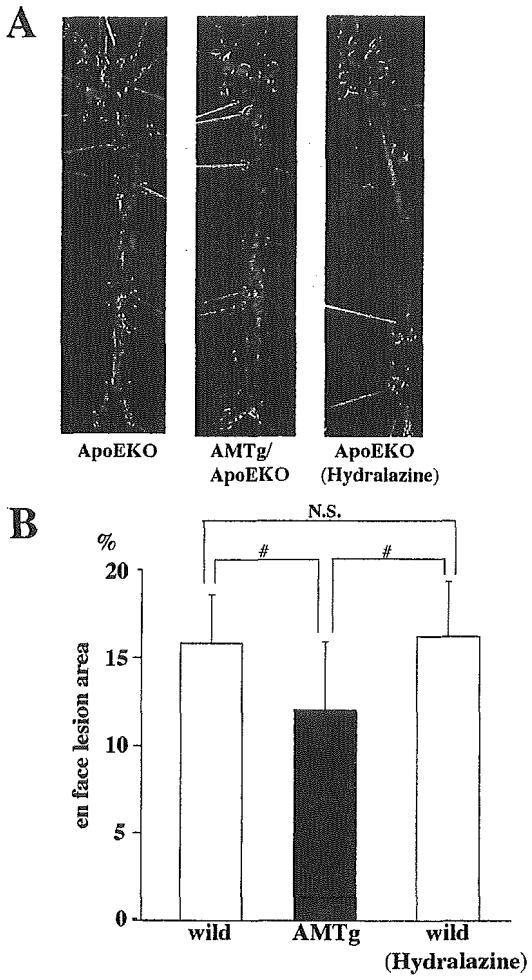

Figure 4. Hypercholesterolemia-induced atherosclerotic lesions. A, Representative photographs of en face atherosclerotic lesions in the entire aorta. B, Percentage of en face atherosclerotic lesions. #$P<0.05$.

(8.36±2.32 versus 4.93±1.07 fmol/mL, respectively; $P<0.01$; n=7 each).

ApoEKO mice and ApoEKO/AMTg mice were fed normal chow until they were 8 weeks old, and then they were fed the atherogenic diet for 2 months. Plasma lipid and lipoprotein concentrations were markedly elevated after an atherogenic diet. However, there were no significant differences in lipid profiles between the AMTg/ApoEKO mice and ApoEKO mice (data not shown). The AMTg/ApoEKO mice (n=16) were significantly more hypotensive than the ApoEKO mice (n=16); systolic blood pressure was 110±15 versus 125±14 mm Hg, respectively ($P<0.01$). Thus, to evaluate the direct effect of hypotension on fatty streak formation, we also evaluated the hydralazine-treated ApoEKO mice, whose systolic blood pressure (104±17 mm Hg) was almost equivalent to that of the AMTg/ApoEKO mice. The representative photographs of en face surface atherosclerotic lesions are shown in Figure 4A. The fatty streak formation of the AMTg/ApoEKO mice (n=12) was significantly smaller than that of the ApoEKO mice (n=12); the en face lesion area was 12.0±3.9% versus 15.8±2.8%, respectively ($P<0.05$, Figure 4B). The same result was observed in the cross-sectional lesion area (223 000±56 000 versus 290 000±45 200 μm^2 for AMTg/ApoEKO mice versus ApoEKO mice, respectively; $P<0.01$). However, the atheromatous lesion formation was not changed in ApoEKO mice when they were treated with hydralazine (en face lesion area 16.2±3.1%, cross-sectional lesion area 301 400±52 100 μm^2; P=NS), suggesting that AM inhibits fatty streak formation via a mechanism other than its hypotensive effect.

Endothelial Function and NO Production

To test endothelial function in ApoEKO/AMTg mice and ApoEKO mice, we examined endothelium-dependent and -independent vasodilatation with the use of aortic rings isolated from mice fed the atherogenic diet (n=8 for each group, Figure 5). As controls, wild-type mice and AMTg mice were used for the aortic ring experiment. There was no significant difference in the tension after preconstriction by phenylephrine among the 4 groups. The relaxation induced by ACh was significantly deteriorated in the apoE-deficient mice compared with the AMTg and wild-type mice. However, endothelium-dependent vasorelaxation was significantly better in ApoEKO/AMTg mice (E_{max} 94.6±2.5%, ED_{50} [−log molar] 7.31±0.14) than in ApoEKO (E_{max} 85.2±4.6%, ED_{50} 6.83±0.25; $P<0.05$). Concerning the 2 control groups, the

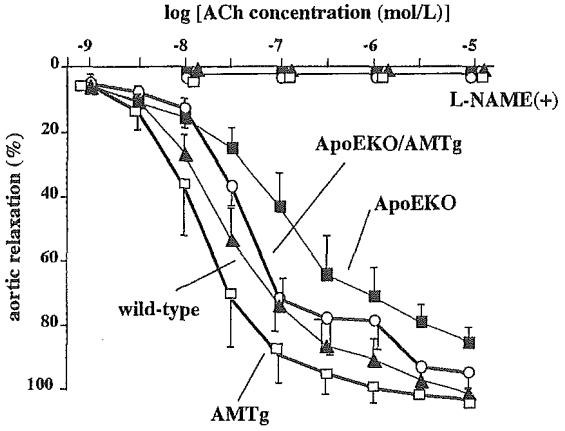

Figure 5. Endothelium-dependent vasorelaxation of aortas from the 4 groups: wild-type (closed triangle), AMTg (open square), ApoEKO (closed square), and AMTg/ApoEKO (open circle) mice (n=8 to 10). Values are mean±SEM. #$P<0.05$.

ED_{50} was also significantly better in AMTg mice (7.39±0.30) than in wild-type mice (7.77±0.16). However, there was no significant difference in E_{max} between AMTg mice (97.7±2.4%) and wild-type mice (99.7±0.4%, P=NS). On the other hand, there was no significant difference in aortic relaxation in response to SNP among the 4 groups. ACh-induced relaxation was almost completely attenuated by L-NAME, suggesting that the vasorelaxation induced by ACh is almost exclusively mediated by endothelium-derived NO. These data suggest that endothelial function was preserved by AM overexpression when damaged by remarkable hypercholesterolemia. Moreover, plasma cGMP levels were also significantly higher in AMTg/ApoEKO mice (7.9±2.0 pmol/mL) than in ApoEKO mice (6.1±1.8 pmol/mL, $P<0.05$), implicating that the steady-state NO production was also upregulated in mice with AM overexpression. On the other hand, the endothelial NO synthase (eNOS) expression levels in aorta detected by reverse transcription–polymerase chain reaction were not different between mice with and without AM overexpression (data not shown), suggesting that the function of eNOS may be regulated by AM at the posttranslational or protein levels.

Discussion

In the present study, we examined the involvement of AM in the formation of vascular lesions by analyzing 2 different experimental models. We evaluated neointimal formation induced by cuff placement and hypercholesterolemia-induced fatty streak formation and found that mice carrying AM transgenes were significantly resistant to vascular injury and fatty streak formation. This is the novel important evidence that supports the vasculoprotective effect of AM in vivo.

Rodents are naturally resistant to neointimal hyperplasia and fatty streak formation. Therefore, several experimental models of neointimal hyperplasia or atherosclerosis have been proposed. In the present study, we adopted the cuff-injury model, in which the endothelial layer remains intact and thrombus formation is very rare because no intravascular manipulation is performed. Using this model, we demonstrated that AM overexpression suppressed neointimal hyperplasia and that BrdU-positive nuclei in the subendothelial area were reduced in the cuff-injured femoral arteries, indicating that the proliferation of VSMCs was downregulated by AM. This evidence is consistent with previous in vitro experiments.[7] On the other hand, there was no significant difference in inflammatory cells around the injured arteries between AMTg and wild-type mice, suggesting that inflammation may not be affected by AM overproduction.

AM can act on endothelial cells to stimulate NO production via calcium-dependent activation of eNOS.[5,23] We tested whether inhibition of NO production diminished the protective effect of AM. Chronic administration of L-NAME abolished the beneficial effect of AM overexpression on vascular injury. Therefore, the vasculoprotective effect of AM was thought to be at least partially mediated by NO. The beneficial effect of NO produced by endothelium has been previously demonstrated in many reports. eNOS knockout mice have been reported to exhibit abnormal intimal hyperplasia after vascular injuries in the cuff placement.[14] eNOS overexpression has also been shown to inhibit neointimal formation significantly in the rat carotid balloon injury model.[24] These previous studies and the present result lead us to postulate that the activation of eNOS by AM may be effective in preventing the formation of vascular proliferative lesions.

Shimizu et al[18] reported that the AM antagonist CGRP(8-37) inhibits neointimal hyperplasia after balloon injury in the rat carotid artery, indicating that endogenous AM in the injured tissue may promote the proliferation of VSMCs. This observation seems to contradict our data. In the present study, AM overexpression inhibited neointimal formation, and CGRP(8-37) infusion partially inhibited the beneficial effect of AM overexpression. This discrepancy may be due to differences in the experimental animals and systems. In our 2 models, the endothelial layers remained intact, and AM was overexpressed mainly in the endothelium. On the other hand, in a rat carotid artery balloon-injury model, the endothelial layers were denuded, and the beneficial effect of AM on endothelial layers was abolished. The injured VSMCs were exposed directly to the blood stream; thus, the phenotypic changes in VSMCs might occur more easily. Concerning other type of cells, AM has an antiproliferative effect of glomerular mesangial cells[25] but stimulates cell proliferation in Swiss 3T3 cells.[26] Therefore, it is likely that the effect of AM on cell proliferation depends on the phenotypes or experimental situations.

To date, apoE knockout mice have been useful in the analysis of cholesterol-induced fatty streak formation. Several experiments on crossbreeding with other genetically altered mice or on pharmacological intervention in ApoEKO mice have been reported.[19,27] Thus, we crossbred AMTg mice with ApoEKO mice to clarify the effect of AM on hypercholesterolemia-induced fatty streak formation. We demonstrated that the presence of AM transgenes suppresses the formation of atheromatous lesions and that this is independent of the blood pressure reduction and lipoprotein profiles. It is worth noting that the vasculoprotective effect of AM was also confirmed in this model.

Endothelial dysfunction is characterized by impaired endothelium-derived NO-mediated vasorelaxation in atherosclerosis in humans and experimental animals. A previous report[28] demonstrated that endothelial dysfunction was impaired in isolated aortic rings of ApoEKO mice compared

with wild-type mice, which was replicated in the present study. In the present study, we found that endothelium-dependent vasodilatation was superior in AMTg/ApoEKO mice compared with ApoEKO mice, although severe dyslipidemia damaged endothelium-dependent vasorelaxation significantly. Moreover, the plasma cGMP level (taken to be indicative of steady-state NO production) was significantly higher in ApoEKO/AMTg mice than in ApoEKO mice. Therefore, our data suggest that the antiatherogenic effect of AM in ApoEKO is at least partially caused by endothelium-derived NO production by chronic AM overexpression.

In summary, we demonstrated the beneficial effects of AM in vivo against vascular injury at least partially via endothelial NO production with the use of genetically altered mice. However, further studies are needed to clarify the precise mechanism of the vasculoprotective effect of AM so that AM can be applied clinically for atherosclerosis and for postangioplasty neointimal hyperplasia.

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Evaluation of the atrial natriuretic peptide gene in stroke

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Abstract

The atrial natriuretic peptide (ANP) gene was, though inconclusive, implied to be etiologically related to stroke in rats and recently in humans. The present study tested the candidacy of ANP for stroke susceptibility by a combination of molecular genetic approaches. First, we undertook an association study using a reported ANP variant, G664A, in two case-control panels independently collected, which involved 970 Japanese subjects. Second, we compared the rat ANP gene sequences and neighboring marker alleles among stroke-prone SHR (SHRSP), normal SHR and WKY of an original inbred colony and we also compared brain ANP expression between SHRSP and normal SHR. In humans, we found no significant association between the 664A variant and stroke in the studied population. In rats, 21 polymorphic sites were identified by direct sequencing of 2170-bp ANP fragments, from which two distinct alleles, SHRSP- and WKY-types, were inferred. From a genealogical point of view, our data indicated that an SHRSP-type allele could not play a determinant role in stroke-proneness. Overall results did not support the disease relevance of ANP, disagreeing with previous reports. Thus, considerable caution should be taken when one attempts to transfer findings in the animal model to humans.

Keywords: Atrial natriuretic factor; Stroke; Genetics; Single nucleotide polymorphism; Association

1. Introduction

Several lines of evidence have supported the substantial involvement of genetic factors in the pathogenesis of stroke [1]. However, studies of the molecular genetics of stroke are not necessarily facile, especially in humans, due to a number of complex features of the disease, e.g. coexistent multifactorial disorders, such as hypertension, diabetes and dyslipidemia. An alternative strategy would be to first explore the predisposition to stroke in the animal model and thereafter, test the corresponding findings in humans, instead of investigating certain candidate genes without any clue. Among animal models thus developed to date, the stroke-prone spontaneously hypertensive rat (SHRSP) is considered of particular interest, since it promotes severe hypertension from the early age of life and also manifests the propensity for stroke on a high salt and low potassium diet [2–4]. Accordingly, this animal has been widely used as a model organism to investigate the etiological relationship between hypertension and stroke.

Recently, two study groups have undertaken genome-wide searches of quantitative trait loci (QTLs) for 'stroke-associated' phenotypes in F2 progeny derived from SHRSP [5,6] and have suggested that predisposition to stroke is at least, in part, under genetic controls independent of hypertension. One group explored genetic susceptibility for latency until the manifestation

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of stroke in F2 progeny between SHRSP and normal (or stroke-resistant) SHR [5], while the other did so for infarct volume after the middle cerebral artery (MCA) occlusion in F2 progeny between SHRSP and Wistar Kyoto (WKY) rats [6]. These genome screens successfully identified four QTLs on three separate rat chromosomes, among which a region around the atrial natriuretic peptide (ANP) gene has drawn substantial attention because markers in the vicinity of ANP on rat chromosome 5 were significantly linked to both phenotypic traits.

Then, the following questions may be asked: (1) whether the ANP gene itself can underlie either (or both) of the 'stroke-associated' phenotypes? (2) whether findings in rats are directly extendable to human stroke? Remarkably, one of the study groups above-mentioned has further proposed ANP as a candidate susceptibility gene based on two lines of evidence. First, SHRSP of a Heidelberg colony (SHRSP/Heidelberg) showed alterations in structure, expression and in vitro function of ANP compared to normal SHR [7]. Second, a case-control study involving 696 white subjects indicated that a single nucleotide polymorphism, G664A, located in exon 1 of the human ANP gene was associated with the risk for stroke [8]. On the contrary, SHRSP of a Glasgow colony (SHRSP/Glasgow) showed no significant differences in either structure or expression of ANP compared to WKY [9]. Rat ANP data from two study groups thus appear discordant and need further confirmation.

Under these circumstances, the present study was designed to evaluate the candidacy of ANP in human and rat stroke. Initially the ANP association was tested in two case-control panels involving 970 Japanese subjects; 270 brain infarction patients and 359 sex- and age-matched controls were included in one panel and 178 patients and 163 controls were included in another panel. This association was primarily tested because ANP could constitute a potential, though not strong, candidate gene for cerebrovascular disorders, whether rat ANP is the susceptibility gene in question or not. Moreover, from a genealogical point of view, disease susceptibility was investigated among SHRSP, normal (or stroke-resistant) SHR and WKY of an original inbred colony, all of which originated from a Wistar rat colony in Japan [4].

2. Methods

2.1. Human subjects

This study was approved by an institutional review committee. Informed consent for participation was obtained from all subjects. Two study panels were independently collected according to classification criteria previously described [10,11]. Briefly, participants in the first panel comprised 270 cases, who were enrolled at the Kitamura Neurosurgery Clinic, Tokyo from September 1996 to May 1997, more than 2 months after the incident of stroke and 359 controls frequency matched by age and sex, who were selected from outpatients at the cardiovascular clinic of the Institute for Adult Diseases, Asahi Life Foundation, Tokyo [10]. Both institutes are in the same area of the megalopolis. The second panel involved three participant groups; (1) 104 subjects with silent brain infarction (SBI) were consecutively enrolled from people undergoing a health screening examination between January 1995 and December 1997 at the Shimane Institute of Health Science; (2) 163 subjects without evidence of SBI on MRI were selected from the same population as controls; and also (3) 74 subjects with symptomatic subcortical infarction were enrolled from outpatients at Shimane Medical University in the corresponding period [11]. Clinical characteristics were defined as a dichotomous phenotype except for age, as depicted in Table 1.

2.2. Genotyping of the G664A polymorphism and statistical analysis

The G664A polymorphism of human ANP was genotyped by the mutagenically separated PCR (MS-PCR) method (Fig. 1) as described in our previous report [12], in which this polymorphism was designated as G191A according to the nucleotide position relative to the transcription start site. Two other single nucleotide polymorphisms (SNPs), C-664G and T1766C, were also genotyped as described previously [12]. The three SNPs had been shown to represent principal ANP polymorphisms in Japanese subjects.

The χ^2-test statistic was calculated between the genotype distribution (or allele frequencies) and stroke status. Because pathological findings of cerebral lesions in SHRSP are similar to those of subcortical (or lacunar) infarction in humans [13], patients with subcortical infarction were analyzed separately in the first panel. Also, because the rat ANP locus is assumed to confer stroke susceptibility independently of hypertension [5,6], each group of cases and controls was stratified by hypertension status to remove its effects on association analysis. Furthermore, confounding influences of six variables listed in Table 1 were assessed in a multiple logistic regression model. The power of a case-control study of the available sample size was determined by calculating the smallest detectable relative risk with 80% power at a 5% Type I error probability [14].

2.3. Experimental animals

We use the name SHR for rats with a low incidence of spontaneous stroke (stroke-resistant SHR or 'SHRSR')