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Excess L-arginine restores endothelium-dependent relaxation impaired by monocrotaline pyrrole

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Abstract

The pyrrolizidine alkaloid plant toxin monocrotaline pyrrole (MCTP) causes pulmonary hypertension in experimental animals. The present study aimed to examine the effects of MCTP on the endothelium-dependent relaxation. We constructed an in vitro disease model of pulmonary hypertension by overlaying MCTP-treated bovine pulmonary artery endothelial cells (CPAEs) onto pulmonary artery smooth muscle cell-embedded collagen gel lattice. Acetylcholine (Ach) induced a relaxation of the control CPAEs-overlaid gels that were pre-contracted with noradrenaline, and the relaxation was inhibited by L-NAME, an inhibitor of NO synthase (NOS). In contrast, when MCTP-treated CPAEs were overlaid, the pre-contracted gels did not show a relaxation in response to Ach in the presence of 0.5 mM L-arginine. Expression of endothelial NOS protein, Ach-induced Ca^{2+} transients and cellular uptake of L-[^{3}H]arginine were significantly smaller in MCTP-treated CPAEs than in control cells, indicating that these changes were responsible for the impaired NO production in MCTP-treated CPAEs. Since cellular uptake of L-[^{3}H]arginine linearly increased according to its extracellular concentration, we hypothesized that the excess concentration of extracellular L-arginine might restore NO production in MCTP-treated CPAEs. As expected, in the presence of 10 mM L-arginine, Ach showed a relaxation of the MCTP-treated CPAEs-overlaid gels. These results indicate that the impaired NO production in damaged endothelial cells can be reversed by supplying excess L-arginine.

Keywords: Monocrotaline; Nitric oxide; L-arginine; Endothelium; Pulmonary hypertension

Introduction

Experimental animal model of pulmonary hypertension (PH) has been created either by exposing animals to hypoxia or a metabolite of the pyrrolizidine alkaloid plant toxin monocrotaline. Hypoxia is known to induce the spasm of pulmonary artery thereby inducing PH. In contrast, cause of PH induced by monocrotaline, especially its toxic metabolite monocrotaline pyrrole (MCTP), is still controversial.

It is well known that MCTP induces megalocytic change in pulmonary artery endothelial cells, which would induce the prolonged activation of vascular remodeling and cause PH (Reindel and Roth, 1991). It was reported that the MCTP-induced inhibition of cdc2 kinase may lead to prolonged cell cycle arrest and subsequent megalocytic change in endothelium (Thomas et al., 1998). Recent reports suggest another possibility that the MCTP-induced megalocytic change may be due to the blockade of Golgi trafficking, thereby leading to the disruption of caveolin-1α (cav-1α)/raft function and mitosis sensor function of Golgi (Mathew et al., 2004; Shah et al., in press). Furthermore, Ito et al. (2000) examined the functional alteration of MCTP-treated vessels and showed the elevation of the resting membrane potential and the impaired endothelium-derived relaxation in pulmonary arteries of

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MCTP-treated rats. It was also reported that the expression level of endothelial NOS (eNOS) protein was altered in PH model rats (Tyler et al., 1999). Another group reported that the expression of eNOS mRNA and protein were not altered in the lungs of MCTP-treated rats but the increased oxidative stress resulted in the low bioavailability of NO (Mathew et al., 2002). Therefore, it can be considered that the elevation of local NO concentration would be a promising approach for the treatment of PH.

Inhalation of NO gas is known to induce pulmonary vasodilation, thereby being regarded as a feasible remedy for cardiovascular diseases including PH (Gianetti et al., 2002; Rubin, 1997). However, technical difficulties of NO inhalation and the short half life of the gas have limited its use mainly to hospitalized patients. Therefore, another approach would be to increase endogenous endothelial NO productivity and/or its action. Sildenafil, an inhibitor of cGMP phosphodiesterase, was reported to reduce pulmonary pressure in PH (Wilkens et al., 2001). Gene transfer of eNOS has also shown to reduce pulmonary artery pressure in animal model of PH of eNOS-deficient mice (Champion et al., 2002) and MCTP-treated rats (Campbell et al., 1999).

In the present study, we have focused on endothelial function, especially the productivity of NO, in calf pulmonary artery endothelial cells (CPAEs) that were treated with MCTP. Endothelial NO production is mainly obtained by the elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that activates eNOS (Moncada et al., 1991). Therefore, we examined the effects of MCTP on NO production, $[Ca^{2+}]_i$ and the expression of eNOS in CPAEs. We have also introduced a novel method to examine endothelium-derived vasorelaxation by using an in vitro model vessel (Kimura et al., 2004) to investigate NO productivity in MCTP-treated CPAEs. Obtained results demonstrate that NO productivity can be restored by increasing the substrate of eNOS, L-arginine, even in damaged endothelial cells.

Methods

Cell culture. CPAEs were purchased from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS). Pulmonary arteries from 1-year calves were obtained from a local slaughterhouse, smooth muscle cells (BPSMCs) were cultured in DMEM with 10% FBS by explant method (Chamley et al., 1977). All cells were stored at −80 °C before use.

Assessment of cell number and cell size. For assessing the effects of MCTP on the number and the size of CPAEs, cells were seeded on 35 mm culture dish at a density of 5000 cells/cm^2, and cultured in the absence or presence of MCTP. Cells were collected by trypsinization, and the cell numbers were manually counted with a hemocytometer.

Cell size of CPAEs was measured with an image analysis software (Photoshop, Adobe Systems Inc., San Jose, CA) from the microscopic images taken with a CCD camera mounted on a microscope (Diaphot TMD; Nikon, Tokyo, Japan).

In vitro model vessel system. Gel contraction assay was performed to examine the production of vasoactive agents in CPAEs. MCTP-treated CPAEs were obtained with trypsinization after being cultured in the presence of 1 μg/ml MCTP for 5 days, and stored at −80 °C before use. BPSMCs were embedded in collagen gel at a density of 4×10^5 cells/ml in 24-well culture plate as previously reported (Kimura et al., 2002). After being cultured for 24 h, control or MCTP-treated CPAEs were overlaid onto the collagen gel at a density of 2×10^4 cells/cm^2, and allowed to spread on the gel surface for further 24 h (see cartoons in Fig. 2).

The culture plate was placed on a hot plate (MP-10DM; Kitazato Supply, Shizuoka, Japan) and kept at 37 °C throughout the measurement. The gel images were captured with a digital camera (QV-800SX, Casio, Tokyo, Japan) every 1 min. Gel contraction was then evaluated by measuring its surface area with an image analysis software (Photoshop, Adobe Systems Inc.). Measured values were normalized to the control gel area that was obtained before the application of noradrenaline.

Measurement of intracellular Ca^{2+} concentration. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured with fura-2 fluorescence. CPAEs were loaded with fura-2/AM (Dojindo, Kumamoto, Japan) and excited at two alternative wavelengths (340 nm and 380 nm) at a rate of approximately 1 Hz. Fluorescent ratio (R) calculated from fluorescence intensities of 510 nm wavelength was converted into apparent Ca^{2+} concentration with an equation,

$$[Ca^{2+}]_i = K_{eff}\ (R - R_{min})/(R_{max} - R),$$

where K_{eff} is the "effective binding constant", R_{min} the fluorescent ratio at zero calcium and R_{max} that at high Ca^{2+}.

ECL Western blotting. Expression of eNOS protein in control and MCTP-treated CPAEs were assessed by chemiluminescence (ECL) Western blotting (ECL phosphorylation detection system, Amersham Pharmacia Biotech, Uppsala, Sweden). A constant amount of cellular protein (50 μg protein per lane) was separated with SDS-PAGE, and eNOS was detected with anti-eNOS polyclonal antibody (StressGen Biotechnologies, Co., San Diego, CA). Expression of β-actin protein was also assessed as an internal control, using monoclonal anti-β-actin antibody (Sigma). Emitted chemiluminescence was detected and analyzed with a lumino image analyzer (FAS-1000, Toyobo, Osaka, Japan).

Measurement of cellular L-[^{3}H]arginine uptake. For the measurement of cellular uptake of L-[^{3}H]arginine (Amershan, Uppsala, Sweden), control and MCTP-treated CPAEs

were seeded on 6-well culture plates at densities of 15,000 and 30,000 cells per well, respectively. To determine the concentration dependence of cellular L-[^{3}H]arginine uptake in MCTP-treated CPAEs, 1 ml of Hanks' balanced salt solution (HBSS) either with 0.3, 1 or 3 μCi/ml L-[^{3}H]arginine was placed on each well. Total uptake of L-[^{3}H]arginine in control CPAEs was determined by placing 1 ml of HBSS with 1 μCi/ml L-[^{3}H]arginine. Other details are descried previously (Kimura et al., 2004).

Drugs and solutions. Modified Krebs solution (1.5 mM Ca^{2+} solution) was used as the standard extracellular solution, containing (in mM): 132 NaCl, 5.9 KCl, 1.2 $MgCl_2$, 1.5 $CaCl_2$, 11.5 glucose, 11.5 HEPES; pH was adjusted to 7.3 with NaOH.

MCTP was prepared from crotaline (Sigma, St. Louis, MO) and o-chloranil (Sigma) with the method described by Mattocks et al. (1989), which would yield practically pure MCTP. It should be noted that the concentration of MCTP used in the present study was that of the refined product. All other drugs used in the present experiment were also obtained form Sigma (St. Louis, MO).

Statistics. Pooled data are given as mean ± SEM, and statistical significance was determined using Student's unpaired *t* test. Probabilities less than 5% ($P < 0.05$) were regarded as significant.

Results

Effects of monocrotaline pyrrole (MCTP) on cell number and cell surface area of CPAEs

Firstly, we examined the effects of MCTP on cell area and cell number in CPAEs. Single application of more than 1 μg/ml of MCTP induced the enlargement of CPAEs (Figs. 1A and Ba) and the reduction in cell number (Fig. 1Bb). MCTP-induced enlargement of CPAEs was irreversible, and persisted even after removing MCTP from culture medium or after subculture. In the following experiments, therefore, we harvested the cells after incubating with 1 μg/ml MCTP for 5 days, and stored at −80 °C until use.

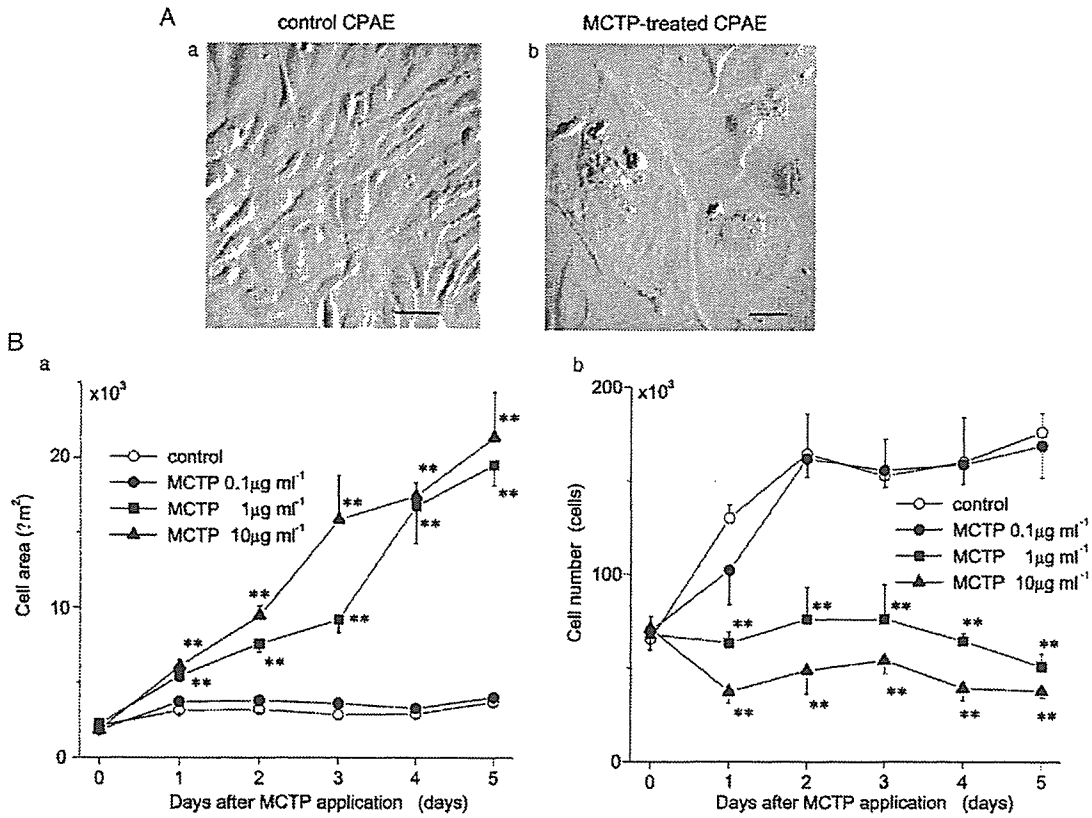

Fig. 1. Effects of monocrotaline pyrrole (MCTP) on cell surface area and cellular proliferation in calf pulmonary artery endothelial cells (CPAEs). (A) Microscopic images of control (a) and MCTP (1 μg/ml)-treated (b) CPAEs. In panel b, CPAEs were incubated with MCTP for 5 days. Each scale bar indicates 50 μm. (B) Time-dependent changes of cell area (a) and cell number (b) in CPAEs. Cells were seeded on 35 mm culture dish at a density of 5000 cells/cm^2, and cultured in the absence and presence of MCTP. $^{**}P < 0.01$ vs. untreated control.

In vitro model vessel using MCTP-treated CPAEs

We have previously reported that cultured vascular smooth muscle cells restore contractility by being embedded in three dimensional collagen gel lattice (Kimura et al., 2002, 2004). Furthermore, we have shown that endothelium-overlaid, smooth muscle cells-embedded gels show relaxation in response to Ach due to the generation of NO (Kimura et al., 2004). Therefore, we then examined the functions of the MCTP-treated CPAEs by using this in vitro model vessel system (see cartoons in Fig. 2).

BTSMCs-embedded collagen gels showed contraction in response to 1 μM noradrenaline. Subsequent application of 1 μM Ach induced a relaxation of the gel when control endothelium was overlaid (Fig. 2A, open circles). Ach-induced relaxation of the gel was not observed in the gels without overlaying CPAEs (not shown), and was significantly inhibited by L-NAME (300 μM, Fig. 2A closed circles), thereby indicating that the relaxation was due to NO production in the overlaid CPAEs. Since this measurement was performed in normal Krebs solution that does not contain L-arginine, it seems that L-arginine was supplied from DMEM in the gel. In contrast, when the same number of MCTP-treated CPAEs was overlaid on the gel, only a trace level of Ach-induced relaxation was observed (Fig. 2B), thereby suggesting that the MCTP suppressed NO productivity in CPAEs.

Ca^{2+} mobilization in MCTP-treated endothelial cells

In order to elucidate the cellular mechanisms for the impairment of NO production in MCTP-treated CPAEs, we then examined the Ach-induced $[Ca^{2+}]_i$ elevation in control and MCTP-treated CPAEs. Ach (1 μM) induced a gradual

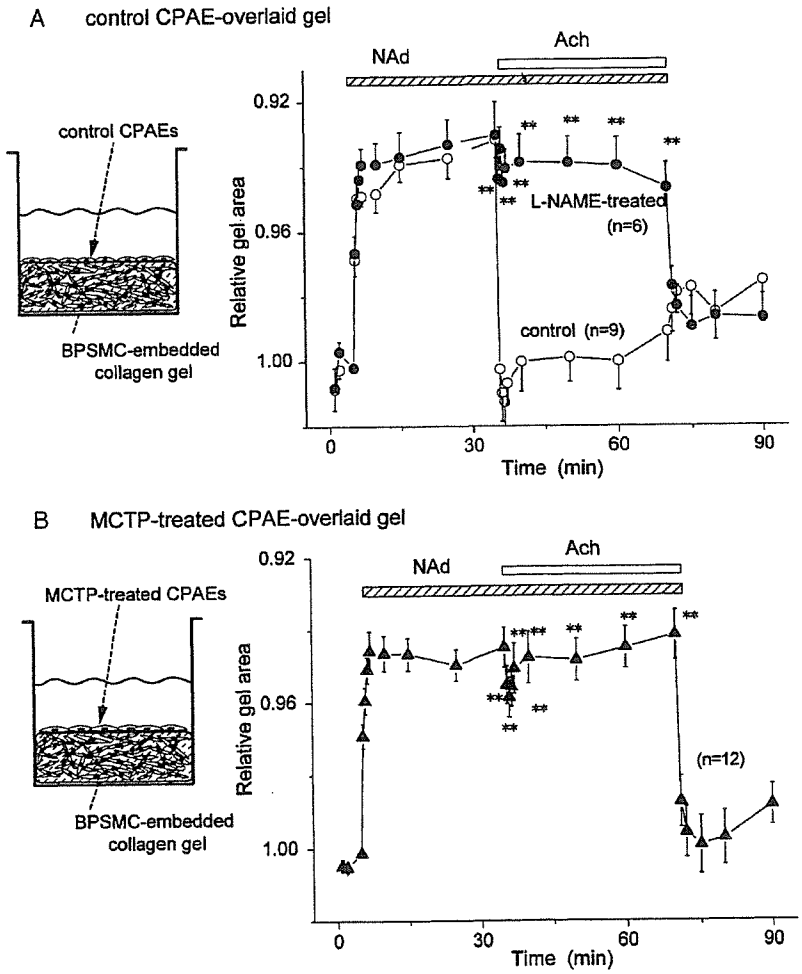

Fig. 2. In vitro model vessel assay of endothelium-dependent relaxation. Bovine pulmonary artery smooth muscle cells (BPSMCs) were embedded in collagen gel lattice, and control or MCTP-treated CPAEs were overlaid (see cartoons). (A) Control CPAEs-overlaid gels showed relaxation in response to 1 μm Ach (open circles). Pretreatment with L-NAME (0.3 mM) inhibited the relaxation (closed circles). $^{**}P < 0.01$ vs. control. (B) When MCTP-treated CPAEs were overlaid, Ach failed to induce a relaxation of the pre-contracted gels. $^{**}P < 0.01$ vs. control in A.

increase in $[Ca^{2+}]_i$ in Krebs solution in control CPAEs (Fig. 3A). Ach (1 μM) showed $[Ca^{2+}]_i$ increase also in MCTP-treated cells (Fig. 3B), but the net increment of $[Ca^{2+}]_i$ (Fig. 3Ca) and time integral of $[Ca^{2+}]_i$ elevation (Fig. 3Cb) were significantly smaller in MCTP-treated cells than in control cells. Therefore this might be one of the causes for the impaired NO production in MCTP-treated CPAEs.

Expression of eNOS protein in MCTP-treated CPAEs

We then examined the expression of eNOS protein in MCTP-treated cells with Western blotting. As shown in Fig. 4, expression of eNOS protein was observed both in control and MCTP-treated CPAEs. However, densitometric analysis of the band revealed that the expression level of eNOS protein was significantly lower in MCTP-treated CPAEs than in control CPAEs. Thus, it seems that the reduced eNOS expression may be another reason for the decreased NO production in MCTP-treated CPAEs.

Cellular uptake of L-[^{3}H]arginine in MCTP-treated and control CPAEs

We then examined the cellular uptake of L-arginine in control and MCTP-treated CPAEs. As shown in Fig. 5A, cellular uptake of L-arginine was significantly lower in MCTP-treated CPAEs than in control cells in the presence of 1 μCi/ml L-[^{3}H]arginine. However, cellular L-arginine uptake was linearly increased according to its extracellular concentration in MCTP-treated CPAEs, and the uptake of control level of L-[^{3}H]arginine was obtained with three times higher concentration of extracellular L-[^{3}H]arginine (3 μCi/ml) in MCTP-treated CPAEs.

Effects of excess L-arginine on endothelium-dependent responses in model vessel

Above results suggest that impaired Ca^{2+} mobilization, reduced eNOS protein expression and lower cellular uptake of L-arginine are responsible for the impairment of NO production in MCTP-treated CPAEs. However, the cellular L-arginine uptake was linearly increased according to its extracellular concentration (Fig. 5A). Therefore, assuming that the expressed eNOS protein was intact, it would be rational to speculate that the sufficient supply of L-arginine may restore NO production even in MCTP-treated CPAEs.

We then performed gel contraction assay in the presence of excess concentration of extracellular L-arginine. As expected, in the presence of 10 mM L-arginine, Ach induced relaxation of the gels that were overlaid with MCTP-treated

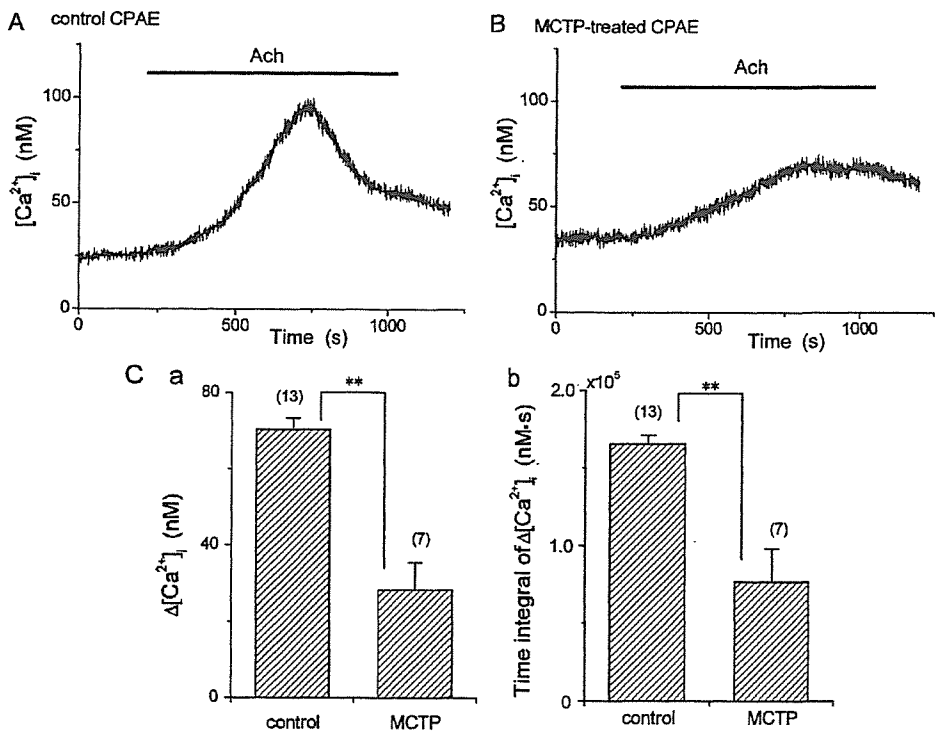

Fig. 3. Ach-induced Ca^{2+} mobilization in control and MCTP-treated CPAEs. (A) Control CPAEs showed gradual increase in $[Ca^{2+}]_i$ in response to 1 μM Ach. Representative $[Ca^{2+}]_i$ trace is shown. (B) Ach also showed an increase in $[Ca^{2+}]_i$ in MCTP-treated CPAEs, but the degree of elevation was smaller than in control cells. Representative $[Ca^{2+}]_i$ trace is shown. (C) Statistical analysis of net peak increment of Ach-induced elevation of $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$), (a) and time integral of $\Delta[Ca^{2+}]_i$ for 15 min (b). MCTP-treated CPAEs showed significantly lower increase in $[Ca^{2+}]_i$ than control cells. $^{**}P < 0.01$.

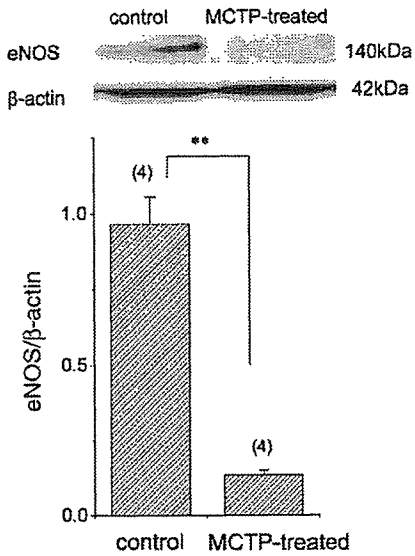

Fig. 4. Expression of endothelial NO synthase (eNOS) protein in control and MCTP-treated CPAEs. Note that the expression of 140 kDa protein is markedly reduced in MCTP-treated cells. Expression of β-action is shown as an internal control. Measurement was repeated for four times, and the representative band images are shown. Mean ± SEM values of the band analysis are shown. $^{**}P < 0.01$.

CPAEs, and the degree of relaxation was comparable to those observed with control CPAEs (Fig. 5B).

Discussion

Various methods have been introduced so far to detect the NO production in vitro, including porphyrinic-based microsensor (Malinski and Taha, 1992), Griess method (Wang et al., 1996), and DAF-2 fluorescence (Kimura et al., 2000). Each method has advantages and disadvantages in its convenience, sensitivity and selectivity. We have recently shown that endothelium-overlaid, smooth muscle-embedded model vessels can be used for detecting NO production in cultured endothelial cells (Kimura et al., 2004). The major advantage of this in vitro model vessel is that it can directly examine the effects of endothelium-derived substances on smooth muscle tonus, and therefore, it enables the detection of not only NO but also other endothelium-derived vasoactive substances. We observed in the present study that control CPAEs-overlaid gels showed relaxation in response to Ach, and L-NAME suppressed it almost completely (Fig. 2A). Therefore, it can be supposed that NO is the main vasorelaxant that was secreted by Ach in CPAEs. We have shown with this model vessel study that MCTP-treated CPAEs did not induce the relaxation of pre-contracted gels (Fig. 2B), thereby indicating that MCTP impairs the NO production in CPAEs. There have been only a few methods for studying PH, and most studies have used disease model animals including MCTP- and hypoxia-treated rats. In contrast, this novel in vitro model vessel is convenient and applicable for examining the effects of various drugs on impaired endothelium.

We have confirmed that MCTP induces the enlargement of pulmonary endothelial cells and the reduction in cell number (Fig. 1). It was reported in CPAEs that these phenomena are due to the inactivation of cdc2 kinase and consequent G2/M phase arrest (Thomas et al., 1998) or to the blockade of Golgi trafficking and subsequent impairment of cav-1α chaperone function (Mathew et al., 2004; Shah et al., in press), and megalocytic change of pulmonary endothelium would induce the prolonged vascular remodeling and causes PH in model animals. The impairment of NO production in MCTP-treated CPAEs, therefore, may be in part due to cell cycle arrest and loss of cav-1α function, which plays an important role in NO production (Minshall et al., 2003). We have further revealed in this study that the impairment of NO production is due to various other cellular mechanisms, including the impaired Ca^{2+} mobiliz-

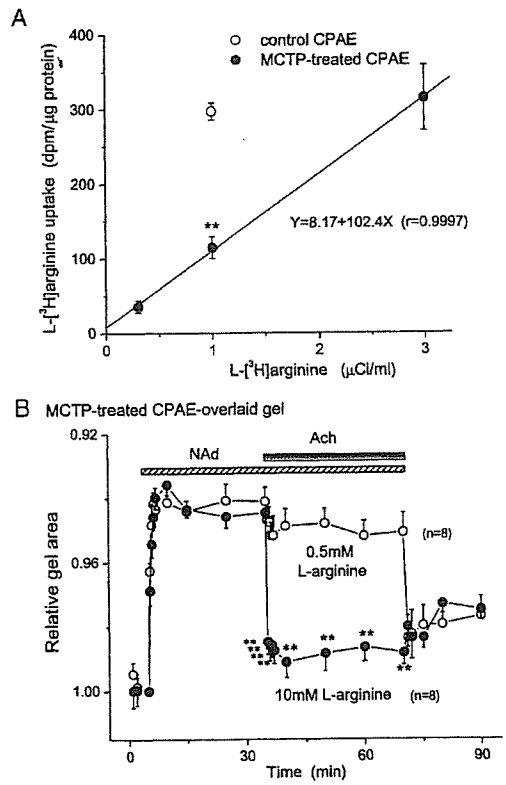

Fig. 5. Effects of high concentration of extracellular L-arginine on its cellular uptake and NO production in MCTP-treated CPAEs. (A) Cellular uptake of L-[^{3}H]arginine was linearly increased according to its extracellular concentration in MCTP-treated CPAEs (closed circles). Note that MCTP-treated CPAEs showed markedly lower uptake of L-[^{3}H]arginine than control CPAEs (open circles). Each symbol represents mean ± SEM value from 6 measurements. $^{**}P < 0.01$ vs. control. (B) High concentration of L-arginine (10 mM) reversed the Ach-induced relaxation of the pre-contracted gels (closed circles). The relaxation was significantly larger than that in the presence of 0.5 mM L-arginine (open circles). $^{**}P < 0.01$ vs. 0.5 mM L-arginine.

ing properties (Fig. 3), lower expression level of eNOS protein (Fig. 4) and reduced cellular uptake of L-arginine (Fig. 5A). Previous study has shown that the expression of eNOS mRNA was rather augmented, but its protein level was decreased in MCTP-treated rat lungs (Tyler et al., 1999). We have also observed that the expression of eNOS mRNA, assessed with RT-PCR, was not different between control and MCTP-treated CPAEs (data not shown). This suggests that the reduced expression of eNOS protein may be due to the impairment of translation but not transcription of its mRNA. Therefore, though it was reported that MCTP binds to DNA and inhibits mRNA transcription (Thomas et al., 1996), we suppose that this is not the only mechanism of action of MCTP on endothelial damage. Actually, recent reports have proposed another possible mechanism for the MCTP-induced impairment of cellular functions, that is, blockade of Golgi transport (Mathew et al., 2004; Shah et al., in press), that would affect cellular integrity including eNOS functions. Therefore, we suppose that the MCTP-induced impairment of cellular functions is due to various cellular and molecular mechanisms.

In control gels, it seems that L-arginine was supplied from DMEM, which contains 0.5 mM L-arginine and was used for constructing the model vessel. We supposed that this L-arginine concentration in the gels might not be sufficient for NO production in MCTP-treated CPAEs. This was indeed the case, that is, endothelium-dependent relaxation was observed in MCTP-treated CPAEs in the presence of excess concentration of L-arginine (10 mM, Fig. 5B). It is well known as "arginine paradox" that excess concentration of L-arginine induces NO production in a concentration-dependent manner at far above the *K*d values of NOS, though the precise mechanisms are still not clarified (Kurz and Harrison, 1997; McDonald et al., 1997; Tsikas et al., 2000). Therefore, we suppose that the restoration of NO production in MCTP-treated CPAEs by excess L-arginine was due to both the arginine paradox phenomenon and the increased cellular uptake of L-arginine.

Dysfunction of pulmonary vascular endothelial cells is thought to play a central role in the pathogenesis of PH not only in experimental animals but also in human (Higenbottam and Laude, 1998; Michelakis, 2003). It is also supposed that the reduction of NO production is responsible in PH caused by MCTP (Mathew et al., 1995) or hypoxia (Millatt et al., 2003) and congenital PH model rat (Le Cras et al., 2000). Therefore, the treatment of PH with drugs modifying NO production has been recently highlighted (Michelakis, 2003). Oral intake and venous infusion of L-arginine have been reported to induce the reduction of pulmonary artery pressure in animal PH models (Mitani et al., 1997) and in PH patients (Mehta et al., 1995), but its detailed mechanism of action has not been clarified and its general use for the treatment of PH is still limited. The present study further revealed that the excess L-arginine would restore NO productivity in damaged endothelial cells with very low level of expression of eNOS protein and impaired Ca^{2+} mobilization. Therefore, other than NO gas inhalation (Gianetti et al., 2002) or oral intake of sildenafil (Wilkens et al., 2001), we suppose that excess supply of cheaper L-arginine might be another considerable maneuver for treating PH.

In conclusion, we have introduced a novel in vitro vessel model with MCTP-treated CPAEs in the present study and revealed that NO production can be restored by excess L-arginine even in damaged endothelial cells.

References

Campbell, A.I., Kuliszewski, M.A., Stewart, D.J., Frasch, H.F., Marshall, C., Marshall, B.E., Tyler, R.C., Muramatsu, M., Abman, S.H., Stelzner, T.J., Rodman, D.M., Bloch, K.D., McMurtry, I.F., 1999. Cell-based gene transfer to the pulmonary vasculature: endothelial nitric oxide synthase overexpression inhibits monocrotaline-induced pulmonary hypertension. Am. J. Respir. Cell Mol. Biol. 21, 567–575.

Chamley, J.H., Campbell, G.R., McConnell, J.D., Groschel Stewart, U., 1977. Comparison of vascular smooth muscle cells from adult human, monkey and rabbit in primary culture and in subculture. Cell Tissue Res. 177, 503–522.

Champion, H.C., Bivalacqua, T.J., Greenberg, S.S., Giles, T.D., Hyman, A.L., Kadowitz, P.J., 2002. Adenoviral gene transfer of endothelial nitric-oxide synthase (eNOS) partially restores normal pulmonary arterial pressure in eNOS-deficient mice. Proc. Natl. Acad. Sci. U.S.A. 99, 13248–13253.

Gianetti, J., Bevilacqua, S., De Caterina, R., 2002. Inhaled nitric oxide: more than a selective pulmonary vasodilator. Eur. J. Clin. Invest. 32, 628–635.

Higenbottam, T.W., Laude, E.A., 1998. Endothelial dysfunction providing the basis for the treatment of pulmonary hypertension: Giles F. Filley lecture. Chest 114, 72S–79S.

Ito, K.M., Sato, M., Ushijima, K., Nakai, M., Ito, K., 2000. Alterations of endothelium and smooth muscle function in monocrotaline-induced pulmonary hypertensive arteries. Am. J. Physiol. 279, H1786–H1795.

Kimura, C., Koyama, T., Oike, M., Ito, Y., 2000. Hypotonic stress-induced NO production in endothelium depends on endogenous ATP. Biochem. Biophys. Res. Commun. 274, 736–740.

Kimura, C., Cheng, W., Hisadome, K., Wang, Y.P., Koyama, T., Karashima, Y., Oike, M., Ito, Y., 2002. Superoxide anion impairs contractility in cultured aortic smooth muscle cells. Am. J. Physiol. 283, H382–H390.

Kimura, C., Oike, M., Ohnaka, K., Nose, Y., Ito, Y., 2004. Constitutive nitric oxide production in bovine aortic and brain microvascular endothelial cells: a comparative study. J. Physiol. (London) 554, 721–730.

Kurz, S., Harrison, D.G., 1997. Insulin and the arginine paradox. J. Clin. Invest. 99, 369–370.

Le Cras, T.D., Kim, D.H., Markham, N.E., Abman, A.S., 2000. Early abnormalities of pulmonary vascular development in the Fawn-Hooded rat raised at Denver's altitude. Am. J. Physiol. 279, L283–L291.

Malinski, T., Taha, Z., 1992. Nitric oxide release from a single cell measured in situ by a porphyrinic-based microsensor. Nature 358, 676–678.

Mathew, R., Zeballos, G.A., Tun, H., Gewitz, M.H., 1995. Role of nitric oxide and endothelin-1 in monocrotaline-induced pulmonary hypertension in rats. Cardiovasc. Res. 30, 739–746.

Mathew, R., Yuan, N., Rosenfeld, L., Gewitz, M.H., Kumar, A., 2002. Effects of monocrotaline on endothelial nitric oxide synthase expression and sulfhydryl levels in rat lungs. Heart Dis. 4, 152–158.

Mathew, R., Huang, J., Shah, M., Patel, K., Gewitz, M., Sehgal, P.B., 2004. Disruption of endothelial-cell caveolin-1α/raft scaffolding

during development of monocrotaline-induced pulmonary hypertension. Circulation 110, 1499–1506.

Mattocks, A.R., Jukes, R., Brown, J., 1989. Simple procedures for preparing putative toxic metabolites of pyrrolizidine alkaloids. Toxicon 27, 561–567.

McDonald, K.K., Zharikov, S., Block, E.R., Kilberg, M.S., 1997. A caveolar complex between the cationic amino acid transporter 1 and endothelial nitric-oxide synthase may explain the "arginine paradox". J. Biol. Chem. 272, 31213–31216.

Mehta, S., Stewart, D.J., Langleben, D., Levy, R.D., 1995. Short-term pulmonary vasodilation with L-arginine in pulmonary hypertension. Circulation 92, 1539–1545.

Michelakis, E.D., 2003. The role of the NO axis and its therapeutic implications in pulmonary arterial hypertension. Heart Failure Rev. 8, 5–21.

Millatt, L.J., Whitley, G.S., Li, D., Leiper, J.M., Siragy, H.M., Carey, R.M., Johns, R.A., 2003. Evidence for dysregulation of dimethylarginine dimethylaminohydrolase I in chronic hypoxia-induced pulmonary hypertension. Circulation 108, 1493–1498.

Minshall, R.D., Sessa, W.C., Stan, R.V., Anderson, R.G., Malik, A.B., 2003. Caveolin regulation of endothelial function. Am. J. Physiol.: Lung Cell. Mol. Physiol. 285, L1179–L1183.

Mitani, Y., Maruyama, K., Sakurai, M., 1997. Prolonged administration of L-arginine ameliorates chronic pulmonary hypertension and pulmonary vascular remodeling in rats. Circulation 96, 689–697.

Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109–142.

Reindel, J.F., Roth, R.A., 1991. The effects of monocrotaline pyrrole on cultured bovine pulmonary artery endothelial and smooth muscle cells. Am. J. Pathol. 138, 707–719.

Rubin, L.J., 1997. Primary pulmonary hypertension. N. Engl. J. Med. 336, 111–117.

Shah, M., Patel, K., Sehgal, P.B., in press. Monocrotaline pyrrole-induced endothelial cell megalocytosis involves a Golgi-blockade mechanism. Am. J. Physiol. Cell Physiol.

Thomas, H.C., Lame, M.W., Wilson, D.W., Segall, H.J., 1996. Cell cycle alterations associated with covalent binding of monocrotaline pyrrole to pulmonary artery endothelial cell DNA. Toxicol. Appl. Pharmacol. 141, 319–329.

Thomas, H.C., Lame, M.W., Morin, D., Wilson, D.W., Segall, H.J., 1998. Prolonged cell-cycle arrest associated with altered cdc2 kinase in monocrotaline pyrrole-treated pulmonary artery endothelial cells. Am. J. Respir. Cell Mol. Biol. 19, 129–142.

Tsikas, D., Boger, R.H., Sandmann, J., Bode-Boger, S.M., Frolich, J.C., 2000. Endogenous nitric oxide synthase inhibitors are responsible for the L-arginine paradox. FEBS Lett. 478, 1–3.

Tyler, R.C., Muramatsu, M., Abman, S.H., Stelzner, T.J., Rodman, D.M., Bloch, K.D., McMurtry, I.F., 1999. Variable expression of endothelial NO synthase in three forms of rat pulmonary hypertension. Am. J. Physiol. 276, L297–L303.

Wang, Y., Shin, W.S., Kawaguchi, H., Inukai, M., Kato, M., Sakamoto, A., Uehara, Y., Miyamoto, M., Shimamoto, N., Korenaga, R., Ando, J., Toyo oka, T., 1996. Contribution of sustained Ca^{2+} elevation for nitric oxide production in endothelial cells and subsequent modulation of Ca^{2+} transient in vascular smooth muscle cells in coculture. J. Biol. Chem. 271, 5647–5655.

Wilkens, H., Guth, A., Konig, J., Forestier, N., Cremers, B., Hennen, B., Bohm, M., Sybrecht, G.W., 2001. Effect of inhaled iloprost plus oral sildenafil in patients with primary pulmonary hypertension. Circulation 104, 1218–1222.

Case report

Successful therapy with argatroban for superior mesenteric vein thrombosis in a patient with congenital antithrombin deficiency

Muta T, Okamura T, Kawamoto M, Ichimiya H, Yamanaka M, Wada Y, Urata M, Kayamori Y, Hamasaki N, Kato K, Eto T, Gondo H, Shibuya T. Successful therapy with argatroban for superior mesenteric vein thrombosis in a patient with congenital antithrombin deficiency. Eur J Haematol 2005: 75: 167–170. © Blackwell Munksgaard 2005.

Abstract: A 38-year-old woman was admitted with superior mesenteric vein (SMV) thrombosis, which was refractory to anticoagulation therapy. The plasma antithrombin activity was decreased and hardly compensated by concentrated antithrombin preparation due to high consumption rate. However, successful anticoagulation was achieved by administration of direct thrombin inhibitor, argatroban. Family studies of antithrombin activity revealed that she had type I congenital antithrombin deficiency. A novel heterozygous mutation in the gene for antithrombin (single nucleotide T insertion at 7916 and 7917, Glu 272 to stop in exon 4) was identified. Argatroban administration would be effective in the treatment of congenital antithrombin deficiency with SMV thrombosis.

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Key words: congenital antithrombin deficiency; superior mesenteric vein thrombosis; argatroban

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It was recently reported that asymptomatic congenital antithrombin deficiency has a prevalence of 1 in 600 in the general population (1). Such deficiency underlied 3–5% in patients with thrombotic diseases (2). Superior mesenteric vein (SMV) is an occasional site of thrombotic manifestation in congenital antithrombin deficiency. The SMV thrombosis causes a short bowel syndrome (3) and sometimes proceeds to a fatal outcome (4).

We describe a congenital antithrombin deficiency patient with SMV thrombosis, who was successfully treated with a direct thrombin inhibitor, argatroban, in combination with concentrated antithrombin preparation.

Case description

A 37-year-old woman was admitted to the Department of Surgery of our hospital due to severe abdominal pain, while she was receiving fertility treatments with a combination of ethinil-estradiol and norgestrel in April 2004. She was diagnosed with SMV thrombosis using computed tomography and magnetic resonance angiography. Intestinal congestion caused extreme swelling of the jejunal wall, resulting in obstructive ileus. Acute pancreatitis and massive ascites were also accompanied. She received continuous infusion of heparin (1200 IU/h), gabexate mesilate (FOY) (1.6 mg/kg/h), and

urokinase (240 000 U/d). The antithrombin activity in the plasma was markedly decreased to 34% (normal, 70–125%), so concentrated antithrombin preparation derived from normal human plasma (Neuart®, Mitsubishi Pharma Co., Tokyo, Japan) was administered at a dose of 60 U/kg/d for five consecutive days. The plasma levels of protein C and protein S activities were within normal limits. Lupus anticoagulant or anticardiolipin antibody was not detected. Her symptoms were alleviated and warfarin was instituted to prevent thrombosis. Heparinization kept an adequate activated partial thromboplastin time (aPTT) between 40 and 100 s. In addition, the thrombo test was controlled around 20% in the short period of warfarinization. However, thrombosis recurred and she developed hypovolemic shock in May. C-reactive protein (CRP) elevated up to 34 mg/dL, and her antithrombin activity markedly decreased to 16.8%. Heparin, FOY, and urokinase were restarted. Soon after administration of concentrated antithrombin preparation, plasma antithrombin activity was elevated to 85.5% but quickly decreased 45.6%. Severe abdominal pain continued and CRP was re-increased. The consumption rate of antithrombin was too fast to control the activity of thrombosis, so argatroban was started at the dose of 1 μg/kg/min and she was referred to our department. Since the consumption rate of antithrombin was too high to control the activity of thrombosis, argatroban was instituted at a dose of 1 μg/kg/min (Fig. 1) after informed consent was obtained. Argatroban was administered to keep the aPTT between 37 and 60 s, although the aPTT was increased transiently. Thereafter, clinical findings gradually improved. The plasma levels of plasmin-α2-plasmin inhibitor complex (PIC), thrombin-antithrombin complex (TAT), d-dimer, and CRP decreased below the estimation limit. However, the plasma activity and the plasma concentration of antithrombin were still decreased to 35.4% and 9.7 mg/dL (normal, 23.6–33.5 mg/dL), respectively. Family studies revealed that her mother had received the treatment of cerebral infarction, and her sister had a history of deep vein thrombosis of the leg during pregnancy. The plasma activity and the antigen concentration of antithrombin of her mother were 60.4% and 16.9 mg/dL, her sister 49% and 12.2 mg/dL, and her nephew 51.4% and 15.7 mg/dL, respectively. The DNA sequence analysis of the patient was performed using the methods described elsewhere (5, 6), which demonstrated a novel insertion mutation, CTG to CTTG at nucleotide positions 7916–7917 in one allele of exon 4 in the antithrombin gene (Fig. 2). This mutation caused deletion of the C-terminal domain of antithrombin and loss of thrombin binding domain. There were no other alterations in the exons and exon–intron boundaries of this gene. As a result, the patient was diagnosed as a heterozygote for type I congenital antithrombin deficiency. Argatroban was administered for 20 d and then replaced by warfarin. When we stopped the administration of argatroban, we introduced

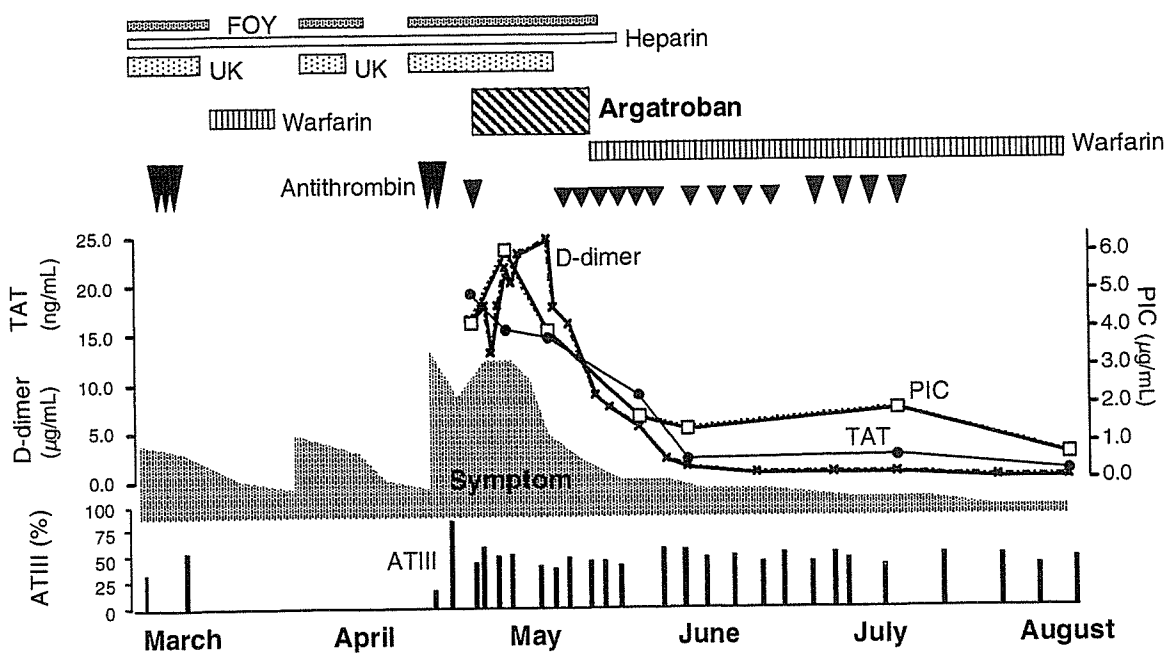

Fig. 1. Clinical course. TAT, thrombin-antithrombin complex; PIC, plasma-α2-plasmin inhibitor complex; FOY, gabexate mesilate; UK, urokinase; antithrombin, concentrated antithrombin preparation; ATIII, antitrhombin activity (trough).

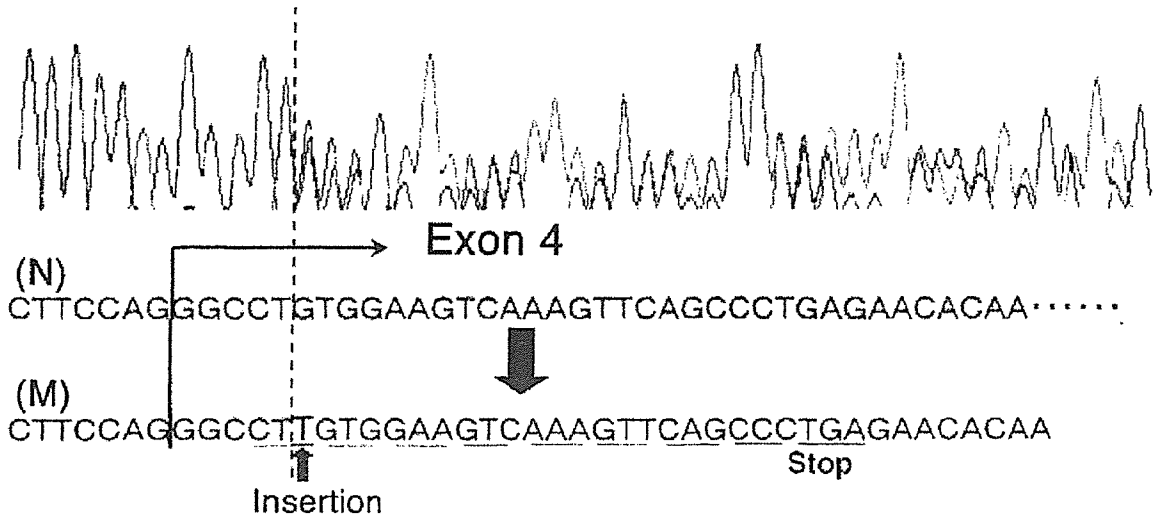

Fig. 2. Direct sequence analysis of the antithrombin gene demonstrated an insertion of T at nucleotide positions 7916–7917 in one allele of exon 4, which caused frameshift and changed the codon 272 to stop. The blue curve indicated the nucleodide 'C', the red curve 'T', the black curve 'G', and the green curve 'A'. The upper line (N) corresponded to the normal allele and the lower line (M) indicated the mutational allele.

concentrated antithrombin preparation at a dose of 30 IU/kg to keep the antithrombin activity up to 70%. Two months later, concentrated antithrombin preparation was combined and she received jujunal anastomosis for obstructive ileus. Thrombosis did not recur by prophylactic administration of warfarin alone thereafter. She discharged with a perfect performance status.

Discussion

Argatroban is a synthetic direct thrombin inhibitor that specifically but reversively binds to the catalytic site of thrombin (7). Argatroban can bind and exert an effect on both free thrombin and fibrin clot-bound thrombin (7). Argatroban is an effective drug for the treatment of acute cerebral infarction (8) and peripheral arterial obstructive disease (9). As for a patient with congenital antithrombin deficiency, argatroban is used during dialysis (10) to prevent clotting in the extracorponeal circuit. In a case report, argatroban was effective for SMV thrombosis caused by acquired antithrombin deficiency due to heavy alcohol consumption (11).

In our patient, a novel point mutation was demonstrated in the gene for antithrombin, which had not previously been reported in the database (12) or in other reports (13–19). Our patient maintained an innate half dose of functional antithrombin, and did not have a history of thrombosis from birth. Fertility treatments with estradiol and norgestrel might have triggered the thrombosis as indicated in the literature (20, 21). The third thrombotic episode was quite severe, and a life threatening. Although a high dose of concentrated antithrombin preparation was introduced, the plasma antithrombin activity promptly decreased and we could not control the progression of thrombosis. However, argatroban administration led to a rapid improvement of thrombosis. Concentrated antithrombin preparation was less needed to keep antithrombin activity. Argatroban may have delay antithrombin consumption and increase antithrombin activity, as suggested by Matsuo *et al.* (7). As for adverse effects of argatroban in our patient, serum levels of transaminase, ALP and gamma GTP were slightly elevated, which did not require specific treatments or discontinuation of argatroban administration. In the literature, 1–3% of patients showed liver dysfunction as adverse events (7).

Our patient indicates that argatroban administration should be considered in congenitally antithrombin-deficient patients complicated with thrombosis who may require immediate anticoagulation. More experience and trials are needed to define the efficacy and safety of argatroban in congenital antithrombin deficiency complicated with thrombosis.

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References

1. Tait RC, Walker ID, Perry DJ, Islam SI, Daly ME, McCall F, Conkie JA, Carrell RW. Prevalence of

antithrombin deficiency in the healthy population. Br J Haematol 1994;**87**:106–112.
2. Perry DJ, Carrell RW. Molecular genetics of human antithrombin deficiency. Hum Mutat 1996;**7**:7–22.
3. Karl R, Garlick I, Zarins C, Cheng E, Chediak J. Surgical implications of antithrombin III deficiency. Surgery 1981;**89**:429–433.
4. Gruenberg JC, Smallridge RC, Rosenberg RD. Inherited antithrombin-III deficiency causing mesenteric venous infarction: a new clinical entity. Ann Surg 1975;**181**:791–794.
5. Watanabe K, Shibuya A, Ishii E, *et al.* Identification of simultaneous mutation of fibrinogen alpha chain and protein C genes in a Japanese kindred. Br J Haematol 2003;**120**:101–108.
6. Kurihara M, Urata M, Iida H, Watanabe K, Wada Y, Wakiyama M, Kinoshita S, Hamasaki N. Characterization of two novel mutations of antithrombin gene observed in Japanese thrombophilic patients. Thrombosis Res 2005;**115**:351–358.
7. Matsuo T, Koide M, Kario K. Development of argatroban, a direct thrombin inhibitor, and its clinical application. Semin Thromb Hemos 1997;**23**:517–522.
8. Urabe T, Tanaka R, Noda K, Mizuno Y. Anticoagulant therapy with a selective thrombin inhibitor for acute cerebral infarction: usefulness of coagulation markers for evaluation of efficacy. J Thromb Thrombolysis 2002;**13**:155–160.
9. Matsuo T, Kario K, Matsuda S, Yamaguchi N, Kakishita E. Effect of thrombin inhibition on patients with peripheral arterial obstructive disease: a multicenter clinical trial of argatroban. J Thromb Thrombolysis 1995;**2**:131–136.
10. Kawada T, Kitagawa H, Hoson M, Okada Y, Shiomura, J. Clinical application of argatroban as an alternative anticoagulant for extracorporeal circulation. Hematol Oncol Clin North Am 2000;**14**:445–457.
11. Dager WE, Gosselin RC, Owings JT. Argatroban therapy for antithrombin deficiency and mesenteric thrombosis: case report and review of the literature. Pharmacotherapy 2004;**24**:659–663.
12. Lane DA, Bayston T, Olds RJ, *et al.* Antithrombin mutation database: 2nd update. Thromb Haemost 1997;**77**:197–211.
13. Nagaizumi K, Inaba H, Amano K, Suzuki M, Arai M, Fukutake K. Five novel and four recurrent point mutations in the antithrombin gene causing venous thrombosis. Int J Hematol 2003;**78**:79–83.
14. Fitches AC, Appleby R, Lane DA, De Stefano V, Leone G, Olds RJ. Impaired cotranslational processing as a mechanism for type I antithrombin deficiency. Blood 1998;**92**:4671–4676.
15. Nakahara Y, Tsuji H, Nakagawa K, *et al.* Detection of two novel mutations (nt2762, exon 2, CAG to TAG, and nt2483 or 2484, exon 2, +A) in individuals with congenital type I antithrombin deficiencies. Blood Coagul Fibrinolysis 1999;**10**:229–231.
16. Beauchamp NJ, Makris M, Preston FE, Peake IR, Daly ME. Major structural defects in the antithrombin gene in four families with type I antithrombin deficiency – partial/complete deletions and rearrangement of the antithrombin gene. Thromb Haemost 2000;**83**:715–721.
17. Yamada T, Yamada H, Morikawa M, Kato EH, Kishida T, Ohnaka Y, Nikaido H, Ozawa T, Fujimoto S. Management of pregnancy with congenital antithrombin III deficiency: two case reports and a review of the literature. J Obstet Gynaecol Res 2001;**27**:189–197.
18. Niiya K, Kiguchi T, Dansako H, Fujimura K, Fujimoto T, Iijima K, Tanimoto M, Harada M. Two novel gene mutations in type I antithrombin deficiency. Int J Hematol 2001;**74**:469–472.
19. Nagaizumi K, Inaba H, Amano K, Suzuki M, Arai M, Fukutake K. Five novel and four recurrent point mutations in the antithrombin gene causing venous thrombosis. Int J Hematol 2003;**78**:79–83.
20. Abet D, Pietri J. Portal and superior mesenteric venous thrombosis secondary to oral contraceptive treatment. Two cases. J Mal Vasc 1982;**7**:59–63.
21. Graubard ZG, Friedman M. Mesenteric venous thrombosis associated with pregnancy and oral contraception. A case report. S Afr Med J 1987;**71**:453.

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REGULAR ARTICLE

Characterization of two novel mutations of the antithrombin gene observed in Japanese thrombophilic patients

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KEYWORDS
Structure and function of antithrombin;
Gene analysis;
Deep vein thrombosis, 20210A, factor V Leiden

Abstract We investigated the molecular basis of reduced functional levels of antithrombin (AT) in two individuals suffering from thromboembolic events. In each case direct sequencing of amplified DNA revealed 13,260–13,262 del in one patient and 2511C>A in the other patient, predicting a heterozygous E381del and P16H, respectively. Both patients had no 20210A allele and factor V Leiden mutation. To understand the molecular mechanism responsible for antithrombin deficiency, stable expression experiments were performed using HEK293 cells transfected with the expression vector containing the wild-type or the mutated recombinant cDNA. In these experiments, the media levels of the two mutated antithrombins were the same as that of wild type, but the specific activity of the E381del mutant decreased significantly compared with that of wild type. These results showed that the E381del mutation was responsible for type II deficiency, whereas the other mutation, P16H, did not produce any definite abnormality which could contribute to antithrombin deficiency.

Abbreviations: AT, antithrombin; PS, protein S; PC, protein C; PT, prothrombin time; APTT, activated partial thromboplastin time; FDP, fibrin degradation product; TAT, thrombinantithrombin complex; PIC, plasmin–plasmin inhibitor complex; HEK293, human embryo kidney 293 cells.

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Antithrombin (AT) is a 58-kDa plasma glycoprotein which is mainly synthesized in hepatocytes and circulates at a concentration of approximately 112–140 mg/l with a half-life of 2 to 3 days [1,2]. It is composed of 432 amino acid residues with four Asn-linked glycosylation sites [3,4]. AT belongs to the serine proteinase inhibitor (serpin) superfamily and plays a critical role in the regulation of the blood coagulation system by inhibiting the final two proteinases of factor Xa and thrombin [2,5–7]. Inhibition of proteinases by AT involves interaction between the active site of the proteinase and the reactive center loop of the inhibitor, which leads to an inactive and stable proteinase–inhibitor complex [8]. The reactive center of AT is held on an exposed peptide loop that extends from 15 residues (P15) on the amino-terminal side of the P1 residue (Arg393) to five residues (P5′) on the carboxy-terminal side [6–9]. After thrombin cleaves the reactive site of Arg393–Ser394, AT undergoes a dramatic conformational change when the cognate proteinases interact directly with the reactive center loop [6–11]. The reactive center loop is incorporated as strand four in the central β-sheet A when the loop interacts with the proteinases, and induces the massive conformational change of AT [8,12]. Inhibition of thrombin by AT is enhanced at least 1000-fold in the presence of heparin [2,13]. The human AT gene is mapped to chromosome 1q23.1–23.9 and comprises seven exons and six introns spanning a total of 13.5 kb of genomic DNA [14].

Since the existence of AT deficiency was first reported in 1965 [15], there has been an increasing line of evidence that a molecular anomaly of AT is an integral risk factor for thrombosis [16–22]. Our laboratory has been directing efforts to identify the causal factors that generate thrombosis by performing a systematic haemostatic investigation [23]. With respect to the AT anomaly, to date, all the coding exons and intron–exon boundaries of the AT gene were analyzed in 6 out of 22 patients having reduced AT activity. Abnormalities of AT gene were detected in two out of six thrombotic patients, with one a deletion mutation and the other a missense mutation. In the present study, we report on the identification and characterization of two novel mutations of the AT gene.

Materials and methods

Patients

Patient 1: A 51-year-old male patient had acute arterial obstruction from a right common iliac artery to a right thigh artery. He had undergone an operation on his right leg to remove the blood clots and a plasty in the right thigh arteries. The patient had a medical history of deep vein thrombosis in both legs at the age of 38. However, there was no suggestion of a family history of thrombosis.

Patient 2: The patient was a 64-year-old female with deep vein thrombosis in the right leg induced by infectious arthritis. A filling defect and deterioration of blood flow in the right femoral vein were observed by venography. She recovered from the deep vein thrombosis by heparin/warfarin treatment for a month. She has been suffering from myeloma for 10 years and impaired function of the liver due to chronic hepatitis B. There was no suggestion of family history of thrombosis. We were unable to study the hematological profile of her family.

Subjects

The subjects consisted of the proband and his daughter in patient 1 and the proband in patient 2. In addition, 50 healthy individuals recruited from the employees of our institution were subjected to this study as normal controls. Prior to the trial, written informed consent was individually obtained from all of them by the attending physicians following full explanation of the aim of the research and guarantee of privacy.

Plasma

Peripheral blood samples collected in 3.13% sodium citrate were centrifuged at 1500×*g* for 20 min, and the resulting supernatant fraction was used to perform clot-based tests. Aliquots of supernatant fractions divided into smaller portions were stored at −80 °C for future use.

Haemostatic examination

The haemostatic profile involved measurements of AT, protein S (PS), protein C (PC), fibrinogen, plasminogen, α2-plasmin inhibitor, heparin cofactor II, lupus anticoagulant, prothrombin time (PT), activated partial thromboplastin time (APTT), thrombotest, fibrin degradation product (FDP), thrombin–antithrombin complex (TAT), and plasmin–plasmin inhibitor complex (PIC) [24]. In addition, protein levels of AT and progressive AT activity were also assessed. AT activity was assayed by chromogenic substrate as heparin-dependent inhibition of bovine thrombin (heparin cofactor activity) using Testzym AT III 2 kit (Daiichi Kagaku, Tokyo, Japan). The reference interval ranged from 80% to 130%. AT

activity independent of heparin (progressive AT activity) was determined by chromogenic substrate after precipitation of fibrinogen by incubating at 56 °C for 15 min as previously described [23].

DNA extraction

Genomic DNA was extracted from peripheral lymphocytes collected from the patients, their relatives, and the healthy individuals using an automated DNA extraction device (NA-1000, KURABO, Osaka, Japan).

Polymerase chain reaction (PCR)

Early studies suggested that there were only six exons but subsequent analysis revealed a 1-kb intron within exon 3 [14]. Although we follow the old nomenclature of AT gene as consisting of exons 1–6, the primers cover all the exons from 1 to 7. Genomic regions of exons 1–6 of the AT gene were each amplified using appropriate primers in a reaction mixture containing 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 2.0 mM $MgCl_2$, 200 μM 4dNTPs, 0.2–0.4 μM primer-set, 0.8–4 ng/μl template DNA, and 0.025 U/μl Extaq (Takara Shuzo, Otsu, Japan). A reaction cycle consisting of sequential incubations for denaturation at 94 °C for 1 min, for annealing at 56–61 °C for 1 min, and for extension at 72 °C for 1 min was repeated twice with denaturation at 94 °C for an additional 4 min being included in the first cycle. Additionally, a reaction cycle consisting of sequential incubations for denaturation at 94 °C for 30 s, annealing at 56–61 °C for 30 s, and extension at 72 °C for 30 s was repeated 30 times, followed by incubation at 72 °C for 10 min. These reactions were performed in an automated device (Gene Amp PCR system 9600R, Roche Diagnostic Systems, Basel, Switzerland). Detection of the G20210A mutation in the prothrombin gene and factor V Leiden was performed as described by Finan et al. [25].

DNA sequencing

The PCR products derived from exons/introns of the AT gene were purified through a Micro Spin™ S-300HR column (Amersham Pharmacia Biotech, Bucks, UK) and processed for pretreatment using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The reaction products were purified through a Centri-Step Spin column (Perkin Elmer Applied Biosystems) and subjected to direct sequencing in an automated sequencer (ABI PRISM 377 sequencer, Perkin Elmer Applied Biosystems). To confirm the presence of mutated AT gene in patient 1, the PCR product of exon 6 was subcloned with the original TA cloning kit (Invitrogen, Carlsbad, CA, USA). For DNA sequencing, the insert DNA was amplified by the colony PCR method. The colony PCR products were then used for sequencing analysis.

Mutagenesis of AT cDNA

The full-length human AT cDNA was prepared from a human liver cDNA library (Uni-ZapRXR Library, Stratagene, CA, USA) by PCR using a mutagenic primer set (sense sequence of 5′-TGTCGACGAT-TAGCGGCCATGTATTC-3′ and antisense sequence of 5′-AACCCG GGAAGAGGTGCAAAG-3′, mutagenic C, C and CCC are underlined), by which new *Sal*1 and *Sma*1 sites are produced in the amino-terminal and carboxyl-terminal regions of the complete AT coding sequence, respectively. The PCR product was sequenced to check it had a proper sequence. An expression vector for the wild-type AT was constructed as follows by inserting the full-length AT cDNA into pC1neo Mammalian Expression Vector (Promega, WI, USA). The 1466-bp PCR product was restricted with *Sal*1 and *Sma*1 and then ligated to a 5466-bp *Sal*1–*Sma*1 restriction fragment of pC1neo Mammalian Expression Vector with T4 DNA ligase. The wild-type AT cDNA-vector construct was transformed into Epricurian Coli XL1-Blue supercompetent cells (Invitrogen). The sequences of DNA from the resulting colonies were verified to be correct by sequence analysis. Mutations were generated by the overlap extension method [26] using the wild-type AT cDNA-vector as a template. The final mutated PCR fragments were also inserted into pC1neo Mammalian Expression Vector as described above and the mutation was confirmed by sequencing of the resulting vector.

Stable expression of recombinant AT

Human embryo kidney 293 (HEK293) cells (Health Science Research Resources Bank, Osaka, Japan) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Rockville, USA) supplemented with 10% fetal calf serum (Gibco BRL), penicillin and streptomycin. After 11 μg of expression vector DNA and 3 μl/DNA μg of liposome reagent (Trans FastTM Transfection Reagent, Promega) were combined and incubated for 15 min at room temperature, the mixture was added to HEK293 cells

(3×105 per 60-mm dish). After 24 h, the medium was replaced with DMEM containing serum and antibiotics. After an additional 48 h, the transformed cells were selected in medium to which 1 mg/ml G418 (Gibco BRL) was added. The medium was replaced every 3 days. When the resistant cells were grown at about 50% confluency, the concentration of G418 in the medium was reduced to 0.6 mg/ml. After 2–3 weeks, the resistant cells were grown at 80–100% confluency and the medium was replaced with serum-free DMEM. After 48 h, the media were harvested and centrifuged at 1500×g for 5 min and the resulting supernatant fraction was stored in aliquots at −80 °C for future use.

Measurement of the activity and the antigen of recombinant AT in the culture medium

Assays of AT activity in the culture media were performed by an amidolytic assay using Testzym AT III 2 kit (Daiichi Kagaku). Assays of AT antigen level in the culture media were performed by Western blotting analysis. The supernatant of the culture media and purified plasma AT (Sigma, Missouri, USA) as a standard were electrophoresed on a 10% polyacrylamide gel for 60 min at 25 mA, and transferred to a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Pharmacia Biotech). The membrane was then immersed in the blocking buffer containing 50 mM Tris–HCl, 100 mM NaCl, 0.05% Tween 20 (pH 7.4) and 5% skim milk for 1 h, and incubated with goat anti-human AT antibody (1:6000) (Enzyme Research Laboratories, Indiana, USA) in the TBS–Tween buffer at 4 °C for 18 h. The membranes were then washed three times for 10 min each with the TBS–Tween buffer, and incubated for 1 h at room temperature with HRP anti-goat immunoglobulin (1:6000) (Amersham Pharmacia Biotech). The membranes were subsequently washed three times for 10 min each with the TBS–Tween buffer. Immunoreactive bands were visualized with an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech), and measured using a luminoimage-analyzer (LAS-1000 plus, Fujifilm, Tokyo, Japan).

Results

Haemostatic examination

Patient 1: AT activity in the presence or absence of heparin in blood samples collected from the patient was nearly half of the normal level at 48% and 49%, respectively. The protein level was within the normal range at 117% (Table 1). All other analytes were within the normal range (Table 1). AT activity of the patient's daughter was within the normal range (data not shown).

Patient 2: AT activity in the presence of heparin and protein level of AT were nearly half of the normal level at 64% and 52%, respectively (Table 1). All other analytes were within the normal range with exception of the FDP and D-dimer, which were 0.0153 and 0.0062 g/l, respectively (Table 1).

Nucleotide sequence of the AT gene

Genomic DNA was extracted from peripheral blood cells of individuals. Genomic regions of exons 1–6 and their exon/intron junctions of the AT gene were amplified using appropriate primers and sequenced.

Patient 1: In direct sequence analysis of the AT gene of patient 1, additional aberrant peaks were observed from nt 13,260 in exon 6, suggesting heterozygous deletion or insertion mutation (Fig. 1). As a consequence of subcloning, deletion of AAG from nucleotide position 13,260 to 13,262, the mutation of which produces a deletion of Glu381, was detected in 7 out of 14 subclones. The remaining subclones were normal implying that the patient was

Table 1 Laboratory examination of the patient

	Reference interval	Patient 1	Patient 2
PT (%)	>70	51	72
APTT (s)	24.0–38.0	24.7	38.6
Fbg (g/l)	2.0–4.0	2.49	4.26
TBT (%)	>60	47	90
HPT (%)	60–120	55	87
PLG (%)	85–145	127	113
a2PI (%)	80–130	96	77
LAC	(−)	(−)	(−)
AT (%)	80–120	48	64
PS (%)	59–128	83	93
PC (%)	75–131	67	72
FDP (g/l)	0.0–0.005	0.0034	0.0153
PIC (g/l)	0.0–0.008	0.0003	0.001
D-D (g/l)	0.0–0.005	NT	0.0062
AT activity (%) Heparin (−)		49	NT
AT activity (%) Heparin (+)	80–120	48	64
AT antigen (%)	80–130	117	52

PT: prothrombin time; APTT: activated partial thromboplastin time; Fbg: fibrinogen; TBT: thrombotest; HPT: hepaplastintest; PLG: plasminogen; α2PI: α2 plasmin inhibitor; LAC: lups anticoagulants; AT: antithrombin; PS: protein S; PC: protein C; FDP: fibrinogen and fibrin degradation product; PIC: plasmin-α2PI complex; D-D: D-dimer; NT: not tested.

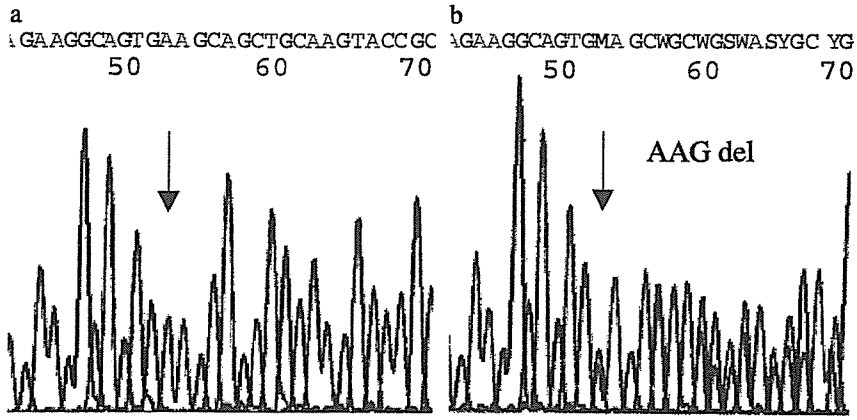

Figure 1 Nucleotide sequences of antithrombin exon 6 from patient 1. A deletion of AAG was observed in the patient's exon 6. (a) Normal control; (b) patient.

heterozygous for the deletion mutation. In his daughter, no mutations were detected in the AT gene.

Patient 2: Direct sequencing of the amplified exon 2 of patient 2 showed a cytosine to adenine transversion mutation at nucleotide position 2511 that converts proline-16 to histidine (P16H). The patient was heterozygous for the mutant (Fig. 2).

We analyzed genomic DNA from 50 healthy individuals for exon 2 and exon 6 of AT molecule to exclude the possibility of the detected mutations being polymorphisms (data not shown).

Secretion of wild-type and mutant ATs in stably transfected HEK293 cells

Wild-type and mutant ATs were expressed in cultured HEK293 cells to confirm whether the identified deletion mutation or amino acid substitution causes an AT deficiency. The AT activities and antigen levels in the culture supernatants were examined by an amidolytic assay and Western blotting analysis, respectively, and expressed as the concentrations of AT where purified plasma AT was used as a standard.

As shown in Fig. 3, both AT mutants of E381del and P16H were secreted normally from the transfected HEK293 cells to media as in the case of wild-type AT. The specific activities of wild-type and P16H mutant were 0.99±0.22 units/ng (mean±S.D., n=5) and 0.90±0.40 units/ng (mean±S.D., n=5), respectively, while the activity of E381del mutant was below a detectable level (Table 2).

Discussion

AT circulates in blood in an inactive form and becomes active upon association with glycosaminoglycans such as heparin and heparan sulphate which interact with the helix D region of AT [27,28]. The unusual long N-terminus region and the carbo-

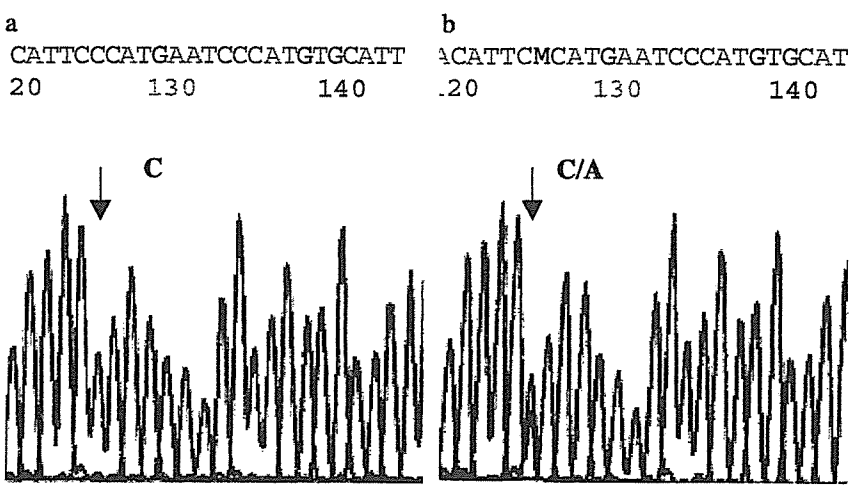

Figure 2 Nucleotide sequences of antithrombin exon 2 from patient 2. A substitution of C to A was observed in the patient's exon 2. (a) Normal control; (b) patient.

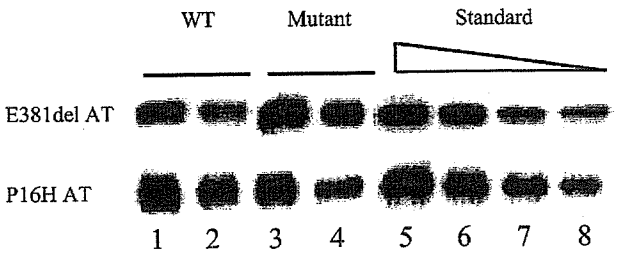

Figure 3 Immunoblot analysis of antithrombin in culture supernatant from E381del mutant (patient 1) under Reducing Conditions. Five microliters of culture supernatant (1:0—1:2 dilution) and standard plasma AT (1.8—14.3 ng of AT) was separated by SDS-PAGE (10% acrylamide) and the gels were electroblotted onto PVDF membranes. Antithrombin was stained with goat anti-human-antithrombin antibody (see Materials and methods). 1: wild; 2: wild (1:2 dilution); 3: mutant, 4: mutant (1:2 dilution); 5—8: standard plasma AT (14.3, 7.3, 3.7, 1.8 ng of AT, respectively).

hydrate attached at Asn135 are specific in AT among the serpin superfamily and affect the heparin binding to AT [8]. The effect of heparin binding to the helix D region is to expel the sheet-inserted residue P15 (Gly379) and P14 (Ser380) from β-sheet A, so that the whole reactive center loop is exposed [29,30]. The allosteric effect between the hinge region (Gly379—Glu381) and the heparin binding region is essential for the heparin activation [31].

The AT deficiency is roughly divided into type I and type II deficiencies. Type I deficiency ("Classical deficiency") has reduced levels of immunologically and functionally determined AT. Type II deficiency has been applied to the cases in which the functionally determined AT is reduced. Type II deficiency is further divided into the heparin binding site (HBS)-type in which heparin binding is abnormal; reactive site (RS)-type in which the reactive center loop is abnormal; and pleiotropic effect (PE)-type in which the influence is pleiotropic [32]. Patient 1 in this study was type II deficiency showing a normal immunological (protein) level and a reduced AT activity (Table 1), and the E381del mutation was detected by base sequencing (Fig. 1). Patient 2 was type I with reduced AT level and reduced activity in plasma (), and base sequencing detected a mutation at P16H on the N-terminal (Fig. 2).

Expression experiments of these AT mutants transfected into HEK293 cells indicated that both of the mutant molecules were secreted normally into culture media (Fig. 3). However, the E381 del mutation had no AT activity, while the P16H mutant had the same specific activity as that of wild type (Table 2). Glutamic acid at 381 (P13) is in the hinge region (P15—P8; Gly379—Thr386) of the reactive center loop and is highly conserved to the same extent as P12 (Ala382) in the serpin superfamily [33,34]. The hinge region and the reactive center loop play pivotal roles for the structural/function relationship of AT [6,8,29,30] and A382T at P12 mutation is known as AT Hamilton or Glasgow II [35—37]. A glutamic acid (Glu381) in the reactive center loop at position P13 has a central role for the allosteric activation of AT by heparin binding [38]. The crystal structure shows that Glu381 contacts stabilize the activated conformation [38]. The loop in almost all the serpins is formed by 17 residues [33] and the tight conservation of the length of serpin reactive center loops is striking when compared with the wide variance in the length of surface loops for other protein families. The inhibitory activity of AT to factor Xa depended upon the length of the N-terminal portion of the reactive center loop, and the deletion of one or two residues lowered the inhibitory activity of AT as well as PAI-1 and PAI-2 [39]. The deletion of residues in the reactive center loop converts the serpin into a substrate [39]. Considering these evidence, we would safely conclude that E381 del mutation had lost the AT activity because the reactive center loop was shortened by deleting glutamic acid at 381.

In the case of P16H mutant of Patient 2, the AT level secreted into the culture media and the specific activity were similar to those of the wild-type (Fig. 3 and Table 2), suggesting that both the protein processing for secretion and the inhibitory activity were normal. Since conservation of proline at position 16 is low in the serpin superfamily [33], its involvement in the retention of the stereostructure of AT and proteinase-inhibitory activity may be low. Incubation of P16H mutant at 40°C did not demonstrate the thermal instability compared to the wild type (data not shown). How do we then explain the fact that Patient 2 exhibited the phenotype of type I deficiency (see Table 2)? In this study, we sequenced all seven exons and the exon—intron boundaries of AT gene by PCR (see Materials and methods). Our method applied in this study, how-

Table 2 AT specific activities in normal pooled plasma and in media secreted from HEK293 cells transfected with wild-type and mutants AT genes

Type of AT genes	Specific activity [arbitrary unit] (units/ng)
Wild-type	0.99±0.22 (n=5)
E381-del mutant	undectectable (n=3)
P16H mutant	0.90±0.40 (n=5)
Normal pooled plasma	1.35±0.33 (n=3)

ever, is not a valid method for detecting partial gene deletion or rearrangement, indicating that we could not exclude a possibility of gene deletion or rearrangement. Patient 2 was a 64-year-old female with deep vein thrombosis in the right leg induced by infectious arthritis. She has been suffering from myeloma for 10 years and also suffering from liver dysfunction due to hepatitis B. Her deep vein thrombosis in the right leg was induced by infectious arthritis in the same leg. She responded to heparin/warfarin treatment during the course of a month, indicating that the deep vein thrombosis was relatively mild. The P16H missense mutation was not due to polymorphism (see Results). Kondo et al. analyzed the molecular deficiency mechanism of heparin cofactor II, and reported a patient whose heparin cofactor II mutant was secreted normally into the culture media of transfected HEK293 cells, although the heparin cofactor II level was decreased in the pateint's plasma [40]. Although the P16H mutant did not show the thermal instability at 40°C, it might be possible that the half-life is shortened due to mutation in some reasons, thereby increasing elimination from the circulation. Regarding the genesis of the decreased plasma AT level in Patient 2, either the P16H mutant may have been secreted into the circulation, but was rapidly degraded, or the mutation may not have been directly involved in the reduction of AT in Patient 2. At the present time, however, we are unable to offer a reasonable explanation that she exhibited the phenotype of type I AT deficiency. It could be due to a complication of myeloma, liver dysfunction and arthritis.

References

[1] Murano G, Williams L, Miller-Andersson M, Aronson DL, King C. Some properties of antithrombin-III and its concentration in human plasma. *Thromb Res* 1980;**18**:259–62.

[2] Mammen EF. Antithrombin: its physiological importance and role in DIC. *Semin Thromb Hemost* 1998;**24**:19–25.

[3] Franzen L-E, Svensson S, Larm O. Structual studies on the carbohydrate portion of human antithrombin. *J Biol Chem* 1980;**255**:5090–3.

[4] Prochownik EV, Markham AF, Orkin SH. Isolation of a cDNA clone for human antithrombin. *J Biol Chem* 1983;**258**: 8389–94.

[5] Huntington JA, Read RJ, Carrell RW. Structure of a serpin–protease complex shows inhibition by deformation. *Nature* 2000;**407**:923–6.

[6] Stein PE, Carrell RW. What do dysfunctional serpin tell us about molecular mobility and diseases? *Struct Biol* 1995;**2**:96–113.

[7] Wright HT, Scarsdale JN. Structual basis for serpin inhibitor activity. *Proteins* 1995;**22**:210–25.

[8] Whisstock J, Skinner R, Lesk AM. An atlas of serpin conformations. *Trends Biochem Sci* 1998;**23**:63–7.

[9] Stratikos E, Gettins PG. Formation of the covalent serpin–proteinase complex involves translocation of the proteinase by more than 70A and full insertion of the reactive center loop into beta-sheet A. *Proc Natl Acad Sci U S A* 1999;**96**:4808–13.

[10] Bjork I, Danielsson A, Fenton JW, Jornvall H. The site in human antithrombin for functional proteolytic cleavage by human thrombin. *FEBS Lett* 1981;**126**:257–60.

[11] Bjork I, Jackson CM, Jornvall H, Lavine KK, Nordling K, Salsgive WJ. The active site of antithrombin. Release of the same proteolytically cleaved form of the inhibitor from complexes with factor χa, factor χa, and thrombin. *J Biol Chem* 1982;**257**:2406–11.

[12] Loebermann H, Tokuoka R, Deisenhofer J, Huber R. Human alpha 1-proteinase inhibitor. Crystal structure analysis of two crystal modifications, molecular model and preliminary analysis of the implications for function. *J Mol Biol* 1984;**177**:531–57.

[13] Rosenberg RD, Damus PS. The purification and mechanism of action antithrombinheparin cofactor. *J Biol Chem* 1973;**248**:6490–505.

[14] Olds RJ, Lane DA, Chowdhury V, De Stefano V, Leone SL, Thein SL. Complete nucleotide sequence of the antithrombin gene: evidence for homologous recombination causing thrombophilia. *Biochemistry* 1993;**32** 4216–24.

[15] Egeberg O. Inherited antithrombin deficiency causing thrombophilia. *Thromb Diath Haemorrh* 1965;**13**: 516–30.

[16] Sas G, Blasko G, Banhegyi D. Abnormal antithrombin III (Antithrombin III "Budapest") as a cause of familial thrombophilia. *Thromb Diath Haemorrh* 1974;**32**:105–15.

[17] Thaler E, Lechner K. Antithrombin III deficiency and thromboembolism. *Clin Haematol* 1981;**10**:369–90.

[18] Finazzi G, Tran TH, Barbui T, Duckert B. Purification of antithrombin 'Vicenza': a molecule with normal heparin affinity and impaired reactivity to thrombin. *Br J Haematol* 1985;**59**:259–63.

[19] Hirsh J, Piovella F, Pini M. Congenital antithrombin III deficiency: incidence and clinical features. *Am J Med* 1989;**87**:34–8.

[20] Caso R, Lane DA, Thompson EA, Olds RJ, Thein SL, Panico M, et al. Antithrombin Vicenza, Ala 384 to Pro (GCA to CCA) mutation, transforming the inhibitor into a substrate. *Br J Haematol* 1991;**77**:87–92.

[21] Demers C, Ginsberg JS, Hirsh J, Henderson P, Blajchman MA. Thrombosis in antithrombin III-deficient persons: report of a large kindred and Literature review. *Ann Intern Med* 1992;**116**:754–61.

[22] Aiach M, Gandrille S, Emmerich J. A review of mutations causing deficiencies of antithrombin, protein C, protein S. *Thromb Haemost* 1995;**74**:81–9.

[23] Tsuda H, Hattori S, Tanabe S, Iida H, Nakahara M, Nishioka S, et al. Screening for aetiology of thrombophilia: a high prevalence of protein S abnormality. *Ann Clin Biochem* 1999;**36**:423–32.

[24] Iida H, Nakahara M, Komori K, Fujise M, Wakiyama M, Urata M, et al. Failure in the detection of aberrant mRNA from the heterozygotic splice site mutant allele for protein S in a patient with protein S deficiency. *Thromb Res* 2001;**102**:187–96.

[25] Finan RR, Tamim H, Ameen G, Sharida HE, Rashid M, Almawi WY. Prevalence of factor V G1691A (factor V-Leiden) and prothrombin G20210A gene mutations in a recurrent miscarriage population. *Am J Hematol* 2002; **71**:300–5.

[26] Ho S, Hunt H, Horton R, Pullen J, Pease L. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 1989;**77:**51–9.

[27] Jin L, Abrahams JP, Skinner R, Petitou M, Pike RN, Carrell RW. The anticoagulant activation of antithrombin by heparin. *Proc Natl Acad Sci U S A* 1997;**94:** 14683–8.

[28] Pike RN, Potempa J, Skinner R, Fitton HL, McGraw WT, Travis J, et al. Heparin dependent modification of the reactive center arginine of antithrombin and cosequent increase in heparin binding affinity. *J Biol Chem* 1997;**272:**19652–5.

[29] Huntington JA, Olson ST, Fan B, Gettins PG. Mechanism of heparin activation of antithrombin. Evidence for reactive center loop preinsertion with expulsion upon heparin binding. *Biochemistry* 1996;**35:**8495–503.

[30] McCoy AJ, Pei XY, Skinner R, Abrahams JP, Carrell RW. Structure of betaantithrombin and the effect of glycosylation on antithrombin's heparin affinity and activity. *J Mol Biol* 2003;**326:**823–33.

[31] Huntington JA, McCoy A, Belzar KJ, Pei XY, Gettins PG, Carrell RW. The conformational activation of antithrombin. A 2.85-Å structure of a fluorescein derivative reveals an electrostatic link between the hinge and heparin binding regions. *J Biol Chem* 2000;**275:**15377–83.

[32] Lane DA, Bayston T, Olds RJ, Fitches AC, Cooper DN, Millar DS, et al. Antithrombin mutation database: 2nd update. For the plasma coagulation inhibitors subcommittee of the Scientific and Standardization Committee of international Society on Thrombosis and Haemostasis. *Thromb Haemost* 1997;**77:**197–211.

[33] Huber R, Carrell RW. Implications of the three-dimensional structure of alpha 1-antitrypsin for structure and function of serpins. *Biochemistry* 1989;**28:**8951–66.

[34] Skriver K, Wikoff WR, Patston PA, Pataton PA, Tausk F, Schapira M, et al. Substrate properties of C1 inhibitor Ma (alanine 434–glutamic acid). Genetic and structural evidence suggesting that the P12-region contains critical determinants of serineprotease inhibitor/substrate status. *J Biol Chem* 1991;**266:**9216–21.

[35] Devraj-Kizuk R, Chui DHK, Prochownik EV, Carter CJ, Ofosu MA, Blajchman MA. Antithrombin III Hamilton: a gene with a point mutation (guanine to adenine) in codon 382 causing impaired serine protease reactivity. *Blood* 1988;**72:**1518–23.

[36] Austin RC, Rachubinski RA, Ofosu FA, Blajchman MA. Antithrombin-III-Hamilton, Ala 382 to Thr: an Antithrombin-IIIvariant that acts a substrate but not an inhibitor of alpha-thrombin and Factor χa. *Blood* 1991; **77:**2185–9.

[37] Ireland H, Lane DA, Thompson E, Walker ID, Blench I, Morris HR, et al. Antithrombin Glasgow II: alanine 382 to threonine mutation in the serpin P12 position, resulting in a substrate reaction with thrombin. *Br J Haematol* 1991;**79:**70–4.

[38] Johnson DJ, Huntington JA. The influence of hinge region residue Glu-381 on antithrombin allostery and metastability. *J Biol Chem* 2004;**279:**4913–21.

[39] Zhou A, Carrell RW, Huntington JA. The serpin inhibitory mechanism is critically dependent on the length of the reactive center loop. *J Biol Chem* 2001;**276:** 27541–7.

[40] Kondo S, Tokunaga F, Kario K, Matsuo T, Koide T. Molecular and cellular basis for type 1 heparin cofactor IIdeficiency (Heparin CofactorIIAwaji). *Blood* 1996;**87:** 1006–12.