Adenovirus-Mediated Transfer of Human Placental Ectonucleoside Triphosphate Diphosphohydrolase to Vascular Smooth Muscle Cells Suppresses Platelet Aggregation In Vitro and Arterial Thrombus Formation In Vivo

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Background—Platelet-rich thrombus formation is a critical event in the onset of cardiovascular disease. Because ADP plays a significant role in platelet aggregation, its metabolism is important in the regulation of platelet activation and recruitment. Ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) is a key enzyme involved in vascular ADP metabolism. We recently isolated 2 isoforms of E-NTPDase from the human placenta. The present study examined whether these isoforms suppress platelet aggregation and thrombus formation after adenovirus-mediated gene transfer to vascular smooth muscle cells (SMCs).

Methods and Results—We constructed adenovirus vectors expressing human placental E-NTPDase isoforms I (AdPlac I) and II (AdPlac II) or bacterial β-galactosidase (AdLacZ). Vascular SMCs infected with AdPlac I expressed significant NTPDase activity and inhibited the platelet aggregation induced by ADP and collagen in vitro. In contrast, SMCs infected with AdPlac II and AdLacZ did not exert antiplatelet effects. To investigate the antithrombotic and antiproliferative effects of placental E-NTPDase isoform I in vivo, we generated thrombosis in rat carotid arteries by systemically administered rose Bengal and transluminal green light 5 days after gene transfer and examined neointimal growth 3 weeks after thrombus formation. Blood flow in AdLacZ-infected arteries rapidly deteriorated and vanished within 96±18 seconds of occlusive thrombus formation. In contrast, blood flow in AdPlac I–infected arteries was preserved for at least 10 minutes during irradiation. In addition, thrombus formation and subsequent neointimal growth were obviously suppressed.

Conclusions—The local expression of placental E-NTPDase in injured arteries might prevent arterial thrombosis and subsequent neointimal growth. (*Circulation*. 2005;111:808-815.)

Key Words: platelets ■ thrombosis ■ genetics ■ muscle, smooth ■ arteries

Thrombus formation is a critical event in the onset of cardiovascular disease. Thrombotic occlusion still occasionally arises after vascular interventions, such as angioplasty and stent implantation. Platelets play a pivotal role in the development of arterial thrombosis. Therefore, antiplatelet agents, including aspirin, ADP receptor blockers, and platelet glycoprotein (GP) IIb/IIIa antagonists, are systemically administered to prevent cardiovascular events.[1] Although some of these agents effectively reduce cardiovascular events,[1] they can also produce systemic hemorrhagic side effects.[2]

Platelet adhesion to injured vascular walls leads to platelet activation and the release of additional agonists such as ADP, serotonin, and thromboxane A_2, which cause further platelet recruitment to injured sites. Because ADP plays a key role in platelet aggregation,[3] its metabolism in the blood is important in the regulation of platelet activation and recruitment. Ectonucleoside triphosphate diphosphohydrolase (E-NTPDase; EC 3.6.1.5, or CD39) is a major metabolic enzyme of ADP in the vasculature.[4] Originally identified in B lymphocytes,[5] E-NTPDase is a membrane-bound enzyme that rapidly hydrolyzes both ATP and ADP to AMP and thereby inhibits platelet aggregation.[6-8] Makita et al[9] purified human placental E-NTPDase (Plac I) as an isoform of CD39 that alternatively differed at the *N*-terminus from that of CD39. Matsumoto et al[10] recently isolated another truncated variant

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of placental E-NTPDase (Plac II). Although these structural differences might affect their enzymatic activities,[11,12] the properties of the 2 isoforms remain unknown.

The present study used adenovirus-mediated gene transfer into vascular smooth muscle cells (SMCs) in vitro and in vivo to investigate whether the 2 isoforms of placental E-NTPDase inhibit platelet aggregation, thrombus formation, and neointimal growth.

Methods

Preparation of Recombinant Adenovirus With Placental E-NTPDase

Replication-defective E1$^-$ and E3$^-$ adenoviral vectors encoding Plac I (AdPlac I) and Plac II (AdPlac II) were generated with the Adeno-X expression system (Clontech) according to the manufacturer's instructions.[13] In brief, cDNA clones of Plac I (1554 bp) and Plac II (921 bp) were isolated at the Department of Blood Transfusion Medicine of Nara Medical University[12] and subcloned into the mammalian expression cassette pShuttle. Recombinant pShuttle was digested and inserted into the Adeno-X viral DNA. The recombinant viruses were propagated in HEK293 cells. Viral titers were determined by limiting dilution as plaque-forming units (PFU).[14] We also constructed control recombinant adenovirus encoding bacterial β-galactosidase (AdLacZ).

Cell Culture

Arterial SMCs were isolated from explanted thoracic aortas of Sprague-Dawley rats. Cells were cultured in SmGM2 growth medium (Sanko Junyaku) containing 5% fetal bovine serum and antibiotics. Confluent cells were immunopositive to anti-smooth muscle actin antibody (Dako Japan).

In Vitro Gene Transfer

Cells from passages 3 to 6 were incubated with the adenovirus vectors in the serum-free SmGM2 medium at the indicated multiplicity of infection (MOI). After incubation for 24 hours at 37°C, cells were washed twice with sterile phosphate-buffered saline and incubated in complete medium until assay.

Western Blots

Four days after infection, cells were lysed in Tris-buffered saline (pH 8.0) containing 150 mmol/L NaCl, 1% Nonidet P-40, 1% Triton X-100, and 1 mmol/L phenylmethylsulfonyl fluoride. After centrifugation at 12 000g for 5 minutes, the supernatant was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on 4% to 12% gradient gels, and then separated proteins were electrophoretically transferred to an Immobilon membrane (Millipore). Nonspecific binding was blocked with 5% skimmed milk, and then the membrane was incubated overnight at 4°C with a monoclonal anti-human placental E-NTPDase antibody (YH34). This novel antibody was produced in mice by a standard procedure at the Department of Blood Transfusion Medicine of Nara Medical University. Purified placental E-NTPDase used as an immunogen was prepared on an immunoadsorbent column as described.[9] Western blotting showed that YH34 bound with high affinity to the purified enzyme (≈80 kDa) under both nonreducing and reducing conditions (data not shown). We determined the immunoglobulin subclass and idiotype of YH34 (IgG_1-κ) by using an isotyping kit (Serotec). We purified YH34 IgG from mice ascites on a protein A–Sepharose CL-4B column (Amersham Bioscience Corp). The antibody recognizes the external domain of both Plac I and Plac II. Specific binding was visualized with a horseradish peroxidase–conjugated goat anti-mouse IgG (Nacalai Tesque) and an enhanced ChemiLuminescence reagent (DuPont NEN).

Analysis of NTPDase Activity

The activity of NTPDase in transfected SMCs was determined by measuring extracellular ATP and ADP concentrations with luciferin-luciferase.[15] In brief, cells (5×10^3) were seeded in 96-well plates and cultured in serum-free SmGM2 medium with 0.1% bovine serum albumin for 2 days. After 2 washes with phosphate-buffered saline, 100 μL of firefly luciferase ATP assay mixture (ATP determination kit, Molecular Probes) was added to each well. Luminescence was measured with a microplate luminometer (Fluoroskan Ascent FL, Labsystems). After 10 minutes of equilibration, 100 nmol/L exogenous ATP was added, and luminescence was measured at 1-minute intervals for up to 15 minutes. ATPase activity, calculated by measuring the degradation of exogenous ATP, was expressed as picomoles of inorganic phosphate per minute per milligram.

We estimated the ADP concentration by converting ADP to ATP.[16] After the cells were washed and the firefly luciferase ATP assay mixture was added, 100 nmol/L exogenous ADP was added to each well. After a 5-minute incubation, 1 U pyruvate kinase and 1 mmol/L phosphoenolpyruvate were added and luminescence was measured. ADPase activity, calculated by measuring the degradation of exogenous ADP, was expressed as picomoles of inorganic phosphate per minute per milligram.

Platelet Aggregation

We evaluated platelet aggregation in the presence of SMCs by using a modification of a described method.[17] Uninfected or infected SMCs detached by EDTA-collagenase were washed 3 times. Blood samples collected in 3.8% sodium citrate (9:1, vol/vol) were centrifuged at 900 or 3000 rpm for 10 minutes to prepare platelet-rich plasma (PRP) or platelet-poor plasma (PPP), respectively. PRP (5×10^8 platelets) and SMCs (5×10^5 cells) were incubated in siliconized cuvettes at 37°C in a PA-20 aggregometer (Kowa) that had been calibrated with PRP and PPP for 0% and 100% transmission, respectively. Thereafter, either ADP (10 μmol/L, final concentration) or collagen (10 μg/mL, final concentration) was added to the cuvettes, and platelet aggregation was measured.

Animal Care

The Animal Care Committee of Miyazaki Medical College (No. 1998-025-6) approved the study protocols. We used 59 male Sprague-Dawley rats weighing 400 to 500 g. The animals received humane care according to the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources and published by the National (Bethesda, Md) Institutes of Health (NIH publication No. 86-23, revised 1985). Aseptic surgery proceeded under general anesthesia induced by an intraperitoneal injection of pentobarbital (50 mg/kg body weight).

In Vivo Gene Transfer Into Injured Arteries

The common carotid arteries of the rat were exposed and isolated by temporary ligation at a distance of 1.5 cm. A 31-gauge needle was inserted into the proximal side of the segment. Thereafter saline was flushed into the segment, and then air was infused at a rate of 50 mL/min for 3.5 minutes to denude endothelial cells.[18] After the segment was filled with 0.1 mL of saline containing AdPlac I or AdLacZ (final titer, 5×10^8 PFU) or saline alone (n=6 each) for 30 minutes, the mixture was aspirated and blood flow was restored.[19] The vessels harvested 2 to 5 days after gene transfer were perfusion-fixed in 4% paraformaldehyde and embedded in paraffin by standard procedures. We detected the expression of human placental E-NTPDase protein by immunohistochemistry (EnVision+ kits) by using the primary monoclonal antibody YH34. The negative control included nonimmune mouse IgG_1 instead of YH34.

NTPDase Activity in Vessels

Carotid arteries without perfusion-fixation were excised and homogenized with a Polytron PT3000 (Kinematica) in Tris-buffered saline (pH 7.4) containing aprotinin and phenylmethylsulfonyl fluoride.[20,21] The homogenates were then incubated with the firefly luciferase ATP assay mixture. Extracellular ATP and ADP concentrations were

determined by measuring luminescence as described earlier. Protein concentrations of homogenates were determined by the bicinchoninic acid assay with bovine serum albumin as the standard.

Arterial Thrombus Formation and Neointimal Growth

Thrombus formation was produced in rat common carotid arteries 5 days after gene transfer by photochemical exposure under anesthesia.[9] Blood flow in the distal side of the arteries was recorded with a transit-time blood flowmeter (T106, Transonic Systems Inc) with a PowerLab system (AD Instruments Pty Ltd). After baseline blood flow was established, rose Bengal (20 mg/kg; Wako) was slowly injected into the jugular vein, and the carotid arteries were irradiated with green light (wavelength, 540 nm) with a xenon lamp (Hamamatsu Photonics) equipped with heat-absorbing and green filters. Blood flow was measured during irradiation for 10 minutes, and then the arteries were perfusion-fixed and stained with hematoxylin and eosin/Victoria blue for histological examination. To evaluate the neointimal growth 3 weeks after thrombus formation, the areas (μm^2) of neointima and media were measured with an image analyzing system (Axio Vision 2.05, Carl Zeiss) by 2 investigators (K.M. and K.H.) who were blinded to the treatment assignment.

Ex Vivo Platelet Aggregation, Prothrombin Time, and Activated Partial Thromboplastin Time

To confirm a systemic antithrombotic effect by local gene transfer, we evaluated platelet aggregation in response to either ADP or collagen ex vivo as described earlier. Prothrombin time and activated partial thromboplastin time were measured with a coagulation timer (Behring Fibrintimer, Behring Diagnostics) before and 5 days after gene transfer.

Statistical Analysis

All data are presented as mean±SE. An unpaired Student *t* test and ANOVA with Bonferroni multiple comparisons were used for comparisons between groups. A value of $P<0.05$ was considered significant.

Results

Expression of Human Placental E-NTPDases and NTPDase Activity of Infected SMCs In Vitro

We examined whether SMCs infected with AdPlac I, AdPlac II, or AdLacZ can produce biologically active E-NTPDases. Figure 1 shows 82- and 50-kDa immunoreactive bands that were detected from 2 days after gene transfer in lysates of cells infected with AdPlac I and AdPlac II, respectively.[9] These proteins persisted for at least 8 days thereafter (data not shown) but were undetectable in lysates of parental SMCs or of those infected with AdLacZ.

The ATPase activities of uninfected SMCs and of those infected with AdLacZ were 77.5±5.5 and 79.3±8.2 pmol $Pi \cdot min^{-1} \cdot mg^{-1}$, respectively (Figure 2A). In contrast, the ATPase activity of SMCs infected with AdPlac I was significantly high at an MOI of 100. We found that the ADPase activity of SMCs infected with AdPlac I was also high in an MOI-dependent manner (Figure 2B); however, the activities of ATPase and ADPase in AdPlac II–infected SMCs were similar to those of parental and AdLacZ-infected SMCs.

Platelet Aggregation With Infected SMCs In Vitro

We investigated whether the expression of Plac I and Plac II in SMCs inhibits platelet aggregation in vitro. Platelet aggregation induced by ADP was significantly and MOI-dependently suppressed in the presence of SMCs infected with AdPlac I, but not when the cells were infected with AdPlac II or AdLacZ (Figure 3A). Collagen-induced platelet aggregation was significantly suppressed in the presence of SMCs infected with AdPlac I at an MOI of 100 but not at that of 10 (Figure 3B). AdPlac I–infected SMCs dose-dependently suppressed platelet aggregation (data not shown), whereas SMCs infected with either AdPlac II or AdLacZ did not affect ADP/collagen-induced aggregation.

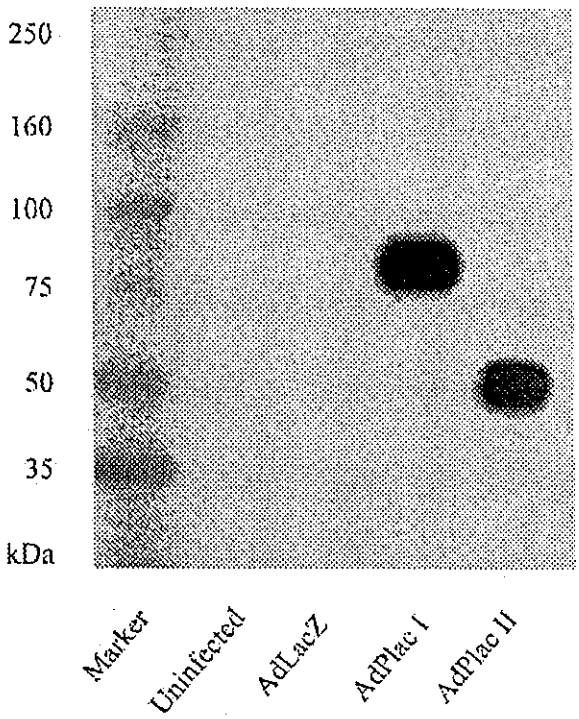

Figure 1. Expression of human placental E-NTPDases in infected SMCs 4 days after gene transfer. Proteins of whole-cell extracts from SMCs infected or not with AdLacZ, AdPlac I, or AdPlac II were separated by SDS-PAGE and immunoblotted against monoclonal anti-human placental E-NTPDase antibody YH34.

Inhibition of Arterial Thrombus Formation and Neointimal Growth in Arteries Infected With AdPlacI In Vivo

Five days after gene transfer, Plac I protein was broadly expressed in the medial SMCs of arteries infected with AdPlac I (Figure 4A) but was undetectable in control arteries infected with AdLacZ (Figure 4C). Arteries infected with AdPlac I were immunonegative for Plac I protein when the antibody was replaced with nonimmune mouse IgG_1 (Figure 4B). Figure 5 shows NTPDase activities in infected arteries (Figure 5). Two days after endothelial denudation with a saline infusion and AdPlac I infection, ATPase and ADPase activities were significantly reduced as compared with those of normal carotid arteries (control) by endothelial denudation. Five days after infection with AdPlac I, however, ATPase and ADPase activities were 2.0- and 1.7-fold higher, respectively.

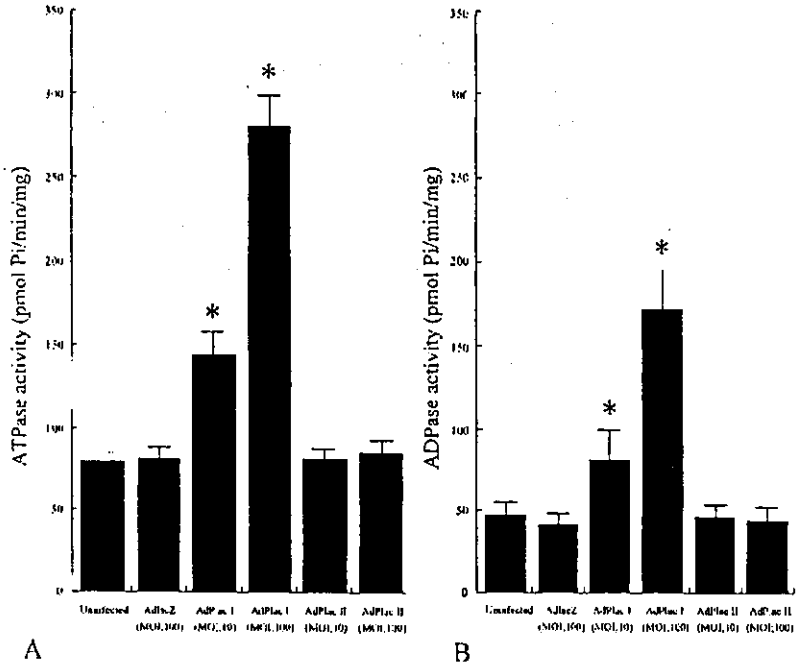

Figure 2. NTPDase activity of infected SMCs. Activities of NTPDase in transfected SMCs were determined by measuring extracellular ATP and ADP concentrations. Activities of ATPase (A) and ADPase (B) were determined in SMCs infected or not with AdLacZ at MOI 100, AdPlac I at MOI of 10 or 100, or AdPlac II at MOI of 10 or 100 (n=5 each; *$P<0.05$ vs AdLacZ).

than those after AdLacZ infection. The numbers of mRNA copies of Plac I per 10^6 copies of the housekeeping gene, glyceraldehyde 2-phosphate dehydrogenase, 2 and 5 days after gene transfer were 15.2±3.5 and 1456±65, respectively. These results indicated that Plac I protein expressed in carotid arteries had biological NTPDase activity in the vascular wall.

Blood flow in arteries infected with AdLacZ rapidly deteriorated after irradiation and vanished within 96±18 seconds (n=6, Figure 6A). In contrast, blood flow in arteries infected with AdPlac I was preserved for at least 10 minutes during green light irradiation (n=6, Figure 6B). Histological analyses revealed that occlusive thrombi, mainly consisting of aggregated platelets, had blocked arteries infected with AdLacZ (Figure 6C), whereas only small mural thrombi had developed in arteries infected with AdPlac I (Figure 6D). The neointimal growth 3 weeks after thrombus formation was significantly reduced by AdPlac I infection. Area of neointima and neointima/media ratio in arteries infected with AdLacZ versus AdPlac I were 97 500±14 000 μm^2 versus 45 300±5900 μm^2 (n=8 each, $P<0.01$) and 1.03±0.13 versus 0.41±0.05 (n=8 each, $P<0.001$), respectively.

Platelet Function and Blood Coagulation in Rats Infected With AdPlac I

Platelet aggregation induced by ADP and collagen, prothrombin time, and activated partial thromboplastin time did not significantly differ between rats infected with AdPlac I and AdLacZ (see Table).

Discussion

We have demonstrated that human placental E-NTPDase isoform I expressed in vascular SMCs hydrolyzed ATP/ADP, prevented platelet aggregation in vitro, and significantly suppressed photochemically induced arterial thrombus formation, as well as subsequent neointimal growth, in vivo. Placental E-NTPDase isoform II expressed in SMCs did not exert antiplatelet effects.

Fresh platelet-rich thrombi frequently develop in association with cardiovascular events, including unstable angina and acute myocardial infarction, as well as immediately after interventions such as angioplasty, stent implantation, and atherectomy.[23,24] Platelet activation induced by ADP plays a pivotal role in arterial thrombus formation.[3] In normal vessels, ADP is rapidly metabolized to AMP by E-NTPDase on the endothelial cell surface, which is subsequently converted to adenosine by the 5′-nucleotidase, also localized on the endothelial cell membrane. Recent in vivo and in vitro gene transfer studies of NTPDase have shown that increased NTPDase activity in the vasculature confers vascular protective effects and also survival benefits on cardiac grafts by blocking thrombotic sequelae.[25,26] Therefore, retaining high NTPDase activity in vascular SMCs should reduce the incidence of thrombus formation after vascular injury.

Vascular E-NTPDase was identified along with CD39, which has 2 putative transmembrane domains and an extracellular domain containing an enzymatically active region.[11,12] The extracellular domain contains 5 apyrase conserved regions (ACRs), of which ACR-1, -4, and -5 are important for maintaining enzymatic activity.[12] Plac I also has 2 putative transmembrane domains and an extracellular domain like CD39, whereas Plac II lacks ACR-5 and a putative transmembrane sequence in the C-terminal region.[10] Our results indicate that ACR-5 is essential for NTPDase activity.

Thrombus formation was almost completely suppressed in arteries infected with AdPlac I. Makita et al[9] showed that placental E-NTPDase blocks platelet aggregation under low shear stress (12 dyne/cm^2) but did not significantly inhibit

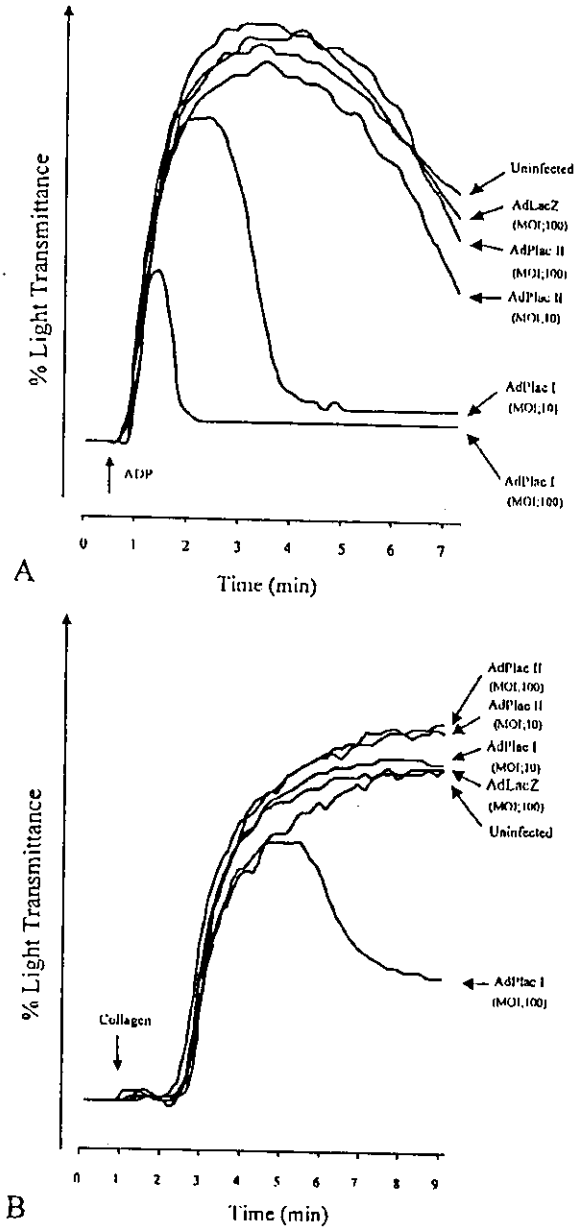

Figure 3. Platelet aggregation in presence of SMCs induced by ADP and collagen. ADP (A) or collagen (B) was added to PRP in presence of uninfected SMCs or SMCs infected with AdLacZ (MOI=100), AdPlac I (MOI=10 and 100), and AdPlac II (MOI=10 and 100), and platelet aggregation was measured.

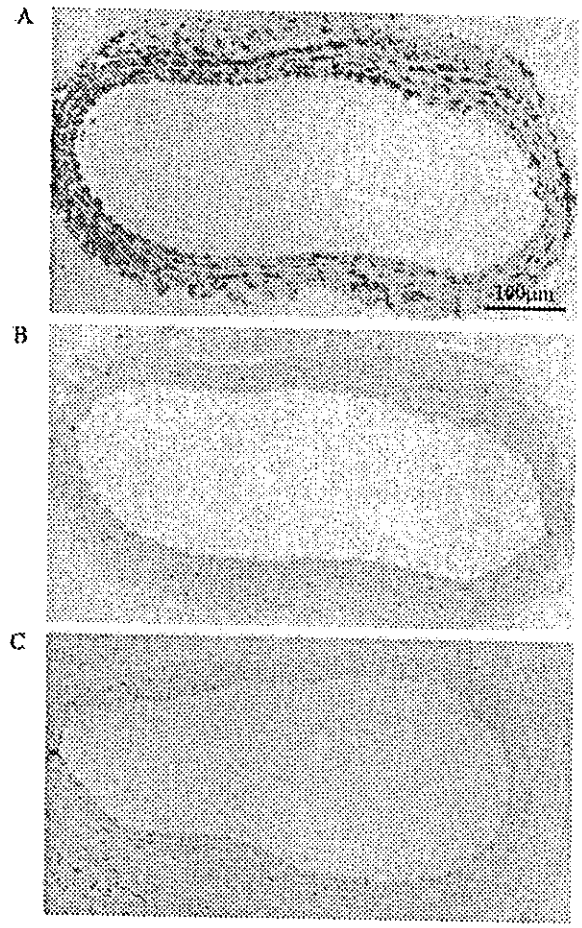

Figure 4. Immunohistochemical staining for human placental E-NTPDase in infected carotid arteries. Representative immunohistochemical microphotographs of rat carotid arteries infected with AdPlac I (A and B) or AdLacZ (C). SMCs in whole wall are immunopositive for human placental E-NTPDase in artery infected with AdPlac I (A) but not with AdLacZ (C). Replacing antibody with nonimmune mouse IgG_1 generated immunonegative reactions (B).

initial aggregation under high shear stress (108 dyne/cm^2), although platelets disaggregated during the later phase in vitro. These results imply that E-NTPDase plays an antithrombotic role under relatively low flow conditions. The finding that this enzyme localizes on syncytiotrophoblasts and in endothelial cells of the umbilical vein, rather than the artery,[9] might be consistent with the results in vitro; however, the present study showed that arterial thrombus formation under high flow conditions was obviously suppressed in vessels infected with AdPlac I. Interactions between von Willebrand factor and the platelet membrane receptors GP Ib and IIb/IIIa are crucial for platelet aggregation when blood flow is high,[27] and ADP and its receptors are key mediators of such interactions.[28,29] Thrombus formation was photochemically induced in the present study.[22] Photochemical exposure produces highly reactive oxygen species that react with cell membrane lipids to cause endothelial cell damage and platelet activation. In this animal model therefore, ADP released from damaged tissue and activated platelets played a critical role in thrombus formation. These lines of evidence indicate that placental E-NTPDase isoform I overexpression in injured vessels is highly antithrombotic, even under high blood flow, which is characteristic of stenosed atherosclerotic arteries.

A key advantage of the local expression of E-NTPDase in the rat model was the absence of systemic side effects.

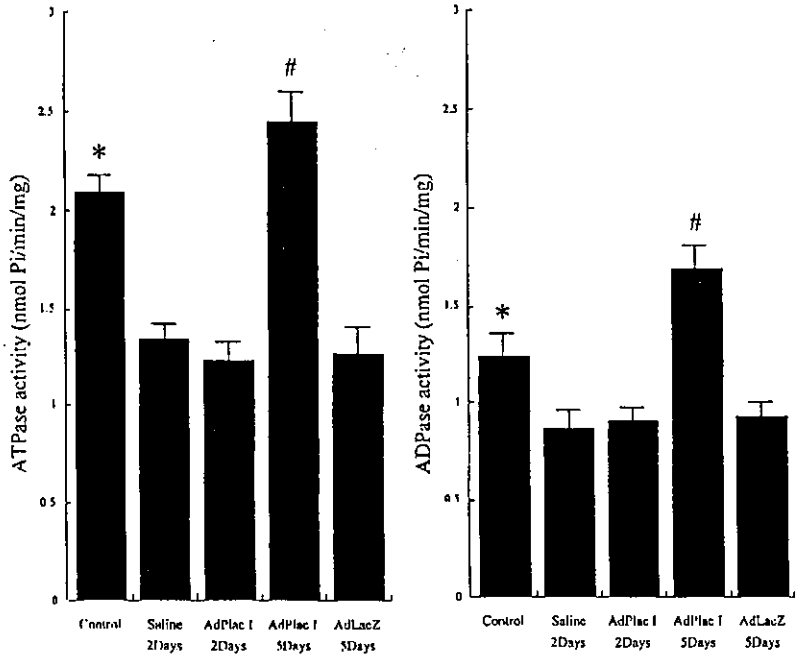

Figure 5. Activities of NTPDase in infected carotid arteries. Homogenized arteries were incubated for 5 minutes with exogenous ATP or ADP, and then ATPase and ADPase activities were determined. Control indicates rat normal carotid artery; saline 2 days, 2 days after endothelial denudation with saline infusion (n=5 each; *$P<0.05$, control vs saline 2 days and AdPlac I 2 days; #$P<0.05$, AdPlac I 5 days vs AdLacZ 5 days).

Animal studies have shown that a new soluble form of the extracellular region of CD39 (solCD39) has systemic antithrombotic effects[30,31]; however, antiplatelet therapy also tends to induce systemic bleeding.[2] This is an important limitation to clinical applicability and indicates a potential advantage of local gene transfer into injured vessels. Our study demonstrated that E-NTPDase expressed on SMCs inhibited platelet aggregation induced not only by ADP but also by collagen, although a high viral titer was required. Thus, local expression of E-NTPDase in diseased arteries should reduce the incidence of cardiovascular events without side effects.

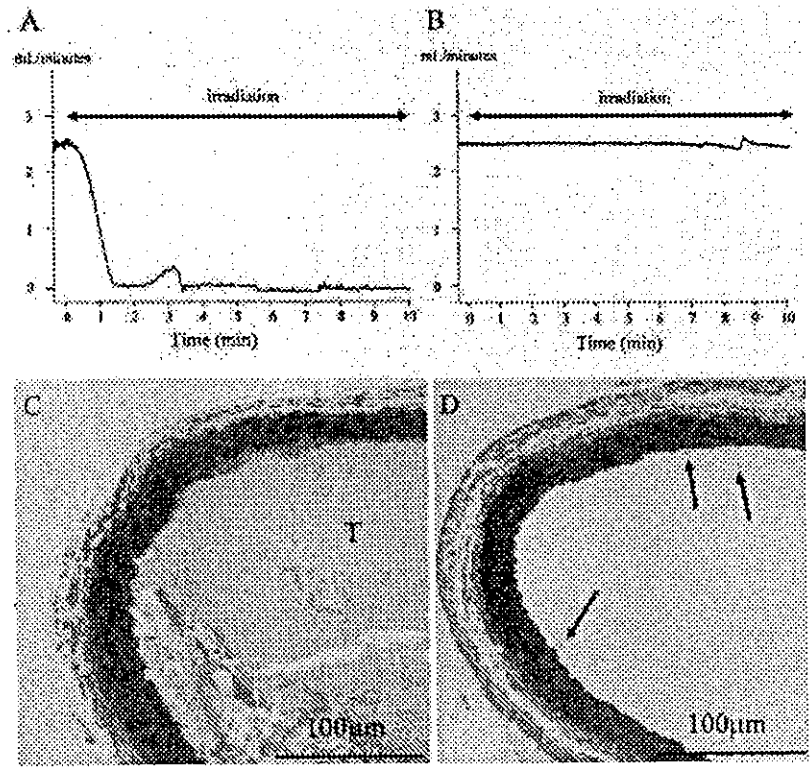

Figure 6. Arterial thrombus formation in arteries injured by air. Thrombus formation induced by photochemical exposure in rat common carotid arteries 5 days after gene transfer. Blood flow of carotid arteries infected with AdLacZ (A) or AdPlac I (B) was recorded during irradiation for 10 minutes. Histologically occlusive thrombus (T) formed in arteries infected with AdLacZ (C), whereas small mural thrombi (arrows) developed in those infected with AdPlac I (D). Results were similar in 6 rats.

Platelet Aggregation and Coagulation Parameters

	Before Virus Infection	AdLacZ	AdPlac I
%, Maximum platelet aggregation			
ADP (10 μmol/L)	83.3±3.8	82.0±3.5	84.0±3.5
Collagen (10 μg/mL)	91.3±5.3	90.8±3.3	89.8±4.3
Coagulation parameters			
Prothrombin time, s	8.6±0.5	8.9±0.2	8.6±0.5
Activated partial thromboplastin time, s	14.6±0.5	14.5±0.5	14.8±0.6

Abbreviations are as defined in text.
n=4 each, $P>0.1$.

The endothelium predominantly expresses E-NTPDase and, to a lesser extent, so do other vascular cells.[32] The enzyme in vascular SMCs would modulate vascular tone via P2 purinoceptors.[33] The present study detected placental E-NTPDase isoform I expression by immunohistochemistry in whole walls of arteries infected with AdPlac I. Antiplatelet ability is thought to depend mainly on the NTPDase activity of luminal SMCs. Gangadharan et al[21] reported that adenovirus-mediated CD39 gene transfer augments NTPDase activity in rabbit iliac arteries injured with balloons but does not significantly reduce platelet deposition on the injured luminal surface. They detected CD39 expression only in luminal SMCs. Although the methods of gene transfer and/or species differences might be relevant factors in explaining this discrepancy, the powerful inhibitory effect against occlusive thrombus formation in AdPlac I–infected arteries might depend not only on antiplatelet function but also on reduced vasoconstriction in injured vessels. We evaluated total NTPDase activities of the vessel wall, which includes potential sources of the enzyme. We could not evaluate native E-NTPDase of rat vascular SMCs. Occlusive thrombi were photochemically induced in all arteries infected with AdLacZ, and the level of E-NTPDase/CD39 mRNA expression in rat carotid arteries was very low (data not shown). These results suggest that native E-NTPDase in SMCs would not have putative antithrombotic roles in vivo.

In addition to thrombus formation, subsequent neointimal growth was suppressed in arteries infected with AdPlac I. Other studies in vitro have revealed that purinergic signaling modulates the proliferation and death of SMCs and endothelial cells.[34] Extracellular ATP and ADP released from platelets, as well as injured or activated SMCs and endothelial cells, is mitogenic for intimal SMCs via P2Y receptors[35] and synergistically acts with growth factors such as platelet-derived growth factor and basic fibroblast growth factor.[35,36] The neointima in the present study was exclusively composed of SMCs and extracellular matrix, suggesting that increased NTPDase activity on SMCs suppresses neointimal growth via the inhibition of SMC proliferation. In addition, thrombus itself contributes to neointimal formation and plaque progression.[37] Taken together, increased NTPDase activity on SMCs directly and/or indirectly might contribute to neointimal growth after thrombosis.

In summary, the present study showed that transfer of the adenovirus-mediated human placental E-NTPDase gene into vascular SMCs eliminates platelet aggregation induced by ADP and collagen as well as occlusive thrombus formation in injured arteries. In the clinical setting of cardiovascular events, not only the development of platelet-rich thrombi but also the rapid activation of the blood coagulation system plays a significant role in thrombus formation. The antithrombotic efficacy of AdPlac I infection should be evaluated in arteries with atherosclerosis.

Acknowledgments

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その他

平成16年度厚生労働科学研究費補助金
難治性疾患克服研究事業
血液凝固異常症に関する調査研究班

第2回班会議
日時：平成17年1月15日（土）午前10時ー4時30分終了予定
場所：慶應義塾大学医学部北里記念図書館2F　北里講堂

プログラム

抄録集

主任研究者　池田　康夫

平成16年度厚生労働科学研究費補助金　難治性疾患克服研究事業

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プログラム

（サブグループ研究報告:30分　各個研究:10分　討論含む）

10：00～　主任研究者　挨拶　池田康夫

10：10～　厚生労働省（疾病対策課）挨拶

10：20～　ITP研究班　…　統括報告　藤村欣吾

サブグループリーダー：藤村欣吾　広島大学医学部
班　　員：桑名正隆　慶應義塾大学医学部
　　倉田義之　大阪大学医学部
研究協力者：藤沢康司　慈恵会医科大学
　　降旗謙一　エスアールエル
　　野村昌作 岸和田市民病院
特別協力者：杉野稔・伊津野孝 東邦大学医学部（疫学班）

10：50～　TTP研究班　…　統括報告　藤村吉博

サブグループリーダー：藤村吉博　奈良県立医科大学
班　　員：　宮田敏行　国立循環器病センター研究所
　　村田　満　慶應義塾大学医学部
　　和田英夫　三重大学医学部
特別協力者：　杉野稔・伊津野孝 東邦大学医学部（疫学班）

11：20～　特発性血栓症研究班　…　統括報告　宮田敏行

サブグループリーダー：宮田敏行　国立循環器病センター研究所
班　　員：　小嶋哲人　名古屋大学医学部
　　坂田洋一　自治医科大学
　　辻　　肇　京都府立医科大学
　　村田　満　慶應義塾大学医学部
　　川崎富夫　大阪大学医学部
研究協力者：猪子英俊　東海大学医学部
特別協力者：杉野稔・伊津野孝 東邦大学医学部（疫学班）

11：50～12：30　昼休み

１２：３０～１３：３０

TTP 班員報告：

松本雅則（藤村吉博）「TMA 患者における血漿 ADAMTS13 抗原解析」

小亀浩市（宮田敏行）「ADAMTS13 の消光性蛍光基質の開発と血栓症の解析への応用」

村田　満　「ADAMTS13 と血栓症」

和田英夫　「TTP の二次アンケート調査の解析途中経過」

１３：３０～１４：３０

特発性血栓症班員報告：

川崎富夫「日本の現状に即した新しい発想の肺塞栓症予防戦略－大阪大学病院の試み」

窓岩清治（坂田洋一）「敗血症 DIC の病態と線溶系因子」

辻　肇　「先天性アンチトロンビン欠損症の遺伝的背景」

小嶋哲人「血栓性素因プロテインＳ欠損所の遺伝子解析」

１４：３０～１５：００　　休　憩

１５：００～１６：００

ITP 班員報告：

桑名正隆「ITP の診断基準改定をめざして」

倉田義之「血小板数著減検体における正確な血小板数測定法の確立
－血小板測定への破砕赤血球の影響についての検討－」

降旗謙一「検査センターにおける ITP 診断検査の準備状況について」

終　了

平成 16 年度厚生労働科学研究費補助金　難治疾患克服研究事業
血液凝固異常症に関する調査研究班
主任研究者：池田康夫
事務局：慶應義塾大学医学部内科池田教授室　Tel: 03-3353-1211　内線 62421

ITP研究グループ　総括報告

広島大学大学院病態薬物治療学講座　血液・腫瘍科　藤村　欣吾

成人特発性血小板減少性紫斑病（ITP）に対する治療ガイドラインの提案

標準的ITP治療は１９８２年に厚生省特発性造血器障害治療研究班からITP治療指針として発表され今日まで定着し汎用されている。

本指針を用いたプロスペクティブな治療研究結果は完全寛解率は約４０％、無反応例は約３０％である。また副腎皮質ホルモン単独による完全寛解は３３％に認められたが、その後の施設間での寛解率は１０～３０％と必ずしも良好ではない。また長期の副腎皮質ホルモン服薬による副作用対策が必要となり、医療情報の普及、意識の向上など従来の指針が医師、患者に受け入れにくい場合も生じている。

さらに免疫学の進歩により発症機序からより的確なITP診断法が提案され、新たな免疫抑制療法の有用性が示唆されている。

また本研究班におけるレトロスペクティブ研究において、ヘリコバクターピロリ菌（HP菌）感染ITP症例に対する除菌療法は未治療例、既治療例を問わず、治療抵抗性を超えて除菌成功例では約６５％に血小板数の増加が認められ、殆どの症例はITPの治療から脱却可能となっている。除菌療法は１週間で、副作用が軽微でありHP菌陽性ITP症例においては有用な治療と考えられた。この除菌効果は米国、北欧などでは認められず本療法を治療に組み入れるガイドラインは本邦で独自に作成される必要がある。

これらの理由によりITP治療ガイドライン（２００４年試案）を作成した。

本ガイドラインの特徴は①ピロリ菌の検査、除菌療法を組み込み、②除菌無効例、ピロリ菌陰性例に対して First line, Second line 治療を示し、③それぞれに治療目標を設定し、④自覚症状、臨床症状、など臨床実態に即したもので、⑤副腎皮質ステロイドの使用期間を極力短くして副作用の発現を少なくする、⑥緊急時、重篤な出血時にはガンマーグロブリン療法を積極的に用いる、などである。

本ガイドラインを履行することによりQOLの改善、全体的なITP治療費の削減が可能になると期待される。

TMA研究グループ　総括報告

藤村吉博　奈良県立医科大学

血栓性微小血管障害症（TMA）は①細血管障害性溶血性貧血、②破壊性血小板減少、③細血管内血小板血栓を主徴とする疾患群で、この中には神経症状優位の血栓性血小板減少性紫斑病（TTP）や腎症状優位の溶血性尿毒症症候群（HUS）が含まれる。近年、TTPの原因として血漿von Willebrand因子特異的切断酵素（VWF-CP、別名ADAMTS13）が同定され、この酵素活性が先天的に欠損もしくは後天的にこの酵素に対する自己阻害抗体（インヒビター）が発生すると、血漿中に超巨大分子量VWFマルチマー（UL-VWFM）が蓄積され、高ずり応力下に血小板凝集・血栓が生じる事が明らかになった。

TMA研究グループ代表である藤村吉博は1998年に本邦で最初にこの酵素活性測定法を確立し（Int J Hematol 2001, Br J Hematol 2001）、平成１６年１０月末で全国の医療施設から依頼された493例のTMA患者についてADAMTS13解析を行った。この結果、同活性著減例はTMA全体の1/3、中等度低下例が1/3、そして軽度低下ないし正常例が残り1/3である事を示した（途中経過をSem Hematol 2004で報告）。著減例の中から先天性TTP（別名Upshaw-Schulman症候群）を２１家系２６症例発見し、うち１０家系については班員の宮田敏行を中心にADAMTS13遺伝子解析を行い、また後天性TTPの３例についても班員の村田満を中心に同遺伝子解析を終了し、これらの結果は既に論文報告（PNAS 2003, Blood 2004, Blood 2004）もしくは投稿中で、同プロジェクトは現在も続行中である。また後天性TTPの中で原因不詳（特発性）のものは自己免疫疾患のカテゴリーに入るが、基礎疾患に続発するものは極めて多彩であり、これらの中で多発性神経症状を呈した血管内リンパ腫とC型肝硬変に合併した症例について、いずれもADAMTS13インヒビターの存在を証明し、これらの合併症としての血小板減少の原因を明らかにした（Neurology 2004, J Hepatology印刷中）。一方で、宮田らはADAMTS13で切断されるVWFサブユニットの73アミノ酸残基（VWF73）を同定し、これは現時点では同酵素の最小基質単位と考えられている（Blood 2004）。 さらにこのVWF73を蛍光標識した合成ペプチドを作成し、短時間でADAMTS13活性を測定しうるFRETS-VWF73アッセイを構築した（論文投稿中）。また藤村らはADAMTS13に対するマウスモノクローナル抗体を作成し、その性状解析後、酵素免疫測定法とWestern blot法によりTTP患者のADAMTS13抗原解析を行った（論文投稿中）。現在、さらに肝臓での産生部位を同定中である。班員の和田英夫は後天性TTPの中で、ADAMTS13活性著減、かつ同インヒビター陽性例では血漿交換療法が卓効する事を自験例で示しその理論を構築した（Transfusion 2002）。この論文は臨床的にも高い評価を得ている。さらに和田はTMAの頻度や治療の標準化を目指し

て、全国の医療施設に対する大規模アンケート調査を行い、 データを集積中である。

本 TMA 研究グループでは、今後の３年間に上記解析を続行すると共に、ADAMTS13 以外の TMA 病因として注目されつつある補体調節因子(factor H、CD46)や血管内皮細胞膜結合糖蛋白質 (CD36, CD39) 等を含めた多角的な解析を行い TMA の系統的診断法の確立を目指す。

特発性血栓症研究グループ　総括報告

宮田敏行　国立

特発性血栓症サブグループの研究

以前に、国立循環器病センターが行った日本人の地域一般住民を対象とした活性測定の結果から推察すると、頻度は、プラスミノーゲン欠乏症 4.29%（このうちプラスミノーゲン異常症、3.87%）、アンチトロンビン欠乏症0.15%、プロテインC欠乏症0.13%、プロテインS欠乏症 1.12%である。これらの欠乏症のうち、アンチトロンビン、プロテイン C、プロテインSの欠乏症は静脈血栓症の危険因子であることが判明している。これらの推定頻度からすると、欠乏症のキャリヤーは全国で100万人を超えることが予測される。本研究は、血栓症の予防や予知に資するため、多施設共同研究として静脈血栓症患者試料を収集するとともに、患者データベースを作成し、静脈血栓症の発症原因を明らかにすることを目的とする。静脈血栓症の遺伝的背景を検討するため、候補遺伝子アプローチおよびゲノム網羅的アプローチをとる。

特発性血栓症サブグループ6施設では、静脈血栓症の遺伝子解析のための研究計画の承認を倫理委員会でうけたあと、161 例の試料の収集を行った。また、「特発性静脈血栓症疾患遺伝子プロジェクト診療情報共通記録用紙」を作成し、臨床情報を収集した。161 例の試料を対象に次の5遺伝子多型のタイピングを行った。ADAMTS13 P475S 変異、プラスミノーゲン A601T 変異 (plasminogen Tochigi mutation)、プロテイン S E155K 変異(protein S Tokushima mutation)、XII 因子 C46T 多型、プラスミノーゲン活性化因子インヒビター4G/5G 多型。正常対照群として、地域一般住民の試料約 1,800 人もしくは約 3,600 人を用いた。タイピング法として、TaqMan 法を用いた。解析の結果、プロテインS E155K 変異が有意に静脈血栓症患者群に高いことが明らかとなった。

TMA 患者における血漿 ADAMTS13 抗原解析

奈良県立医科大学　藤村　吉博、○松本　雅則

【緒言】TTP や HUS などの TMA 患者での ADAMTS13 活性やインヒビター測定については多くの報告があるが、これら患者における ADAMTS13 抗原解析については全く報告がない。我々は、recombinant ADAMTS13 を用いて同モノクローナル抗体(mAb)を 3 種類作成した。うち、2 種類（A10, C7）は奈良医大で、1 種類(WH2-11-1)は化血研で作成されたものである。これらの抗体の性状を検討し、Western blot 法(WB)により ADAMTS13 抗原を解析した。

【方法】対象は、健常人 60 人、Upshaw-Schulman 症候群(USS)患者 19 家系 24 症例およびその家族 49 人、特発性 TTP 96 症例、薬剤性 TTP 11 症例、O157:H7 による HUS 8 症例。非還元条件下 WB では A10 を、還元条件下 WB では WH2-11-1 を用いた。

【結果】ADAMTS13 mAb の認識部位は、A10:ディスインテグリンドメイン、WH2-11-1:TSP-1 の 4 番目、C7: TSP-1 の 7/8 番目とすべて異なっていた。いずれの抗体も非還元 WB で血漿中の抗原と反応し、うち WH2-11-1 のみが還元 WB でも抗原と反応した。A10 は IgG 終濃度 50 ug/ml で完全に酵素活性を阻害した。C７は 100 ug/ml でも部分阻害しか示さず、WH2-11-1 は阻害活性を認めなかった。非還元および還元 WB では、先天性、後天性 TTP ともに ADAMTS13 抗原量の低下と分解亢進像が確認された。一方、O157:H7 陽性 HUS では、このような分解亢進像は認められなかった。

【まとめ】TTP 患者では、血漿 ADAMTS13 の分解亢進によって同酵素活性が低下していることが予想され、今後この分解亢進がどのような機序か検討する予定である。

ADAMTS13 の消光性蛍光基質の開発と血栓症の解析への応用

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血栓性血小板減少性紫斑病（TTP）は、先天性もしくは後天性の ADAMTS13 欠乏症により起こる。ADAMTS13 は、フォンビルブランド因子（VWF）切断酵素活性を示す血漿プロテアーゼであり、超巨大 VWF マルチマーを適度に切断することにより、過度の血小板凝集を抑制している。本酵素の活性測定法は数種類報告されているものの、時間がかかり精度にも問題があった。そこで私達は、VWF の A2 ドメインのアミノ酸配列をもとに基質領域の絞り込みを試み、73 残基が最小であることを見出し、これを VWF73 と命名した（Kokame et al, Blood, 2004）。本法は５時間で測定を終了できるものの、ウエスタンブロット法を使用するために操作がやや煩雑で、更なる改良が待たれていた。そこで今回、VWF73 に蛍光共鳴エネルギー転移（FRET 原理）に基づいた蛍光基と消光基を導入することにより、ADAMTS13 の新しい基質 FRETS-VWF73 の合成に成功した。本基質は、正常血漿の用量依存性および反応時間依存性の蛍光強度の増大を示し、96 穴プレートを用いて１時間以内での定量が可能となった。反応条件を検討した結果、5mM Bis-Tris, 25mM $CaCl_2$, 0.005% Tween 20, pH 6.0 が最も適した緩衝液であった。VWF マルチマーの減少を観察する SDS-アガロース電気泳動法（Furlan 法）と本法は R^2=0.75 を示し、良い相関関係を示した。TTP 患者血漿では蛍光の増大を示さず、HUS・DIC・ITP の患者血漿では蛍光の増大が見られた。本法を用いて、一般住民（男性 167 名、女性 214 名）の活性を測定したところ、活性は 30%ー150%以上の広い分布を示し、男性の平均活性値は女性より有意に低く、男女ともに加齢による活性の減少を示した。FRETS-VWF73 は、日本・米国・ヨーロッパで販売が開始され、ADAMTS13 活性の測定に広く使用されると期待される。

ADAMTS13と血栓症

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Von Willebrand factor切断酵素（VWF-CP：ADAMTS-13）の著明な活性低下はHUS/TTPの発症に関与することが知られている。一方、本酵素の軽度の低下が血栓症の発症に及ぼす影響については必ずしも明確ではない。軽度の低下は各種後天的要因や疾患のみならず、先天的な要因、特にADANTS13遺伝子多型によっても起こりうる。VWFと血栓性疾患の関連に関しては、血中VWFの上昇は心筋梗塞の危険因子あるいは予後不良因子であり、動物実験でVWFに対するモノクローナル抗体が動脈血栓の発症を抑えたとの報告もあるが、VWF活性を生理的に制御する筈のADAMTS13と、体内でのVWF活性や血小板機能との関連はこれまで充分に検討されていなかった。これまでの国立循環器病センターや我々の研究で、頻度の高い一塩基置換が日本人で数種類同定されており、これらの多型が血栓性疾患に及ぼすインパクトを見極める事は日本人における血栓症の原因を探る上で甚だ重要である。

ここでは、（1）ADAMTS13遺伝子多型 *vs* ADAMTS13活性、（2）ADAMTS13活性 *vs* 血中VWF（抗原量、活性）、（3）ADAMTS13遺伝子多型 *vs* 血中VWF（抗原量、活性）、（4）血中VWF *vs* ずり依存性血小板機能、（5）ADAMTS13遺伝子多型 *vs* ずり依存性血小板機能、など、in vivoにおける関連について健常人検体を用いて検討した。さらにADAMTS13の産生組織に関して、特異抗体による免疫組織学的方法等を用いて正常細胞や培養細胞について検討した。これらの成績について報告する。

TTP 二次アンケート調査の解析途中経過

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血栓性血小板減少性紫斑病（TTP）ならびに/溶血性尿毒症症候群(HUS)に対する 2 次調査の解析結果を報告する。

回収された 182 例の内訳は、女性 103 例、男性 79 例であり、原因ならびに基礎疾患は、家族性 17 例、薬剤性 6 例、妊娠 2 例、自己免疫性疾患 16 例、悪性腫瘍 9 例、移植症例 9 例、病原性大腸菌性 60 例、原発性 60 例であった。死亡率は全体で 19.8%であり、それぞれ家族性 5.9%、薬剤性 16.7%、妊娠 0%、自己免疫性疾患 31.3%、悪性腫瘍 85.7%、移植症例 77.8%、病原性大腸菌性 1.7%、原発性 23.1%で、悪性腫瘍や移植による TTP/HUS が最も悪く、典型的 TTP と考えられる自己免疫性ならびに原発性の死亡率は 23−31%であった。

ADAMTS13 活性は 70 例で測定されており、10%以下が 37 例(52.9%)、10-50%が 19 例(27.1%)、50%以上が 14 例(20%)であった。家族性では 6 例で ADAMTS13 活性は著減し、3 例が中等度低下し、2 例が正常値であった。ADAMTS13 の著明減少は、薬剤性、妊娠、悪性腫瘍、移植例では認められず、自己免疫性では 50%、原発性では 61%に認められた。1 例のみ測定された病原性大腸菌性では、ADAMTS13 活性が著明減少していた。

先天性ならびに後天性とも、ADAMTS13 の低下例と非低下例が見られたが、自己免疫性ならびに原発性 TTP/HUS の約 50%以上が ADAMTS13 の著減により発症したことが示唆された。また、TTP/HUS 例での ADAMTS 測定はいまだ 50%以下であり、今後さらに ADAMTS13 測定の普及が必要と考えられた。

また、網血小板(RP)は骨髄での血小板産生を反映し、特発性血小板現象性紫斑病（約 80 例）での検討では、感度 80%、特異度 90%、NPV85%であり、小数例での検討であるが TTP でも有意に増加した。