diphtheria toxin A fragment expression cassette was inserted into downstream of the 3′-homologous region. The vector was introduced into 129/Sv-derived R-CMTI-1A embryonic stem cells by electroporation. Cells were selected in medium containing G418 (Invitrogen, Carlsbad, CA) and screened by polymerase chain reaction (PCR) and Southern blot analyses. Targeted cells were microinjected into C57BL/6 blastocysts to generate chimeric mice. The resulting male chimeras were bred to wild-type 129/Sv females to produce heterozygous F1 offspring on the 129/Sv genetic background. Heterozygotes were interbred to obtain homozygous mice. Male mice aged 8 to 12 weeks were used for phenotypic analyses. Pregnant female mice aged 8 months were used for renal histology analysis. Female mice aged 15 to 20 weeks (20-30 g) were used for in vivo thrombosis experiments. All animal procedures were performed in accordance with institutional guidelines and were approved by the Animal Care and Use Committee of the National Cardiovascular Center Research Institute.

Genotypic analysis

gDNA, isolated from ear or kidney, was used for genotyping by PCR or Southern blot analyses. For PCR analysis, DNA amplification was performed using a mixture of 3 primers: an intron 2–specific forward primer (5′-ACCCTATCTCTGGCCTGTATTCCT-3′), an intron 3–specific reverse primer (5′-TACTGACTTGTGACCACAAGCCCT-3′), and a neo cassette-specific reverse primer (5′-ATCGAGTCTAGCTTGGCTGGACGT-3′). For Southern blot analysis, a 580-bp fragment upstream of the 5′-homologous region was generated by PCR with primers 5′-TGTCTGCAAGTGCAGTGAGAGGCA-3′ and 5′-AATGAAGATGGCACCAGTGAGGAT-3′ and used for the synthesis of a fluorescein-labeled probe. The probe was hybridized to *Hin*dIII-digested gDNA and detected using a CDP-*Star* detection module (Amersham, Piscataway, NJ).

RT-PCR analysis

Total RNA was prepared from liver using ISOGEN reagent (Nippon Gene, Tokyo, Japan) and subjected to 1-step reverse transcription-PCR (RT-PCR; Qiagen, Hilden, Germany). An exon 21/22-specific sense primer (5′-TTGTGGGAGAGGTCTGAAGGAACT-3′) and an exon 24/25-specific antisense primer (5′-ACAGGAGACAGAGCACTCTGTCCA-3′) were used to amplify ADAMTS13 mRNA.

In situ hybridization

In situ hybridization was performed as described.[17] A 435-bp mouse *Adamts13* cDNA fragment (nucleotides: 679-1113) was used to synthesize digoxigenin-labeled sense and antisense RNA probes by in vitro transcription with a DIG RNA labeling mix (Roche, Basel, Switzerland). The probe was hybridized to liver sections and detected using an anti-DIG AP conjugate (Roche) and NBT/BCIP solution (Roche). Sections were counterstained with Kernechtrot solution.

Measurement of plasma ADAMTS13 activity

With the mice under ether anesthesia, blood was collected from the retro-orbital plexus into tubes containing a 0.1 volume of 3.8% sodium citrate. Plasma was prepared from blood by centrifugation at 800*g* for 15 minutes at room temperature. ADAMTS13 activity was measured using a recombinant substrate, GST-mVWF73-H, as described.[16,18] Activity was also measured using a fluorogenic substrate, FRETS-VWF73 (Peptide Institute, Minoh, Japan).[19]

VWF multimer analysis

Plasma samples, diluted in sodium dodecyl sulfate (SDS) sample buffer (10 mM Tris-HCl, 2% SDS, 2 mM EDTA, 0.02% bromphenol blue, and 43.5% glycerol, pH 6.8) were electrophoresed on a 1% agarose gel (Agarose IEF; Amersham) at a constant current of 15 mA at 4°C. After transfer to a nitrocellulose membrane (Bio-Rad, Hercules, CA) by capillary blotting, the membrane was incubated in peroxidase-conjugated rabbit anti–human VWF (1:500, Dako, Glostrup, Denmark) in 5% skim milk to detect VWF multimers. Bound antibody was detected with Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer, Boston, MA) on an image analyzer (Fujifilm, Tokyo, Japan). The chemiluminescent intensities of each lane were scanned using Image Gauge software (Fujifilm); the relative intensity profiles were shown.

Hematologic analysis

Blood cell counts and hematocrit were determined using an automatic cell counter (KX-21NV; Sysmex, Kobe, Japan). Peripheral blood smears were stained with May-Grünwald-Giemsa and examined under light microscopy. Plasma haptoglobin levels were analyzed using a mouse haptoglobin enzyme-linked immunosorbent assay (ELISA) test kit (Life Diagnostics, West Chester, PA).

Plasma VWF antigen was measured by ELISA using antibodies against human VWF. Plasma samples in 1% BSA were applied to rabbit anti–human VWF-coated (Dako) ELISA plates for 2 hours at room temperature. Bound VWF was detected by incubation with peroxidase-conjugated rabbit anti–human VWF (1:4000, Dako) in 1% BSA for 1 hour. Bound antibody was detected using a SureBlue Reserve TMB Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD); the absorbance at 450 nm was measured. A standard curve was constructed from the pooled plasma of 129/Sv mice.

Plasma fibrinogen levels were also measured by ELISA using rabbit anti–human fibrinogen (Dako) and peroxidase-conjugated goat anti–mouse fibrinogen (Nordic Immunological Laboratories, Tilburg, The Netherlands) antibodies. Plasma factor VIII (FVIII) activity was measured using a Testzym FVIII Kit (Daiichi Pure Chemicals, Tokyo, Japan). To assess the ELISA and FVIII activity data, the levels measured in wild-type mice were arbitrarily defined as 100%.

Histologic analysis

The kidneys of pregnant female mice were fixed in phosphate-buffered 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin and eosin or periodic acid-Schiff reagent. VWF antigen was detected using an ENVISION+ system (Dako) with rabbit anti–human VWF (Dako).

Coagulation tests and bleeding assay

The prothrombin time (PT) and activated partial thromboplastin time (APTT) of plasma samples were determined using Thrombocheck PT (Sysmex) and Thrombocheck APTT (Sysmex) reagents, respectively. Bleeding analysis was performed on mice anesthetized with sodium pentobarbital (50 μg/g). Tails were amputated 3 mm from the tip and immersed in 1 mL PBS at 37°C for 15 minutes. Blood loss was estimated from the comparison of the absorbance of the PBS at 562 nm with that of PBS containing known volumes of mouse blood.

Platelet aggregation analysis

Platelet aggregation was measured using an aggregometer (MC Medical, Tokyo, Japan) as described.[20] Platelet counts in platelet-rich plasma (PRP) were adjusted to $3.0 \times 10^5/\mu L$ by adding platelet-poor plasma (PPP). Aggregation was initiated by addition of acid-insoluble type I collagen (MC Medical) or botrocetin to PRP. PPP was used as a standard indicating 100% aggregation.

Perfusion assay with a parallel plate flow chamber

Platelet thrombus formation in flowing blood on immobilized collagen was analyzed using a parallel plate flow chamber as described.[21,22] Acid-insoluble type I collagen-coated (Sigma, St Louis, MO) glass coverslips were placed in a flow chamber. The chamber was mounted on a fluorescence microscope (Axiovert S100; Carl Zeiss, Oberkochen, Germany) equipped with a 40 ×/0.75 numeric aperture objective lens (Carl Zeiss) and a CCD camera system (DXC-390; Sony, Tokyo, Japan). Blood was collected into tubes containing argatroban (240 μM; Mitsubishi Chemical Corporation, Tokyo, Japan). The fluorescent dye mepacrine (10 μM; Sigma) was added to the blood. Whole blood samples were aspirated through the chamber and across the collagen-coated coverslip by a syringe

pump (Harvard Apparatus, South Natic, MA) at a constant flow rate producing a wall shear rate of 750 s^{-1}. The shear rate was calculated from the assumption that the viscosity of mouse blood is equal to that of human blood. To analyze the cumulative thrombus volume, image sets at 1.0-μm z-axis intervals within a defined area (156.4 × 119.6 μm) was captured using MetaMorph software (version 6.1.4; Universal Imaging, West Chester, PA). After blind deconvolution of image sets processed by AutoDeblur software package (version 8.0.2; AutoQuant Imaging, Troy, NY), 3-dimensional volumetric measurements of thrombi were accomplished using VoxBlast software (version 3.0; Vartek, Fairfield, IA).

In vivo thrombosis model

A mixture of 600 ng/g collagen (Nycomed, Roskilde, Denmark) and 60 ng/g epinephrine (Sigma) was injected into tail vein of mice.[23] Blood was collected 15 minutes after the injection and platelet counts were determined.

Statistical analysis

Statistical significance was assessed by the Student *t* test or the χ^2 test. Differences were considered to be significant at *P* below .05.

Results

Generation of ADAMTS13-deficient mice

We previously reported 2 strain-specific forms of the mouse *Adamts13* gene.[16] In the 129/Sv strain, the *Adamts13* gene contains 29 exons, as in human *ADAMTS13*, encoding a protein with a similar domain organization as human ADAMTS13. Several strains of mice, including the C57BL/6 strain, harbor a retrotransposon insertion, encoding a variant form of ADAMTS13 that lacks the C-terminal domains. Therefore, we generated and analyzed ADAMTS13-deficient mice on a 129/Sv genetic background.

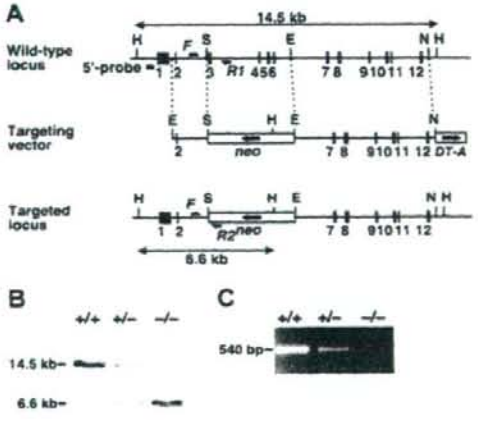

Figure 1. Targeted disruption of the mouse *Adamts13* gene. (A) Structure of the targeted locus in the mouse *Adamts13* gene. Exons are represented by filled boxes. A neomycin-resistance cassette (neo), in the opposite transcriptional orientation, and a forward-oriented diphtheria toxin A fragment expression cassette (DT-A) are indicated. Homologous fragments are indicated by dotted lines; the *Hin*dIII fragments detected by Southern analysis of the wild type and targeted alleles are indicated by double-headed arrows. The sites of primers used for the genotyping PCR (F, R1, and R2) are indicated by arrows. H indicates *Hin*dIII; S, *Sal*; E, *Eco*RI; N, *Nco*I. (B) Southern blot analysis. gDNA from offspring obtained from heterozygous intercrosses was digested with *Hin*dIII and detected with the 5'-specific probe (wild type: 14.5 kb; targeted allele: 6.6 kb). (C) RT-PCR analysis. Total RNA isolated from mouse liver was reverse-transcribed and amplified using the *Adamts13*-specific primer set to generate a 540-bp fragment.

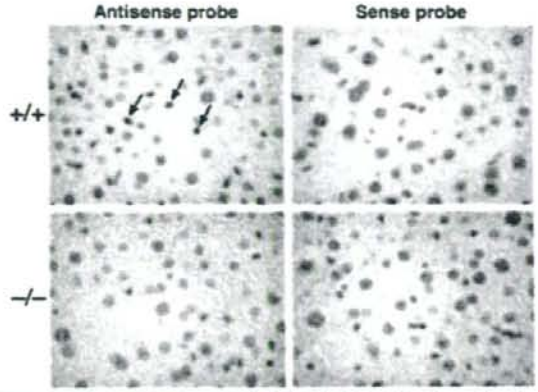

Figure 2. In situ hybridization analysis of ADAMTS13 mRNA. Liver sections from *Adamts13*$^{+/+}$ (top panels) and *Adamts13*$^{-/-}$ (bottom panels) mice were hybridized to the antisense (left panels) or sense (right panels) *Adamts13* RNA probes. The hybridized sections were counterstained with Kernechtrot solution. Typical positive signals are indicated by arrows.

The *Adamts13* gene was disrupted using a targeting vector that eliminated exons 3-6, encoding the catalytic domain (Figure 1A). The expected structure of the targeted locus was confirmed by PCR (data not shown) and Southern blotting (Figure 1B). Elimination of ADAMTS13 mRNA in *Adamts13*$^{-/-}$ mice was verified by RT-PCR of total RNA from liver (Figure 1C), the primary site of synthesis.[16] In situ hybridization analysis also confirmed the loss of ADAMTS13 mRNA in *Adamts13*$^{-/-}$ mice (Figure 2). Because ADAMTS13 is expressed in hepatic stellate cells,[24,25] we detected hybridization with an antisense probe in the nonparenchymal liver cells of *Adamts13*$^{+/+}$ mice. According to their morphology, these cells were hepatic stellate cells. Specific hybridization was not detected in sections from *Adamts13*$^{-/-}$ mice.

No ADAMTS13 enzymatic activity could be detected in plasma samples of *Adamts13*$^{-/-}$ mice by either qualitative (Figure 3A) or quantitative (Figure 3B) methods using GST-mVWF73-H and FRETS-VWF73, respectively, as substrates. Enzymatic activity in *Adamts13*$^{+/-}$ mice was reduced to approximately 35% that seen in *Adamts13*$^{+/+}$ mice (Figure 3B).

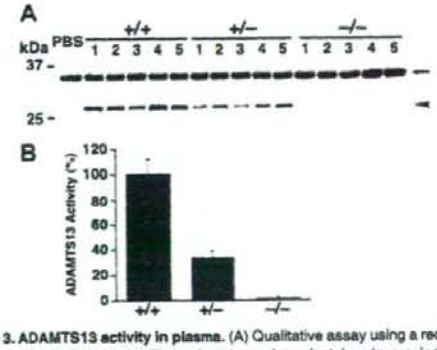

Figure 3. ADAMTS13 activity in plasma. (A) Qualitative assay using a recombinant substrate, GST-mVWF73-H. The substrate and product bands are indicated by arrows and arrowheads, respectively. (B) Quantitative assay using a fluorogenic substrate, FRETS-VWF73. Data are mean ± SD from 4 mice for each genotype. The average activity measured in wild-type mice was defined as 100%.

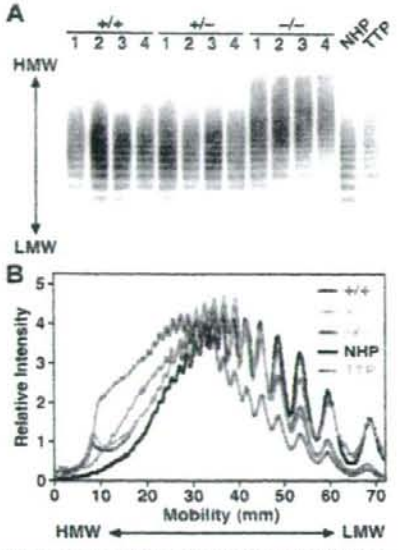

Figure 4. Analysis of plasma VWF multimers. (A) VWF multimer patterns. Plasma samples (1 μL/lane) from *Adamts13*$^{+/+}$, *Adamts13*$^{+/-}$, and *Adamts13*$^{-/-}$ mice were electrophoresed on SDS-agarose gels and transferred to nitrocellulose membranes. VWF multimers were detected with anti-VWF antibodies. Normal human plasma (NHP) and ADAMTS13-deficient TTP patient plasma (TTP) were analyzed in parallel (0.2 μL/lane). (B) Relative intensities of plasma VWF multimers. The chemiluminescent intensities of the VWF multimer patterns (A) were scanned using image analysis software. HMW indicates high molecular weight; LMW, low molecular weight.

Accumulation of UL-VWF multimers in plasma

In humans, genetic defects in ADAMTS13 lead to the accumulation of UL-VWF multimers in plasma. Analysis of VWF-multimer patterns in plasma detected UL-VWF multimers in *Adamts13*$^{-/-}$ mice (Figure 4), suggesting ADAMTS13 deficiency supports the accumulation of plasma UL-VWF multimers. Because the laddering patterns of VWF multimers in *Adamts13*$^{+/+}$ and *Adamts13*$^{+/-}$ mice were similar, less than half of the normal plasma ADAMTS13 activity (Figure 3B) was sufficient to regulate VWF multimer size. VWF multimers in mice were larger than those in humans (Figure 4B); the multimer sizes seen in *Adamts13*$^{+/+}$ mice were similar to those observed in patients with TTP.

No TTP symptoms in ADAMTS13-deficient mice

Genotyping of 195 offspring of *Adamts13*$^{+/-}$ intercrosses showed the expected 1:2:1 mendelian distribution of *Adamts13*$^{+/+}$ (52 of 195), *Adamts13*$^{+/-}$ (91 of 195), and *Adamts13*$^{-/-}$ (52 of 195). Thus, ADAMTS13 deficiency did not cause embryonic lethality. *Adamts13*$^{-/-}$ mice were viable and fertile. To date, 4 *Adamts13*$^{-/-}$ mice exhibited lateral flexion of upper body; one of them had a cloudy eye. Further study is required to uncover whether this rare phenotype is caused by ADAMTS13 deficiency. Although pregnancy is a triggering event for TTP,[26] deficient females survived pregnancy, delivering viable offspring in normal-sized litters. No significant difference in blood cell counts (Table 1) or plasma haptoglobin levels (Table 2) was observed between *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice. Peripheral blood smears from *Adamts13*$^{-/-}$ mice did not show erythrocyte fragmentation (data not shown), demonstrating a lack of spontaneous thrombocytopenia and hemolytic anemia in *Adamts13*$^{-/-}$ mice. The renal histology of *Adamts13*$^{-/-}$ mice during pregnancy did not exhibit microvascular thrombi deposition or excessive accumulation of VWF antigen (data not shown). Thus, *Adamts13* disruption in mice did not cause TTP-like symptoms.

Table 1. Blood cell counts

	Adamts13$^{+/+}$	*Adamts13*$^{-/-}$
Red blood cell count, $\times 10^{12}$/L	8.19 ± 0.41	7.97 ± 0.25
Hemoglobin level, g/L	129 ± 5	126 ± 4
Hematocrit concentration	.426 ± .021	.422 ± .008
Platelet count, $\times 10^{9}$/L	512 ± 42	532 ± 62

Values are mean ± SD of 7 mice in each genotype.

Increased thrombogenesis in ADAMTS13-deficient mice

Plasma VWF antigen levels in *Adamts13*$^{-/-}$ mice were elevated in comparison with those from *Adamts13*$^{+/+}$ mice (Table 2). The activity of plasma FVIII, which correlates with VWF levels, was also significantly increased in *Adamts13*$^{-/-}$ mice (Table 2). The plasma fibrinogen levels, however, were comparable between *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice (Table 2). PT and APTT suggested the coagulant state in *Adamts13*$^{-/-}$ mice was normal (Table 2). To investigate the effects of ADAMTS13 deficiency on hemostasis in vivo, we measured blood loss after tail transection. There were no significant differences in blood loss between *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice (Table 2), suggesting UL-VWF multimers did not impair hemostasis.

To uncover a latent prothrombotic state caused by the presence of UL-VWF multimers in *Adamts13*$^{-/-}$ mice, we investigated platelet aggregation under static or flow conditions. We examined agonist-induced platelet aggregation under static conditions. Aggregation responses to botrocetin and collagen in *Adamts13*$^{-/-}$ mice were indistinguishable from those seen in *Adamts13*$^{+/+}$ mice (Figure 5). Thus, an UL-VWF–mediated prothrombotic state could not be detected in *Adamts13*$^{-/-}$ mice under static conditions.

Focusing on thrombus formation under flow, whole blood was perfused over a collagen-coated surface in a parallel plate flow chamber. Even though mice have smaller platelets than humans, thrombus formation was more prominent in mice than in humans, under our flow chamber system. The maximum shear rate to follow up thrombus formation in mouse blood was 750 s^{-1} and we selected this rate for comparing thrombogenesis between the groups. Cumulative thrombus volume was recorded every 0.5 minute after beginning perfusion (Figure 6). Until 3.5 minutes of perfusion, thrombus formation progressed slowly; the thrombus volume did not differ between the *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ groups. After 3.5 minutes, the thrombus grew rapidly in *Adamts13*$^{-/-}$ mice; the thrombus volume at 5.5 minutes was significantly higher in *Adamts13*$^{-/-}$ mice than in *Adamts13*$^{+/+}$ mice. Thus, ADAMTS13 deficiency in mice does not affect the

Table 2. Hematologic and coagulation parameters

	Adamts13$^{+/+}$	*Adamts13*$^{-/-}$
Haptoglobin, %	100 ± 67	103 ± 69
VWF antigen, %	100 ± 23	129 ± 31*
FVIII activity, %	100 ± 10	146 ± 22†
Fibrinogen, %	100 ± 5	98 ± 7
PT, s	16.1 ± 0.8	16.0 ± 1.0
APTT, s	44.2 ± 3.7	43.3 ± 2.5
Blood loss, μL	12.5 ± 8.4	9.5 ± 3.1

Values are mean ± SD of 12 mice in each genotype except for the blood loss, where it is mean ± SD of 18 mice.

*$P < .05$ when compared with *Adamts13*$^{+/+}$ mice

†$P < .001$ when compared with *Adamts 13*$^{+/+}$ mice.

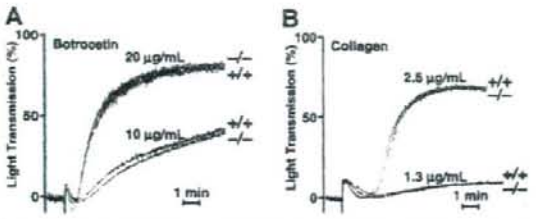

Figure 5. Platelet aggregation under static condition. (A) Botrocetin-induced aggregation. Pooled PRP samples from *Adamts13*$^{+/+}$ or *Adamts13*$^{-/-}$ mice were treated with botrocetin at a final concentration of 10 or 20 μg/mL. Aggregation was measured using an aggregometer at 37°C with constant stirring. (B) Collagen-induced aggregation. Pooled PRP samples were treated with acid-insoluble type I collagen at a final concentration of 1.3 or 2.5 μg/mL. Bars indicate 1 minute. The results of 3 typical experiments are shown.

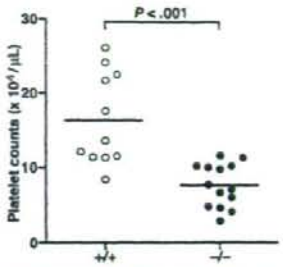

Figure 7. Platelet counts following collagen plus epinephrine challenge. Mice were given injections of 600 ng/g collagen plus 60 ng/g epinephrine via tail vein and platelet counts were measured 15 minutes after injection. Symbols represent platelet counts of a single mouse. Bars represent the mean values of groups. Platelet counts after the challenge were significantly lower in *Adamts13*$^{-/-}$ mice (n = 14) than *Adamts13*$^{+/+}$ mice (n = 11) at 7.7 ± 2.9 × 10⁴/μL and 16.4 ± 6.2 × 10⁴/μL, respectively (mean ± SD; $P < .001$), whereas platelet counts without challenge were not different between groups (*Adamts13*$^{-/-}$, 86.2 ± 13.2 × 10⁴/μL; *Adamts13*$^{+/+}$, 83.7 ± 3.3 × 10⁴/μL; mean ± SD of 4 mice).

initial adhesion of platelets to collagen, but enhances thrombus growth under shear stress.

To evaluate in vivo consequence of a lack of ADAMTS13, we examined a model of collagen-induced thrombosis. Under the conditions we examined, the mortality was not different between *Adamts13*$^{+/+}$ and *Adamts13*$^{-/-}$ mice (1 of 12 and 1 of 15 died, respectively, $P = .87$ by χ^2 test). However, platelet counts of treated mice were significantly lower in *Adamts13*$^{-/-}$ mice than in *Adamts13*$^{+/+}$ mice (Figure 7), whereas platelet counts of untreated mice were not different between groups. These results indicate that ADAMTS13 deficiency generates prothrombotic state in vivo as well as in vitro.

Discussion

This study suggests 2 perspectives on the etiology of TTP. First, deficiency in ADAMTS13 alone is sufficient to generate UL-VWF multimers in plasma, leading to a prothrombotic state. Second, ADAMTS13 deficiency is insufficient to produce the typical symptoms of TTP in mice. ADAMTS13 deficiency may induce TTP only when combined with other triggering factors.

Under static conditions, platelet aggregation responses to collagen and botrocetin were indistinguishable in ADAMTS13-deficient mice from those seen in wild-type mice, although the plasma VWF multimer size was larger in ADAMTS13-deficient mice. This result is consistent with the previous report that botrocetin is active on rodent platelets, reacting to a broad spectrum of high to low molecular weight VWF multimers.[27] Under flow conditions, however, thrombus formation on a collagen surface was enhanced in ADAMTS13-deficient mice. Although initial platelet adhesion to immobilized collagen was not affected, the growth rate of thrombus was significantly faster in ADAMTS13-deficient mice. In an in vivo thrombosis model, ADAMTS13-deficient mice were more sensitive to collagen-induced thrombocytopenia than wild-type mice, confirming in vitro observation in the flow chamber study. Thus, it was concluded that ADAMTS13 deficiency produces the prothrombotic state. Further study will be necessary to elucidate whether this prothrombotic state is ascribable to hyperreactivity of UL-VWF multimers in ADAMTS13-deficient mice.

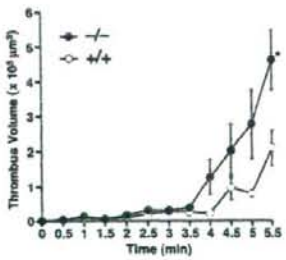

Figure 6. Thrombogenesis on collagen surface under flow. Whole blood from *Adamts13*$^{+/+}$ or *Adamts13*$^{-/-}$ mice containing mepacrine-labeled platelets was perfused over an acid-insoluble type I collagen-coated surface at a wall shear rate of 750 s^{-1}. The cumulative thrombus volume, analyzed using a multidimensional imaging system, was measured every 0.5 minute until 5.5 minutes. Data are the mean ± SEM of 5 mice for each genotype. *Significant differences at $P < .05$ in comparison with *Adamts13*$^{+/+}$ mice.

Although prolonged coagulation time was not observed, plasma levels of VWF antigen and FVIII activity were elevated in ADAMTS13-deficient mice, potentially reflecting endothelial damage induced by undetectable platelet aggregates. Alternatively, the plasma clearance rate of VWF multimers without cleavage by ADAMTS13 might be slower than cleaved VWF multimers. High levels of VWF antigen are also seen in the plasma of patients with low ADAMTS13 activity.[28]

ADAMTS13 deficiency in mice did not cause a major defect in hemostasis that would lead spontaneously to typical TTP symptoms. ADAMTS13 deficiency may cause a milder prothrombotic state in mice than in humans. The plasma VWF multimer sizes in wild-type mice were larger than those seen in humans, comparable to those in human TTP patients (Figure 4B). Mice lacking VWF exhibit milder tendencies to bleed than patients with type 3 von Willebrand disease.[29] Thus, the dependence of platelet aggregation on VWF might differ in laboratory mice from humans.

Alternatively, ADAMTS13 deficiency may not be sufficient for the development of TTP, even in humans. There is a large variation in the phenotypes of TTP patients with ADAMTS13 deficiency. Most TTP patients with congenital ADAMTS13 deficiency had their first acute episode in the newborn period or early infancy. Only a number of exceptional cases remain asymptomatic until adulthood.[30] Patients with identical *ADAMTS13* genotypes, but different symptoms, have also been described,[31,32] suggesting that the etiology of TTP cannot be explained by a single defect in ADAMTS13. Secondary triggering factors may promote the pathogenic platelet thrombus formation that results in TTP. Indeed,

Motto et al[32] independently reported generation of ADAMTS13-deficient mice and revealed that the injection of shigatoxin, a substance toxic to endothelium, provoked TTP-like symptoms in the ADAMTS13-deficient mice. In the present study, we observed enhanced thrombus formation on collagen surface under flow and promoted thrombocytopenia induced by the injection of a mixture of collagen and epinephrine in ADAMTS13-deficient mice. Genetic defects or environmental factors may stimulate endothelial activation or damage via TTP triggers, such as oxidative stress,[33] infection,[34] antiendothelial cell antibodies,[35] or complement dysfunction.[36,37] ADAMTS13-deficient mice may be useful to identify TTP triggers.

Acknowledgments

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Adiponectin Acts as an Endogenous Antithrombotic Factor

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Objective—Obesity is a common risk factor in insulin resistance and cardiovascular diseases. Although hypoadiponectinemia is associated with obesity-related metabolic and vascular diseases, the role of adiponectin in thrombosis remains elusive.

Methods and Results—We investigated platelet thrombus formation in adiponectin knockout (APN-KO) male mice (8 to 12 weeks old) fed on a normal diet. There was no significant difference in platelet counts or coagulation parameters between wild-type (WT) and APN-KO mice. However, APN-KO mice showed an accelerated thrombus formation on carotid arterial injury with a He-Ne laser (total thrombus volume: $13.36\pm4.25\times10^7$ arbitrary units for APN-KO and $6.74\pm2.87\times10^7$ arbitrary units for WT; $n=10$; $P<0.01$). Adenovirus-mediated supplementation of adiponectin attenuated the enhanced thrombus formation. In vitro thrombus formation on a type I collagen at a shear rate of 250 s^{-1}, as well as platelet aggregation induced by low concentrations of agonists, was enhanced in APN-KO mice, and recombinant adiponectin inhibited the enhanced platelet aggregation. In WT mice, adenovirus-mediated overexpression of adiponectin additionally attenuated thrombus formation.

Conclusion—Adiponectin deficiency leads to enhanced thrombus formation and platelet aggregation. The present study reveals a new role of adiponectin as an endogenous antithrombotic factor. **(*Arterioscler Thromb Vasc Biol.* 2006;26:224-230.)**

Key Words: acute coronary syndromes ■ obesity ■ platelets ■ thrombosis

Obesity is associated with insulin resistance, accelerated atherothrombosis, and cardiovascular diseases.[1,2] Recent studies have revealed that adipose tissue is not only a passive reservoir for energy storage but also produces and secretes a variety of bioactive molecules, known as adipocytokines, including tumor necrosis factor (TNF) α, leptin, resistin, and plasminogen activator inhibitor type-1.[2–4] Dysregulated production of adipocytokines participates in the development of obesity-related metabolic and vascular diseases.[2–4]

Adiponectin is an adipocytokine identified in the human adipose tissue cDNA library, and Acrp30/AdipoQ is the mouse counterpart of adiponectin (reviewed in reference[5]). Adiponectin, of which mRNA is exclusively expressed in adipose tissue, is a protein of 244 amino acids consisting of 2 structurally distinct domains, an N-terminal collagen-like domain and a C-terminal complement C1q-like globular domain. Adiponectin is abundantly present in plasma (5 to 30 μg/mL), and its plasma concentration is inversely related to the body mass index.[5] Plasma adiponectin levels decrease in obesity, type 2 diabetes, and patients with coronary artery disease (CAD).[5–9] Indeed, adiponectin (APN) knockout (KO) mice showed severe diet–induced insulin resistance.[10] In cultured cells, we have demonstrated that human recombinant adiponectin inhibited the expression of adhesion molecules on endothelial cells, the transformation of macrophages to foam cells, and TNF-α production from macrophages.[5,11] Furthermore, APN-KO mice showed severe neointimal thickening in mechanically injured arteries.[12] Adenovirus-mediated supplementation of adiponectin attenuated the development of atherosclerosis in apolipoprotein E–deficient mice as well as postinjury neointimal thickening in APN-KO mice.[12,13] These data suggest the antiatherogenic properties of adiponectin, and, hence, hypoadiponectinemia may be associated with a higher incidence of vascular diseases in obese subjects. Although it is also possible that an altered hemostatic balance may contribute to the pathogenesis of acute cardiovascular events in such patients, the roles of adiponectin in hemostasis and thrombosis remains elusive.

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Here we have provided the first evidence that adiponectin affects thrombus formation, and, hence, hypoadiponectinemia may directly contribute to acute coronary syndrome. Our data indicate a new role of adiponectin as an antithrombotic factor.

Methods

Mice

APN-KO male mice (8 to 12 weeks old) were generated as described previously.[10,12] We analyzed mice backcrossed to C57BL/6 for 5 generations.[10,12]

Preparation of Mouse Platelets and Measurement of Coagulation Parameters

Mouse platelet-rich plasma (PRP) was obtained as described previously.[14] Coagulation parameters were measured by SRL Inc.

Platelet Aggregation Study, Adhesion Study, and Flow Cytometry

Platelet aggregation and platelet adhesion study was performed as described previously.[14] Integrin $\alpha_{IIb}\beta_3$ activation and α-granule secretion of wild-type (WT) and APN-KO platelets were detected by phycoerythrin-conjugated JON/A monoclonal antibody (mAb), which binds specifically to mouse-activated $\alpha_{IIb}\beta_3$ (Emfret Analytics) and FITC-conjugated anti-P-selectin mAb (Becton Dickinson), respectively.[14]

Assessment of Atherosclerosis and Bleeding Time Measurement

Assessment of atherosclerosis was performed as described previously.[15] The tail of anesthetized mice (nembutal 65 mg/kg; 8 to 12 weeks old) was transected 5 mm from the tip and then immersed in 0.9% isotonic saline at 37°C. The point until complete cessation of bleeding was defined as the bleeding time.

He-Ne Laser–Induced Thrombosis

The observation of real-time thrombus formation in the mouse carotid artery was performed as described previously.[15] Anesthetized mice (nembutal 65 mg/kg) were placed onto a microscope stage, and the left carotid artery (450 to 500 μm in diameter) was gently exposed. Evans blue dye (20 mg/kg) was injected into the left femoral artery via an indwelt tube, and then the center of the exposed carotid artery was irradiated with a laser beam (200 μm in diameter at the focal plane) from a He-Ne laser (Model NEO-50MS; Nihon Kagaku Engineering Co, Ltd). Thrombus formation was recorded on a videotape through a microscope with an attached CCD camera for 10 minutes. The images were transferred to a computer every 4 s, and the thrombus size was analyzed using Image-J software (National Institutes of Health). We calculated thrombus size by multiplying each area value and its grayscale value together. We then regarded the total size values for an individual thrombus obtained every 4 s during a 10-minute observation period as the total thrombus volume and expressed them in arbitrary units.

Flow Chamber and Perfusion Studies

The real-time observation of mural thrombogenesis on a type I collagen-coated surface under a shear rate of 250 s^{-1} was performed as described previously.[16] Briefly, whole blood obtained from anesthetized mice was anticoagulated with argatroban, and then platelets in the whole blood were labeled by mepacrine. Type I collagen-coated glass cover slips were placed in a parallel plate flow chamber (rectangular type; flow path of 1.9-mm width, 31-mm length, and 0.1-mm height). The chamber was assembled and mounted on an epifluorescence microscope (Axiovert S100 inverted microscope, Carl Zeiss Inc) with the computer-controlled z-motor (Ludl Electronic Products Lts). Whole blood was aspirated through the chamber, and the entire platelet thrombus formation process was observed in real time and recorded with a video recorder.

Preparation of Adenovirus and Recombinant Adiponectin

Adenovirus producing the full-length mouse adiponectin was prepared as described previously.[10] Plaque-forming units (1×10^8) of adenovirus-adiponectin (Ad-APN) or adenovirus-β-galactosidase (Ad-βgal) were injected into the tail vein. Experiments were performed on the fifth day after viral injection. The plasma concentrations of adiponectin were measured by a sandwich ELISA. Mouse and human recombinant proteins of adiponectin were prepared as described previously.[11,17]

RT-PCR

Total cellular RNA of platelets from WT or APN-KO mice was obtained, and contaminated genomic DNA was removed using a QuantiTect Reverse-Transcription kit (QIAGEN). One microgram of total RNA was used as a template for RT-PCR as described previously.[18] For the amplification of transcripts of mouse adiponectin receptors AdipoR1 and AdipoR2, the following primers were used: mouse AdipoR1 5′-ACGTTGGAGAGTCATCCCGTAT-3′ (sense) and 5′-CTCTGTGTGGATGCGGAAGAT-3′ (antisense) and mouse AdipoR2 5′-TGCGCACACATTTCAGTCTCCT-3′ (sense) and 5′-TTCTATGATCCCCAAAAGTGTGC-3′ (antisense).[19,20] For human platelet isolation, PRP obtained from 50 mL of whole blood was passed through a leukocyte removal filter as described previously.[21] This procedure removed >99.9% of the contaminated leukocytes.[21] For human AdipoR1 and AdipoR2, the following primers were used: human AdipoR1 5′-CTTCTACTGCTCCCCACAGC-3′ (sense) and 5′-GACAAAGCCCTCAGCGATAG-3′ (antisense) human AdipoR2 5′-GGACCGAGCAAAAGACTCAG-3′ (sense) and 5′-CACCCAGAGGCTGCTACTTC-3′ (antisense). In addition, total cellular RNA obtained from a megakaryocytic cell line, CMK, and that from a human monocytic cell line, THP-1 (positive control)[22] was examined in parallel. RT-PCR samples omitting reverse transcriptase were used as negative controls.

Statistical Analysis

Results were expressed as mean±SD. Differences between groups were examined for statistical significance using Student *t* test.

Results

Characteristics of Adiponectin-Deficient Mice and Assessment of Atherosclerotic Lesions

The basal profiles of APN-KO male mice have been previously described.[10,12] To exclude the effects of diet on APN-KO mice, we used APN-KO male mice (8 to 12 weeks old) fed on a normal diet in this study. There were no differences in platelet counts, PT, APTT, and plasma fibrinogen concentrations (Table I, available online at http://atvb.ahajournals.org). Histological analyses revealed that neither Oil Red O staining of the inner surface of whole aorta nor elastin-van Gieson staining of transverse sections of carotid arteries showed any apparent atherosclerotic lesions in WT or APN-KO mice (data not shown).

Bleeding Time in APN-KO Mice

To examine the effects of adiponectin deficiency on thrombosis and hemostasis, we studied bleeding time in APN-KO mice. The bleeding time in APN-KO mice was slightly but significantly shorter (96.9±34.9 s; n=30; $P<0.05$) than that in WT mice (130.9±52.1 s; n=30).

Enhanced Thrombus Formation in APN-KO Mice and Adiponectin Adenovirus Ameliorates the Thrombogenic Tendency

We next examined the effect of adiponectin deficiency on thrombus formation using the He-Ne laser-induced carotid

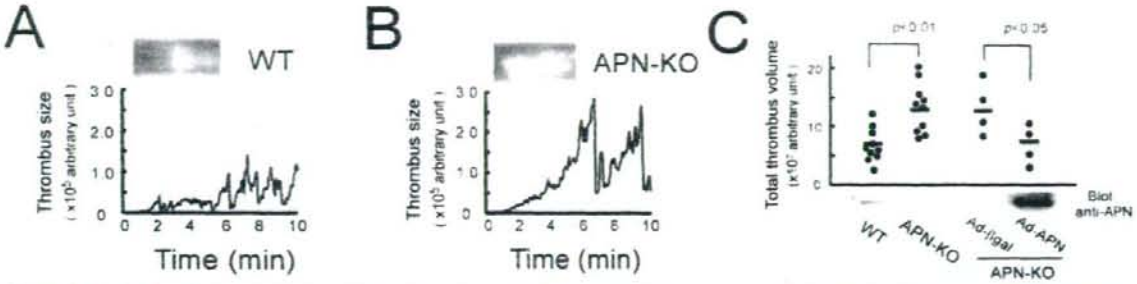

Figure 1. He-Ne laser–induced thrombus formation and adenovirus-mediated supplementation of adiponectin. Anesthetized mice were injected with Evans blue dye followed by irradiation with the He-Ne laser at the exposed left carotid artery. The representative time course of thrombus formation in (A) WT or (B) APN-KO mice is shown. (C) The total thrombus volume was significantly larger in APN-KO mice ($n=10$; $P<0.01$). In another set of experiments, administration of adenovirus-producing mouse adiponectin (Ad-APN) significantly attenuated the total thrombus volume, as compared with control adenovirus (Ad-βgal)-infected APN-KO mice ($n=4$; $P<0.05$). Plasma adiponectin levels detected in immunoblots are shown in the lower panel.

artery thrombus model. Endothelial injury of the carotid artery was induced by the interaction of Evans blue dye with irradiation from the He-Ne laser. In WT mice, thrombus formation started 61.0±25.0 s after the initiation of He-Ne laser irradiation ($n=10$). When the thrombi reached a certain size, they frequently ruptured and detached themselves from the wall because of increased shear stress. Thus, thrombus formation in this in vivo model showed a cyclic fluctuation, and complete occlusion was not observed (Figure 1). During a 10-minute observation period, the cycles of thrombus formation were 8.5±2.3 in WT mice. In APN-KO mice, there was no significant difference in the initiation time for thrombus formation (54.8±8.9 s; n=10; $P=0.46$). However, the cycles of thrombus formation during the 10-minute observation period were significantly fewer (5.4±2.0; n=10; $P<0.01$) in APN-KO mice. The thrombi in APN-KO mice grew larger and appeared to be stable and more resistant to the increased shear stress. Accordingly, the total thrombus volume was significantly larger in APN-KO mice ($6.74\pm2.87\times10^7$ arbitrary units in WT mice and $13.36\pm4.25\times10^7$ arbitrary units in APN-KO mice; n=10; $P<0.01$).

To confirm that adiponectin deficiency is responsible for the enhanced thrombus formation in APN-KO mice, we injected Ad-βgal or Ad-APN into APN-KO mice. On the fifth day after adenoviral injection, we confirmed the elevated plasma adiponectin level in Ad-APN-infected APN-KO mice in an ELISA assay (48.7±6.8 μg/mL; n=4), as well as in an immunoblot assay. In the carotid artery thrombus model, the total thrombus volume in Ad-βgal-infected APN-KO was $12.94\pm4.67\times10^7$ arbitrary units, which was compatible with that of APN-KO mice shown in Figure 1. In contrast, Ad-APN infection significantly decreased the total thrombus volume in APN-KO mice ($6.23\pm3.09\times10^7$ arbitrary units; n=4; $P<0.05$). These results indicate that adiponectin deficiency is responsible for the thrombogenic tendency in vivo.

Platelet-Thrombus Formation on Immobilized Collagen Under Flow Conditions

Because endothelial function may affect in vivo thrombus formation, we next performed in vitro mural thrombus formation on a type I collagen-coated surface under flow conditions. Figure 2 shows thrombus formation during a

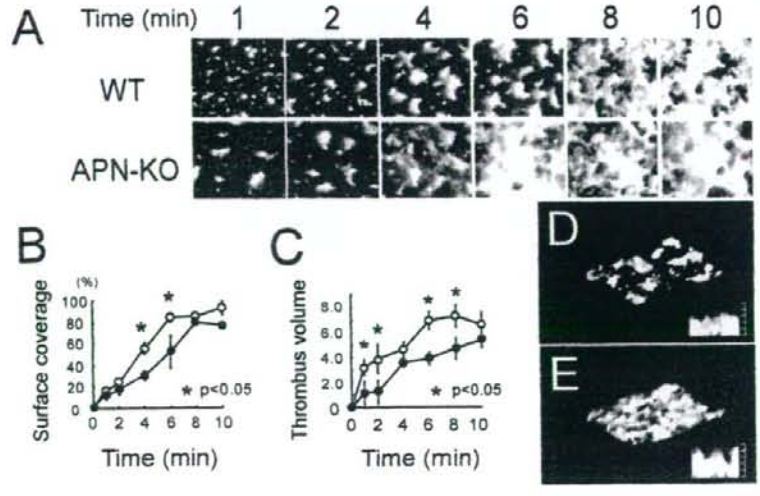

Figure 2. Thrombogenesis on a type I collagen-coated surface under flow conditions. (A) Mepacrine-labeled whole blood obtained from WT (top) or APN-KO mice (bottom) was perfused on a type I collagen-coated surface at a shear rate of 250 s^{-1}. (B) Platelet surface coverage (%) and (C) thrombus volume are shown at indicated time points. (●, WT; ○, APN-KO; *$P<0.05$). Shown are representative 3D images of thrombus formation at 6-minute perfusion in whole blood obtained from (D) WT and (E) APN-KO mice. Each inserted figure shows thrombus height.

10-minute perfusion of mouse whole blood anticoagulated with thrombin inhibitor at a low shear rate (250 s^{-1}). In whole blood obtained from WT mice, the thrombus fully covered the collagen-coated surface after 8 to 10 minutes of perfusion. In contrast, the thrombus grew more rapidly and fully covered the surface at 6 minutes in APN-KO mice. At 1 and 2 minutes of perfusion, there was no apparent difference in the initial platelet adhesion to the collagen surface between WT and APN-KO mice, whereas the platelet aggregate formation was significantly enhanced in APN-KO, even at 1 minute. We additionally examined the possibility that adiponectin might inhibit platelet adhesion onto collagen, because adiponectin binds to collagen types I, III, and V.[23] However, mouse recombinant adiponectin (40 μg/mL) did not inhibit the adhesion of platelets onto collagen, indicating that the inhibitory effect of adiponectin is not mediated by the inhibition of platelet binding to collagen (data not shown). At a high shear rate (1000 s^{-1}), the thrombus grew rapidly and fully covered the surface within 3 to 4 minutes. Under such strong stimuli, we did not detect any difference in thrombus formation between WT and APN-KO mice, probably because of the full activation of platelets.

Adiponectin Inhibits the Enhanced Platelet Aggregation in APN-KO Mice

In platelet aggregation studies, PRP obtained from APN-KO mice showed significantly enhanced platelet aggregation in response to low doses of agonists (ADP 2.5 μmol/L, collagen 2.5 μg/mL, and protease-activated receptor 4–activating peptide [PAR4-TRAP] 75 μmol/L), as compared with WT mice (Figure 3). The maximal platelet aggregation was achieved at higher concentrations of agonists, and the enhanced platelet aggregation in APN-KO mice was not apparent at these high doses of agonists, probably because of the full activation of platelets.

To confirm the inhibitory effect of adiponectin on platelet aggregation in vitro, we mixed 1 volume of PRP obtained from APN-KO mice with 4 volumes of platelet-poor plasma (PPP) obtained from APN-KO mice injected with either Ad-βgal or Ad-APN to adjust platelet counts to $300\times10^3/\mu L$. As shown in Figure 4A, the in vitro supplementation of PPP containing adiponectin attenuated the enhanced platelet aggregation. Similarly, in vitro administration of mouse recombinant adiponectin (40 μg/mL) to PRP from APN-KO mice attenuated the enhanced platelet aggregation (Figure 4B).

Expression of Adiponectin Receptors in Platelets and Effects of Adiponectin Deficiency on $\alpha_{IIb}\beta_3$ Activation and P-Selectin Expression

To reveal the effect of adiponectin on platelets, we examined whether platelets possess transcripts for adiponectin receptors AdipoR1 and AdipoR2 by using RT-PCR. As shown in Figure 5A, platelets from APN-KO, as well as WT mice, contained mRNAs for AdipoR1 and AdipoR2. We also confirmed that the human megakaryocytic cell line CMK, as well as carefully isolated human platelets, possessed mRNAs for AdipoR1 and AdipoR2. We next examined the effects of adiponectin deficiency on $\alpha_{IIb}\beta_3$ activation and α-granule secretion at various concentrations of agonists by flow cytometry. However, neither the platelet $\alpha_{IIb}\beta_3$ activation induced by ADP nor P-selectin expression induced by PAR4-TRAP showed significant difference between WT and APN-KO mice (n=4; Figure 5B and 5C).

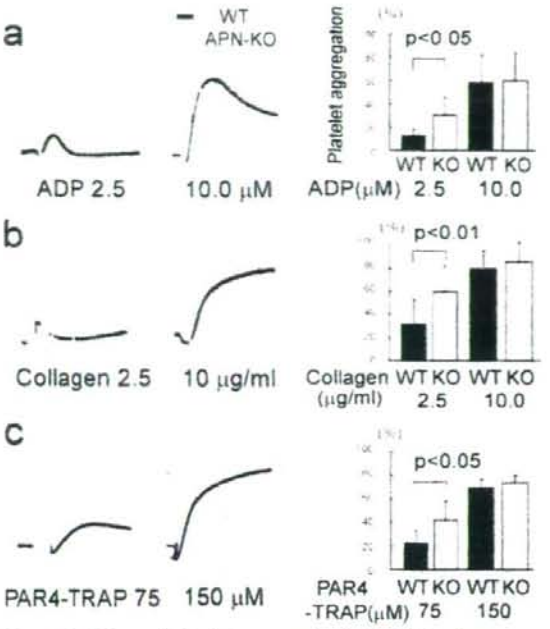

Figure 3. Enhanced platelet aggregation in APN-KO mice. Platelet aggregation in PRP obtained from WT or APN-KO mice. PRP ($300\times10^3/\mu L$) obtained from WT (black line) or APN-KO mice (gray line) was stimulated with ADP (a; n=4), collagen (b; n=4), or PAR4-TRAP (c; n=3). As compared with WT mice, platelet aggregation was enhanced in APN-KO mice at low concentrations of agonists.

Adiponectin Adenovirus Attenuates Thrombus Formation in WT Mice

Because WT mice have large amounts of adiponectin in their plasma, we, therefore, examined whether adiponectin overexpression could additionally inhibit thrombus formation, as well as platelet function, in WT mice. After the administration of Ad-APN or Ad-βgal into WT mice, the plasma adiponectin levels in Ad-APN-infected mice reached ≈4 times higher than those in Ad-βgal-infected WT mice (8.5±0.6 μg/mL for Ad-βgal and 37.0±14.8 μg/mL for Ad-APN; n=5). As shown in Figure 6A, platelet aggregation in PRP induced by collagen or PAR4-TRAP was significantly attenuated by the overexpression of adiponectin. Similarly, in vitro administration of human recombinant adiponectin (40 μg/mL) to human PRP attenuated the platelet aggregation response to 2.5 μg/mL collagen (Figure 6B). Moreover, in the He-Ne laser–induced carotid artery thrombus model, the overexpression of adiponectin significantly inhibited thrombus formation in WT mice ($4.38\pm0.75\times10^7$ arbitrary units for Ad-βgal and $2.75\pm0.61\times10^7$ arbitrary units for Ad-APN; n=7; $P<0.05$; Figure 6C).

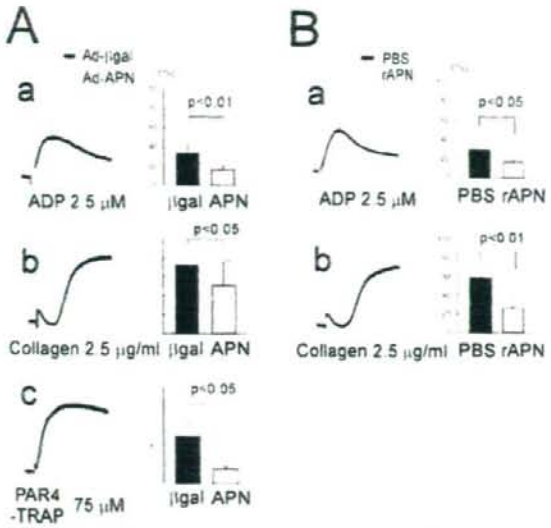

Figure 4. Effects of in vitro supplementation of adiponectin or recombinant adiponectin on the enhanced platelet aggregation in APN-KO mice. (A) One volume of PRP from APN-KO mice was mixed with ≈4 volumes of PPP from APN-KO mice injected with Ad-βgal (black line) or Ad-APN (gray line) to obtain a platelet concentration of $300\times10^3/\mu L$. Platelets were stimulated with indicated agonists (n=4). (B) Mouse recombinant adiponectin (40 μg/mL, gray line) or PBS (black line) was added to PRP from APN-KO mice. Platelets were adjusted to 300×10^3 platelets/μL and stimulated with indicated agonists (n=4).

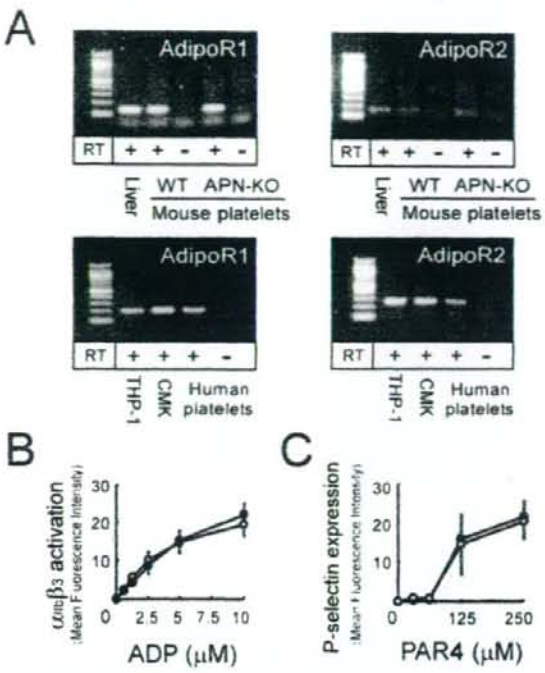

Figure 5. Expression of adiponectin receptors and effects of adiponectin deficiency on platelet function. (A, top) Expressions of transcripts for adiponectin receptors, AdipoR1 (133-bp fragments) and AdipoR2 (156-bp fragments), in platelets from WT or APN-KO mice were examined by RT-PCR. The liver was used as a positive control. (Bottom) Expressions of transcripts for adiponectin receptors, AdipoR1 (196-bp fragments) and AdipoR2 (243-bp fragments), in CMK cells, as well as human platelets, were examined by RT-PCR; 100-bp DNA Ladder (New England Biolabs) was used as a marker. Effects of adiponectin deficiency on (B) $\alpha_{IIb}\beta_3$ activation and (C) α-granule secretion. PRP obtained from WT (●) or APN-KO (○) mice in the presence of phycoerythrin-JON/A mAb or FITC-anti-P-selectin mAb was stimulated with the indicated agonist and then analyzed by flow cytometry without any washing. There were no significant differences in platelet $\alpha_{IIb}\beta_3$ activation or P-selectin expression between WT and APN-KO mice (n=4).

Discussion

In the present study, we have newly revealed an antithrombotic effect of adiponectin. APN-KO male mice (8 to 12 weeks old) fed on a normal diet showed no significant differences in platelet counts and coagulation parameters compared with WT mice. In the He-Ne laser–induced carotid artery thrombus model, APN-KO mice showed an accelerated thrombus formation, and adenovirus-mediated supplementation of adiponectin attenuated this enhanced thrombus formation. Platelet aggregometry and the real-time observation of in vitro thrombus formation on a type I collagen-coated surface under flow conditions showed the enhanced platelet function in APN-KO mice. Moreover, adenovirus-mediated overexpression of adiponectin attenuated in vivo thrombus formation, as well as the in vitro platelet aggregation response, even in WT mice. Thus, the present data strongly suggest that adiponectin possesses an antithrombotic potency.

We have demonstrated that low concentrations of adiponectin are associated with the prevalence of CAD in men, which is independent of well-known CAD risk factors.[8] Pischon et al[9] have recently shown that high concentrations of adiponectin are associated with a lower risk of myocardial infarction in men, which is also independent of inflammation and glycemic status and can be only partly explained by differences in blood lipids. These clinical studies suggest that the protective effect of adiponectin on the development of CAD may be primary rather than secondary through the protection of metabolic abnormalities, such as insulin resistance. Indeed, APN-KO mice fed on a normal diet did not show any abnormalities in plasma glucose, insulin, or lipid profiles.[10,12] Although the atherosclerotic and thrombotic processes are distinct from each other, these processes appear to be interdependent, as shown by the term *atherothrombosis*. The interaction between the vulnerable atherosclerotic plaque, which is prone to disruption, and thrombus formation is the cornerstone of acute coronary syndrome (ACS).[24] In this context, our present data strongly suggest that adiponectin deficiency (or hypoadiponectinemia) may directly contribute to the development of ACS by enhanced platelet thrombus formation.

Although APN-KO fed on a normal diet showed no significant differences in major metabolic parameters, they showed delayed clearance of FFA in plasma, elevated plasma TNF-α concentrations (≈40 pg/mL in APN-KO; ≈20 pg/mL in WT), and elevated CRP mRNA levels in white adipose tissue.[12,25] In addition, recombinant adiponectin increased NO production in vascular endothelial cells.[26] To rule out any

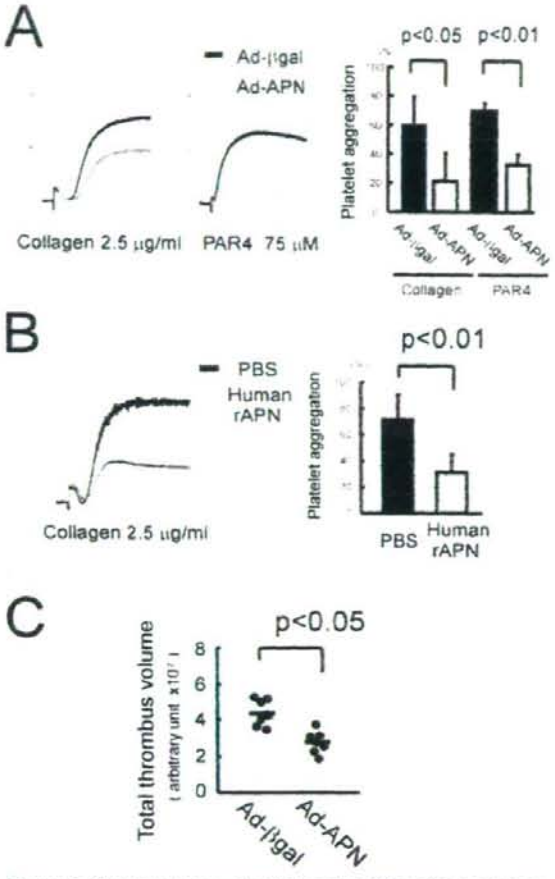

Figure 6. Overexpression of adiponectin additionally attenuates thrombus formation in WT mice. (A) Platelet aggregation in PRP obtained from WT mice injected with either Ad-βgal or Ad-APN. PRP ($300\times10^3/\mu L$) obtained from WT mice injected with either Ad-βgal (black line) or Ad-APN (gray line) was stimulated with collagen or PAR4-TRAP (n=4). Administration of Ad-APN significantly attenuated platelet aggregation in WT mice. (B) Human recombinant adiponectin (40 μg/mL, gray line) or PBS (black line) was added to PRP ($300\times10^3/\mu L$) from control subjects. Platelets were stimulated with collagen (n=7). (C) He-Ne laser–induced thrombus formation in WT mice injected with either Ad-βgal or Ad-APN. Administration of Ad-APN in WT mice additionally reduced the total thrombus volume in the carotid artery thrombus model (n=7, $P<0.05$).

effect of adiponectin on vascular cells, we examined in vitro thrombus formation on a type I collagen-coated surface under flow conditions, as well as platelet aggregation in APN-KO mice. Thus, the enhanced platelet function in APN-KO mice was still evident even in the absence of vascular cells. Moreover, human and mouse recombinant adiponectin attenuated the aggregation response obtained from control human subjects and from APN-KO mice, respectively. Thus, adiponectin inhibits platelet function. However, the mechanism by which adiponectin attenuates platelet aggregation and arterial thrombus formation in vivo remains unclear. During thrombogenesis, platelets adhere to altered vascular surfaces or exposed subendothelial matrices, such as collagen, and then become activated and aggregate to each other.[16] The thrombus formed in APN-KO mice appeared to be stable and more resistant to the increased shear stress, without affecting the initiation time for thrombus formation in carotid artery injury experiments, as well as in flow chamber perfusion experiments. In addition, preincubation of collagen with recombinant adiponectin did not inhibit platelet adhesion on collagen under static conditions. Thus, it is unlikely that the inhibitory effect of adiponectin is mediated by the inhibition of platelet binding to collagen. These characteristics are quite distinct from C1q-TNF–related protein-1, which belongs to the same family as adiponectin and inhibits thrombus formation by interfering with platelet–collagen interaction.[27] We confirmed that transcripts for AdipoR1 and AdipoR2 were present in mouse and human platelets and CMK cells. Although the platelet–platelet interaction appeared to be enhanced in APN-KO mice, we did not detect any difference in agonist-induced $\alpha_{IIb}\beta_3$ activation or P-selectin expression between APN-KO and WT mice by flow cytometry. Based on these results, it is possible that adiponectin may inhibit $\alpha_{IIb}\beta_3$-mediated intracellular postligand binding events. Alternatively, previous studies have shown that adiponectin is physically associated with many proteins, including α_2-macroglobulin, thrombospondin-1 (TSP-1), and several growth factors.[5,23,28] Interestingly, TSP-1, after secretion from platelet α granules, may participate in platelet aggregation by reinforcing interplatelet interactions through direct fibrinogen-TSP-fibrinogen and TSP-TSP crossbridges.[29,30] In this context, it is also possible that it may interfere with interplatelet interactions in platelet aggregation. Additional studies to clarify the mechanism of adiponectin are currently under way.

In conclusion, our present study revealed that adiponectin acts as an endogenous antithrombotic factor. Although it is possible that the in vivo antithrombotic effect of adiponectin may be partly mediated by its action on vascular cells, our present data clearly indicate that adiponectin affects platelet function in the absence of vascular cells. In addition, the overexpression of adiponectin in WT mice attenuates in vivo thrombus formation, as well as the in vitro platelet aggregation response. Our data provide a new insight into the pathophysiology of ACS in nonobese, as well as obese, subjects, and adiponectin (and its derivatives) may be a new candidate for an antithrombotic drug.

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外科周術期輸血トリガー値に関する考察

——心臓血管外科周術期を中心に

Perioperative transfusion threshold, adopted mainly in cardiovascular surgery

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◎血液製剤の安全性が向上し，無輸血手術が最重要課題であった時代から，安全性が高い確率で担保できる血液製剤をいかに効果的に利用できるかが外科周術期輸血の重要課題となりつつある．この流れのなかで，外科周術期輸血トリガー値を探索する臨床試験，とくにランダム化比較試験の報告が増加している．製剤ごとにこれら報告について，心臓血管外科手術周術期を中心に詳細に検討してみた．各施設で，主観ではなく客観的な根拠に基づいた血液製剤の適正使用を検討する参考となれば幸いである．

Key word：輸血トリガー値，心臓血管外科周術期，エビデンス，臨床試験，使用指針

近年，同種血液製剤の感染症スクリーニングにNAT(核酸増幅法)が導入され，安全性が飛躍的に向上した．B型肝炎伝播は30〜40万本に1回起こるリスクとなり，C型肝炎，HIV感染症については無視できるレベルまで低下している．また，保存前白血球除去の導入によりさらに安全性の向上が見込まれる．よって，輸血による感染症伝播が問題視され，無輸血手術が最重要課題であった時代から，安全性が高い確率で担保できる血液製剤をいかに効果的に利用するかが重要となる時代にシフトしつつある．この流れのなかで，外科手術などにおける輸血トリガー値を模索する臨床試験，とくにランダム化比較試験が倫理上許されることとなり，近年多くの報告がなされるようになった．今後，患者予後をエンドポイントとした臨床試験を積み重ね，適正な輸血トリガー値を設定する試みが重要となる．

平成15年(2003)7月に血液法『安全な血液製剤の安定供給の確保等に関する法律』が施行され，その第8条に，医療関係者の責務として「血液製剤の適正な使用に努めなければならない」と規定されている．適正輸血を推進するためには外科手術周術期輸血療法に関して科学的根拠に基づいた，患者に対して最少のリスクとなるような血液製剤の使用基準の確立が望まれる．平成17年(2005)9月に厚生労働省医薬安全局血液対策課により改訂された『血液製剤の使用指針』のなかで，血液製剤の外科的適応に関して各科術式に対して巻末に記載がなされ，文献が掲載されている．

本稿では心臓血管外科周術期を中心に，改訂された『血液製剤の使用指針』を考慮に入れながら，最近報告されたエビデンスをもとにして，詳細に血液製剤の使用基準について検討してみたい．

赤血球製剤

心臓血管外科疾患に対する貧血許容限界はいまだ確立されていない．冠動脈バイパス手術，弁置換術，大血管外科手術，小児手術など，疾患や患者や状況により異なると考えられる．改訂された『血液製剤の使用指針』のなかでは術前の輸血について，「術前の貧血は必ずしも投与の対象とはならない．慣習的に行われてきた術前投与のいわゆる10/30ルール〔ヘモグロビン(Hb)値10 g/dl，ヘマトクリット(Ht)値30%以上にすること〕は近年では根拠のないものとされている」と記載されている．また，「人工心肺使用時には血液希釈が起こる．

人工心肺中の Hb 値についての上限及び下限は明らかではない．人工心肺離脱後は Hb 値が 7～8 g/dl 以上(＜10 g/dl)になるようにすることが多い」と記述されている．また，循環器疾患への配慮として以下のような記述がある．「通常は Hb 値が 7～8 g/dl 程度であれば十分な酸素の供給が可能であるが，冠動脈疾患あるいは肺機能障害や脳循環障害のある患者では，Hb 値を 10 g/dl 程度に維持することが推奨される」．

1．成人心臓外科

心臓手術患者が除外されているものの重症患者における輸血トリガー値について画期的な報告がなされた．集中治療が必要な高リスク患者に対して“血管内容量が正常範囲内であればどこまで低い Hb を許容できるか”に関して，カナダで実施された多施設共同ランダム化比較対照試験(TRICC trial[1])である．16 歳以上の 838 名の intensive care unit(ICU)患者を対象として，Hb 7 g/dl を開始基準とし，Hb を 10 g/dl 未満に管理する群(restrictive strategy 群)と Hb 10 g/dl を開始基準として 10～12 g/dl に維持する群(liberal strategy 群)とが比較検討されている．その結果，主要評価項目である 30 日間死亡率には差がなかったが，restrictive strategy 群が liberal strategy 群より院内死亡率が低く，患者の予後を改善する可能性があることが示された．また，55 歳未満や APACHE Ⅱ score が 20 以下に限ると 30 日間死亡率も著明に低いことが報告されている．したがって，高リスク群であっても Hb を 10 g/dl 以上に維持する必要がない可能性が示唆されている．

この TRICC trial のサブグループ解析として，心臓血管疾患合併患者 357 名および虚血性心疾患合併患者 257 名を対象とした報告がある[2]．30 日間死亡率，60 日間死亡率，院内死亡率および ICU 死亡率に restrictive strategy 群と liberal strategy 群で有意差がなかったと報告されている．ただし，虚血性心疾患患者に関する統計学的検出力は 17.0%のため，急性心筋梗塞や不安定狭心症では解釈に注意が必要であると思われる．同じ TRICC trial のサブグループ解析で，人工呼吸器離脱に関する検討が行われた[3]．人工呼吸管理を受けた 713 名について人工呼吸器離脱に関して検討され，restrictive strategy 群と liberal strategy 群で有意差がなく，人工呼吸管理を要する時間は Hb にはよらないが，輸血により延長すると報告された．

ランダム化比較試験ではなく観察研究として，人工心肺中の貧血許容限界について冠動脈バイパス術患者を対象とした報告がある[4]．冠動脈バイパス術患者の人工心肺中の最低 Ht は院内死亡率に対する独立危険因子であり，最低 Ht が 14%以下になると死亡率が増加する．さらに，高リスク患者(ショック，腎不全，心室性不整脈，心臓手術の既往，ニトログリセリン持続静注，うっ血性心不全，腸骨動脈の狭窄性疾患，高齢者)では，最低 Ht が 17%以下になると死亡率が増加すると報告されており，人工心肺中であっても，とくに高リスク群では，ある程度 Hb を維持する必要があることが示唆されている．

同様の報告が 2001 年にもなされている[5]．冠動脈バイパス術人工心肺中の最低 Ht は，院内死亡率，術中術後の大動脈内バルーンパンピング(IABP)使用および再ポンプに有意に関係していた．術中術後の脳血管障害および再開胸止血には関係していなかった．Ht 23%から危険性は高まり，Ht 19%未満では Ht 25%以上と比較して約 2.4 倍危険性が高くなると報告されている．

人工心肺中の Hb について，さらに 1,760 症例の人工心肺使用患者での後ろ向き観察研究が行われ，人工心肺中の 24%未満の Ht 最低値は腎障害リスクの増加と関連しており，とくに人工心肺の使用時間が長期になり(90 分以上)，輸血が行われるとリスクが増加すると報告されている[6]．

手術後，ICU 入室時における貧血許容限界に対する検討もなされている[7]．冠動脈バイパス術後では従来考えられていたのとは逆に，Q 波心筋梗塞および IABP を要する重症左心機能不全は Ht が高いほど増加すること，さらに不安定狭心症，緊急手術あるいは心臓手術の既往をもつ高リスク患者に限ると，Ht 34%以上の場合に死亡率がもっとも高く，Ht が 25～33%の場合がもっとも低かったと報告されている．また，術後 24 時間以内の最低 Hb が死亡率や合併症発症率に与える影響を検討したコホート研究[8]によると，Hb は死亡率や心筋梗塞を含めた循環動態に関する合併症および中

枢神経系合併症には影響しなかった．しかし，最低 Hb が低いほど腹部臓器合併症，腎合併症および入院日数が増加していた(腎合併症は 65 歳以上の高齢者では影響はなかった)．

これらおもに冠動脈バイパス術を対象になされた報告を考慮すると，Hb 10 g/dl を超える過剰な輸血や，無輸血にこだわるあまり極端に Hb を低下させることは，患者予後を悪くする可能性が示唆される．したがって，通常 Hb が 8 g/dl になれば輸血するタイミングを考慮し，Hb 10 g/dl を超えて輸血する必要はないと考えられる．

2. 小児心臓外科

小児心臓外科については，①症例によるバリエーションが大きいため，コントロールされた臨床研究を実施しにくい，②施設間の比較検討が行いにくい，③採血による貧血が問題となり，臨床研究の実施に困難を伴う，④臨床研究のインフォームドコンセントが得られにくい，などの理由から小児心臓外科の輸血療法に関するエビデンスが少なく，ガイドラインの作成が困難であると思われる．しかし最近，小児心臓外科に関してもいくつか興味深い報告がなされている．とくに，小児の場合は余命が長いため長期予後が問題となり，これらに焦点をあてた報告がなされている．

先天性心疾患の術後患者 243 名において，5 歳時点での神経学的発達に及ぼす影響に対する前向き観察研究がある[9]．同意取得率は 36% とやはり小児での臨床試験の困難さがうかがえる．結果は以下のとおりである．人工心肺を用いた症例が 82% で，人工心肺時間は full-scale IQ には影響しなかったが，performance IQ を悪化させる独立危険因子であった．人工心肺中最低 Ht は，家庭環境スコアを調整すると，両心室疾患では危険因子とならなかったと報告されているが，人工心肺中最低 Ht に関しては単心室疾患も含めた解析が行われていない．人工心肺中最低中枢温は危険因子とならなかったが，超低体温下循環停止時間は full-scale IQ を悪化させる傾向がみられた．

別の前向き観察研究[10]では 18 名の 3〜17 歳の認知機能が正常な心房中隔欠損症(ASD)患者を対象とし，手術前および術後 6 カ月で評価が行われた．人工心肺中は，最低 Ht 26.6±4.6%，最低中枢温 31.4±1.6℃，最低ポンプ流量 2,207±210 ml/min/m^2で，認知機能の低下は認められなかったと報告されている．しかし，結果のグラフでは明らかな低下がみられる患者が存在しているので，結果の解釈には注意が必要であると思われ，対象患者数が少ないため，明確な低下は認められなくてもわずかな低下の存在は否定できず，長期観察によりはじめて明らかとなる可能性があると考えられる．

後ろ向き観察研究の報告もあり[11]，ASD 患者に対しての治療として手術群 26 名とカテーテル治療群 19 名での精神運動発達テストに関する比較検討が行われた．年齢および両親の IQ を調整すると full-scale IQ をはじめとする複数のテストで手術群のほうが悪い結果となった．人工心肺中最低 Ht が full-scale IQ ともっとも強い相関を示し，Ht が高いほど IQ が高くなる傾向にあったと報告されている．

さらに最近，驚嘆すべきランダム化比較試験が行われた[12]．9 カ月未満の乳児で，出生時体重 2.3 kg 以上の先天性心疾患患者 147 名が対象となっている．人工心肺中最低 Ht を 21.1%±2.4% と低く抑えた群 74 名と 27.7%±3.2% と高くした群 73 名でのランダム化比較試験である．術後 1 歳時点での神経学的発達を調べたスコアは，Ht を低く抑えた群で有意に低く，健常人の 2 SD 以下の低いスコアの患者が明らかに多かったと報告されている．

また著者らは，チアノーゼ性心疾患の代表である Fallot 四徴症心内修復術症例で 7 歳未満の患者 236 名を対象とした後ろ向きコホート研究を実施した．人工心肺中最低 Hb は術後 30 日での死亡ならびに合併症発症に対する独立危険因子であり，人工心肺中最低 Hb 6 g/dl 以上 10 g/dl 以下の患者と比較すると，6 g/dl 未満の患者で 2.6 倍，10 g/dl を超える患者で 3.8 倍も危険であるとのデータを得ている[13]．

したがって，先天性心疾患患者において術中赤血球輸血のトリガー値をあまり低くしすぎると，合併症の増加ならびに精神発達という長期予後に影響を与える可能性がある．

3. 輸血の長期予後に与える影響

輸血が長期予後に与える影響についての報告もなされている．1施設における後ろ向き観察研究で，初回冠動脈バイパス術単独患者1,915名を対象として解析した結果，術中，術後に輸血を受けた患者は術後5年での死亡率が2倍高かったと報告されている[14]．現在，心臓血管外科手術の手技などが飛躍的に向上し，冠動脈バイパス術の術後1年生存率は90%を超える時代となっている[15]．今後，長期予後も見据えた輸血療法の適応を検討する必要があると思われる．

アルブミン製剤

改訂された『血液製剤の使用指針』では，「出血量が循環血液量の50%以上になり，人工膠質液や赤血球輸血を行っても血圧が維持しがたく，血清アルブミン値が3.0 g/dl未満の場合，等張アルブミン製剤の適応とされる」，また，「通常，心臓手術時の人工心肺の充填には，主として細胞外液補充液が使用される．なお，人工心肺実施中の血液希釈で起こった低アルブミン血症は，血清アルブミンの喪失によるものではなく一時的なものであり，利尿により術後数時間で回復するため，アルブミン製剤を投与して補正する必要はない．人工心肺を用いる場合で術前に血清アルブミン値が低い症例や体重10 kg未満の小児の場合は等張アルブミン製剤で人工心肺を充填することがある」と，アルブミン製剤の適応について記述されている．

1998年，Cochrane Injuries Group Albumin Reviewers[16]によって循環血液量低下，熱傷，低アルブミン血症についてすでに公表された30のランダム化試験についてのメタ解析がなされた．その結果，どの病態においてもアルブミンの投与により生存率を高めることはなく，むしろ全体ではアルブミン投与群で死亡率が高いと報告され，アルブミンの有効性，安全性に疑問を投げかけた衝撃的な報告がなされた．

一方，2001年にはさらにアルブミンの適応例（手術，腹水など）を増やし，55のランダム化試験をメタアナリシスで検討した結果ではCochrane Injuries Group Albumin Reviewersの結果と異なり，アルブミンが死亡率を増加させるという結果は認められないと報告された[17]．

人工心肺使用心臓手術における術後出血量についてヒドロキシエチルスターチ（HES）投与とアルブミン投与が与える影響について，16試験（n=653）を対象としたメタ解析によって比較検討した報告がある[18]．患者の術後出血量はHESを投与されるよりアルブミン投与で有意に少なかったことが示された．1,000 ml以上の術後24時間の出血は，アルブミン群で19%，HES群で33%に認められたと報告されている．

“Solucient Clinical Pathways Database”を利用して，冠動脈バイパス術を実施した患者19,578例の退院データを分析した報告がある[19]．このうち8,084例（41.3%）がアルブミン投与を受けていた．アルブミン投与を受けた患者の死亡率は2.47%であり，非蛋白コロイドを受けた患者の死亡率は3.03%であった（p=0.02）．多変量ロジスティック回帰分析によって死亡率のオッズはアルブミン群のほうが25%低いと報告された（オッズ比0.80，95%信頼区間0.67～0.96）．

低アルブミン血症が急性疾患の予後に関する独立した危険因子であるかどうかを検討した報告もある[20]．291,433例の患者（心臓手術，非心臓手術，腎障害などの急性重症患者）を対象に，予後予測因子として低アルブミン血症を評価している90のコホート研究のメタ解析が行われた．別に，535例を対象に低アルブミン血症の治療に関する9つの比較対象試験に対するメタ解析が行われた．低アルブミン血症は不良転帰に関する用量依存性の独立危険因子であり，アルブミンが1 g/dl低下するごとに，死亡率が137%，罹病率89%，ICU滞在期間28%，入院期間71%と有意なオッズ比の上昇が認められた．比較対象試験における用量依存性の分析から，アルブミン投与により血清アルブミンが3 g/dlを超えると合併症発生率が低下することが示唆された．

2004年には画期的なアルブミンに対するエビデンスが報告された．ICU管理患者を対象（心臓手術後，肝移植後，熱傷患者は除外）とした生食群とアルブミン使用群を比較する多施設共同二重盲験ランダム化比較試験が実施された[21]．6,997患者がエントリー（生食群3,500，アルブミン3,497）され，

28日死亡率，単臓器，多臓器不全の発生率，ICU滞在，病院滞在日数，人工呼吸を必要とした日数のいずれにも両群間で有意差はなかった．また患者を，外傷の有無，重症敗血症の有無，ARDSの有無に分けた解析においても，両群の死亡率には有意差が認められなかったと報告された．

しかしその後，The Sepsis Occurrence in Acutely ill Patients(SOAP)studyのサブ解析[22]として，3,147名を対象とした観察研究においてコックスの比例ハザードモデルを用いて解析した結果，アルブミンは30日生存率を有意に下げていた．さらに，propensity scoreに基づきマッチされた339ペアの解析でも，ICU(34.8% vs. 20.9%)ならびに院内死亡率(41.3% vs. 27.7%)はアルブミン使用患者でいずれも高かったとの報告がなされた．

アルブミンは有害であるとの1998年のCochrane Injuries Group Albumin Reviewersの衝撃的な報告がなされて以来，アルブミンの有効性についてさまざまな検討がなされているが，いまだ，アルブミンの有効性に関しては明確ではない．しかし，Cochrane Injuries Groupの報告ほどアルブミンは有害ではなく，症例によってはとくに心臓血管外科周術期ではアルブミン製剤の投与は患者予後を改善させる可能性が高いと現時点では考えられる．

新鮮凍結血漿(FFP)

改訂された『血液製剤の使用指針』では，FFPの適応は「PTおよび/またはAPTTが延長している場合〔①PTは(ⅰ)INR2.0以上，(ⅱ)30%以下，②APTTは(ⅰ)各医療機関における基準の上限の2倍以上，(ⅱ)25%以下とする〕」とされる．また，大量輸血時では「消費性凝固障害が併存しているかを検討し，凝固因子欠乏による出血傾向があると判断された場合に限り，新鮮凍結血漿の適応がある」と記述されている．

しかし，aPTT，PT(PT-INR)に関して，出血を予測できるcutoff値がどのレベルであるかについていまだ明確なエビデンスはない[23]．

FFPに関するランダム化比較試験を集めたシステマティックレビューの報告が最近なされている．肝疾患，心臓血管外科，ワーファリン治療，DIC，大量出血患者などを対象としたFFPの効果に対する57のランダム化比較試験について検討したものである[24]．人工心肺離脱後のFFPの投与に関する10試験を検討した結果では，FFP投与が術後出血ならびに輸血量を明らかに減じたという結果は得られなかったと報告されている．しかし，投与されたFFPは6〜15 ml/kgで予防的になされたものであり，登録された患者数はFFP群で12〜60と少ないため，この解析が意味をもつかどうか不明であるとされている．また，心臓手術周術期での凝固異常や出血を抑制するためのFFPの予防的投与に関するランダム化比較試験を集めたメタ解析[25]でも，上記と同様の結果が報告されている．

FFPには正常レベルの凝固因子しか含まれておらず(しかも抗凝固剤で少し希釈されている)，FFPを用いて凝固系を改善させるためにはかなりの量のFFPを輸血する必要がある．しかし，大量のFFPを輸血するとその循環動態に与える影響は無視できなくなる．したがって，心臓血管外科手術における大量出血，凝固異常の補正には，欧米で使用されているクリオプレチピテートがFFPより有効である可能性が高い．今後，研究デザインが困難ではあるものの，予防目的ではなく治療目的としたFFPやクリオプレチピテートの有効性に関する，よく組織された，十分なパワーをもったランダム化比較試験などによって，FFPの使用指針の妥当性が検討される必要がある．

濃厚血小板製剤

改訂された『血液製剤の使用指針』の人工心肺使用手術時の周術期管理では，「術中・術後を通して血小板数が3万/μl未満に低下している場合には，血小板輸血の適応である．ただし，人工心肺離脱後の硫酸プロタミン投与後に血算及び凝固能を適宜検査，判断しながら，必要に応じて5万/μl程度を目処に血小板輸血開始を考慮する．なお，複雑な心大血管手術で長時間(3時間以上)の人工心肺使用例，再手術などで広範な癒着剝離を要する例，及び慢性の腎臓や肝臓の疾患で出血傾向をみる例のなかには，人工心肺使用後に血小板減少あるいは機能異常によると考えられる止血困難な

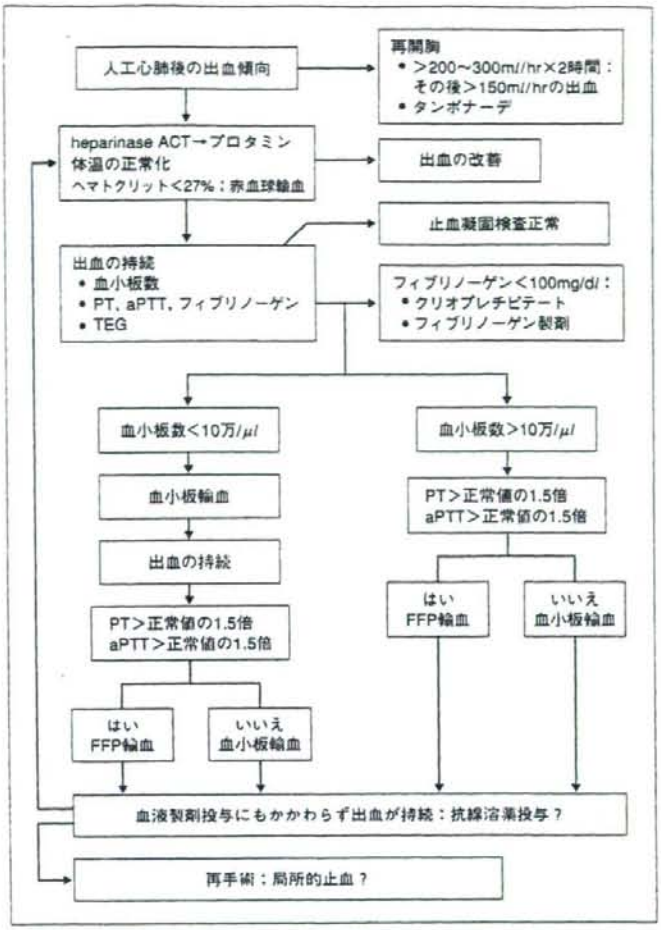

図 1　人工心肺離脱後に出血傾向を伴う患者における輸血アルゴリズム(文献[28]より一部改変)

人工心肺離脱後に出血傾向を伴う患者における濃厚赤血球，新鮮凍結血漿(FFP)，濃厚血小板，クリオプレチピテート投与による止血を行うための提案されている輸血アルゴリズムの 1 例．ACT：activated coagulation time，PT：prothrombin time，aPTT：activated partial thromboplastin time，TEG：thromboelastogram．

出血(oozing など)をみることがある．凝固因子の欠乏を伴わず，このような病態を呈する場合には，血小板数が 5 万/μl～10 万/μl になるように血小板輸血を行う」とされている．

血液疾患(造血器腫瘍治療)では血小板輸血のトリガー値に対するランダム化比較試験が積み重ねられ，エビデンスに基づいたガイドラインの策定が進んでいるが，心臓外科における血小板輸血のトリガー値に関する明確なエビデンスはいまだ存在しない．この標準化のために人工心肺離脱後に出血傾向を伴う患者において，検査所見に基づいた輸血アルゴリズムを用いて血小板輸血などの適応を決定する試みがなされ，経験的な方法に比べ輸血量や出血量の減少に対して効果があったとい

う報告がなされている[26,27]．

現在提唱されている人工心肺離脱後の出血傾向に対する輸血アルゴリズムの一例[28]を示す(図1)．今後このようなアルゴリズムの妥当性について，ランダム化比較試験などによって患者予後改善をエンドポイントとして検討され，その結果としてのエビデンスに基づいたガイドラインの作成が待ち望まれる．

おわりに

近年，上述したように心臓血管外科領域においても輸血トリガー値に関してのさまざまな臨床試験の報告がなされるようになった．今後は安全性が高い確率で担保できるようになった血液製剤をいかに効果的に使用するかが重要なテーマとなる．そのためには輸血トリガー値に関して，よく組織された，十分なパワーをもったランダム化比較試験などによる，患者予後改善をエンドポイントとしての検討がますます重要となる．

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