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

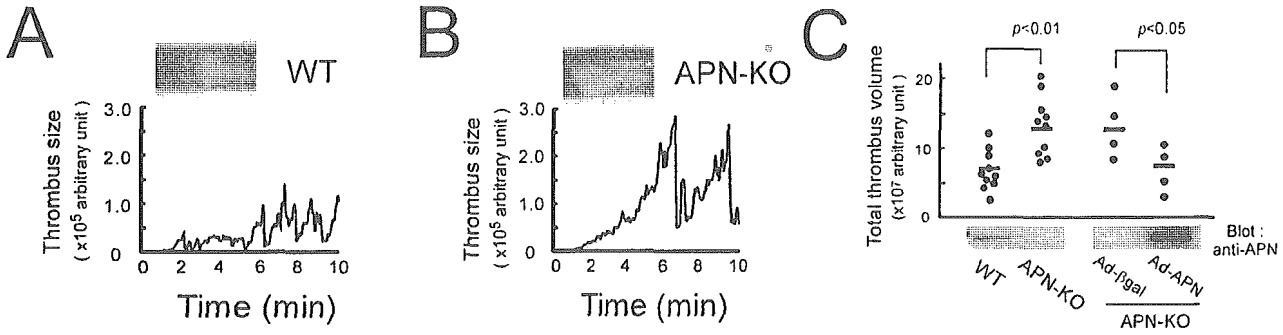

Figure 1. He-Ne laser–induced thrombus formation and adenovirus-mediated supplementation of adiponectin. Anesthetized mice were injected with Evans blue dye followed by irradiation with the He-Ne laser at the exposed left carotid artery. The representative time course of thrombus formation in (A) WT 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

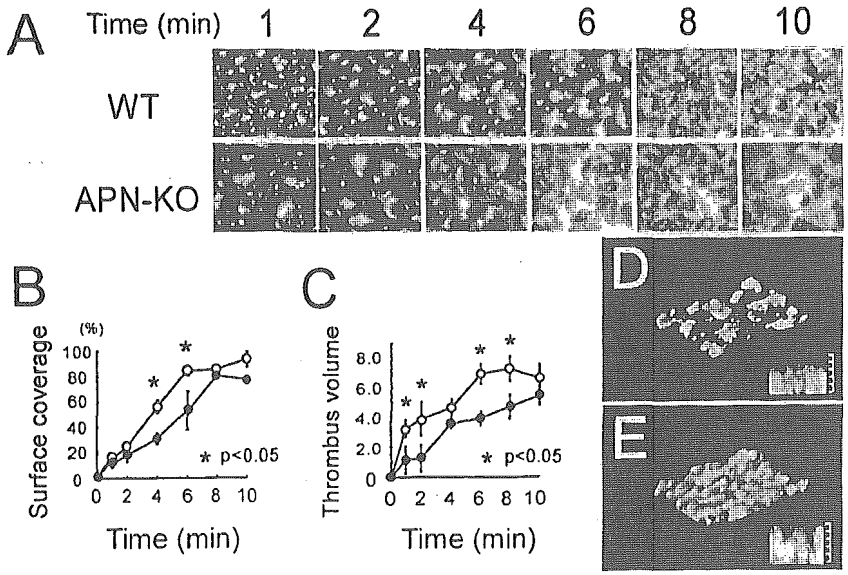

Figure 2. Thrombogenesis on a type I collagen-coated surface under flow conditions. (A) Mepacrine-labeled whole blood obtained from WT (top) or APN-KO mice (bottom) was perfused on a type I collagen-coated surface at a shear rate of 250 s^{-1}. (B) Platelet surface coverage (%) and (C) thrombus volume 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).

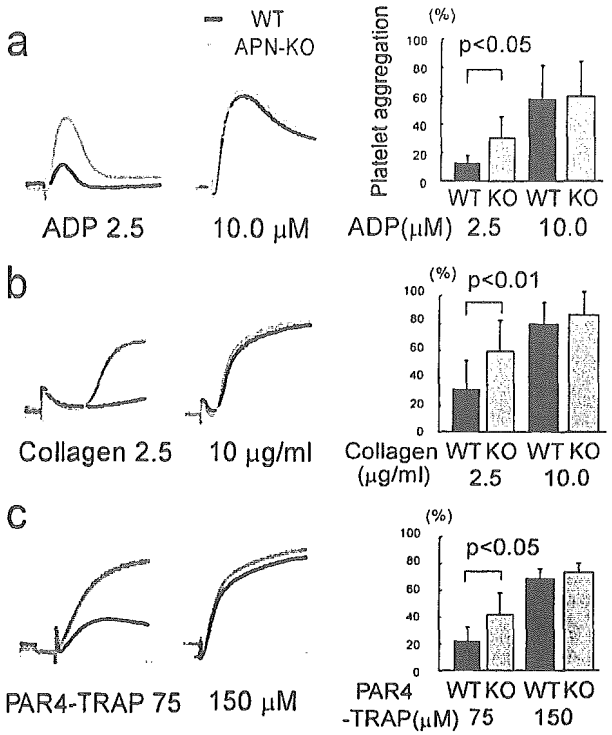

Figure 3. Enhanced platelet aggregation in APN-KO mice. Platelet aggregation in PRP obtained from WT or APN-KO mice. PRP ($300\times10^3/\mu L$) obtained from WT (black line) or APN-KO mice (gray line) was stimulated with ADP (a; n=4), collagen (b; n=4), or PAR4-TRAP (c; n=3). As compared with WT mice,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

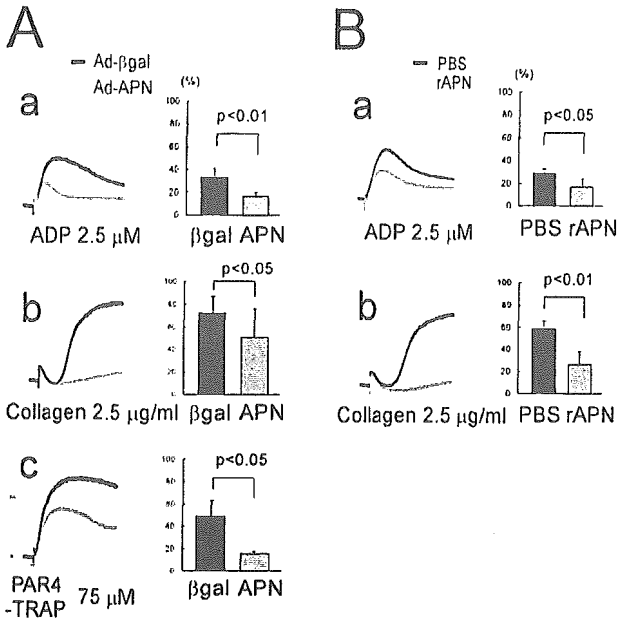

Figure 4. Effects of in vitro supplementation of adiponectin or recombinant adiponectin on the enhanced platelet aggregation in APN-KO mice. (A) One volume of PRP from APN-KO mice was mixed with ≈4 volumes of PPP from APN-KO mice injected with Ad-βgal (black line) or Ad-APN (gray line) to obtain a platelet 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).

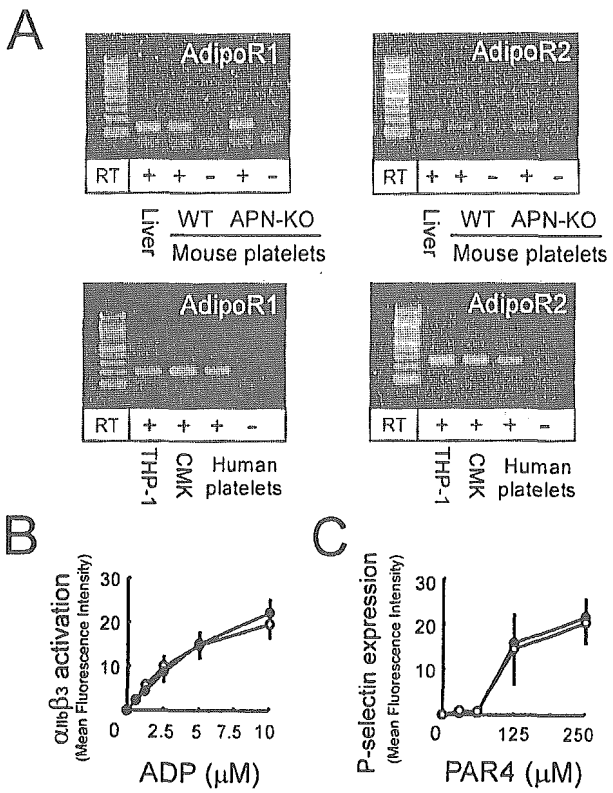

Figure 5. Expression of adiponectin receptors and effects of adiponectin deficiency on platelet function. (A, top) Expressions of transcripts for adiponectin receptors, AdipoR1 (133-bp fragments) and AdipoR2 (156-bp fragments), in platelets from WT or APN-KO mice were examined by RT-PCR. The liver was 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

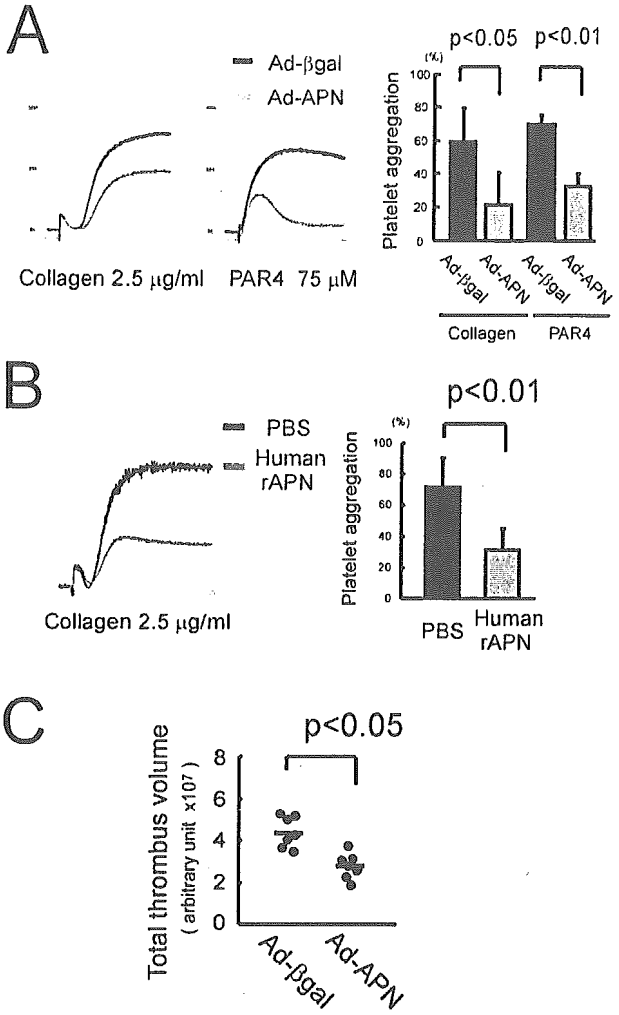

Figure 6. Overexpression of adiponectin additionally attenuates thrombus formation in WT mice. (A) Platelet aggregation in PRP obtained from WT mice injected with either Ad-βgal or Ad-APN. PRP ($300\times10^3/\mu L$) obtained from WT mice injected with either Ad-βgal (black line) or Ad-APN (gray line) 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.

Acknowledgments

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IV. 資料（Protocol）

平成17年度厚生労働科学研究
(循環器疾患等総合研究事業)

弓部大動脈全置換術における超低体温療法と中等度低体温療法の多施設共同前向き研究

JSTAR－Ⅰ

(Japanese Study of Total Arch Replacement)

Protocol

主任研究者: 国立循環器病センター 心臓血管外科医長　　荻野 均

Study Code:
Version: 1.801
Date: 2006/1/23

試験実施計画書の主な改訂記録

版番号	作成（改訂）年月日
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略号および用語の定義

AD	Adrenalin	アドレナリン
ADP	Adenosine diphosphate	アデノシンニリン酸
APTT	Activated partial thromboplastin time	活性化部分トロンボプラスチン時間
Asc.Ao	ascending aorta	上行大動脈
AT-III	Antithrombin III	抗トロンビンIII
AxA	Axillary artery	腋窩動脈
BCA	Brachiocephalic artery	腕頭動脈
BT	Bladder temperature	膀胱温
CI	Cardiac index	心係数
CK-MB	Creatinine Kinase - Myocardial Band	クレアチニンキナーゼ、心筋由来
CO	cardiac output	心拍出量
Cr	Creatinine	クレアチニン
CRC	Clinical Research Coordinator	臨床試験コーディネーター
CRF	Case Report Form	症例報告書
CT	Computed Tomography	コンピュータ断層撮影法
DOA	Dopamine	ドーパミン
DOB	Dobutamine	ドブタミン
ECG	Electrocardiogram	心電図
FA	Femoral artery	大腿動脈
FDP	Fibrin degradation product	フィブリン分解産物
FFP	Fresh frozen plasma	新鮮凍結血漿
FiO_2	Friction of inspired oxygen	吸入気酸素濃度
HD	Hemodialysis	血液透析
HEC	Hospital Ethical Committee	倫理委員会
IABP	Intraaortic balloon pumping	大動脈内バルーンパンピング
ICU	Intensive care unit	集中治療室
INR	International Normalized Ratio	国際標準比
IRB	Institutional Review Board	施設の臨床試験審査委員会
IVC	Inferior vena cava	下大静脈
LCCA	Left common carotid artery	左総頸動脈
LOS	Low cardiac output syndrome	低心拍出量状態
LSCA	Left subclavian artery	左鎖骨下動脈
MAP	Mannitol adenine phosphate	濃厚赤血球
MRI	Magnetic resonance imaging	核磁気共鳴画像
NAD	Noradrenalin	ノルアドレナリン
NPT	nasopharyngeal temperature	鼻咽頭温
PAI-1	Plasminogen activator	パイワン
PCWP	Pulmonary capillary wedge pressure	肺動脈楔入圧
PMI	Perioperative myocardial infarction	周術期心筋梗塞

PO2	Pressure of oxygen	酸素分圧
PT	Prothrombin time	プロトロンビン時間
RA	Right atrium	右房
RCP	Retrograde cerebral perfusion	逆行性脳灌流
RT	Rectal temperature	直腸温
SCP	Selective (antegrade) cerebral perfusion	選択的（順行性）脳灌流
SVC	Superior vena cava	上大静脈
TAT	Thrombin antithrombin	トロンビン・アンチトロンビン
TT	Tympanic temperature	鼓膜温

目次

1. 研究の目的

1. 1. 背景

近年、高齢化が進み、大動脈疾患に対する手術件数は冠動脈手術と共に増加の一途をたどっている。しかしながら、通常の開心術に比べ、高い手術侵襲度、手術の困難さ、患者の高齢化、多岐にわたる併存疾患、大量出血などの問題があり、その手術成績の向上は急務である。特に、弓部大動脈瘤に対する人工血管置換術(弓部全置換術)はその中心をなし、生命予後に止まらず、高次機能を含め脳保護法は未だに重要な課題である。 我が国では、脳保護法として、従来からの超低体温循環停止に加え、補助手段として選択的順行性脳灌流(SCP)と逆行性脳灌流(RCP)が開発され臨床応用されてきた。最近では、時間的制約の少ない点から、より生理的なSCPが定着し広く用いられている。この SCP を用いた場合、脳の灌流が維持されており、必ずしも超低体温を用いる必要がない。その観点から、最近になり生理的条件に近付けた 28℃中等度低体温下手術が試みられ、有効性が報告されつつある (**文献1－4**)。弓部全置換術においても、通常の開心術の進歩、発展と同様に、(超)低体温の弊害である体外循環時間の延長、臓器の温度較差、非生理的環境、それに基づく全身浮腫、肺障害、出血傾向などが回避でき、早期回復や出血が少ないなど「warm surgery」の利点が期待できうる。しかしながら、前述の報告では、比較対照群がなく単独での報告であり、従来からの超低体温下弓部全置換と比べどの程度の有用性、安全性があるかは明確にされていない(**文献1－4**)。最近の我々の学会発表(H Ogino、AHA in 2004、 K Minatoya、AATS in 2005)においても、以前の症例に用いた超低体温法を対照群とした比較検討に過ぎず、未だ厳密な検証に至っていない。したがって、中等度低体温下手術の有効性を明らかにするためには、ランダム化比較試験に基づく厳密な比較検討が必要とされる。

1. 2. 主要目的

最終目的は、28℃中等度低体温下弓部全置換術と 20℃超(深度)低体温下弓部全置換術の二群間でランダム化比較試験を行い、中等度低体温下弓部置換術の優位性を明らかにすることである。本研究では、まず、中等度低体温下弓部全置換術と超低体温下弓部全置換術における多施設共同前向き調査研究を行い、それぞれの弓部全置換術の特徴を明らかにすることを目的とする。将来的に、得られた中等度低体温下弓部全置換術の利点のいくつかを主要項目として設定し、より厳密に二群間でランダム化比較試験を行う予定である。

2. 対象患者

2．1． 選択基準

(1) 待機的胸骨正中切開下弓部全置換術患者

2．2． 除外基準

(1) 緊急患者(大動脈瘤破裂、急性大動脈解離)

(2) 再手術(再胸骨正中切開)患者

★ 術前ワーファリン：術前3日前までに中止し、ヘパリンに変更することが望ましい

★ 抗血小板薬：術前1週間前に服用中止、パナルジンは2週間前に中止することが望ましい

3．研究デザイン

3．1． 研究デザイン

多施設共同・前向き調査研究

比較群

28℃群： 膀胱温(BT)28℃下に SCP 灌流圧 ≧50 mmHg

20℃群： BT20℃下に SCP 灌流圧 30～50 mmHg

各施設の基準において、20℃群ないしは 28℃群を選択する。

登録期間は倫理委員会承認後から 2006 年11月までの約一年間とする。

症例の追跡期間は登録から術後 1 ヶ月もしくは退院時とする。

3．2． 症例数

登録期間に到達可能な症例数とする。

(参考) 参加施設の過去 1 年間の弓部全置換手術件数

国立循環器病センター	67 例(単独+待機 47)
東北大学	20 例(単独 17)
浜松医科大学	24 例(待機 17, 緊急7)

神戸大学	38 例
小倉記念病院	45 例(非解離 29, うち待機 26, 単独 16, 75 歳未満 11)

3. 3. 評価項目

1) 28℃中等度低体温下弓部全置換群と20℃超低体温下弓部全置換群における、術後 30 日以内死亡(手術死亡)、および脳・脊髄障害、心臓障害、肺障害、腎障害、出血、感染、などの合併症の発生割合

2)下記項目の評価

① 手術: 循環停止時間、心筋虚血時間、選択的脳灌流時間、体外循環時間、手術時間、麻酔時間

② 出血: 術中出血量、総輸血量(MAP、FFP)、総血小板輸血、24 時間ドレーン排液量

③ 循環: CO/CI、PCWP、帰室時 DOA/B、NAD、AD の投与量
術後 72 時間のカテコラミン総投与量
血清乳酸値

④ 呼吸: ICU 帰室時 PO_2/FiO_2 ratio、抜管時期

⑤ 脳: 覚醒時期、脳高次機能

⑥ 血液: 血小板、PT-INR、APTT、フィブリノーゲン、TAT、D-dimer、FDP、AT-III、プロテイン C、PAI-1

⑦ 回復: ICU 滞在日数、術後入院期間、入院治療費

3)合併症の定義:

1. 死亡(30 日以内)

2. 合併症の発生(72 時間以内)

(1)脳障害(**文献5**):

1)一時的脳障害(Temporary neurological dysfunction)
重度せん妄(severe delirium)、錯乱(confusion)、激昂(agitation)、記憶障害、などで退院までに軽快、改善するもの

2)永久的脳障害(Permanent neurological dysfunction)
CT、MRI 上明らかとなる脳梗塞を含め、退院まで持続する局所的ないしは広範囲脳障害

(2)脊髄障害:対麻痺、不全対麻痺、膀胱直腸障害

(3)心臓障害(**文献6**):1)ないしは2)

1)低心拍出量症候群(LOS): a)ないしはb)

a)大動脈バルーンパンピング(IABP)を必要とする循環不全

b)ドーパミン(DOA)/ドブタミン(DOB)以外に、6時間以上持続し 0.1γ/kg/min 以上のノルアドレナリン(NAD)ないしはアドレナリン(AD)を必要とする循環不全

2)周術期心筋梗塞(PMI): 以下の二項目以上を満たす場合

a) 心筋由来酵素上昇(CK-MB)≧30μg/L, トロポニン≧20 μg/L

b) 新たな壁異常

c) ECG 上、新しい Q 波の出現

(4)肺障害(**文献7**):1)から4)のいずれか

1)72 時間以上の人工呼吸

2)再挿管

3)気管切開

4)抗生剤を必要とする肺炎

(5)腎障害(**文献7**):1)から3)のいずれか

1)術前クレアチニン(Cr)≦1.0 mg/dl → 最高 Cr≧2.0 mg/dl

2)術前クレアチニン＞1.0 → 術前 Cr の 100%以上の上昇

3)血液透析(HD)の使用

(6)出血:1)から3)のいずれか

1)濃厚赤血球輸血＞20 単位

2)血小板輸血≧30 単位

3)出血再開胸

(7)感染(**文献7**):1)から3)のいずれか

1)胸骨骨髄炎、縦隔炎

2)人工血管感染

3)敗血症

3．4 研究の手順

3．4．1．インフォームドコンセントの手順

本研究は対象患者の自発的同意のもとに行う。対象患者には説明者(医師)が、多施設共同で超低体温下弓部全置換術と中等度低体温下弓部全置換術の前向き調査研究を行う意義、各々の長所、短所、各々の手術の利益、不利益を口頭および文章で説明を行い、同意文書による同意を得る。いずれの段階においても、同意を撤回拒否でき、拒否による不利益はないものとする。

3. 4. 2. 有害事象、合併症の報告の手順

担当医師は、有害事象および重篤な合併症が発生した時点で、中央事務局に連絡する。
合併症、有害事象が発生した場合には日常診療の場合と同様に最善の治療を実施する。

4. 麻酔

麻酔方法: プロポフォール(propofol)持続投与と中等量フェンタニール(fentanyl)20-50μg/kgによる全身麻酔

前投薬: 入室1時間前にジアゼパム(diazepam)5-10mg 内服

麻酔導入: 就眠薬 ミダゾラム(midazoram)0.5-1.5mg/kg, プロポフォール 1-1.5mg/kg, チオペンタール(thiopental)3-5mg/kg
麻薬 フェンタニール 3-10μg/kg
筋弛緩薬 ベクロニウム(vecuronium) 0.1-0.2mg/kg

麻酔維持: 原則としてプロポフォールの持続投与(4-8mg/kg/hr)で維持を行い、循環の安定に必要と判断される場合、吸入麻酔の使用も可とする。
適宜ベクロニウムとフェンタニールを追加投与しフェンタニールの総投与量は 20-50μg/kg 程度とする。

5. 手術方法

1) 胸骨正中切開
2) 体外循環(CPB):
送血:上行大動脈(asc.Ao)±腋窩動脈(AxA)±大腿動脈(FA)の組み合わせ
Asc.Ao
Asc.Ao＋AxA
Asc.Ao＋FA
Asc.Ao＋AxA＋FA
AxA＋FA
脱血:上・下大静脈(S・IVC)ないしは右房(RA)
3) 温度:
脳: 鼻咽頭温(NPT)ないしは鼓膜温(TT) 28℃/20℃
脊髄、腹部臓器: 膀胱温(BT)ないしは直腸温(RT) 28℃/20℃
深部温として、BT を基本とする。

NPT/TT と BT の解離が5℃以内のなるように全身冷却する。

4) 中心冷却: α-stat

5) SCP:

RAxA(10-16Fr) or BCA (14-18Fr)+LCCA(12-14Fr)+LSCA(12-14Fr)

6) SCP 灌流圧:左右浅側頭動脈圧、左右橈骨動脈圧ないしは SCP カニュラ先端圧

20℃ 30-50 mmHg → 10 ml/kg/min が目安

28℃ ≧50 mmHg → 15～25 ml/kg/min が目安

7) 最低ヘモグロビン:6.0g/dl 以上を維持(これ以下では輸血開始を前提とする)

8) 弓部分枝再建:ウーブン(woven)ないしはニッティッド(knitted)ダクロン(Dacron)4分枝人工血管を用いて個別再建

9) 吻合順: a)ないしはb)

a) 末梢側吻合(循環停止下)→ 左鎖骨下動脈(LSCA) → 中枢側吻合→左総頸動脈(LCCA)/腕頭動脈(BCA)

b) 末梢側吻合(循環停止下)→ 中枢側吻合 → LSCA/LCCA/BCA

10) 復温時期および速度: 規定しない

ただし、弓部分枝再建終了までは 32℃以下とする

6. 観察・検査項目

時期	術前	術中	術直後(ICU)	術後 3 週(退院直前)※	術後6カ月
同意	○				
患者背景	○				
手術情報		○			
高次機能	○			○	△
頭部 MRI	○			○	
心機能	○		○		
呼吸機能	○		○		
腎機能	○		○		
出血		○	○		
血液検査	○	○	○	○	
胸部 CT	○			○	
死亡・合併症	→	→	→	→	→

○: 必須、△: 必須ではないが、可能であれば調査する

※: 術後 3～5 週を許容する

1） 患者背景

2） 手術情報(麻酔記録ないしは体外循環記録)

④ 最低 NPT, 最低 BT, 最低末梢温(℃)

⑤ 脳灌流圧(SCP 圧)の推移: 浅側頭動脈圧、橈骨動脈ないしはカニュラ先端圧

⑥ SCP 灌流量の推移

⑦ 最低ヘモグロビン

⑧ 循環停止時間、心筋虚血時間、SCP 時間、体外循環時間、手術時間、麻酔時間

⑨ 術中出血量

⑩ 輸血量(MAP、FFP)、血小板輸血量

3） 術後情報(ICU)

① 帰室時体温: 血液温(Swan－Ganzカテーテル)、末梢温

② 帰室時心機能: 心拍出量(CO)/心係数(CI), 肺動脈楔入圧(PCWP)

③ 帰室時 DOA/B, NAD, AD の投与量

④ 術後 72 時間のカテコラミン総投与量

⑤ 帰室時呼吸機能: PO_2/FiO_2 ratio

⑥ 血清乳酸値最高値

⑦ 術後出血量:24 時間ドレーン排液量

⑧ 覚醒時期:離握手の可能な時期

⑨ 抜管時期

⑩ ICU 滞在日数

4） 脳機能 (術前:1月以内、術後:3～5週後)

① 術前後高次機能(試験時間:40 分)

(1) auditory verbal learning test	言語性記憶
(2) Trail-making A	選択的注意
(3) Trail-making B	選択的注意
(4) Grooved pegboard	巧緻運動
(5) Digit Span	注意
(6) ベントン視覚記銘力検査	視覚性記憶

② 術前後の頭部 MRI:T1 強調画像、T2 強調画像、Flair 画像の三画像、(可能なら T2*強調画像)

③ 術前頭部単純 CT: 脳梗塞発生時の対照として

5） 血液・凝固能(術前、術中、術翌朝)

① 血小板数

② PT-INR、APTT、フィブリノーゲン、TAT、D-dimer、FDP