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## Figure legends

### 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

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 (RBCs)、新鮮凍結血漿 (FFP)、濃厚血小板 (PLTs)、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 (文献28より一部改変の上引用)

## 人工弁・補助循環における 抗血小板療法

### はじめに

人工物が血液に触れる状況である人工弁装着術後や補助循環施行時には、血栓形成を引き起こす危険性が高い。また、生体における病的血栓症は、抗血栓性を低下させるアテローム硬化などの血管壁の変化、血管狭窄部位での異常な高ずり応力や血流のうっ滞などの血流の変化、血漿抗血栓性因子や血小板凝集阻害因子の低下、組織因子の発現が異常に亢進した血球の増加、血漿抗線溶因子の亢進や血液粘稠度の上昇などの血液成分の変化、などが引き金となり発生するため、人工弁装着術後や補助循環施行時にも配慮が必要である。

抗血栓療法として、抗血小板薬と抗凝固薬が用いられる。動脈内での血栓形成には、アスピリンなどの抗血小板薬が使用される。これは、動脈内においては、凝固因子が段階的に活性化されてフィブリン形成に至る凝固系よりも、豊富で速い血流に抗して血管壁の損傷・変性部位に粘着・凝集する性質をもつ血小板の関与が大きいためである。これに対して、肺血栓塞栓症に結びつく深部静脈血栓症や、心原性脳塞栓症を惹起する左房内血栓などは、発症リスクとして血流のうっ滞が特徴的である。このような血流うっ滞下で形成される血栓に対しては、抗凝固薬がより効率的に抑制するとされている。生体で形成される血栓の多くは混合血栓であるが、動脈血栓は血小板系が、静脈血栓は凝固系が中心的役割を演じているといえる。

人工弁装着術や補助循環施行患者においては、基礎疾患として血栓塞栓症をもつ場合が少なくない。さらに機械弁、グラフトなど、異物が挿入されるために抗血小板療法や抗凝固療法が必要となる場合が多い。しかし、人工心肺を用いた手術では、血小板機能低下、凝固障害、線溶系亢進をきたすため、術後早期は出血傾向に傾く。したがって、術後の抗凝固、抗血小板療法は、出血のリスクと血栓塞栓症のリスクとのバランスを考慮することが重要となる。

ここでは、人工弁・補助循環における抗血小板療法の現況について概説する。

## I. 人工弁装着術後における抗凝固・抗血小板療法

わが国において年間1万例以上の人工弁置換術が行われるようになり、その成績も安定してきている。人工弁術後にはワルファリンカリウム（ワルファリン）を用いた抗凝固療法が一般的<sup>1)</sup>で、用いる人工弁、手術後の時期および部位によって以下のコントロールが示されている<sup>2)</sup>。

1) 人工弁置換術後3カ月以内：PT-INR(prothrombin time-international normalized ratio：プロトロンビン時間の国際標準化比) 2.0～3.0

2) 人工弁置換術後3カ月以降

機械弁●大動脈弁置換術

危険因子なし 二葉弁, Medtronic Hall\*弁：PT-INR 2.0～2.5

他のディスク弁, Starr-Edward\*弁：PT-INR 2.0～3.0

危険因子あり：PT-INR 2.0～3.0

●僧帽弁置換術：PT-INR 2.0～3.0

生体弁+危険因子あり：PT-INR 2.0～3.0

(危険因子：心房細動, 血栓塞栓症の既往, 左心機能の低下, 凝固亢進状態)

また、上記の治療を行っているにもかかわらず血栓塞栓症を発症した患者に対しては、PT-INRを2.5～3.5にコントロールする、あるいは抗血小板薬としてアスピリンまたはジピリダモールを併用することを検討する<sup>3)</sup>。

日本人における人工弁に関するワルファリンコントロールについては、血栓や出血性のイベントの報告<sup>4)</sup>があり、今後さらに至適PT-INRについて検討が必要と考えられる。

また、脳梗塞発症予防に対しては抗血小板薬、特に低用量アスピリンの効果が期待されている<sup>5)</sup>。現在、虚血性脳血管障害のリスクをもつ機械弁置換術後の心房細動例に対する、ワルファリンによるPT-INR 2.0～3.0のコントロールと低用量アスピリンの併用効果について、無作為化比較対照試験が行われている。

## II. 補助循環患者における抗凝固・抗血小板療法

現在用いられる補助循環としては、IABP (intra-aortic balloon pumping：大動脈内バルーンポンピング), PCPS(ECMO)(percutaneous cardiopulmonary support：経皮的心肺補助法 [extracorporeal membrane oxygenator：膜型人工肺による機械的呼吸循環補助]), VAS (ventricular assist system：補助人工心臓)がある。このなかで、前2者は急性期において簡

便に用いられる循環補助手段であり、通常ヘパリンによる抗凝固療法が行われる。これに対し、VASは強力な長期にわたる循環補助が必要な場合に適応されており、安定状態で継続するため、ワルファリンや抗血小板薬による治療が必要となる。現在行われている治療を次に示す。

## 1. IABP

IABP挿入後、ヘパリン(未分画)(1万単位/日)を投与する。なお、活性化凝固時間(ACT: activated clotting time)を200秒前後に維持するのが望ましい。

## 2. PCPS (ECMO)

PCPS挿入時に全身ヘパリン化(100単位/kg)を行う。

駆動開始後、ヘパリンコーティング回路を用いる場合には、ACTを180～200秒前後に維持するようヘパリンを投与する。通常回路を用いる場合には、ACTを250～300秒に維持する。

なお、離脱を図るために流量を2L/min以下にする場合には、目標ACTを増加する。

## 3. VAS (補助人工心臓)

現在わが国で用いられる主なVASとしては、体外設置方式の東洋紡型と、体内植込み型のNovacor<sup>®</sup>およびHeartMate-VE<sup>®</sup>がある。VASにおける血栓形成に関与する主な因子として、血液接触面の性状と用いられる人工弁がある。血液接触面に関しては、通常smooth surface(平滑面)が用いられ、HeartMate-VE<sup>®</sup>ではrough surface(粗面)が用いられている。また、最近ではsmooth surfaceにおいても各種のコーティング(ヘパリンなど)を施し、抗血栓性の向上が図られている。また、人工弁に関しては、体外設置型では機械弁が用いられ、体内植込み型では生体弁が用いられている。

抗血栓療法として、血液接触面をsmooth surfaceにしているものでは、従来ワルファリンおよびヘパリンによる抗凝固療法が主として行われてきた。筆者らも、東洋紡型VASにおいて、当初ワルファリン(PT-INRの目標値: 2～3)あるいはヘパリンによる抗凝固療法による管理を行った。しかし、早期に血液ポンプ内に血栓形成を認めることが多く、頻回の血液ポンプ交換が必要であった。装着術後早期の血栓を検討すると、白色血栓が多く、白血球数 $1\text{万}/\text{mm}^3$ 以上および血小板数 $10\text{万}/\text{mm}^3$ 以上になった場合に発生しやすかった。そこで、PT-INRの目標値を3～4とするとともに抗血小板療法を併用するようにしたところ、血栓形成の危険性が減少した<sup>1)</sup>。これに対し、rough surfaceを持つHeartMate-VE<sup>®</sup>では血栓形成の危険性が少ないとされ、通常抗血小板療法のみが行われる。

VAS症例の増加に伴い、安定した抗血栓療法がより望まれるようになってきている。ワルファリンに関しては、PT-INRによるコントロールが可能であるが、抗血小板薬のコントロールが今

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後の課題であり、各種血小板機能検査によるコントロールが検討されている<sup>9)</sup>。筆者らは、ずり応力下血小板血栓形成能(後述)を用いている。

以下に筆者らの抗凝固および抗血小板療法について述べる。

##### (1) 東洋紡型 LVAS (left ventricular assist system : 左心補助人工心臓) (機械弁, 血液接触面 : smooth surface)

術中はヘパリンを用い、その後硫酸プロタミン(プロタミン)で中和を行う。再手術時などではアプロチンを併用する。装着早期には、外科的出血がコントロールされるまで原則的に抗凝固療法を行わない。早期抜管が可能な場合には、初期からワルファリンによる抗凝固療法と抗血小板薬による抗血小板療法を行う。

経口が開始された段階でワルファリンを開始、当初の目標 PT-INR は 2 とし、その後 3~4 を目標値とする。経口摂取ができない場合には、経鼻胃管からの投与を試みる。早期に経口や経鼻胃管から投与できない場合には、外科的出血が落ち着いた段階で低分子ヘパリンを投与する。なお、PT-INR が目標値以下の場合、表 1 に示すようにワルファリン増量による調整と、低分子ヘパリン(フラグミン<sup>®</sup>)を併用する。また、PT-INR が目標値以上の場合、ワルファリンの減量/休薬と凍結血漿の投与を表 1 のプロトコールに従って行う。

表 1 LVAS 装着患者の抗凝固療法

PT-INR	ワルファリン	フラグミン <sup>®</sup> (単位/kg/時間)	凍結血漿
< 2	増量	10	(-)
2~2.5	増量	7.5	(-)
2.5~3	増量	5	(-)
3~4	【目標域】	(-)	(-)
4~5	減量/休薬	(-)	(-)
5~5.5	休薬	(-)	(考慮)
5.5<	休薬	(-)	投与

また、経口摂取不良となった場合、発熱、疼痛などにより鎮痛解熱薬を投与した場合、および感染症を伴った場合には、PT-INR の上昇に注意する必要がある。PT-INR を補正する場合、通常ビタミン K 投与は行わない。これは、ビタミン K でリバースした場合、リバウンドがあり、さらに再度の PT-INR コントロールに難渋するためである。出血を伴った場合には、ワルファリン投与を中止し凍結血漿を投与することに加え、乾燥ヒト血液凝固第Ⅴ因子複合体製剤の使用を考慮する。

併用する抗血小板療法としては、経口摂取が開始された段階で、外科的出血を考慮した上、

アスピリン 81 mg/日あるいはバイアスピリン<sup>®</sup> 100 mg/日で開始する。また、血小板数が 10 万/mm<sup>3</sup> を越えた場合には可及的早期に投与を開始する。投与開始 4 日から 1 週後に血小板機能検査（ずり応力下血小板血栓形成能）を行い、必要に応じ投与量の追加あるいは減量を行う。その後適宜、血小板機能検査を行い、アスピリンの投与量を調整する。

### (2) Novacor<sup>®</sup> LVAS (生体弁, 血液接触面: smooth surface)

術中はヘパリンを用い、体外循環離脱後プロタミンで中和を行う。術後早期は、外科的出血がコントロールされてから抗凝固療法を開始する。経口摂取開始後は、ワルファリン:PT-INR を 2.5 ~ 3.5 に維持する。抗血小板薬として欧米では、アスピリンを 100 ~ 200 mg (状況により ~ 600 mg) / 日投与し、必要に応じて、ジピリダモール (150 mg × 2 ~ 4 回 / 日) や塩酸チクロピジン (チクロピジン) (200 ~ 400 mg/日) を併用することが勧められている。筆者らは、前項の東洋紡 VAS と同様の対応を行っている。

### (3) HeartMate-VE<sup>®</sup> (生体弁, 血液接触面: rough surface)

術中はヘパリンを用い、体外循環離脱後プロタミンで中和を行う。術後外科的出血がコントロール(ドレーンからの出血量 < 60 mL/h)されてから抗血小板療法を開始する。通常アスピリン (81 ~ 100 mg/日) で開始し、血小板機能測定の結果を参考に調整する。また、必要に応じてジピリダモール (75mg × 3/日) を併用する。

流量低下や経口摂取不良時など臨床所見に応じて、ヘパリンあるいはワルファリンによる抗凝固療法を併用する。

## Ⅲ. ずり応力下血小板血栓形成能

血小板機能の検討において、測定環境の重要性が指摘されている。すなわち、血小板は生体内では血管内皮近傍を流れ、血流の特性による速度勾配によって生じるずり応力に絶えずさらされながら循環している。血管内皮細胞は nitric oxide (一酸化窒素), prostaglandin (プロスタグランジン) などを産生し、血小板粘着が起らないように血管のダイナミクスを支えている。しかし、いったん血管内皮に外傷などで損傷が起ると、血管内皮下組織 (主にコラーゲンからなる) と反応する形で血小板粘着が生じ、それに引き続いて血小板凝集、血栓形成が引き起こされる。この際、ずり応力依存性に血小板粘着、凝集が引き起こされることが明らかとなっている<sup>10)</sup>。

したがって、生体内での血小板機能の評価においては、血流存在下 (ずり応力下) における血小板の機能を常に考慮する必要がある<sup>11)</sup>。これらの点を踏まえ、筆者らは、抗血小板薬の効果



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を、できる限り生理的状況下に近い形で検討するために、全血を用い流動状況下で検討する方法をとっている。すなわち、平行板型フローチャンバーを用い、患者全血を対象として、コラーゲン固相表面上でのずり応力下血小板血栓形成能を測定することで、血小板機能を評価している。

現在、この新しい評価系の有効性を確認するための臨床試験を実施している<sup>12)</sup>。

## おわりに

人工物を血液に接触させる人工弁装着あるいは補助循環においては、血栓形成のコントロールが重要であり、人工弁や人工物の血液接触面の生体適合性の向上を目指して、材料や血液接触面の性状(rough surface, ヘパリンコーティングなど)の検討が行われているが、血液の血栓形成能の制御も必要である。従来ワルファリンが中心であったが、血栓形成における血小板の役割の重要性が認識されるにつれ、抗血小板薬の併用が検討されている。人工心臓においては、抗血小板薬の併用は必須となっているが、人工弁においては今後の検討が待たれる状況である。

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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:  $13.36 \pm 4.25 \times 10^7$  arbitrary units for APN-KO and  $6.74 \pm 2.87 \times 10^7$  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  $250 \text{ s}^{-1}$ , 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.<sup>1,2</sup> 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)  $\alpha$ , leptin, resistin, and plasminogen activator inhibitor type-1.<sup>2-4</sup> Dysregulated production of adipocytokines participates in the development of obesity-related metabolic and vascular diseases.<sup>2-4</sup>

Adiponectin is an adipocytokine identified in the human adipose tissue cDNA library, and Acrp30/AdipoQ is the mouse counterpart of adiponectin (reviewed in reference<sup>5</sup>). 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  $\mu\text{g/mL}$ ), and its plasma concentration is inversely related to the body mass index.<sup>5</sup> Plasma adiponectin levels decrease in

obesity, type 2 diabetes, and patients with coronary artery disease (CAD).<sup>5-9</sup> Indeed, adiponectin (APN) knockout (KO) mice showed severe diet-induced insulin resistance.<sup>10</sup> 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- $\alpha$  production from macrophages.<sup>5,11</sup> Furthermore, APN-KO mice showed severe neointimal thickening in mechanically injured arteries.<sup>12</sup> 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.<sup>12,13</sup> 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, hypo-adiponectinemia 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.<sup>10,12</sup> We analyzed mice backcrossed to C57BL/6 for 5 generations.<sup>10,12</sup>

### Preparation of Mouse Platelets and Measurement of Coagulation Parameters

Mouse platelet-rich plasma (PRP) was obtained as described previously.<sup>14</sup> 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.<sup>14</sup> Integrin  $\alpha_{IIb}\beta_3$  activation and  $\alpha$ -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.<sup>14</sup>

### Assessment of Atherosclerosis and Bleeding Time Measurement

Assessment of atherosclerosis was performed as described previously.<sup>15</sup> 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.<sup>15</sup> Anesthetized mice (nembutal 65 mg/kg) were placed onto a microscope stage, and the left carotid artery (450 to 500  $\mu$ m in diameter) was gently exposed. Evans blue dye (20 mg/kg) was injected into the left femoral artery via an indwelled tube, and then the center of the exposed carotid artery was irradiated with a laser beam (200  $\mu$ m 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.<sup>16</sup> 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 Ltd). 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.<sup>10</sup> Plaque-forming units ( $1 \times 10^6$ ) of adenovirus-adiponectin (Ad-APN) or adenovirus- $\beta$ -galactosidase (Ad- $\beta$ gal) 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.<sup>11,17</sup>

### 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.<sup>18</sup> For the amplification of transcripts of mouse adiponectin receptors AdipoR1 and AdipoR2, the following primers were used: mouse AdipoR1 5'-ACGTTGGAGAGTCATCCCCTAT-3' (sense) and 5'-CTCTGTGTGGATGCGGAAGAT-3' (antisense) and mouse AdipoR2 5'-TGCGCACACATTCAGTCTCCT-3' (sense) and 5'-TTCTATGATCCCCAAAAGTGTGC-3' (antisense).<sup>19,20</sup> For human platelet isolation, PRP obtained from 50 mL of whole blood was passed through a leukocyte removal filter as described previously.<sup>21</sup> This procedure removed >99.9% of the contaminated leukocytes.<sup>21</sup> 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)<sup>22</sup> was examined in parallel. RT-PCR samples omitting reverse transcriptase were used as negative controls.

### Statistical Analysis

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

## Results

### Characteristics of Adiponectin-Deficient Mice and Assessment of Atherosclerotic Lesions

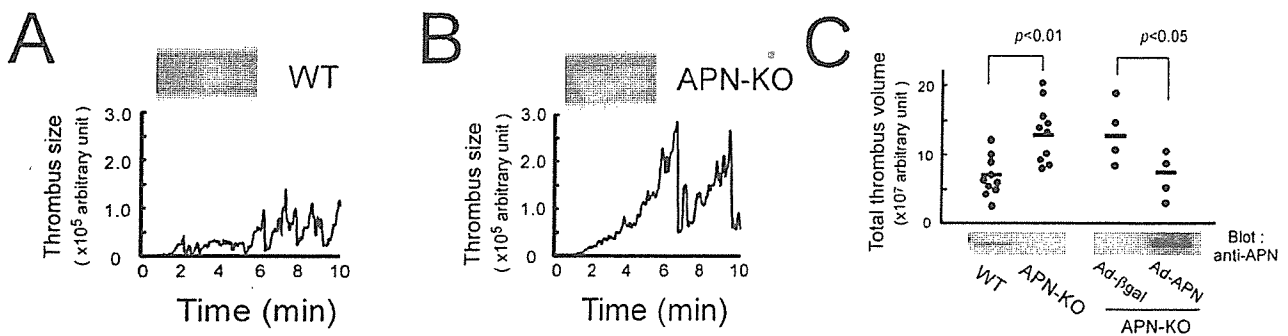
The basal profiles of APN-KO male mice have been previously described.<sup>10,12</sup> To exclude the effects of diet on APN-KO mice, we used APN-KO male mice (8 to 12 weeks old) fed on a normal diet in this study. There were no differences in platelet counts, PT, APTT, and plasma fibrinogen concentrations (Table 1, 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 ( $96.9 \pm 34.9$  s;  $n=30$ ;  $P<0.05$ ) than that in WT mice ( $130.9 \pm 52.1$  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



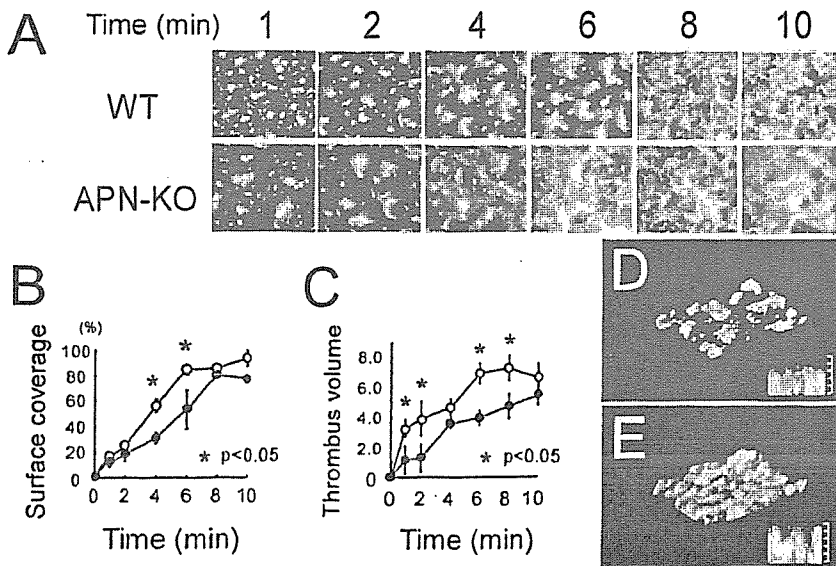
**Figure 1.** He-Ne laser-induced thrombus formation and adenovirus-mediated supplementation of adiponectin. Anesthetized mice were injected with Evans blue dye followed by irradiation with the He-Ne laser at the exposed left carotid artery. The representative time course of thrombus formation in (A) 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- $\beta$ gal)-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  $61.0 \pm 25.0$  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  $8.5 \pm 2.3$  in WT mice. In APN-KO mice, there was no significant difference in the initiation time for thrombus formation ( $54.8 \pm 8.9$  s;  $n=10$ ;  $P=0.46$ ). However, the cycles of thrombus formation during the 10-minute observation period were significantly fewer ( $5.4 \pm 2.0$ ;  $n=10$ ;  $P<0.01$ ) 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 ( $6.74 \pm 2.87 \times 10^7$  arbitrary units in WT mice and  $13.36 \pm 4.25 \times 10^7$  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- $\beta$ gal 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 ( $48.7 \pm 6.8$   $\mu$ g/mL;  $n=4$ ), as well as in an immunoblot assay. In the carotid artery thrombus model, the total thrombus volume in Ad- $\beta$ gal-infected APN-KO was  $12.94 \pm 4.67 \times 10^7$  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 ( $6.23 \pm 3.09 \times 10^7$  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. ( $\bullet$ , WT;  $\circ$ , 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 ( $250 \text{ s}^{-1}$ ). 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.<sup>23</sup> However, mouse recombinant adiponectin ( $40 \mu\text{g/mL}$ ) 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 ( $1000 \text{ s}^{-1}$ ), 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.

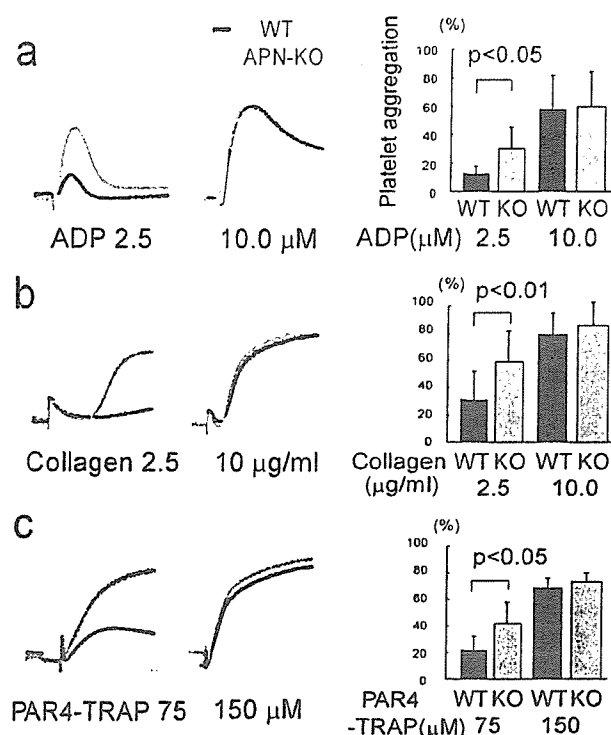
#### Adiponectin Inhibits the Enhanced Platelet Aggregation in APN-KO Mice

In platelet aggregation studies, PRP obtained from APN-KO mice showed significantly enhanced platelet aggregation in response to low doses of agonists (ADP  $2.5 \mu\text{mol/L}$ , collagen  $2.5 \mu\text{g/mL}$ , and protease-activated receptor 4-activating peptide [PAR4-TRAP]  $75 \mu\text{mol/L}$ ), 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- $\beta\text{gal}$  or Ad-APN to adjust platelet counts to  $300 \times 10^3/\mu\text{L}$ . 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 ( $40 \mu\text{g/mL}$ ) 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 $\alpha_{\text{IIb}}\beta_3$ 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  $\alpha_{\text{IIb}}\beta_3$  activation and  $\alpha$ -granule secretion at various concentrations of agonists by flow

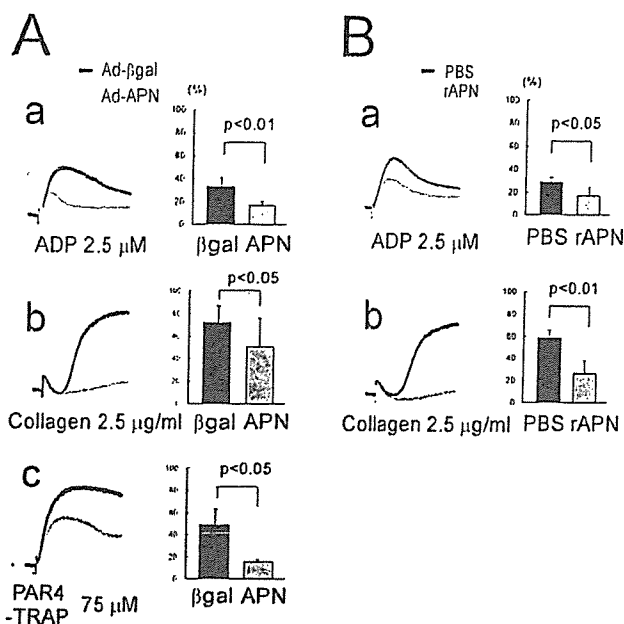


**Figure 3.** Enhanced platelet aggregation in APN-KO mice. Platelet aggregation in PRP obtained from WT or APN-KO mice. PRP ( $300 \times 10^3/\mu\text{L}$ ) obtained from WT (black line) or APN-KO mice (gray line) was stimulated with ADP (a;  $n=4$ ), collagen (b;  $n=4$ ), or PAR4-TRAP (c;  $n=3$ ). As compared with WT mice, platelet aggregation was enhanced in APN-KO mice at low concentrations of agonists.

cytometry. However, neither the platelet  $\alpha_{\text{IIb}}\beta_3$  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).

#### 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- $\beta\text{gal}$  into WT mice, the plasma adiponectin levels in Ad-APN-infected mice reached  $\approx 4$  times higher than those in Ad- $\beta\text{gal}$ -infected WT mice ( $8.5 \pm 0.6 \mu\text{g/mL}$  for Ad- $\beta\text{gal}$  and  $37.0 \pm 14.8 \mu\text{g/mL}$  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 ( $40 \mu\text{g/mL}$ ) to human PRP attenuated the platelet aggregation response to  $2.5 \mu\text{g/mL}$  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 ( $4.38 \pm 0.75 \times 10^7$  arbitrary units for Ad- $\beta\text{gal}$  and  $2.75 \pm 0.61 \times 10^7$  arbitrary units for Ad-APN;  $n=7$ ;  $P < 0.05$ ; Figure 6C).

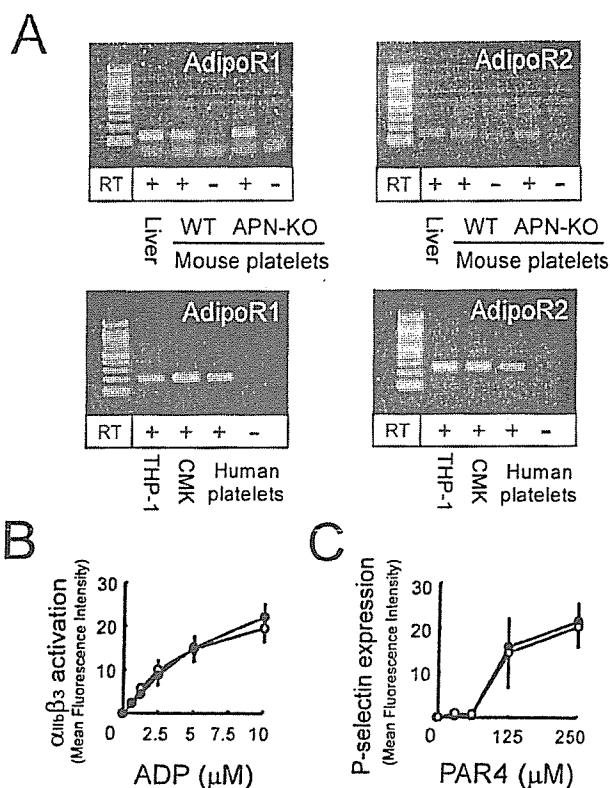


**Figure 4.** Effects of in vitro supplementation of adiponectin or recombinant adiponectin on the enhanced platelet aggregation in APN-KO mice. (A) One volume of PRP from APN-KO mice was mixed with  $\approx 4$  volumes of PPP from APN-KO mice injected with Ad- $\beta$ gal (black line) or Ad-APN (gray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

### 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.

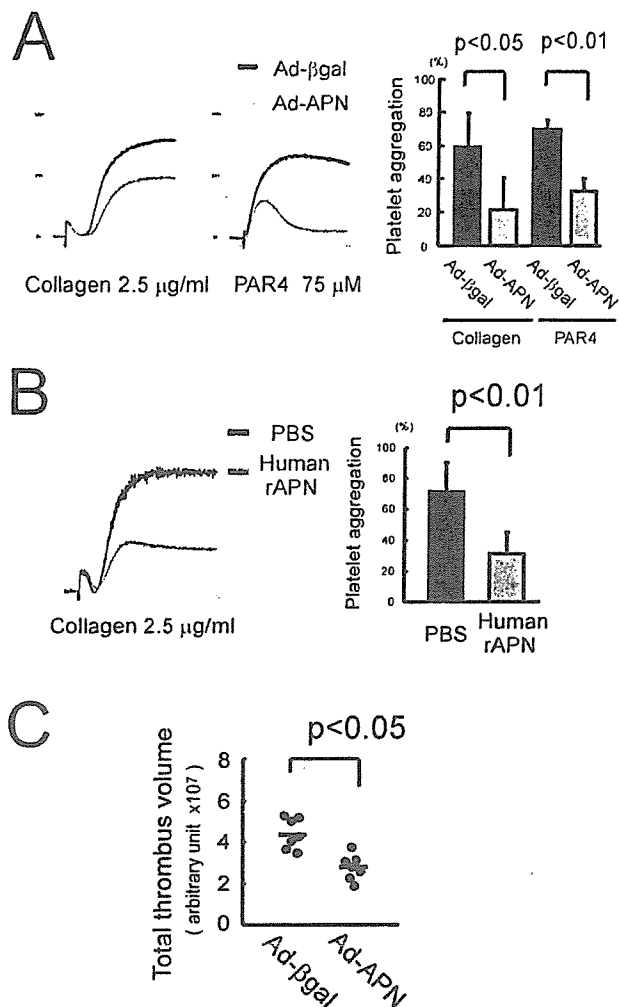
We have demonstrated that low concentrations of adiponectin are associated with the prevalence of CAD in men, which is independent of well-known CAD risk factors.<sup>6</sup> Pischon et al<sup>9</sup> have recently shown that high concentrations of adiponectin are associated with a lower risk of myocardial 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



**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)  $\alpha$ -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 ( $n=4$ ).

show any abnormalities in plasma glucose, insulin, or lipid profiles.<sup>10,12</sup> 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).<sup>24</sup> 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- $\alpha$  concentrations ( $\approx 40 \text{ pg}/\text{mL}$  in APN-KO;  $\approx 20 \text{ pg}/\text{mL}$  in WT), and elevated CRP mRNA levels in white adipose tissue.<sup>12,25</sup> In addition, recombinant adiponectin increased NO production in vascular endothelial cells.<sup>26</sup> 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 ( $300 \times 10^3/\mu\text{L}$ ) 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 ( $40 \mu\text{g}/\text{mL}$ , gray line) or PBS (black line) was added to PRP ( $300 \times 10^3/\mu\text{L}$ ) 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.<sup>16</sup> 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.<sup>27</sup> 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  $\alpha_2$ -macroglobulin, thrombospondin-1 (TSP-1), and several growth factors.<sup>5,23,28</sup> Interestingly, TSP-1, after secretion from platelet  $\alpha$  granules, may participate in platelet aggregation by reinforcing interplatelet interactions through direct fibrinogen-TSP-fibrinogen and TSP-TSP crossbridges.<sup>29,30</sup> 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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## 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

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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 <sub>2</sub>	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