factor VII, activated prothrombin complex concentrates) for high inhibitor levels (>5 Besthesda U). On the other hand, immunosuppressive agents (eg, corticosteroid, cyclophosphamide, azathioprine, rituximab) or IVIG has been used to suppress the generation of coagulation inhibitors. Other approaches are plasmapheresis and immunoadsorption using a protein A–Sepharose column to remove coagulation inhibitors, but the indications for these therapies are limited.

Evaluation of the response to one therapeutic modality in the management of coagulation inhibitors is not always easy, for a number of reasons. First, there are only a few inhibitor patients, and thus it is almost impossible to conduct a randomized clinical trial. There have been only a few such trials on acquired coagulation inhibitors [25,45]. This situation influences the evaluation of efficacy because cases of unsuccessful treatment with IVIG may not have been reported, with only successful cases having been evaluated. Second, most patients present with life-threatening bleeding and are treated with several different therapies simultaneously or sequentially. It is difficult, therefore, to assess the outcome of any single modality. Third, it is known that spontaneous fluctuation or disappearance of the inhibitor may occur [72].

As is shown in Table 1, the efficacy of IVIG therapy alone is not very high (ie, 30%). Moreover, the CR rates for combination therapy with IVIG plus glucocorticoid and/or cyclophosphamide (IVIG plus prednisolone/dexamethasone, 73%; IVIG plus prednisolone/dexamethasone and cyclophosphamide, 74%) did not differ from those of immunosuppressive agents without IVIG (prednisolone/dexamethasone plus cyclophosphamide, 75%) (Table 2). However, the clinical benefits of IVIG include a rapid response and fewer adverse effects, which are frequently observed with the chronic administration of glucocorticoid or other immunosuppressive agents. Regarding the use of cyclophosphamide in particular, it is possible for cytotoxicity to induce myelosuppression and secondary malignancy. Thus, IVIG therapy should be considered for acute massive bleeding in patients with acquired coagulation inhibitors because of its faster action. On the other hand, IVIG therapy costs approximately US $10,000 for a 5-day infusion, which is much more costly than other treatments except rituximab. These considerations taken together suggest that the use of IVIG for the management of acquired coagulation inhibitors might be limited, because whether a given treatment is used depends on the balance between cost and benefit.

7. Conclusion

For patients with acquired coagulation inhibitors against factor VIII, the efficacy of IVIG therapy alone was estimated to be 30% in 35 cases. On the other hand, the response to combination therapy with IVIG plus immunosuppressive agents (eg, corticosteroid, cyclophosphamide) seems to be better (ie, 70% in 45 cases) than IVIG as single-agent therapy. IVIG may be considered as one choice of treatment for acquired coagulation inhibitors, especially when a rapid response is required without myelosuppression, but its use alone would be limited because of its lower efficacy and high cost.

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Unblinded Pilot Study of Autologous Transplantation of Bone Marrow Mononuclear Cells in Patients With Thromboangiitis Obliterans

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Background—The short-term clinical benefits of bone marrow mononuclear cell transplantation have been shown in patients with critical limb ischemia. The purpose of this study was to assess the long-term safety and efficacy of bone marrow mononuclear cell transplantation in patients with thromboangiitis obliterans.

Methods and Results—Eleven limbs (3 with rest pain and 8 with an ischemic ulcer) of 8 patients were treated by bone marrow mononuclear cell transplantation. The patients were followed up for clinical events for a mean of 684±549 days (range 103 to 1466 days). At 4 weeks, improvement in pain was observed in all 11 limbs, with complete relief in 4 (36%). Pain scale (visual analog scale) score decreased from 5.1±0.7 to 1.5±1.3. An improvement in skin ulcers was observed in all 8 limbs with an ischemic ulcer, with complete healing in 7 (88%). During the follow-up, however, clinical events occurred in 4 of the 8 patients. The first patient suffered sudden death at 20 months after transplantation at 30 years of age. The second patient with an incomplete healing of a skin ulcer showed worsening of the lesion at 4 months. The third patient showed worsening of rest pain at 8 months. The last patient developed an arteriovenous shunt in the foot at 7 months, which spontaneously regressed by 1 year.

Conclusions—In the present unblinded and uncontrolled pilot study, long-term adverse events, including death and unfavorable angiogenesis, were observed in half of the patients receiving bone marrow mononuclear cell transplantation. Given the current incomplete knowledge of the safety and efficacy of this strategy, careful long-term monitoring is required for future patients receiving this treatment. (***Circulation.*** **2006;114:2679-2684.)**

Key Words: angiogenesis ■ collateral circulation ■ endothelium ■ peripheral vascular diseases

The clinical consequences of severe peripheral arterial disease or critical limb ischemia include rest pain and the loss of tissue integrity in the distal limb.[1–3] Therapeutic options for such patients are limited. These conditions are often refractory to conservative measures and are typically unresponsive to drug therapy. When vascular obstruction involves a long segment or is widespread, percutaneous revascularization may not be feasible. Surgical therapy, consisting of arterial bypass or amputation, is complicated by variable morbidity and mortality, and its effectiveness depends on the short- and long-term patencies of the conduit employed. Therapeutic angiogenesis thus constitutes a potential alternative treatment strategy for such patients.[4,5]

Previous investigators have suggested that endothelial progenitor cells, originating from bone marrow, circulate in adult peripheral blood and participate in postnatal neovascularization.[6–8] Subsequent experiments have shown that bone marrow or bone marrow–derived cells have the potential to stimulate angiogenesis and thereby modulate the hemodynamic deficit in ischemic limbs in vivo.[9,10] The Therapeutic Angiogenesis by Cell Transplantation (TACT) study first demonstrated that the magnitude of angiogenesis stimulated by these cells is sufficient to constitute a therapeutic benefit in patients with critical limb ischemia.[11] In that study, the investigators injected bone marrow mononuclear cells (BM-MNCs) into the ischemic limb of patients and documented a significant improvement in the hemodynamic deficit as well as the relief of ischemic symptoms. Although the TACT

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study established the concept of using BM-MNCs for therapeutic angiogenesis, limited information is available about the long-term safety and efficacy of this strategy.

The purpose of the present study was to determine the long-term safety and clinical impact of BM-MNC transplantation for "no-option" patients with thromboangiitis obliterans.

Methods

Patients

Eight patients with thromboangiitis obliterans were treated with an autologous transplantation of BM-MNCs between March 2002 and September 2004. The diagnosis of thromboangiitis obliterans was based on the criteria proposed by Olin[12]: (1) onset before age 45; (2) current (recent) history of tobacco use; (3) the presence of distal-extremity ischemia (infrapopliteal or infrabrachial) indicated by claudication, rest pain, ischemic ulcers, or gangrene; (4) exclusion of autoimmune or connective tissue diseases, hypercoagulable states, and diabetes mellitus; (5) exclusion of a proximal source of emboli by echocardiography and arteriography; and (6) consistent arteriographic findings in the clinically involved and noninvolved limbs.

Patients qualified for cell transplantation if they had chronic limb ischemia, with rest pain or a nonhealing ischemic ulcer, present for a minimum of 4 weeks without evidence of improvement in response to conventional drug therapy; showed angiographic evidence of vasculopenia in the affected limb; and were not candidates for percutaneous or surgical revascularization. The exclusion criteria included severe concurrent illness, the presence of proliferative diabetic retinopathy, and a history or clinical evidence of a malignant disorder.

All the patients involved in the present study received continuous medical therapy for >2 months before BM-MNC transplantation to confirm that conventional measures would be insufficient to achieve improvement in rest pain or skin ulcer/gangrene. During this period, no surgical therapies such as bypass grafting, extensive debridement, skin grafting, or limb amputation were performed. In addition, the patients were admitted to the hospital for a minimum of 1 month before BM-MNC transplantation to exclude the likelihood of spontaneous improvement in ischemic symptoms resulting from an enrollment bias. It should be also pointed out that the patients remained in the hospital and received the same therapy for at least 1 month after BM-MNC transplantation to avoid changes in their treatment.

BM-MNC Transplantation

While the patients were under general anesthesia, marrow cells were aspirated from the ileum. BM-MNCs were sorted on an AS-104 blood-cell separator (Fresenius HemoCare, Redmond, Wash) and concentrated to a final volume of ≈50 mL. After bone marrow cells were sorted on the AS-104 blood-cell separator, a small fraction of the cells was used for BM-MNC counting; the concentration of BM-MNCs in the final product was determined by using a microscope counting chamber after May-Giemsa staining. By using another fraction of cells, the number of $CD34^+$ cells in the BM-MNCs was also determined by fluorescence-activated cell sorting (FACS SCAN flow cytometer; Becton Dickinson, San Jose, Calif). The cells were incubated with the FITC-conjugated mouse monoclonal antibody against human CD34 (clone 581; Becton Dickinson) according to manufacturer's instructions.

For each patient, ≈100 aliquots of BM-MNCs (0.5 mL per aliquot) were administered via a syringe with a 27-gauge needle. Injection was performed into 9 lower limbs in 7 of the patients and the bilateral hands in 1. Injection sites were arbitrarily selected according to angiographic findings (ie, the degree of vasculopenia) and included calf muscles such as the soleus and gastrocunemius muscles as well as the sole muscles of the foot. For the patient with hand ischemia, injection was performed in palm muscles.

Assessment of Short-Term Outcome

Ischemic pain was assessed with a visual analog pain scale (VAS) with 10 levels. Ischemic ulcers were documented by color photography. Resting ankle-brachial pressure index (ABI) was calculated as the quotient of absolute ankle pressure and brachial pressure (the patient who received BM-MNC transplantation in his hands was excluded from ABI analysis). Angiographic assessment was performed with magnetic resonance angiography, computed tomographic angiography, or digital subtraction angiography. Adverse events were defined as death, limb amputation, pathological angiogenesis, recurrence/worsening of ischemic symptoms (ie, rest pain, skin ulcer, gangrene), myocardial infarction, stroke, and malignant disease.

Assessment of Long-Term Outcome

The mean length of follow-up was 684±549 days (range 103 to 1466). Patients were followed up by history analysis, physical examination, routine blood testing, ABI, and angiography at prescribed intervals during the first year, after which they were contacted at an outpatient clinic or by telephone to track events.

Data Analysis

All data are presented as mean±SD (range) or frequencies (percentage).

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Diagnosis

The diagnosis of thromboangiitis obliterans was made according to the criteria described above. Among the 8 patients, only patient 6 did not completely fulfill the criteria; ie, this patient had no history of tobacco use (Table 1). Laboratory screening excluded the possibility of other underlying diseases, however, including autoimmune and connective tissue diseases. It should be also pointed out that patient 6 had diabetes mellitus at the time of cell transplantation but not at the onset of thromboangiitis obliterans. With the typical characteristic angiographic findings of thromboangiitis obliterans, such as multiple segmental arterial involvement (skip lesions) and "cork-screw" collateral vessels, we diagnosed patient 6 as having thromboangiitis obliterans, even though the patient did not have a history of tobacco use.

Patient Characteristics

The demographic and clinical data of the 8 patients are shown in Table 1. The mean age of the patients enrolled was 46±14 years (range 28 to 63). Seven patients (88%) were male. One patient had undergone prior femoral-tibial artery bypass grafting, and 1 had undergone sympathetic ganglion block. These treatments were performed >1 year before BM-MNC transplantation. Seven patients (88%) had a history of smoking, all of whom stopped smoking at least 1 month before transplantation.

Short-Term Outcome

The total volume of cells aspirated from the ileum was 728±72 mL (range 600 to 800) per patient, and the total volume of injected BM-MNCs was 45±7 mL (range 30 to 50) per patient. Total number of injected BM-MNCs was $3.5\pm0.8\times10^9$ (range 2.0 to 4.7×10^9), and that of $CD34^+$ cells was $6.8\pm2.6\times10^7$ (range 2.4 to 9.7×10^7).

TABLE 1. Patient Characteristics

Patient	Age	Sex	Fontaine Stage	Previous Treatment	DM	HT	HLP	Smoking	BM-MNC ($\times 10^9$)	$CD34^+$ in BM-MNC ($\times 10^7$)	ABI, Baseline	ABI, 1 Month	VAS, Baseline	VAS, 1 Month
1	63	M	III(lt)	Bypass graft	−	−	+	+	3.0	6.6	0.34	0.55	5	0
2	31	M	IV(rt)	Medical	−	−	−	+	4.7	9.7	0.49	0.39	5	1
3	52	M	IV(lt)	Medical	−	−	−	+	4.1	9.0	0.65	0.67	7	2
4	28	M	IV(lt)	Sympathetic ganglion block	−	−	−	+	2.0	6.8	0.50	0.26	5	0
5	32	M	IV(rt)	Medical	−	−	−	+	3.8	2.4	−	−	5	2
			IV(lt)								−	−	5	2
6	55	F	IV(lt)	Medical	+	+	−	−	3.4	4.0	0.53	0.51	4	3
7	63	M	III(rt)	Medical	−	+	−	+	3.0	9.1	1.10	0.91	5	3
			IV(lt)								0.76	0.94	5	3
8	43	M	IV(rt)	Medical	−	+	−	+	3.6	6.8	1.00	1.04	5	0
			III(lt)								1.00	1.07	5	0

DM indicates diabetes mellitus; HT, hypertension; and HLP, hyperlipidemia.

Angiographic assessment at 4 weeks after transplantation revealed an apparent increase in limb vascularity in 3 of the 8 (38%) patients (4 of the 11 limbs) (Figure 1). Hemodynamic assessment also failed to document evidence of improved collateral development. Specifically, an increase in ABI (>0.1) was observed in 2 of 7 (29%) patients (2 of 8 limbs), whereas a decrease in ABI (>0.1) was observed in 2 of 7 (29%) patients (2 of 8 limbs). As a result, mean ABI measured at 4 weeks (0.71±0.30) did not differ from that at the baseline (0.70±0.27). Because 2 patients had sites of arterial occlusion distal to the ankle, they showed normal ABIs before treatment. Even after the exclusion of these 2 patients, ABI showed no changes between before (0.55±0.15) and after transplantation (0.55±0.24).

In contrast to the angiographic and hemodynamic results, improvement in limb status was observed in all 8 patients (100%). Improvement in VAS was observed in all 11 limbs, with a decrease from a mean of 5.1±0.7 to 1.5±1.3. Furthermore, complete pain relief was achieved in 4 of the 11 limbs (36%). Improvement in skin ulcers was also observed in all 8 limbs (100%), with complete healing in 7 (88%). Although surgical amputations of the distal limb were performed in 2 patients at 1 month, these operations were intentionally scheduled to be performed after transplantation with the expectation of sufficiently improving the limb perfusion to distally advance the site of amputation (Table 2; Figure 2A and 2B).

Figure 1. Digital subtraction angiography at (A) baseline and (B) 1 month after cell transplantation. Arrows indicate newly visible collateral vessels at the calf level.

Long-Term Outcome

The mean follow-up period was 684±549 days (range 103 to 1466). At the final follow-up, VAS score remained unchanged from that observed at 1 month after transplantation in 5 of the 8 patients (63%). The mean VAS score at follow-up also remained low (2.3±1.9) compared with that observed at baseline (5.1±0.7).

In contrast to the pain scale results, adverse events were observed in as many as 4 patients (50%) (Table 2). At age 30

TABLE 2. Adverse Outcomes After Autologous Transplantation of BM-MNCs in Patients With Thromboangiitis Obliterans

Adverse Outcomes	30 Days	Final Follow-Up
Death	0	1 (13)
Major amputation	0	0
Minor amputation	2 (25)*	0
Unexpected angiogenesis	0	1 (13)
Recurrence/worsening of skin ulcer/gangrene	0	2 (25)†
Recurrence/worsening of pain	0	1 (13)
Cardiovascular event	0	0
Cerebrovascular event	0	0
Malignancy	0	0

Values are expressed as n (%).

*Amputation was intentionally scheduled to be performed at 1 month after transplantation.

†One patient was the same one who developed unexpected angiogenesis.

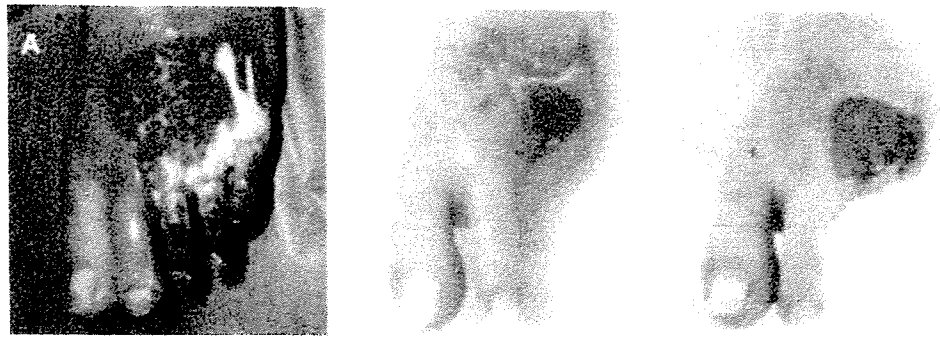

Figure 2. Skin ulcer at (A) 1 month, (B) 2 months, and (C) 4 months after cell transplantation. The patient had far-advanced gangrene, and total limb integrity could not be fully preserved. The patient received a prescheduled amputation of the distal limb at 1 month, and the skin ulcer continued to improve thereafter. At 4 months, however, the skin lesion began to enlarge.

years, patient 4 suddenly died of an unknown cause at 20 months after transplantation. This patient had previously been a smoker, but had stopped smoking before cell transplantation. He had no history of diabetes, hypertension, or hyperlipidemia. Furthermore, ^{201}thallium myocardial scan performed before BM-MNC transplantation showed no signs of myocardial ischemia. After cell transplantation, his limb pain disappeared within 1 week and his skin ulcer resolved by 1 month. Thereafter, he was completely free of limb symptoms. Twenty months after cell transplantation, however, he was found dead at his home. He had never experienced chest pain up to the time of his death. Because no autopsy was performed, the cause of his death remains unknown.

Patient 6 showed worsening of an ischemic ulcer at 4 months. The patient had far-advanced gangrene, and total limb integrity could not be fully preserved. The patient underwent a prescheduled amputation of the distal limb at 1 month (see Short-Term Outcome) (Figure 2A), and the skin ulcer continued to improve thereafter (Figure 2B). At 4 months, however, the skin lesion began to increase in size (Figure 2C). The patient subsequently received a second round of cell therapy.

In patient 7, despite complete healing of the skin ulcer, rest pain did not completely resolve after transplantation, with a VAS score of 3 at 1 month. At 8 months, the patient experienced worsening of rest pain (VAS score=4). After a combination of exercise training and maximal drug therapy, the pain improved and became well tolerated.

Patient 8 experienced swelling and recurrence of the skin ulcer in his foot at 7 months. Computed tomographic angiography documented an early venous return of contrast material in his right limb (Figure 3B) that was not observed at the baseline (Figure 3A). Ultrasound examination disclosed an arterialized waveform in the dorsal vein at the base of his third toe, suggesting the presence of an arteriovenous shunt. By 1 year, the swelling and skin ulcer had spontaneously regressed. The systolic pulsatile component in the venous waveform was found to be diminished on ultrasound examination, and early venous filling had disappeared on computed tomographic angiography (Figure 3C).

Discussion

In the present unblinded and uncontrolled pilot study, we documented that the transplantation of BM-MNCs was associated with an improvement in ischemic symptoms for up to 4 years. Indeed, VAS scores improved from 5.1 ± 0.7 to 2.3 ± 1.9 at follow-up. Furthermore, skin ulcers remained completely healed in 6 of 7 patients. In this regard, the present findings extend previous observations[11] by establishing the potential long-term benefit of BM-MNC transplantation for the treatment of arterial insufficiency.

It should be noted, however, that half of the patients suffered adverse events during follow-up. Such a high rate of adverse events cannot be explained by the natural course of the disease itself. In general, the prognosis of patients with thromboangiitis obliterans is directly related to tobacco

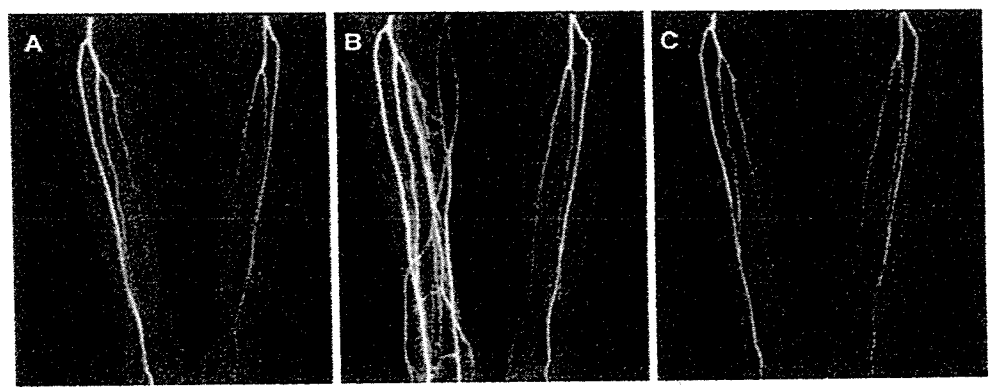

Figure 3. Computed tomographic angiography at (A) baseline, (B) 7 months, and (C) 1 year after cell transplantation. Early venous return of contrast material observed at 7 months spontaneously regressed by 1 year.

use.[12,13] Patients who are able to stop smoking avoid the recurrence of the disease and amputation.[14] In addition, unlike those with atherosclerosis of the extremities, these patients rarely show involvement of visceral vessels and do not appear to be at an increased risk of stroke or myocardial infarction. The mortality rates are thus not higher than those of age- and sex-matched populations.[15,16] It is entirely possible that the high rate of adverse events observed in our patients may have been directly related to BM-MNC transplantation rather than to the progression of the disease itself.

Patient 4 suddenly died at 20 months after transplantation at the age of 30 years. The deaths of patients receiving BM-MNC transplantation were previously reported in the TACT study, in which 2 of 25 patients died of acute myocardial infarction within 24 weeks after transplantation.[11] The patients' backgrounds in the TACT study, in terms of age and comorbidity, may have been totally different from those of our study, in which only patients with thromboangiitis obliterans were recruited. As mentioned above, in patients with thromboangiitis obliterans, coronary involvement is rare, and they usually do very well as long as they discontinue smoking.[12–16] Patient 4 had no risk factors for atherosclerosis and stopped smoking before BM-MNC transplantation. Furthermore, ^{201}thallium scintigraphy performed before transplantation documented no sign of myocardial ischemia. Considering the patient's background and the natural course of the disease,[12–16] the possibility that his death was related to BM-MNC transplantation cannot be excluded. In this regard, several studies have suggested the possible role of BM-MNCs in atherogenesis. A recent report by Silvestre et al,[17] for example, demonstrated that the transplantation of BM-MNCs into ischemic limbs of apolipoprotein E–knockout mice led to a significant increase in atherosclerotic plaque size at a distant site. More recently, George et al[18] have also shown that an intravenous injection of bone marrow cells into apolipoprotein E–knockout mice results in an increase in atherosclerotic lesion size, whereas an injection of endothelial progenitor cells influences plaque stability. These reports indicate that attempts to enhance neovascularization by using BM-MNCs could also enhance unwanted plaque growth and instability, thus suggesting the possibility that our young patient died of an acute coronary event due to accelerated atherogenesis after BM-MNC transplantation.

We also encountered the development of an arteriovenous shunt, which could be a potential consequence of BM-MNC transplantation. Indeed, concerns have been raised about the potential adverse effects of cell transplantation, ie, unregulated differentiation and proliferation. Wakitani et al[19] reported that teratoma formation could occur after embryonic stem cell transplantation. Yoon et al[20] documented intramyocardial calcification after the transplantation of bone marrow cells in rats. Our observations may provide another cautionary example of unregulated differentiation and proliferation. Although the arteriovenous shunt in our case was self-limited, it may represent unwanted angiogenesis; thus, careful monitoring is warranted for future patients who receive BM-MNC transplantation.

Worsening or recurrence of ischemic symptoms was observed in 3 patients. The short-term outcome at 1 month was poor in these patients except in the patient with an arteriovenous shunt. The improvement in rest pain was not substantial in 1 patient. Healing of the skin ulcer was incomplete in another. It is anticipated that a poor response 1 month after BM-MNC transplantation could result in a poor long-term outcome. It is important to note that, in the latter patient with incomplete healing of the skin ulcer, the angiographic improvement of the collateral network at 1 month remained unchanged at 5 months when worsening of the skin ulcer was observed. It is suggested that the temporal sequence of improvement in ischemic limb status does not necessarily parallel the temporal evolution of collateral development.

Conclusions

In this unblinded and uncontrolled pilot study, long-term adverse events after BM-MNC transplantation, including death and unfavorable angiogenesis, were observed in half of the patients with thromboangiitis obliterans. Given the current incomplete knowledge of the safety and efficacy of this strategy, careful long-term monitoring is required for future patients receiving BM-MNC transplantation.

Sources of Funding

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Disclosures

None.

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CLINICAL PERSPECTIVE

The favorable short-term outcome of bone marrow mononuclear cell transplantation (BM-MNC) transplantation has been established in patients with critical limb ischemia. However, the long-term outcome of this treatment strategy has not been determined yet. In our case series, we documented that long-term adverse events, including death and unfavorable angiogenesis, were observed in 4 of 8 patients receiving BM-MNC transplantation. The first patient suffered sudden death at 20 months after transplantation at 30 years of age. The second patient with incomplete healing of a skin ulcer showed worsening of the lesion at 4 months. The third patient had worsening of rest pain at 8 months. The last patient developed an arteriovenous shunt in the foot at 7 months, which spontaneously regressed by 1 year. Given the current incomplete knowledge on the safety and efficacy of this strategy, it is suggested that careful long-term monitoring is required in patients receiving BM-MNC transplantation. To our knowledge, this is the first report on the long-term outcome of transplantation of BM-MNCs for critical limb ischemia, and the first that documents the development of unfavorable angiogenesis and sudden death after therapeutic angiogenesis.

Complete deficiency in ADAMTS13 is prothrombotic, but it alone is not sufficient to cause thrombotic thrombocytopenic purpura

Fumiaki Banno, Koichi Kokame, Tomohiko Okuda, Shigenori Honda, Shigeki Miyata, Hisashi Kato, Yoshiaki Tomiyama, and Toshiyuki Miyata

ADAMTS13 is a plasma metalloproteinase that regulates platelet adhesion and aggregation through cleavage of von Willebrand factor (VWF) multimers. In humans, genetic or acquired deficiency in ADAMTS13 causes thrombotic thrombocytopenic purpura (TTP), a condition characterized by thrombocytopenia and hemolytic anemia with microvascular platelet thrombi. In this study, we report characterization of mice bearing a targeted disruption of the *Adamts13* gene. ADAMTS13-deficient mice were born in the expected mendelian distribution; homozygous mice were viable and fertile. Hematologic and histologic analyses failed to detect any evidence of thrombocytopenia, hemolytic anemia, or microvascular thrombosis. However, unusually large VWF multimers were observed in plasma of homozygotes. Thrombus formation on immobilized collagen under flow was significantly elevated in homozygotes in comparison with wild-type mice. Thrombocytopenia was more severely induced in homozygotes than in wild-type mice after intravenous injection of a mixture of collagen and epinephrine. Thus, a complete lack of ADAMTS13 in mice was a prothrombotic state, but it alone was not sufficient to cause TTP-like symptoms. The phenotypic differences of ADAMTS13 deficiencies between humans and mice may reflect differences in hemostatic system functioning in these species. Alternatively, factors in addition to ADAMTS13 deficiency may be necessary for development of TTP. (Blood. 2006;107:3161-3166)

Introduction

Thrombotic thrombocytopenic purpura (TTP) is a life-threatening systemic disease, characterized by anemia, thrombocytopenia, and microvascular thrombosis.[1-4] Hemolysis, the cause of the anemia, generates pointed red cell fragments, schistocytes. Thrombocytopenia is caused by the consumption of platelets in thrombi, which cause renal and neurologic dysfunction. Without treatment, the mortality rate of affected patients exceeds 90%, but plasma exchange reduces the death rate to approximately 20%.[5]

Our understanding of TTP pathophysiology increased considerably with the identification of ADAMTS13, which specifically cleaves the Tyr^{1605}-Met^{1606} peptidyl bond of von Willebrand factor (VWF).[6-10] VWF is a large glycoprotein that mediates platelet adhesion to vascular lesions. It is mainly synthesized in endothelial cells and secreted into the blood as "unusually large" VWF (UL-VWF) multimers, the highly active forms for platelet adhesion and aggregation.[11,12] ADAMTS13 cleaves UL-VWF multimers into smaller forms under flow, limiting platelet thrombus formation under normal conditions. Severe deficiency in ADAMTS13 activity is observed in most patients with TTP, allowing UL-VWF multimers to persist in the circulation.[1-4] UL-VWF multimers mediate enhanced platelet clumping under shear stress, which is thought to cause the clinical symptoms of TTP. Congenital TTP is associated with mutations in the *ADAMTS13* gene, whereas acquired TTP results from the production of autoantibodies against ADAMTS13. A number of causative mutations for congenital TTP have been identified within the *ADAMTS13* gene.[3,4] In vitro expression studies have confirmed the deleterious effects of mutant ADAMTS13 on proteolytic activity or secretion.[13-15]

Here, we generated a mouse model of ADAMTS13 deficiency by a gene-targeting approach, to further understand the pathophysiologic process of TTP. We found that the complete deficiency in ADAMTS13 is not sufficient to produce in mice the typical TTP phenotype. Other triggers may be needed to provoke the disease.

Materials and methods

Generation of ADAMTS13-deficient mice

The isolation of λ phage genomic clones containing *Adamts13* has been previously described.[16] The targeting vector was constructed from a 12.3-kb fragment including exons 3-12, in which the 3.6-kb *SalI-EcoRI* region containing exons 3-6 was replaced by a neomycin resistance cassette. A

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F.B. designed research, performed research, analyzed data, and wrote the paper; K.K. designed research, performed research, and wrote the paper; T.O. contributed vital analytical tools and interpreted the data; S.H. contributed vital analytical tools and interpreted the data; S.M. contributed vital analytical tools and interpreted the data; H.K. performed research, contributed vital analytical tools, and interpreted the data; Y.T. contributed vital analytical tools and interpreted the data; and T.M. designed research and wrote the paper.

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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 *Hind*III-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.

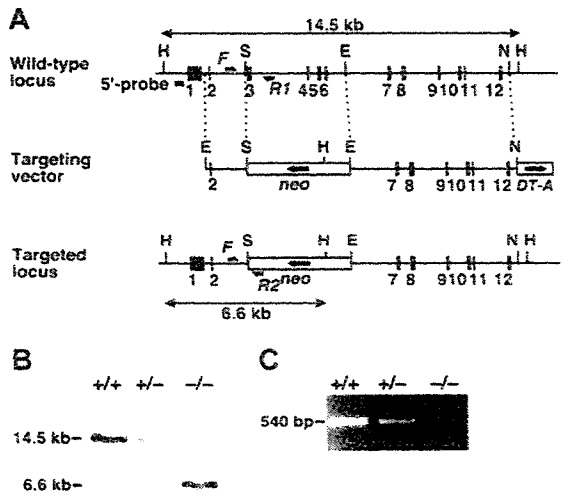

Figure 1. Targeted disruption of the mouse *Adamts13* gene. (A) Structure of the targeted locus in the mouse *Adamts13* gene. Exons are represented by filled boxes. A neomycin-resistance cassette (neo), in the opposite transcriptional orientation, and a forward-oriented diphtheria toxin A fragment expression 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*I; 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.

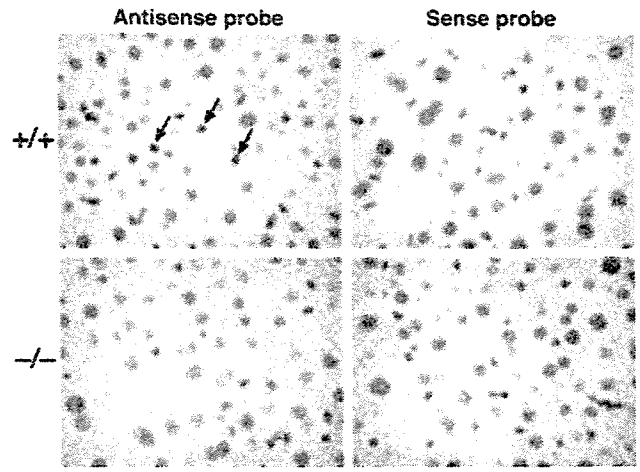

Figure 2. In situ hybridization analysis of ADAMTS13 mRNA. Liver sections from *Adamts13*$^{+/+}$ (top panels) and *Adamts13*$^{-/-}$ (bottom panels) mice were hybridized to the antisense (left panels) or sense (right panels) *Adamts13* RNA probes. The hybridized sections were counterstained with Kernechtrot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

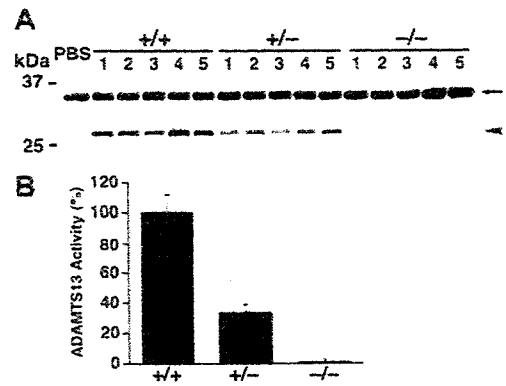

Figure 3. ADAMTS13 activity in plasma. (A) Qualitative assay using a recombinant substrate, GST-mVWF73-H. The substrate and product bands are indicated by arrows and arrowheads, respectively. (B) Quantitative assay using a fluorogenic substrate, FRETS-VWF73. Data are mean ± SD from 4 mice for each genotype.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

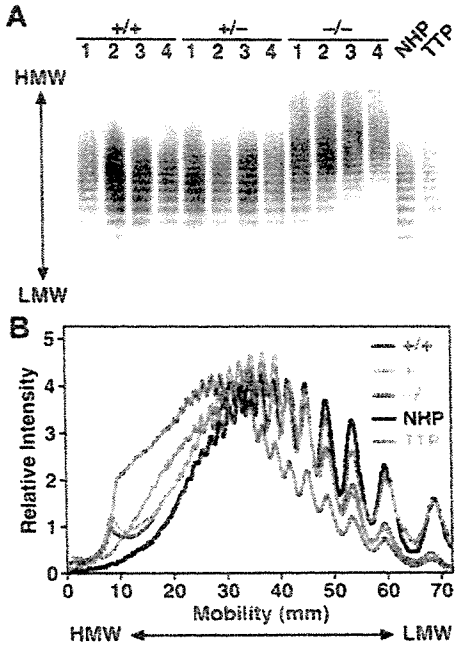

Figure 4. Analysis of plasma VWF multimers. (A) VWF multimer patterns. Plasma samples (1 μL/lane) from *Adamts13*$^{+/+}$, *Adamts13*$^{+/-}$, and *Adamts13*$^{-/-}$ mice were electrophoresed on SDS-agarose gels and transferred to nitrocellulose membranes. VWF multimers were detected with anti-VWF antibodies. 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.

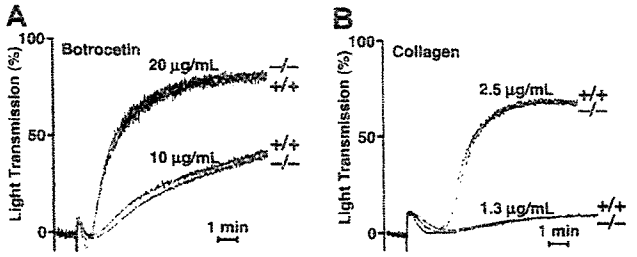

Figure 5. Platelet aggregation under static condition. (A) Botrocetin-induced aggregation. Pooled PRP samples from *Adamts13*$^{+/+}$ or *Adamts13*$^{-/-}$ mice were treated with botrocetin at a final concentration of 10 or 20 μg/mL. Aggregation was measured using an aggregometer at 37°C with constant 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.

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

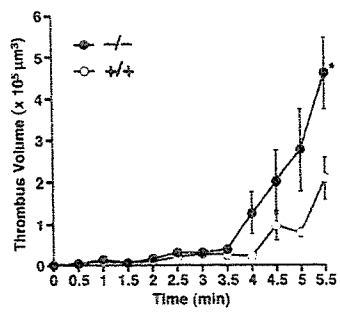

Figure 6. Thrombogenesis on collagen surface under flow. Whole blood from *Adamts13*$^{+/+}$ or *Adamts13*$^{-/-}$ mice containing mepacrine-labeled platelets was perfused over an acid-insoluble type I collagen-coated surface at a wall shear rate of 750 s^{-1}. The cumulative thrombus volume, analyzed 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.

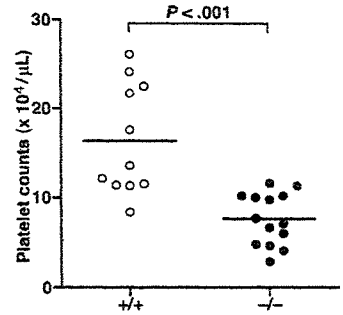

Figure 7. Platelet counts following collagen plus epinephrine challenge. Mice were given injections of 600 ng/g collagen plus 60 ng/g epinephrine via tail vein and platelet counts were measured 15 minutes after injection. Symbols represent platelet counts of a single mouse. Bars represent the mean values 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 × 10^4/μL and 16.4 ± 6.2 × 10^4/μL, respectively (mean ± SD; $P < .001$), whereas platelet counts without challenge were not different between groups (*Adamts13*$^{-/-}$, 86.2 ± 13.2 × 10^4/μL; *Adamts13*$^{+/+}$, 83.7 ± 3.3 × 10^4/μL; mean ± SD of 4 mice).

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.

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

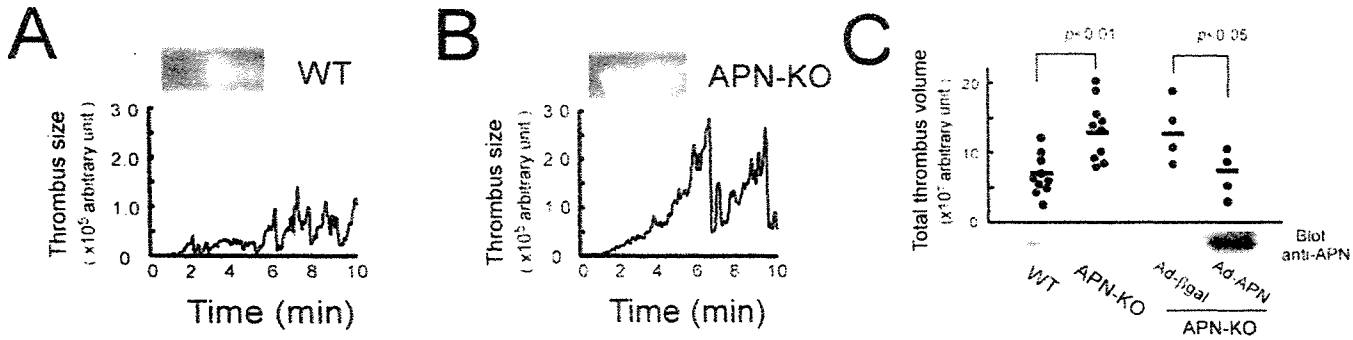

Figure 1. He-Ne laser–induced thrombus formation and adenovirus-mediated supplementation of adiponectin. Anesthetized mice were injected with Evans blue dye followed by irradiation with the He-Ne laser at the exposed left carotid artery. The representative time course of thrombus formation in (A) WT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

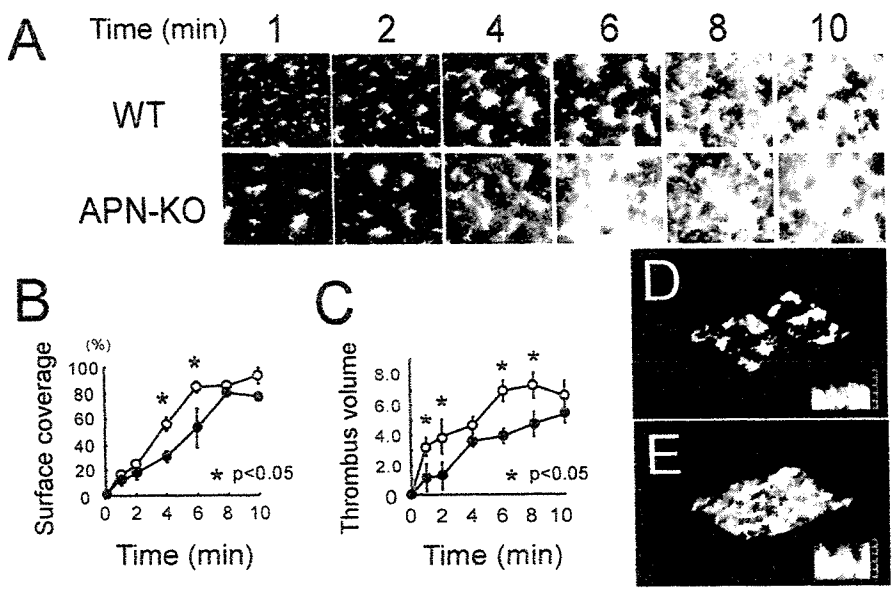

Figure 2. Thrombogenesis on a type I collagen-coated surface under flow conditions. (A) Mepacrine-labeled whole blood obtained from WT (top) or APN-KO mice (bottom) was perfused on a type I collagen-coated surface at a shear rate of 250 s^{-1}. (B) Platelet surface coverage (%) and (C) thrombus volume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

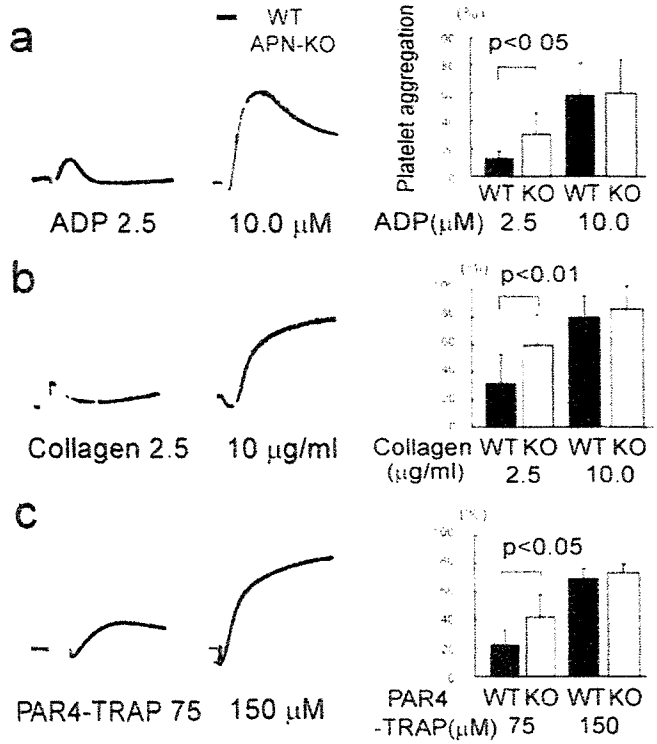

Figure 3. Enhanced platelet aggregation in APN-KO mice. Platelet aggregation in PRP obtained from WT or APN-KO mice. PRP ($300\times10^3/\mu L$) obtained from WT (black line) or APN-KO mice (gray line) was stimulated with ADP (a; n=4), collagen (b; n=4), or PAR4-TRAP (c; n=3). As compared with WT mice,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

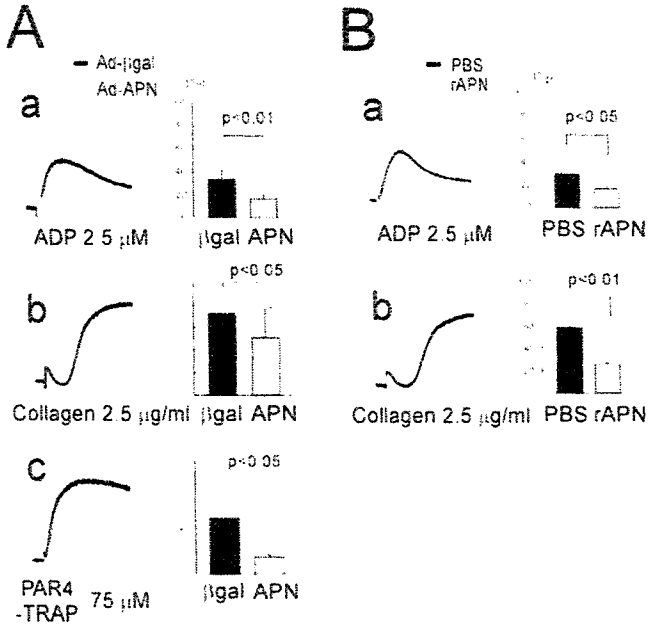

Figure 4. Effects of in vitro supplementation of adiponectin or recombinant adiponectin on the enhanced platelet aggregation in APN-KO mice. (A) One volume of PRP from APN-KO mice was mixed with ≈4 volumes of PPP from APN-KO mice injected with Ad-βgal (black line) or Ad-APN (gray line) to obtain a platelet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

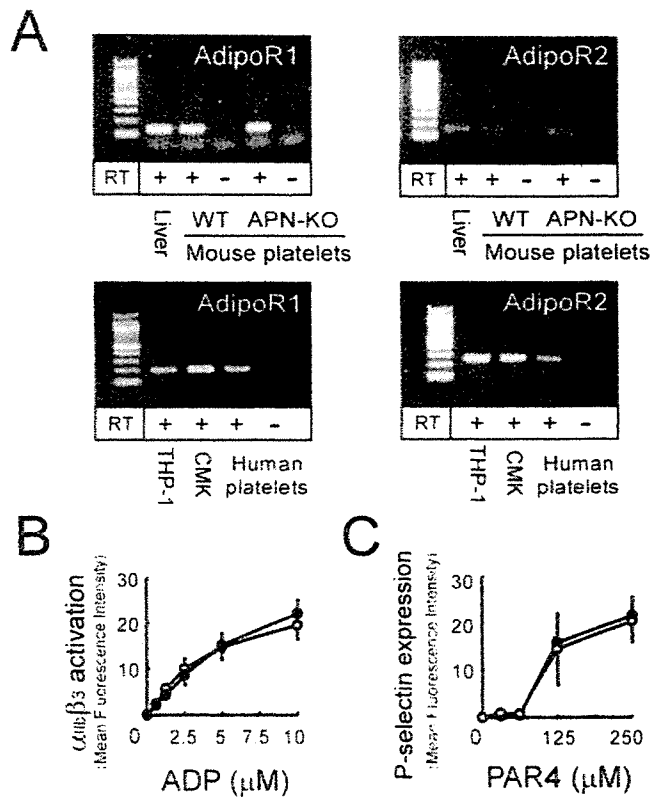

Figure 5. Expression of adiponectin receptors and effects of adiponectin deficiency on platelet function. (A, top) Expressions of transcripts for adiponectin receptors, AdipoR1 (133-bp fragments) and AdipoR2 (156-bp fragments), in platelets from WT or APN-KO mice were examined by RT-PCR. The liver was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