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

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

Methods and Results—We constructed adenovirus vectors expressing human placental E-NTPDase isoforms I (AdPlac I) and II (AdPlac II) or bacterial /H9252-galactosidase (AdLacZ). Vascular SMCs infected with AdPlac I expressed significant NTPDase activity and inhibited the platelet aggregation induced by ADP and collagen in vitro. In contrast, SMCs infected with AdPlac II and AdLacZ did not exert antiplatelet effects. To investigate the antithrombotic and antiproliferative effects of placental E-NTPDase isoform I in vivo, we generated thrombosis in rat carotid arteries by systemically administered rose Bengal and transluminal green light 5 days after gene transfer and examined neointimal growth 3 weeks after thrombus formation. Blood flow in AdLacZ-infected arteries rapidly deteriorated and vanished within 96±18 seconds of occlusive thrombus formation. In contrast, blood flow in AdPlac I–infected arteries was preserved for at least 10 minutes during irradiation. In addition, thrombus formation and subsequent neointimal growth were obviously suppressed.

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

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

Thrombus formation is a critical event in the onset of cardiovascular disease. Thrombotic occlusion still occasionally arises after vascular interventions, such as angioplasty and stent implantation. Platelets play a pivotal role in the development of arterial thrombosis. Therefore, antiplatelet agents, including aspirin, ADP receptor blockers, and platelet glycoprotein (GP) Ib/IIa antagonists, are systemically administered to prevent cardiovascular events.1 Although some of these agents effectively reduce cardiovascular events,3 they can also produce systemic hemorrhagic side effects.2 Platelet adhesion to injured vascular walls leads to platelet activation and the release of additional agonists such as ADP, serotonin, and thromboxane A2, which cause further platelet recruitment to injured sites. Because ADP plays a key role in platelet aggregation,3 its metabolism in the blood is important in the regulation of platelet activation and recruitment. Ectonucleoside triphosphate diphosphohydrolase (E-NTPDase; EC 3.6.1.5, or CD39) is a major metabolic enzyme of ADP in the vasculature.4 Originally identified in B lymphocytes,5 E-NTPDase is a membrane-bound enzyme that rapidly hydrolyzes both ATP and ADP to AMP and thereby inhibits platelet aggregation.6–8 Makita et al9 purified human placental E-NTPDase (Plac I) as an isoform of CD39 that alternatively differed at the N-terminus from that of CD39. Matsumoto et al10 recently isolated another truncated variant.
of placental E-NTPDase (Plac II). Although these structural differences might affect their enzymatic activities,\textsuperscript{11,12} the properties of the 2 isoforms remain unknown.

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

Methods

Preparation of Recombinant Adenovirus With Placental E-NTPDase

Repllication-defective E1\textsuperscript{−} and E3\textsuperscript{−} adenoviral vectors encoding Plac I (AdPlac I) and Plac II (AdPlac II) were generated with the Adeno-X expression system (Clontech) according to the manufacturer’s instructions.\textsuperscript{13} In brief, cDNA clones of Plac I (1554 bp) and Plac II (921 bp) were isolated at the Department of Blood Transfusion Medicine of Nara Medical University\textsuperscript{10} and subcloned into the mammalian expression cassette pShuttle. Recombinant pShuttle was digested and inserted into the Adeno-X viral DNA. The recombinant viruses were propagated in HEK293 cells. Viral titers were determined by limiting dilution as plaque-forming units (PFU).\textsuperscript{14} We also constructed control recombinant adenovirus encoding bacterial \(\beta\)-galactosidase (AdLacZ).

Cell Culture

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

In Vitro Gene Transfer

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

Western Blots

Four days after infection, cells were lysed in Tris-buffered saline (pH 8.0) containing 150 mmol/L NaCl, 1% Nonidet P-40, 1% Triton X-100, and 1 mmol/L phenylmethylsulfonyl fluoride. After centrifugation at 12 000 g for 5 minutes, the supernatant was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions on 4% to 12% gradient gels, and then separated proteins were electrophoretically transferred to an Immobilon membrane (Millipore). Nonspecific binding was blocked by incubation in 5% skimmed milk, and then the membrane was incubated overnight at 4\(^\circ\)C with a monoclonal anti-human placental E-NTPDase antibody (YH34). This novel antibody was produced in mice with the primary standard procedure at the Department of Blood Transfusion Medicine of Nara Medical University. Purified placental E-NTPDase used as an immunogen was prepared on an immunoadsorbent column as described.\textsuperscript{9} Western blotting showed that YH34 bound with high affinity to the purified enzyme (~80 kDa) under both nonreducing and reducing conditions (data not shown). We determined the immunoglobulin subclass and idiotype of YH34 (IgG1-\(\kappa\)) by using an isotyping kit (Serotec). We purified YH34 IgG from mice ascites on a protein A–Sepharose CL-4B column (Amersham Bioscience Corp). The antibody recognizes the external domain of both Plac I and Plac II. Specific binding was visualized with a horseradish peroxidase–conjugated goat anti-mouse IgG (Nacalai Tesque) and an enhanced Chemiluminescence reagent (DuPont NEN).

Analysis of NTPDase Activity

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

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

Platelet Aggregation

We evaluated platelet aggregation in the presence of SMCs by using a modification of a described method.\textsuperscript{17} Uninfected or infected SMCs detached by EDTA-collagenase were washed 3 times. Blood samples collected in 3.8% sodium citrate (9:1, vol/vol) were centrifuged at 900 or 3000 rpm for 10 minutes to prepare platelet-rich plasma (PRP) or platelet-poor plasma (PPP), respectively. PRP (5 \(\times\) 10\textsuperscript{7} platelets) and SMCs (5 \(\times\) 10\textsuperscript{3} cells) were incubated in siliconized cuvettes at 37\(^\circ\)C in a PA-20 aggregometer (Kowa) that had been calibrated with PRP and PPP for 0% and 100% transmission, respectively. Thereafter, either ADP (10 \(\mu\)mol/L, final concentration) or collagen (10 \(\mu\)g/mL, final concentration) was added to the cuvettes, and platelet aggregation was measured.

Animal Care

The Animal Care Committee of Miyazaki Medical College (No. 1998-025-6) approved the study protocols. We used 59 male Sprague-Dawley rats weighing 400 to 500 g. The animals received humane care according to the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National (Bethesda, Md) Institutes of Health (NIH publication No. 86-23, revised 1985). Aseptic surgery proceeded under general anesthesia induced by an intraperitoneal injection of pentobarbital (30 mg/kg body weight).

In Vivo Gene Transfer Into Injured Arteries

The common carotid arteries of the rat were exposed and isolated by temporary ligation at a distance of 1.5 cm. A 31-gauge needle was inserted into the proximal side of the segment. Thereafter saline was flushed into the segment, and then air was infused at a rate of 50 mL/min for 3.5 minutes to denude endothelial cells.\textsuperscript{18} After the segment was filled with 0.1 mL of saline containing AdPlac I or AdLacZ (final titer, 5 \(\times\) 10\textsuperscript{8} PFU) or saline alone (n = 6 each) for 30 minutes, the mixture was aspirated and blood flow was restored.\textsuperscript{19} The vessels harvested 2 to 5 days after gene transfer were perfusion-fixed in 4% paraformaldehyde and embedded in paraffin by standard procedures. We detected the expression of human placental E-NTPDase protein by immunohistochemistry (EnVision+ kits) by using the primary monoclonal antibody YH34. The negative control included nonimmune mouse IgG\(_1\) instead of YH34.

NTPDase Activity in Vessels

Carotid arteries without perfusion-fixation were excised and homogenized with a Polytron PT3000 (Kinematica) in Tris-buffered saline (pH 7.4) containing aprotinin and phenylmethylsulfonyl fluoride.\textsuperscript{20,21} The homogenates were then incubated with the firefly luciferase ATP assay mixture. Extracellular ATP and ADP concentrations were
determined by measuring luminescence as described earlier. Protein concentrations of homogenates were determined by the bicinchoninic acid assay with bovine serum albumin as the standard.

**Arterial Thrombus Formation and Neointimal Growth**

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

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

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

**Statistical Analysis**

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

**Results**

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

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

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

**Platelet Aggregation With Infected SMCs In Vitro**

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

**Inhibition of Arterial Thrombus Formation and Neointimal Growth in Arteries Infected With AdPlac I In Vivo**

Five days after gene transfer, Plac I protein was broadly expressed in the medial SMCs of arteries infected with AdPlac I (Figure 4A) but was undetectable in control arteries infected with AdLacZ (Figure 4C). Arteries infected with AdPlac I were immunonegative for Plac I protein when the antibody was replaced with nonimmune mouse IgG, but when the antibody was replaced with nonimmune mouse IgG, (Figure 4B). Figure 5 shows NTPDase activities in infected arteries and neointimal growth in arteries infected with AdPlac I 3 weeks after thrombus formation. The areas (μm$^2$) of neointima and media were measured with an image analyzing system (Axio Vision 2.05, Carl Zeiss) by 2 investigators (K.M. and K.H.) who were blinded to the treatment assignment.
than those after AdLacZ infection. The numbers of mRNA copies of Plac I per 10^6 copies of the housekeeping gene, glyceraldehyde 2-phosphate dehydrogenase, 2 and 5 days after gene transfer were 15.2 ± 3.5 and 1456 ± 65, respectively. These results indicated that Plac I protein expressed in carotid arteries had biological NTPDase activity in the vascular wall.

Blood flow in arteries infected with AdLacZ rapidly deteriorated after irradiation and vanished within 96 ± 18 seconds (n = 6, Figure 6A). In contrast, blood flow in arteries infected with AdPlac I was preserved for at least 10 minutes during green light irradiation (n = 6, Figure 6B). Histological analyses revealed that occlusive thrombi, mainly consisting of aggregated platelets, had blocked arteries infected with AdLacZ (Figure 6C), whereas only small mural thrombi had developed in arteries infected with AdPlac I (Figure 6D). The neointimal growth 3 weeks after thrombus formation was significantly reduced by AdPlac I infection. Area of neointima and neointima/media ratio in arteries infected with AdLacZ versus AdPlac I were 97 ± 500 μm² versus 45 ± 300 ± 5900 μm² (n = 8 each, P < 0.01) and 1.03 ± 0.13 versus 0.41 ± 0.05 (n = 8 each, P < 0.001), respectively.

**Platelet Function and Blood Coagulation in Rats Infected With AdPlac I**

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

**Discussion**

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

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

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

Thrombus formation was almost completely suppressed in arteries infected with AdPlac I. Makita et al showed that placental E-NTPDase blocks platelet aggregation under low shear stress (12 dyne/cm²) but did not significantly inhibit
initial aggregation under high shear stress (108 dyne/cm²), although platelets disaggregated during the later phase in vitro. These results imply that E-NTPDase plays an anti-thrombotic role under relatively low flow conditions. The finding that this enzyme localizes on syncytiotrophoblasts and in endothelial cells of the umbilical vein, rather than the artery,⁹ might be consistent with the results in vitro; however, the present study showed that arterial thrombus formation under high flow conditions was obviously suppressed in vessels infected with AdPlac I. Interactions between von Willebrand factor and the platelet membrane receptors GP Ib and IIb/IIIa are crucial for platelet aggregation when blood flow is high,²⁷ and ADP and its receptors are key mediators of such interactions.²⁸,²⁹ Thrombus formation was photochemically induced in the present study.²² Photochemical exposure produces highly reactive oxygen species that react with cell membrane lipids to cause endothelial cell damage and platelet activation. In this animal model therefore, ADP released from damaged tissue and activated platelets played a critical role in thrombus formation. These lines of evidence indicate that placental E-NTPDase isoform I overexpression in injured vessels is highly antithrombotic, even under high blood flow, which is characteristic of stenosed atherosclerotic arteries.

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

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

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

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<th>Before Virus Infection</th>
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<th>AdPlac I</th>
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<td>% Maximum platelet aggregation</td>
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<td>ADP (10 μmol/L)</td>
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<td>Collagen (10 μg/mL)</td>
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<td>Coagulation parameters</td>
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<td>Prothrombin time, s</td>
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<td>Activated partial thromboplastin time, s</td>
<td>14.6±0.5</td>
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Abbreviations are as defined in text.

n=4 each, P>0.1.

The endothelium predominantly expresses E-NTPDase and, to a lesser extent, so do other vascular cells. The enzyme in vascular SMCs would modulate vascular tone via P2 purinoceptors. The present study detected placental E-NTPDase isoform 1 expression by immunohistochemistry in whole walls of arteries infected with AdPlac I. Antiplatelet ability is thought to depend mainly on the NTPDase activity of luminal SMCs. Gangadharan et al11 reported that adenovirus-mediated CD39 gene transfer augments NTPDase activity in rabbit iliac arteries injured with balloons but does not significantly reduce platelet deposition on the injured luminal surface. They detected CD39 expression only in luminal SMCs. Although the methods of gene transfer and/or species differences might be relevant factors in explaining this discrepancy, the powerful inhibitory effect against occlusive thrombus formation in AdPlac I-infected arteries might depend not only on antiplatelet function but also on reduced vasoconstriction in injured vessels. We evaluated total NTPDase activities of the vessel wall, which includes potential sources of the enzyme. We could not evaluate native E-NTPDase of rat vascular SMCs. Occlusive thrombi were photochemically induced in all arteries infected with AdLacZ, and the level of E-NTPDase/CD39 mRNA expression in rat carotid arteries was very low (data not shown). These results suggest that native E-NTPDase in SMCs would not have putative anti-thrombotic roles in vivo.

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

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

Acknowledgments

This study was supported by grants-in-aid for scientific research (Nos. 14570153, 15590305, and 16590284 to Dr Asada; 14570869 and 16591212 to Dr Tamura; and 15591017, H14-002 to Dr Fujimura) and by the 21st Century COE program (Life Science) from the Ministry of Education, Science, Sports, and Culture of Japan (to Dr Asada).

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Circulation. 2005;111:808-815; originally published online February 7, 2005;
doi: 10.1161/01.CIR.0000155239.46511.79
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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