Supplement Material

Methods

Effects of Human pE-NTPDase on rabbit platelet aggregation

We had previously purified human pE-NTPDase glycoprotein\(^1\), and in this study we evaluated the effects of human pE-NTPDase on rabbit platelet aggregation induced by ADP as previously described.\(^{1,2}\) Rabbit platelet-rich plasma (3×10\(^8\) platelets/ml) was incubated in siliconized cuvettes at 37\(^\circ\)C for 5 minutes with or without purified human pE-NTPDase (1.5 \(\mu\)g/ml, final concentration). ADP (1 \(\mu\)g/ml, final concentration) was then added to the cuvettes and platelet aggregation was measured with the use of NKK Hematracer-1 (SSR Engineering, Tokyo).

Cationization of Gelatin and Preparation of Cationic Gelatin-Coated Stens

Ethylenediamine and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt were added to 250 ml of 100 mM phosphate-buffered solution (pH 5.0) containing 5 g of gelatin. After dialysis, the solution was freeze-dried to obtain cationic gelatin hydrogel. The cationic ratio was determined by the conventional trinitrobenzenesulfonate (TNBS) method.\(^{3,4}\) Normal stainless steel stents (bare metal stents, BMS) were incubated in aqueous solutions of 10% cationic gelatin for 5 minutes, and left at 4\(^\circ\)C overnight. The cationic gelatin hydrogel was cross-linked to the stents in HCl-acetone (3:7, v/v) containing 0.31 mg/ml glutaraldehyde at 4\(^\circ\)C for 24 hours. The stents were immersed in 100 mM glycine solution at 4\(^\circ\)C for 24 hours to remove residual aldehyde. Stents were rinsed three times with double distilled water and freeze-dried. The successful coating of stents with cationic gelatin hydrogel was confirmed by coomassie brilliant blue staining.
Preparation of Gene-Eluting Stents

Gelatin-coated stents were incubated in plasmid DNA solutions (pE-NTPDase or Lac Z (1 mg/ml)) so that they would absorb the plasmid. Five gelatin-coated stents were steeped in $^{125}$I-labeled plasmid DNA solution (1 mg/ml) to measure the amount of plasmid DNA absorbed. After drying, the radioactivity was measured with a gamma counter (ARC-301B, Aloka). The mean amount of plasmid DNA content in five gelatin-coated stents calculated by radioactivity was 78.3 ± 5.1 µg, which suggests that each gene eluting stent accommodated almost same amount of plasmid DNA.

Repeated-Balloon Injury of Rabbit Femoral Arteries

On two occasions separated by a 4-week interval, the rabbits were anesthetized with pentobarbital sodium (25 mg/kg body weight, i.v.) and subjected to balloon injury of the right femoral artery (FA). Briefly, the first injury was induced by fluoroscopically inserting a Fogarty 2F balloon catheter (Edwards Lifesciences, CA, USA) via the right anterior tibial artery into the right femoral artery, where it was inflated to 1.5 atm. The inflated balloon was then pulled back a distance of 1.0 cm three times. Four weeks later, formation of a stenotic lesion at the site of injury was confirmed angiographically, and then the second injury was induced. In this case, a PTCA balloon catheter (2.75 mm-diameter, 15 mm-length) was inserted via the introducer sheath in the right carotid artery to a site just distal to the stenotic lesion, and a second injury was induced in the same manner as the first. After the second injury, platelet-rich thrombus formation similar to ACS was induced.

In Vivo Stent Implantation

Angiography of rabbit FAs was performed 5 minutes after the second injury to confirm that no occlusion of the injured site or flow delay indicating distal occlusion occurred. Immediately following, 500 U of heparin was administered via a sheath in
the right carotid artery, and a stent was implanted in the injured right FA by balloon inflation (at 12 atm for 20 seconds). Successful stent implantation was confirmed by angiography in all rabbits.

**X-Gal stain**

For the BMS, gelatin-coated stent, and Lac Z stent groups, βGalactosidase expression in the FA at days 3 and 7 (n=3 in each group, respectively) post stent implantation was quantified with X-Gal stain as previously described to confirm successful gene transfer and protein expression.

**Evaluation of Patency of Stent Implanted Arteries**

Peak flow velocity in FAs was measured by continuous Doppler method transcutaneously on day 3 post stent implantation (n=10 for the BMS, gelatin-coated stent, and pE-NTPDase stent groups, and n=4 for the Lac Z stent group). Based on differences in peak flow velocity between the stent implanted right FAs and the contralateral normal FAs, we defined differences <30% as patent, >30% as stenosis, and no flow as occlusion, in accordance with a previous study. Angiography of FAs was carried out to evaluate the patency of the stent implanted arteries on days 3 and 7 post stent implantation (n=10 and 12 on each day for the BMS, gelatin-coated stent, and pE-NTPDase stent groups, n=4 and 6 on each day for the Lac Z stent group). In all rabbits, the stent implanted site of the right FA and the size-matched contralateral normal FA were harvested after angiography for the evaluation of gene and protein expression, NTPDase activity, and histological examination.

**RNA Extraction and RT-PCR**

In the BMS, gelatin-coated stent, and pE-NTPDase stent groups (n=4), E-NTPDase mRNA expression in whole FAs of stent implanted site, and endothelial nitric oxide
synthase (eNOS) mRNA expression in the neointimal tissues of stent implanted FAs on days 3 and 7 post stent implantation were evaluated by real-time polymerase chain reaction (PCR) as previously described.\(^7\) RNA was isolated from the whole FAs of stent implanted site using TRIzol Reagent (Invitrogen, Carlsbad, CA), after which cDNA was generated from total RNA by using a SuperScript II Reverse Transcriptase Kit (Invitrogen). Real-time polymerase chain reaction (PCR) was then performed in an ABI-Prism 7700 (Applied Biosystems, Foster City, CA) using QuantiTect SYBR Green PCR Kit (Qiagen). The sense and antisense primers were as follows:

5’CATGAATTCCATGGGCAAGGGAACCAAGGACCTGAC3’ and 5’AGCACAATCCCCATCTTAACG3’ for human E-NTPDase,

5’CTACCCCTTTGACTTCCAGGG3’ and 5’CTTGGCCAGTTTCTGCCACAG3’ for total E-NTPDase including both endogenous rabbit E-NTPDase and exogenous human E-NTPDase, 5’ACCTGTGTGACCCTCACC3’ and 5’GGGGACAGGAAATAGTTGACC3’ for eNOS, and

5’GATGACCCAGATCATGTGGT3’ and 5’AGGTCCAGACGCAGGATG3’ for β-actin. The primers for total E-NTPDase were generated to have high homology to human, rat, and mouse E-NTPDase, because the sequence of rabbit E-NTPDase has not been determined. The mRNA levels of human or total E-NTPDase and eNOS were normalized to β-actin for each sample.

**Western Blotting**

To evaluate the expression of human pE-NTPDase protein in whole FAs of stent implanted site on days 3 and 7 post stent implantation (n=3 and 4 on each day, respectively, for the BMS, gelatin-coated stent, and pE-NTPDase stent groups), western blotting was performed as previously described\(^8\) with a primary antibody against FLAG (SIGMA-ALDRICH), because there is no specific antibody to human E-NTPDase for western blotting.
Measurement of NTPDase Activity

Rabbit FAs were homogenized in Tris-buffered saline (pH 7.4) containing aprotinin and phenylmethylsulfonyl fluoride. To determine whether human pE-NTPDase protein expressed in the arteries treated with the pE-NTPDase stent exhibited normal enzymatic activity, NTPDase activity was measured by luciferin-luciferase bioluminescence assay using an ATP assay system (TOYO B-Net CO, LTD) on days 3 and 7 post stent implantation (n=3 and 4 on each day, respectively, for the BMS and pE-NTPDase stent groups). Finally, NTPDase activity in the stent implanted FAs was expressed as a ratio to the activity in the contralateral normal FA.

Histological Examination

The stent implanted FAs were fixed in 4% paraformaldehyde. To prepare cross-sections of stent-implanted FAs, fixed arteries were embedded in plastic resin (Technovit 8100, Heraeus Kulzer, Armonk) according to the manufacturer’s instructions, separated transversely into 3 parts, and cut into 5 µm sections in each part. Each section was stained with hematoxylin and eosin for histological analysis (n=4 in each group). The sections were also examined immunohistochemically with the antibodies against human pE-NTPDase (YH34)\textsuperscript{1,2}, α-smooth muscle actin (MCA1906H, AbD Serotec), and macrophages (RAM11, DAKO Japan). Additionally, part of the thrombi in the BMS and gelatin-coated stent groups were extracted, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 3 µm sections, and immunostaining for GP IIb/IIIa (Affinity Biologicals Inc) was performed.
References


**Figure legends**

Supplemental Figure I

Platelet aggregation induced by ADP (1.0 μg/ml) in rabbit platelet-rich plasma without (A) or with (B) human pE-NTPDase (1.5 μg/ml).

Supplemental Figure II

X-Gal staining of stent implanted FA sections. FAs from the LacZ stent group analyzed on days 3 and 7 exhibited strong X-Gal staining. This was not observed in the BMS or gelatin-coated stent groups.

Supplemental Figure III

Typical images of peak flow velocity at the distal site of stent implantation on day 3. In the BMS, gelatin-coated stent, and LacZ stent groups, the peak flow velocity in the stent implanted FAs is faster than in the contralateral normal FAs in cases of stenosis (A) or undetectable in cases of occlusion (B). In contrast, in the pE-NTPDase stent group, all treated FAs showed blood flow patterns similar to the contralateral normal FAs (C). Dotted lines indicate the peak flow velocity of the contralateral normal FA in each rabbit.

Supplemental Movie I

Representative angiography on day 7 indicating the occlusion of right FA implanted with the stent lacking the pE-NTPDase gene.

Supplemental Movie II

Representative angiography on day 7 showing the patency of right FA implanted with the pE-NTPDase gene-eluting stent.
Supplemental Figure I

A

% Light Transmittance

ADP

B

% Light Transmittance

pE-NTPDase

ADP
Supplemental Figure II

BMS  gelatin-coated stent  LacZ stent

day 3  day 7
Supplemental Figure III

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pE-NTPDase (-)

pE-NTPDase (+)