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ORIGINAL ARTICLE

Impaired platelet function in a patient with $P2Y_{12}$ deficiency caused by a mutation in the translation initiation codon

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Summary. In this study, we have identified a patient (OSP-1) with a congenital $P2Y_{12}$ deficiency showing a mild bleeding tendency from her childhood and examined the role of $P2Y_{12}$ in platelet function. At low concentrations of agonists OSP-1 platelets showed an impaired aggregation to several kinds of stimuli, whereas at high concentrations they showed a specifically impaired platelet aggregation to adenosine diphosphate (ADP). ADP normally induced platelet shape change and failed to inhibit PGE_1-stimulated cAMP accumulation in OSP-1 platelets. Molecular genetic analysis revealed that OSP-1 was a homozygous for a mutation in the translation initiation codon (ATG to AGG) in the *P2Y*$_{12}$ gene. Heterologous cell expression of wild-type or mutant $P2Y_{12}$ confirmed that the mutation was responsible for the deficiency in $P2Y_{12}$. OSP-1 platelets showed a markedly impaired platelet spreading onto immobilized fibrinogen. Real-time observations of thrombogenesis under a high shear rate (2000 s^{-1}) revealed that thrombi over collagen were small and loosely packed and most of the aggregates were unable to resist against high shear stress in OSP-1. Our data suggest that secretion of endogenous ADP and subsequent $P2Y_{12}$-mediated signaling are critical for platelet aggregation, platelet spreading, and as a consequence, for stabilization of thrombus.

Keywords: $\alpha_{IIb}\beta_3$, initiation codon, mutation, $P2Y_{12}$ deficiency, platelets, thrombogenesis.

Introduction

Platelets play a crucial role not only in a hemostatic plug formation, but also in a pathologic thrombus formation, particularly within atherosclerotic arteries subjected to high shear stress [1,2]. As an initial step in thrombogenesis, platelets adhere to exposed subendothelial matrices such as von Willebrand factor (VWF) and collagen, then become activated and aggregate to each other. These processes are primarily mediated by platelet surface glycoproteins such as GPIb-IX-V, $\alpha_2\beta_1$, GPVI, and $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) [3,4]. In addition, several mediators such as adenosine diphosphate (ADP), thromboxane A_2, and thrombin cause further platelet activation and recruitment of circulating platelets to the injury sites through activation of $\alpha_{IIb}\beta_3$ and subsequent binding of VWF and fibrinogen.

Recent studies have demonstrated a critical role for ADP in arterial thrombogenesis [5–7]. ADP is actively secreted from platelet dense granules on platelet activation and is passively released from damaged erythrocytes and endothelial cells. Platelets possess at least two major G protein-coupled ADP receptors that are largely responsible for platelet responses to ADP: $P2Y_1$ and $P2Y_{12}$ [6]. $P2Y_1$ is the G_q-coupled receptor responsible for mediating platelet shape change and reversible platelet aggregation through intracellular calcium mobilization [8,9], whereas $P2Y_{12}$ is the G_i-coupled receptor responsible for mediating inhibition of adenylyl cyclase and sustained platelet aggregation [10–12]. $P2Y_{12}$ is the therapeutic target of efficacious antithrombotic agents, such as ticlopidine, clopidogrel, and AR-C compounds [5,6], and its congenital deficiency results in a bleeding disorder [13,14]. The analyses of patients with $P2Y_{12}$ deficiency as well as $P2Y_{12}$-null mice would provide more precise information about the role of $P2Y_{12}$ in platelet function than those using $P2Y_{12}$ inhibitors. To date, four different families with a defect in the expression or the function of $P2Y_{12}$ have been characterized [10,13–16]. In this study, we have described a patient with the congenital $P2Y_{12}$ deficiency due to a homozygous mutation in the translation initiation codon and analyzed the role of $P2Y_{12}$ in platelet aggregation, platelet spreading onto immobilized fibrinogen, and thrombogenesis on a type I collagen-coated surface under a high shear rate. Our present data have demonstrated a crucial role of $P2Y_{12}$ in various platelet functions.

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Materials and methods

Patient history

The proband (OSP-1) is a 67-year-old Japanese female with a lifelong history of easy bruising. She (OSP-1) was born from non-consanguineous parents who had no hemorrhagic diathesis. Although she showed massive bleeding during delivery of her son, she had no history of transfusions. Patient OSP-1 showed normal platelet count, normal coagulation tests (prothrombin time and activated partial thromboplastin time) and slightly elevated plasma fibrinogen (398 mg dL^{-1}). Ivy bleeding time of the patient was consistently prolonged (> 15 min). Clot retraction by MacFarlane's method was normal (50%; normal values 40%–70%). Her son never suffered from a bleeding tendency. Informed consent for analyzing their platelet function and molecular genetic abnormalities was obtained from OSP-1, her husband and their son.

Preparation of platelet-rich plasma and washed platelet suspension

Platelet-rich plasma (PRP) for aggregation studies was prepared by a centrifugation of whole blood anticoagulated with citrate at 250 *g* for 10 min and then the platelet count was adjusted at 300×10^6 mL^{-1} by platelet-poor plasma. Washed platelets were prepared as previously described [17]. In brief, 6 volumes of freshly drawn venous blood from the patient, her husband, son or healthy volunteers were mixed with 1 volume of acid–citrate–dextrose (ACD; National Institutes of Health Formula A, NIH, Bethesda, MD, USA) and centrifuged at 250 *g* for 10 min to obtain PRP. After incubation with 20 ng mL^{-1} prostaglandin E1 (PGE_1; Sigma-Aldrich, St Louis, MO, USA) for 15 min, the PRP was centrifuged at 750 *g* for 10 min, washed three times with 0.05 mol L^{-1} isotonic citrate buffer containing 20 ng mL^{-1} PGE_1 and resuspended in an appropriate buffer.

Platelet aggregometry

Platelet aggregation using PRP was monitored by a model PAM-6C platelet aggregometer (Mebanix, Tokyo, Japan) at 37 °C with a stirring rate of 1000 r.p.m. as previously described [18]. Protease-activated receptor 1-activating peptide (PAR1 TRAP, SFLLRNPNDKYEPF) and adenosine 3′,5′-diphosphate (A3P5P) were purchased from Sigma-Aldrich Corp. $P2Y_{12}$ antagonist, AR-C6993MX (2-propylthio-D-fluoromethylene adenosine 5-triphosphate) was a kind gift from AstraZeneca (Loughborough, UK).

Flow cytometry and measurement of intracellular cAMP

Flow cytometric analysis using various monoclonal antibodies (mAbs) specific for platelet membrane glycoproteins was performed as previously described [19].

For measuring intracellular cAMP levels, samples of 200 μL of washed platelets (60×10^6) in Walsh buffer (137 mM of NaCl, 2.7 mM of KCl, 1.0 mM of $MgCl_2$, 3.3 mM of NaH_2PO_4, 3.8 mM of HEPES, 0.1% of glucose, 0.1% of BSA, pH 7.4) were incubated with 1 μmol L^{-1} PGE_1 for 15 min, and then platelets were stimulated with ADP or epinephrine. After incubation for 15 min. total cellular cAMP levels were measured using the Biotrak cAMP enzyme immunoassay system from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

Platelet adhesion assay

Adhesion study was performed as previously described [20]. In brief, non-treated polystyrene 10 cm dishes were coated with 100 μg mL^{-1} human fibrinogen in 5 mL of phosphate-buffered saline (PBS) at 4 °C overnight. After washing with PBS, dishes were blocked with PBS containing 1% of bovine serum albumin (BSA) for 90 min at 37 °C. Aliquots (1 mL) of washed platelets (25×10^6 mL^{-1}) were added to the fibrinogen-coated dishes and incubated at 37 °C. After incubation for 40 min, adherent platelets were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100 and stained with TRITC-conjugated phalloidin. Platelet morphology and degrees of spreading were determined by fluorescence microscopy (Olympus, Tokyo, Japan).

Platelet thrombus formation under flow conditions

The real-time observation of mural thrombogenesis on a type I collagen-coated surface under a high shear rate (2000 s^{-1}) was performed as previously described [21]. In brief, type I collagen-coated glass coverslips 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 a microscope (BX60; Olympus, Tokyo, Japan) equipped with epifluorescent illumination (BX-FLA; Olympus) and a charge-coupled device (CCD) camera system (U-VPT-N; Olympus). Whole blood containing mepacrine-labeled platelets obtained from OSP-1 or control subjects was aspirated through the chamber by a syringe pump (Model CFV-3200, Nihon Kohden, Tokyo, Japan) at a constant flow rate of 0.285 mL min^{-1}, producing a wall shear rate of 2000 s^{-1} at 37 °C.

Amplification and analysis of platelet RNA

Total cellular RNA of platelets was isolated from 20 mL of whole blood, and $P2Y_1$ or $P2Y_{12}$ mRNA was specifically amplified by reverse transcription-polymerase chain reaction (RT-PCR), as previously described [22]. The following primers were constructed based on the published sequence of $P2Y_{12}$ cDNA and used for the first round PCR for $P2Y_{12}$ cDNA: Y12F1, 5′-GGCTGCAATAACTACTACTTACTGG-3′ [sense, nucleotide(nt) −74 to −50]; Y12R4, 5′-CAGGACAGTGTAGAGCAGTGG-3′ (antisense, nt 85 to 105) [10].

Allele-specific restriction enzyme analysis (ASRA)

Genomic DNA was isolated from mononuclear cells using SepaGene kit (Sanko Junyaku Co Ltd, Tokyo, Japan). Amplification of the region around the initiation codon of the $P2Y_{12}$ gene was performed by using primers *Bsr*DI-GF, 5′-CTTTTGTTCTCTAGGTAACCAACAAGCAA-3′ (sense, the mismatched base is underlined), and Y12R4 (antisense described above) using 250 ng of DNA as a template. These primers can be found in GenBank accession no. AC024886.20 and the sense primer corresponds to 127558–127585. PCR products were then digested with restriction enzyme *Bsr*DI. The resulting fragments were electrophoresed in a 6% polyacrylamide gel.

Construction of $P2Y_{12}$ expression vectors and cell transfection

The full-length cDNA of wild-type (WT) and mutant $P2Y_{12}$ was amplified by RT-PCR using primers Y12-*Hin*dIII-F, 5′-GAATTCAAGCTTCAAGAAATGCAAGCCGTCGACA-ACCTC-3′ (sense, nt −6 -21 for WT, *Eco*RI and *Hin*dIII sites introduced at the 5′ end were underlined) or Y12-*Hin*dIII-F2, 5′-GAATTCAAGCTTCAAGAAAGGCAAGCCGTCGAC-AACCTC-3′ (sense, nt −6 −21 for mutant), and Y12H-Not-R, 5′-TCTAGAGCGGCCGCTCAATGGTGATGGTGATGA-TGCATTGGAGTCTCTTCATT-3′ (antisense, nt 1012–1029, His × 6 were introduced before stop codon, *Not*I and *Xba*I sites introduced at the 5′ end were underlined). The amplified fragments were digested with *Hin*dIII and *Not*I, and the resulting 1059-bp fragments (nt −9 −1050) were extracted using QIAquick gel extraction kit (Qiagen, GmbH, Germany). These fragments were inserted into the pcDNA3 (Invitrogen, San Diego, CA, USA) digested with *Hin*dIII and *Not*I. The fragments inserted were characterized by sequence analysis to verify the absence of any other substitutions and the proper insertion of the PCR cartridge into the vector.

A total of 10 μg of WT or mutant $P2Y_{12}$ construct was transfected into human embryonic kidney 293 cells (HEK293 cells, 10^6 cells) using the calcium phosphate method as previously described [22]. Transfectants were lyzed by 1% Triton X-100 PBS containing protease inhibitors 2 days after transfection, and proteins were separated by 7.5% SDS-PAGE. After transferred onto a PVDF membrane, expressed proteins were detected by rabbit anti-His tag antibody.

Results

Platelet aggregation studies

We first examined the expression of platelet membrane glycoproteins in OSP-1 by flow cytometry. The patient's platelets (OSP-1 platelets) normally express GPIb-IX, $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), $\alpha_2\beta_1$, and CD36 (data not shown). Fig. 1 shows platelet aggregation of PRP in response to various agonists. The aggregation of OSP-1 platelets induced by 20 μM of ADP was markedly impaired with only a small and transient

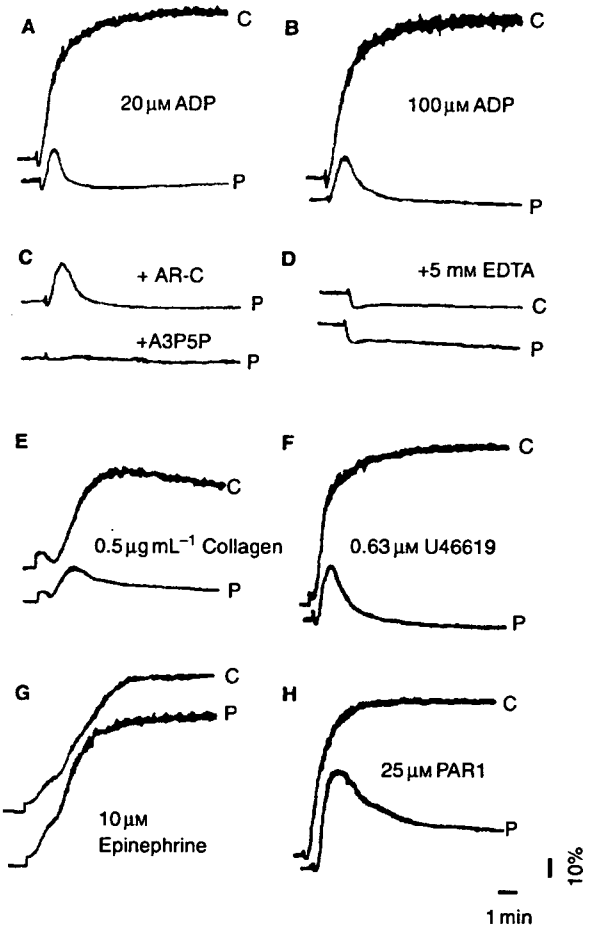

Fig. 1. Platelet aggregation induced by various agonists. Platelet aggregation was induced by various agonists in citrated PRP from patient OSP-1 (labeled 'P') or a control subject (labeled 'C'). Agonists used are (A) 20 μM of ADP, (B) 100 μM of ADP, (C) 20 μM of ADP in the presence of 1 μM of AR-C69931MX ('AR-C'), a specific $P2Y_{12}$-antagonist, or 1 mM of A3P5P ('A3P5P'), a specific $P2Y_1$-antagonist, (D) 20 μM of ADP in the presence of 5 mM of EDTA, (E) 0.5 μg mL^{-1} of collagen, (F) 0.63 μM of U46619, (G) 10 μM of epinephrine, and (H) 25 μM of PAR1-TRAP.

aggregation (Fig. 1A), and the aggregation was still impaired even at 100 μM of ADP (Fig. 1B). As compared with control platelets, the aggregation of OSP-1 platelets was also impaired with a transient aggregation in response to low concentrations of collagen (0.5 μg mL^{-1}, Fig. 1E), U46619 (0.63 μM, Fig. 1F), or PAR1 TRAP (25 μM, Fig. 1H). In response to 1.3 mg mL^{-1} ristocetin (not shown) or 10 μM of epinephrine (Fig. 1G), OSP-1 platelets aggregated normally. When OSP-1 platelets were stimulated with 20 μM of ADP in the presence of 5 mM of EDTA, the light transmission decreased equivalent to control platelets suggesting that OSP-1 platelets changed shape normally (Fig. 1D). We then examined effects of ADP receptor antagonists on the aggregation of OSP-1 platelets induced by 20 μM of ADP. A total of 1 mM of A3P5P, a specific $P2Y_1$ antagonist, abolished the residual response of OSP-1 platelets to ADP, whereas 1 μM of AR-C69931MX, a specific $P2Y_{12}$

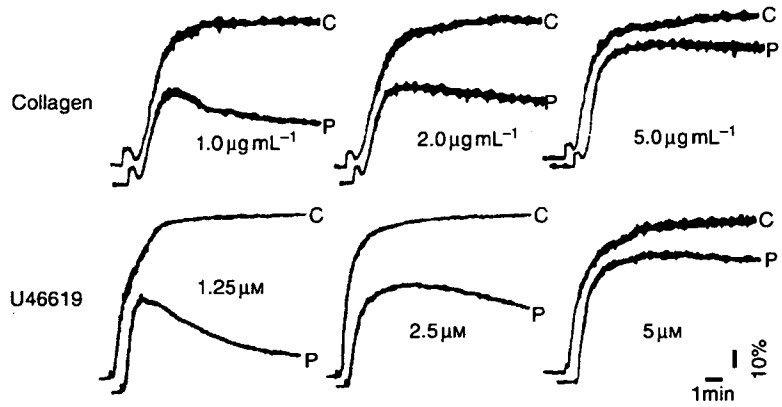

Fig. 2. Platelet aggregation induced by collagen or U46619 at various concentrations. Platelet aggregation in citrated PRP from patient OSP-1 (labeled 'P') or a control subject (labeled 'C') was induced by various concentrations of collagen or U46619. At high concentrations of collagen or U46619, OSP-1 platelets aggregate almost normally.

antagonist, did not induce an additional inhibition on the platelet aggregation (Fig. 1C). These data suggest that the impaired response of the patient's platelets may be due to an abnormality in signaling evoked by ADP and that $P2Y_{12}$-mediated signaling rather than $P2Y_1$-mediated signaling may be completely defective in patient OSP-1.

We also examined the aggregation of OSP-1 platelets induced by higher concentrations of agonists. As shown in Fig. 2, the aggregation response of OSP-1 platelets improved as the concentrations of agonists increased, and they aggregated almost normally in response to high concentrations of collagen (5 μg mL^{-1}), U46619 (5 μM), or PAR1 TRAP (100 μM) (not shown). In addition, we confirmed that 1 μM of AR-C69931MX conferred essentially the same defect on the aggregation of control platelets in response to U46619 as that of OSP-1 platelets and did not further inhibit OSP-1 platelet aggregation induced by 5 μg mL^{-1} of collagen, 5 μM of U46619, or 100 μM of PAR1 TRAP (data not shown). These data indicated that at high concentrations of agonists OSP-1 platelets showed the specifically impaired aggregation to ADP.

Effect of ADP on PGE_1-stimulated cAMP accumulation in platelets

To determine whether $P2Y_{12}$-mediated signaling is specifically impaired, we examined an inhibitory effect of ADP on 1 μM of PGE_1-stimulated cAMP accumulation in platelets from the patient, her husband, their son, and healthy unrelated controls. ADP inhibited intracellular cAMP levels in platelets from the patient's husband, son and healthy unrelated controls (not shown) by approximately 80%, whereas the inhibition was only 15% in the patient's platelets (Fig. 3). In contrast to ADP, epinephrine normally inhibited cAMP accumulation in platelets from the patient as well as her husband and son. These results strongly suggest that the defect could be due to an abnormality in G_i coupling ADP receptor, $P2Y_{12}$.

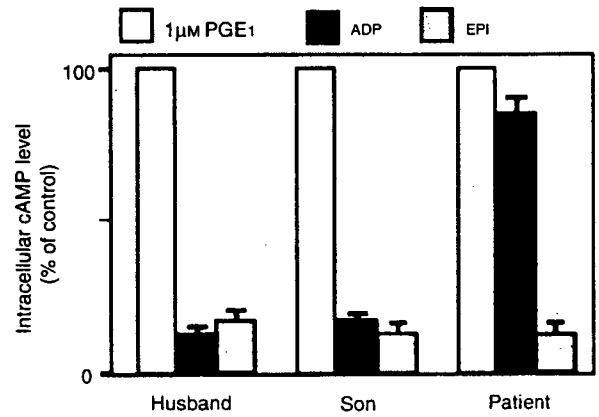

Fig. 3. Effect of ADP or epinephrine on the inhibition of PGE_1-induced cAMP accumulation in platelets. Washed platelets from patient OSP-1, husband or son were incubated with 1 μM of PGE_1 for 15 min and stimulated with 20 μM of ADP or 10 μM of epinephrine. Intracellular cAMP levels were expressed as a percent of cAMP levels in the absence of agonists. Results in OSP-1 are the mean of two experiments.

Nucleotide sequence analysis of cDNA and genomic DNA of $P2Y_{12}$

To reveal a molecular genetic defect in OSP-1, we analyzed the entire coding regions of both $P2Y_1$ and $P2Y_{12}$ cDNAs amplified from platelet mRNA by RT-PCR. A single nucleotide substitution (T → G) was identified within the translation initiation codon (ATG → AGG) in the patient's $P2Y_{12}$ cDNA (Fig. 4A). This substitution was also confirmed by reverse sequencing. No other nucleotide substitutions were detected within the coding region of either $P2Y_{12}$ or $P2Y_1$ cDNA from the patient. OSP-1 appeared homozygous for the substitution, and the substitution was not detected in 20 control subjects.

Nucleotide sequence analysis of PCR fragments from the patient's genomic DNA also suggested the homozygosity of the substitution (data not shown). To further confirm the homo-

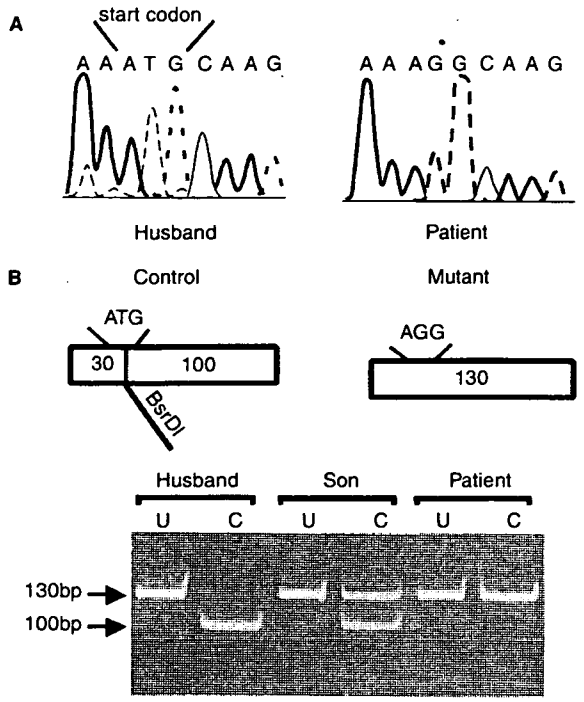

Fig. 4. Sequence analysis of $P2Y_{12}$ cDNA and restriction enzyme analysis of the $P2Y_{12}$ gene. (A) cDNA obtained by RT-PCR from platelet mRNA was analyzed by sequencing using a sense primer Y12F1. (B) PCR was performed to generate 130-bp fragments including initiation codon of $P2Y_{12}$ as described in Materials and methods. Undigested (U) or digested (C) PCR products with *Bsr*DI were analyzed on a 6% polyacrylamide gel. In patient OSP-1, the T → G mutation at position 2 abolishes a *Bsr*DI restriction site.

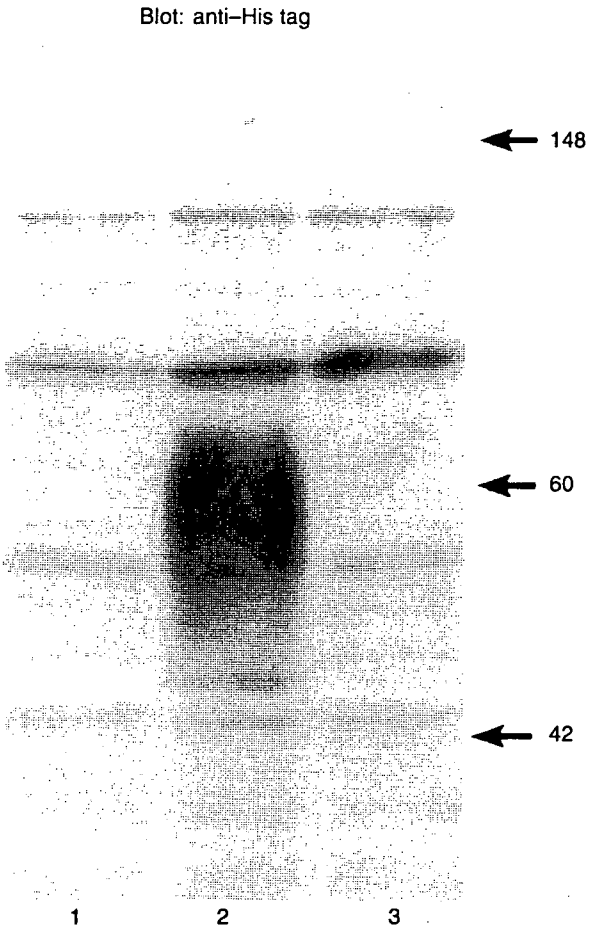

Fig. 5. Expression of $P2Y_{12}$ in HEK293 cells transfected with WT or mutant His-tag attached $P2Y_{12}$. Wild-type or mutant $P2Y_{12}$ construct was transfected into HEK293 cells using the calcium phosphate method. Transfectants were lyzed by 1% Triton X-100 PBS containing protease inhibitors 2 days after transfection. Cell lysates from mock transfectant (lane 1), cells transfected with WT $P2Y_{12}$ (lane 2) or mutant $P2Y_{12}$ (lane 3) were separated by 7.5% SDS-PAGE, and immunoblot was performed by anti-His-tag antibodies.

zygosity, allele-specific restriction enzyme analysis (ASRA) was performed. The region around the initiation codon of the $P2Y_{12}$ gene was amplified by PCR using primers *Bsr*DI-GF and Y12R4. A restriction site for *Bsr*DI would be abolished by the T → G substitution. As shown in Fig. 4B, ASRA clearly indicated that the patient and her son were homozygous and heterozygous for the substitution, respectively. These results also confirm that the substitution is inheritable.

Heterologous cell expression of WT and mutant $P2Y_{12}$

As the substitution at the translation initiation codon might induce an alternative translation starting at downstream ATGs leading to an expression of shorter form of $P2Y_{12}$, we decided to investigate effects of the substitution found in the patient on the expression of $P2Y_{12}$. Expression vectors encoding WT and mutant $P2Y_{12}$ in which His-tag was attached at the C-terminal portion of $P2Y_{12}$ were constructed as described in the Materials and methods. Wild-type or mutant $P2Y_{12}$ construct was transfected into HEK 293 cells, and then expressed proteins were analyzed 48 h after transfection in an immunoblot assay employing anti-His antibodies. As shown in Fig. 5, WT $P2Y_{12}$ protein with an apparent molecular weight of ~60 KDa was expressed in 293 cells as a His-tag-positive protein. In sharp contrast, the mutant $P2Y_{12}$-expression vector failed to express any His-tag-positive protein. These results provide strong evidence that the T → G substitution at the translation initiation codon of $P2Y_{12}$ cDNA is responsible for the $P2Y_{12}$ deficiency.

Platelet spreading on immobilized fibrinogen

As it has been well documented that release of endogenous ADP is required for full platelet spreading onto immobilized fibrinogen [23], we next analyzed the patient's platelet spreading in order to evaluate the role of $P2Y_{12}$. Control platelets adhered to fibrinogen underwent morphological changes ranging from filopodia protrusion to complete spreading, and 50.5% ± 21.3% of the adherent platelets spread ($n = 3$) (Fig. 6A). In sharp contrast, the patient's platelets showed an

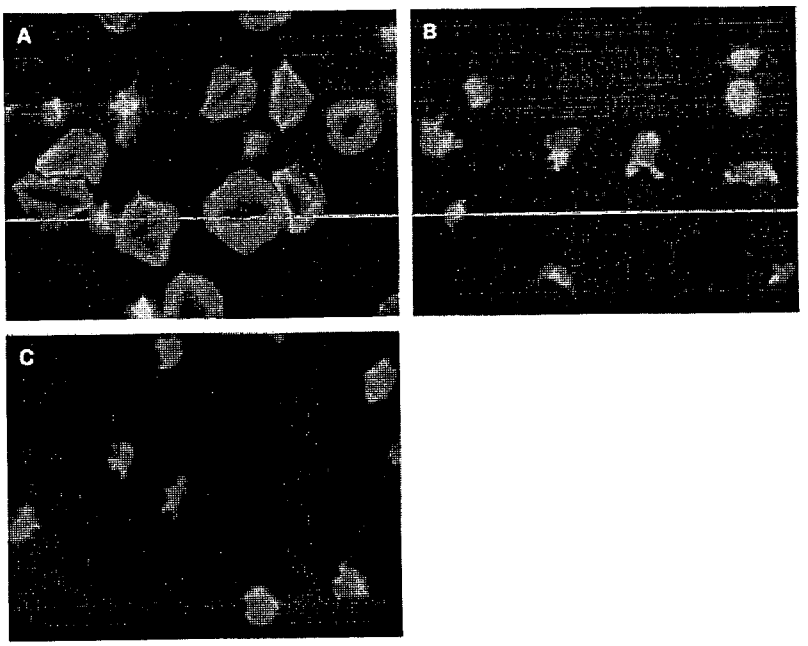

Fig. 6. Platelet spreading on immobilized fibrinogen. (A,B) Washed platelets from a control subject were applied onto fibrinogen-coated polystyrene dishes and incubated at 37 °C for 40 min without any inhibitor (A) or with 1 μM of AR-C69931MX (B). (C) Washed platelets from the patient were applied onto fibrinogen-coated polystyrene dishes and incubated at 37 °C for 40 min without any inhibitor. Adherent platelets were then fixed, permeabilized and stained with TRITC-conjugated phalloidin. Platelet morphology was analyzed by fluorescence microscopy.

impaired spreading and only 2.3% ± 1.4% of the adherent platelets spread ($n = 3$, $P < 0.001$, Fig. 6C). Similar results were obtained with control platelets in the presence of 1 μM of AR-C69931MX (6.2% ± 2.2%, $n = 3$, $P < 0.001$, Fig. 6B). In addition, 1 mM of A3P5P also markedly inhibited platelet spreading ($n = 3$, 10.1% ± 2.2%, $P < 0.001$, not shown). These results suggest that both $P2Y_{12}$ and $P2Y_1$ are necessary for platelet spreading.

Platelet-thrombus formation on immobilized collagen under flow conditions

To investigate the role of $P2Y_{12}$ in thrombus formation, we observed the real-time process of mural thrombogenesis on a type I collagen-coated surface under flow conditions with high shear rate (2000 s^{-1}) using the whole blood from OSP-1. Real-time observation revealed that thrombi formed on type I collagen were unstable. As platelet aggregates of the patient were loosely packed each other and unable to resist against high shear stress, most of the aggregates at the apex of the thrombi came off the thrombi constantly. On the other hand, most of thrombi formed by control platelets were densely packed with higher fluorescent intensity and were stable with constant growth during observation (Video 1 and 2).

As shown in Fig. 7A, the area covered with patient platelets after 7 min of flow was greater than that of control platelets (91.8% ± 0.3% vs. 82.2% ± 1.4%, $n = 3$, $P < 0.01$). However, thrombi formed by OSP-1 platelets were loosely packed, whereas thrombi were large and densely packed in controls. The overall fluorescent intensity of thrombi of OSP-1 platelets was lower than that of control platelets. Three-dimensional analysis revealed the striking difference in size and shape of individual thrombus formed after 10 min between the patient and control platelets (Fig. 7B). Thrombi formed by control platelets were large in size, clearly edged and surrounded by thrombus-free areas. On the other hand, individual thrombus formed by patient platelets was mostly small and appeared to be a thin layer of platelet aggregates. Thrombus height at the plateau phase was 10.2 ± 0.4 μm, which was less than half of controls (21.2 ± 0.4 μm).

Discussion

$P2Y_{12}$ coupled with $G\alpha_i$, primarily with $G\alpha_{i2}$, consists of 342 amino acid residues with seven transmembrane domains (TM), and its deficiency is responsible for congenital bleeding diathesis [10–16]. To date, five mutations responsible for a defect in the expression or the function of $P2Y_{12}$ in four different families have been demonstrated [10,15,16]. Patient ML possessed a mutation consisting of a two nucleotide deletion at amino acid 240 (near the N-terminal end of TM6), which would lead to a premature termination of $P2Y_{12}$ [10,14]. A two nucleotide deletions at amino acid 98 (next to the N-terminal end of TM3) and a single nucleotide deletion occurring just beyond TM3 were identified in other two families, both of which would lead to a premature termination of $P2Y_{12}$ [13,15]. However, in these reports expression studies had not been performed to show the direct association between these mutations and the $P2Y_{12}$ deficiency [10,15]. Patient AC, whose platelets expressed dysfunctional $P2Y_{12}$ with normal

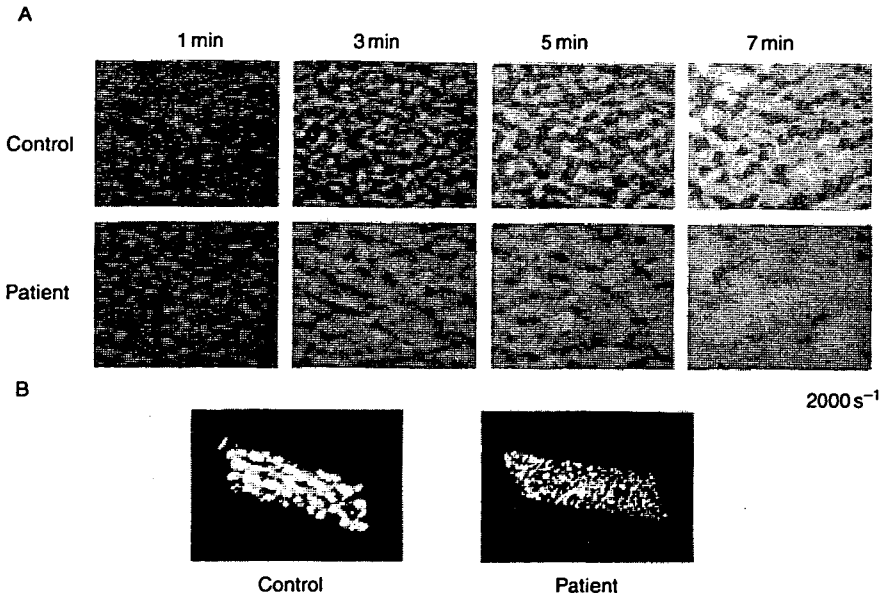

Fig. 7. Thrombus formation on immobilized collagen under flow conditions. (A) Whole blood containing mepacrine-labeled platelets obtained from the patient or control subjects was aspirated through a chamber with type I collagen-coated coverslips. Thrombi formed under a high shear rate (2000 s^{-1}) at indicated time points were observed using a microscope equipped with epifluorescent illumination and a CCD camera system. (B) Three-dimensional structure of thrombi formed after 10 min flow by platelets from the patient or a control subject was analyzed.

binding capacity of 2-methylthioadenosine 5′-diphosphate (2MeS-ADP), was compound heterozygous for Arg256 → Gln in TM6 and for Arg265 → Trp in the third extracellular loop of $P2Y_{12}$. Platelets from patient AC showed an increased platelet aggregation at high dose ADP compared with low dose ADP, suggesting the presence of residual receptor function [16]. In this study, we described a patient (OSP-1) with congenital bleeding diathesis bearing a novel homozygous mutation within the translation initiation codon (ATG → AGG) of the $P2Y_{12}$ gene. Consistent with previous studies, the aggregation of OSP-1 platelets with $P2Y_{12}$ deficiency was impaired to various agonists such as collagen, U46619, and PAR1 TRAP at low concentrations, but almost normal at high concentrations [11–14]. These findings confirmed the critical role of $P2Y_{12}$-mediated signaling evoked by endogenous ADP in platelet aggregation induced by low concentrations of agonists. In contrast to patient AC with residual $P2Y_{12}$-mediated signaling, the impaired platelet aggregation in OSP-1 in response to ADP was neither improved even at 100 μM of ADP stimulation nor reduced by adding 1 μM of AR-C69931MX, suggesting a complete loss of the $P2Y_{12}$ function. Family study confirmed that patient OSP-1 was homozygous for the mutation, and our expression study demonstrated that the mutation is responsible for the $P2Y_{12}$ deficiency.

A number of examples of mutations in the translation initiation codons have been demonstrated in various diseases [24]. Although some cases having mutations in the initiation codons did not express any related abnormal protein, Pattern *et al.* showed an abnormal $G\alpha_s$ protein possibly synthesized as a result of initiation at downstream ATGs due to a mutation at an initiation codon (ATG → GTG) in patients with Albright's hereditary osteodystrophy [24,25]. In OSP-1, we detected the T → G mutation at position +2, and our expression study denied the possibility that the substitution might induce an alternative translation at downstream ATGs leading to an expression of shorter form of $P2Y_{12}$.

As to platelet spreading onto immobilized fibrinogen, OSP-1 platelets showed the impaired platelet spreading. Similarly, A3P5P inhibited the platelet spreading. It has been well documented that release of endogenous ADP is required for full platelet spreading onto immobilized fibrinogen [23], and Obergfell *et al.* [26] have demonstrated that the platelet spreading requires sequential activation of Src and Syk in proximately to $\alpha_{IIb}\beta_3$. In contrast to the ADP-induced platelet shape change shown in OSP-1 platelets in the platelet aggregometer, our data indicated that both $P2Y_{12}$ and $P2Y_1$ were necessary for the full spreading onto immobilized fibrinogen.

Employing clopidogrel or AR-C69931 MX as an inhibitor for $P2Y_{12}$, several studies examined the role of $P2Y_{12}$ in thrombogenesis under flow conditions [27–30]. However, some discrepancy between the studies was pointed out and non-specific effects of these inhibitors were not completely ruled out [28–30]. As patient OSP-1 was deficient in $P2Y_{12}$ as shown in this study, it would be informative to examine the real-time process of thrombogenesis on a type I collagen-coated surface under a high shear rate (2000 s^{-1}) employing whole blood obtained from OSP-1. Our data demonstrated that $P2Y_{12}$-deficiency led to the loosely packed thrombus and the impaired thrombus growth with enhancing adhesion to collagen, which was consistent with the study by Remijn *et al.* [30] employing patient ML's platelets. The increase in platelet adhesion to

collagen was probably due to the impaired platelet consumption by the growing thrombi [27,30]. Moreover, our real-time observation indicated that the loosely packed aggregates were unable to resist against high shear stress, and then most of the aggregates at the apex of the thrombi came off the thrombi. In contrast, Andre *et al.* [12] did not detect significant differences in *ex vivo* thrombus volume formed over human type III collagen under a shear rate of 871 s^{-1} between $P2Y_{12}^{-/-}$ and WT mice. Although Andre *et al.* used non-anticoagulated mouse blood instead of anticoagulated blood, Roald *et al.* [27] demonstrated that clopidogrel significantly reduced the thrombus volume over type III collagen employing non-anticoagulated human blood. Loosely packed platelet thrombi with swollen non-degranulated platelets were detected following clopidogrel intake, whereas densely packed thrombi with partly fused platelets were detected before clopidogrel intake by electron microscopy [27]. Thus, it is likely that differences between human and mouse, rather than those between non-anticoagulated and anticoagulated blood, may account for the discrepancy. Nevertheless, they showed that *ex vivo* thrombi were loosely packed and that only small and unstable thrombi were formed in $P2Y_{12}^{-/-}$ mice without reaching occlusive size in mesenteric artery injury model *in vivo* [12].

Our present study has demonstrated the novel mutation responsible for the $P2Y_{12}$ deficiency and suggested that secretion of endogenous ADP and subsequent $P2Y_{12}$-mediated signaling is critical for platelet aggregation, platelet spreading, and as a consequence, for stabilization of thrombus. Mild bleeding tendency observed in patient OSP-1 further emphasizes the efficacy of $P2Y_{12}$ receptor as a therapeutic target for thrombosis.

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Supplementary material

The following supplementary material is available online at http://www.blackwell-synergy.com/loi/jth:

Figure S1. Perfusion study using control platelets. A real-time movie of platelets perfused over type-I collagen shows that thrombi formed by control platelets are densely packed and stable. This 5-second movie was taken at 5-minute perfusion under a high shear rate (2000 s^{-1}).

Figure S2. Perfusion study using OSP-1 platelets. A real-time movie of platelets perfused over type-I collagen shows that thrombi formed by the patient OSP-1 platelets are loosely packed and unstable. Newly formed aggregates on the top of thrombi keep on moving toward downstream and some aggregates came off the thrombi. This 5-second movie was taken at 5-minute perfusion under a high shear rate (2000 s^{-1}).

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Surgical Management of Distal Arch Aneurysm: Another Approach With Improved Results

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Background. Surgical treatment for distal arch aneurysm carries the risk of stroke. Although left thoracotomy has been used for repair of distal arch aneurysm as a standard approach, we have performed total arch replacement under deep hypothermia with circulatory arrest through a midsternotomy for this subset of aneurysms.

Methods. From January 1998 to February 2003, 119 patients underwent elective total arch replacement (mean age, 72.3 ± 6.0 years) for distal arch aneurysm under deep hypothermia with circulatory arrest. Antegrade selective cerebral perfusion was used for brain protection. Arch vessels were independently reconstructed using quadrifurcated grafts. Concomitant procedures included tricuspid annuloplasty in 1 patient, aortic valve operations in 2, sinotubular junction plication in 6, and coronary artery grafting in 22.

Results. The early mortality rate was 0.84% (1 of 119). The mean duration of circulatory arrest was 67.1 ± 19.7 minutes. Perioperative stroke rate was 0.84% (1 of 119). This stroke occurred 9 days postoperatively in an 81-year-old man with a history of cerebral infarction. Other complications were reexploration for bleeding in 1 patient (0.84%) and respiratory failure in 6 (5.0%).

Conclusions. This operative approach for distal arch aneurysm featured a low mortality rate and low risk of perioperative stroke. Concomitant cardiac surgery could be performed routinely in standard fashion. Distal arch aneurysms that do not involve a large segment of the descending thoracic aorta can thus be repaired with low mortality and few cerebral complications through a midsternotomy.

(Ann Thorac Surg 2006;81:1353–7)

Aneurysms that arise in the distal aortic arch and spare the innominate artery but not the left common carotid or subclavian artery can be approached through a standard left thoracotomy [1]. There is, however, a subset of distal arch aneurysms that do not involve a large segment of the descending thoracic aorta. This subset of aneurysms can be approached through midsternotomy. Although few reports are available concerning stroke after descending aortic surgery through left thoracotomy, stroke does indeed occur after surgery of the descending aorta, and is as frequent as for surgery of the ascending aorta [2]. We began to perform total arch replacement for distal arch aneurysms through a midsternotomy instead of replacement of diseased aorta through a left thoracotomy in our institution in the early 1990s, with a decrease in operative morbidity [3]. In this study, we evaluated recent surgical outcomes of total arch replacement for distal aortic arch aneurysm through a standard midsternotomy.

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Patients and Methods

Patients

From January 1998 to February 2003, 119 consecutive patients underwent elective total arch replacement for distal aortic arch aneurysm under deep hypothermia with circulatory arrest, and accounted for 64.3% of all patients (185) undergoing elective total arch replacement. Mean age was 72.3 ± 6.0 years, and there were 20 women. All patients underwent surgery on an elective basis. All aneurysms were atherosclerotic, and 50 patients (42%) had saccular type and 69 (58%) had fusiform type aneurysms.

Operative Techniques

The skin incision extended from the suprasternal notch to a point equidistant between the xiphoid process and umbilicus. To expose the left subclavian artery, a left hemicollar incision was added (Fig 1A). All operative maneuvers were performed through a midsternotomy.

The femoral artery or ascending aorta was used as a site of cannulation for arterial return. Ascending cannulation is preferable when atherosclerotic change in the ascending aorta is minimal on epiaortic echography. However, femoral arterial cannulation is used when the ascending aorta exhibits severe atherosclerotic change. Additional cannulation of the right axillary artery was employed in 97 cases (81.5%). Reperfusion and rewarming were always performed in antegrade fashion through

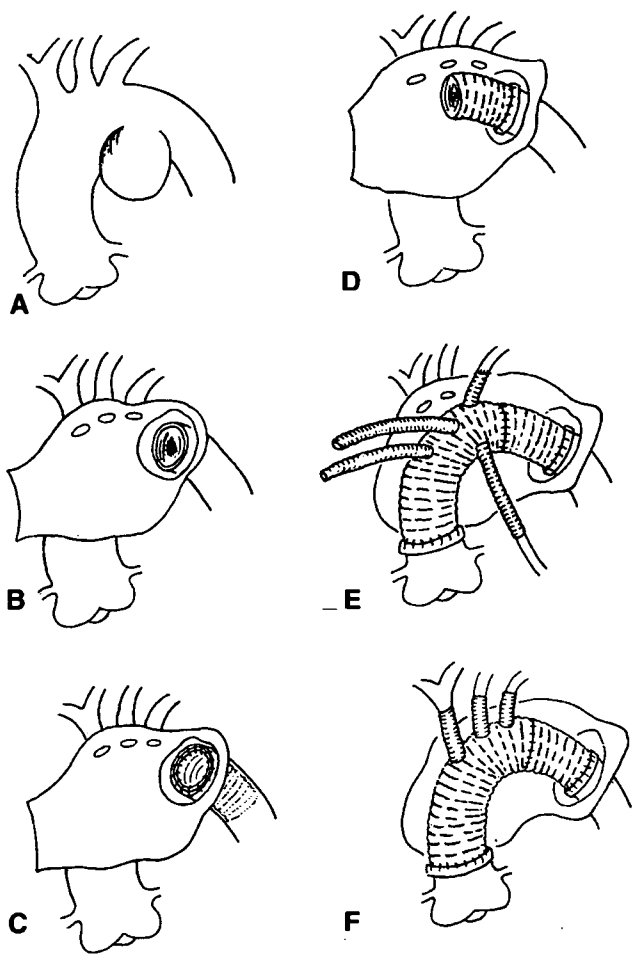

Fig 1. (A) The left subclavian artery. (B) Complete transaction of the descending aorta distal to the left subclavian artery. (C) A short graft was introduced into the lumen of the descending aorta, and then sewn to the aortic wall. (D) The short graft was pulled out of the descending aorta. (E) Quadrifurcated graft was anastomosed to the short graft, and then left subclavian artery was reconstructed. Proximal anastomosis to the ascending aorta was followed. (F) The left internal carotid artery, and the brachiocephalic artery were reconstructed.

the side branch of the quadrifurcated graft. For graft replacement, collagen woven or a gelatin-impregnated knitted Dacron (C. R. Bard, Haverhill, Pennsylvania) graft was used. Quadrifurcated graft was used for graft replacement. Arch vessels were independently reconstructed using the quadrifurcated graft, and the en-bloc repair technique was not used at all.

Open distal anastomosis was consistently performed with complete transaction of the descending aorta distal to the left subclavian artery (Fig 1B). The stepwise technique for distal anastomosis was employed in most cases. A short graft was introduced into the lumen of the descending aorta from the stump, and then sewn to the aortic wall with a running suture with 3-0 or 4-0 polypropylene (Fig 1C). The short graft was then pulled out of the descending aorta (Fig 1D). At the suture line, the graft was inverted circumferentially and fixed to the aortic wall properly. In rare cases, bleeding is observed from this suture line, but hemostasis is easily achieved with an additional stitch. Finally, quadrifurcated graft was anastomosed to the short graft with running sutures of 2-0 or 3-0 polypropylene (Fig 1E). That was followed by anastomosis of the left subclavian artery, proximal anastomosis to the ascending aorta, the left internal carotid artery, and final anastomosis of the brachiocephalic artery (Fig 1F).

Antegrade cerebral perfusion was performed with an ordinary arterial cannula in the right axillary artery, or with a balloon-tip cannula inserted directly into the brachiocephalic artery from inside the aortic arch, and in the left common carotid artery. The left subclavian artery was usually clamped, except in patients with a dominant left vertebral artery. Cerebral perfusion flow was maintained at 300 to 500 mL/min; the mean pressure in the superficial temporal arteries ranged from 40 to 60 mm Hg, and the nasopharyngeal temperature ranged from 20° to 26°C. We have, since 1997, gradually elevated hypothermc circulatory arrest temperature from 20° to 26°C for aortic arch surgery. Monitoring of perfusion pressure bilaterally in the superficial temporal arteries was performed using standard methods. Neurologic monitoring, including electroencephalography, was not performed during surgery.

Concomitant Procedures

Concomitant procedures included tricuspid annuloplasty in 1 patient, aortic valve operations in 2, sinotubular junction plication in 6, and coronary artery grafting in 22. These procedures could be performed routinely in standard fashion through a midsternotomy.

Results

The mean duration of circulatory arrest was 67.1 ± 19.7 minutes. A second pump run after weaning from cardiopulmonary bypass was required in 4 patients because of bleeding at the distal anastomosis. In these cases, hemostasis was accomplished through midsternotomy without an additional incision for left thoracotomy. The early mortality rate was 0.84% (1 of 119). This patient was a 72-year-old man with a history of myocardial infarction, and the reason for death was low output syndrome due to perioperative myocardial infarction. Perioperative stroke rate was 0.84% (1 of 119). This stroke occurred 9 days postoperatively in an 81-year-old man with a history of cerebral infarction. He had exhibited atrial fibrillation postoperatively, and atrial fibrillation was thought to have been the reason for the stroke. Seven patients (5.9%) had transient neurologic deficit, and 4 (3.4%) had hoarseness. Other complications were reexploration for bleeding in 1 patient (0.84%) and respiratory failure in 6 (5.0%). All patients who survived surgery underwent postoperative computed tomography, which revealed no problems with grafts including branches for arch vessels.

Comment

Distal arch aneurysms have been considered a subset of aneurysms of the descending thoracic aorta. Left thoracotomy has therefore been used as a standard approach for replacement of the diseased segment in patients with them. However, the distal arch can also be approached through a midsternotomy.

Stroke is a devastating complication of aortic surgery. The occurrence of it after descending aorta surgery seems somewhat counterintuitive, as the descending aorta, which is downstream from the head vessels, might not be expected to be a significant source of air or particulate matter [2]. Surgical treatment for distal aortic arch aneurysm through left thoracotomy does, however, carry the risk of stroke. Although reports on the risk of stroke after descending thoracic aneurysm repair are limited, some [4, 5] have indicated that it is 3.3% to 8.1%. Manipulation of the aortic arch for proximal control in descending aortic operations is thought to be one factor predisposing to stroke. The proximal clamp must be placed close to the subclavian artery with or without bypass or shunt in most strategies for descending aortic repair [6].

Surgery of the aortic arch also features the risk of stroke. It is usually performed under deep hypothermic circulatory arrest with or without cerebral perfusion. Techniques for brain protection have gradually improved in the last 10 years, and the rate of stroke has significantly decreased [7, 8]. Another factor possibly reducing neurologic complications is right axillary artery perfusion. Since we began using this type of perfusion, the rate of stroke in our patients has dramatically decreased. The antegradely perfused aortic flow through the axillary artery conflicts with retrogradely perfused femoral artery flow in the descending aorta. Cerebral embolism caused by retrograde femoral artery perfusion or direct insertion of a perfusion cannula from atheromatous brachiocephalic artery can be prevented with axillary artery perfusion [9]. However, because the size of the artery was only enough for cannulas of 12F to 16F in size, this axillary perfusion could not sustain total body perfusion. In the present series, the rate of stroke was only 0.8%, and the one stroke that occurred was thought to be due to postoperative atrial fibrillation. This is the lowest incidence of stroke among reports on the repair of distal arch aneurysm [10].

During the same period in our institution, 81 consecutive patients underwent descending aortic aneurysm repair under partial cardiopulmonary bypass through a left thoracotomy and 24 consecutive patients underwent descending aortic aneurysm repair under deep hypothermic circulatory arrest through a left thoracotomy. Although no paraplegia developed in either group, 2 patients (2.5%) in the former group and 3 patients (12.5%) in the latter group suffered stroke postoperatively. In fact, the rate of stroke was lower in our group, for which meticulous brain protection technique was used, than in these other groups of patients.

Distal anastomosis to the descending aorta should be performed within a limited period under hypothermic circulatory arrest. Technically, the distal anastomosis is the most strenuous part of total arch replacement performed through a midsternotomy. The suture line is sometimes quite deep from the midline, and it is difficult to place an additional stitch for hemostasis. Therefore, the anastomosis should be secured and safely performed within a limited amount of time. There have been several reports on alternative methods of exposure of the distal aortic arch for surgical intervention [11–13]. These alternatives have been introduced for distal anastomosis, which is usually difficult, especially in patients with diseased descending aorta. These approaches generally require longer incisions and cannot be considered less invasive. Another alternative is stent grafting. Sueda and colleagues [14] report that transaortic endovascular stent grafting was an effective alternative approach for distal arch aneurysm. For unclear reasons, however, it features the risk of paraplegia. Moreover, long-term follow-up data have not yet been reported for it.

Complete transection of the descending aorta for open distal anastomosis is important for preventing injury of the left recurrent nerve. We believe that the inclusion technique of anastomosis carries the risk of left recurrent nerve injury, and that complete dissection of the stump is of key importance in avoiding such injury. In this series, only 4 patients had mild hoarseness, but no serious recurrent nerve palsy followed.

We have used the stepwise technique routinely for distal anastomosis for the reasons noted above. Though the anastomosis requires an additional 10 to 15 minutes, performance of it is much easier than direct suturing even with limited exposure, and features less risk of bleeding. With this stepwise technique, no additional incision is needed for exposure, unless repair of a longer segment of the descending aorta is necessary. Only when the anastomosis is difficult with the use of this stepwise technique should an additional left thoracotomy be considered. In this series, additional left thoracotomy was not required, even though some patients required another pump run for hemostasis. The difficulty of anastomosis was appropriately evaluated preoperatively by computed tomography or magnetic resonance imaging. When the aneurysm does not extend beyond the level of the left pulmonary artery, distal anastomosis of the graft can, in our experience, be performed through a midsternotomy.

It might still be questioned whether total arch replacement for distal arch aneurysm is excessive, since the transverse arch and a part of the ascending aorta, which are not diseased, are also replaced. We believe, however, that there are several advantages to complete total arch replacement for patients with distal arch aneurysm. First, the risk of embolization as a cause of neurologic deficits can be reduced. Many patients with distal arch aneurysm have severe atherosclerotic change in the thoracic aorta, which is often widespread. We often encounter severe atherosclerotic change at the origin of the arch vessels in patients with distal arch aneurysm. Thus, the ascending and transverse aorta are, in addition to the diseased

distal arch, potential sources of embolus. Therefore, total arch replacement with a quadrifurcated graft may decrease the risk of embolization from arch vessels. Second, when the distal arch aneurysm is of saccular type, patch closure might be considered. However, because of the high incidence of pseudoaneurysm or residual aneurysm after patch repair for saccular aneurysm of the aortic arch, graft replacement of the aorta is generally recommended [15]. The third advantage is that concomitant surgery such as coronary artery bypass grafting and valve surgery are possible using the usual approaches. Twenty-two patients (18.5%) required concomitant coronary artery bypass grafting in this series, and all coronary targets were easily accessible. Most cardiovascular surgeons are familiar with the midsternotomy approach and can deal with unexpected situations during operation.

Hagl and colleagues [16] noted the superiority of the midsternal approach compared with left thoracotomy. They reported that the more respiratory disturbances resulted from approaching the aorta through a left thoracotomy than through midsternotomy. Although their report was limited to octogenarians and our series featured a slightly younger population, it may well be that elderly patients have increased risk of respiratory complications after operations performed through a left thoracotomy.

In conclusion, advanced brain protection and meticulous procedures can reduce the rate of stroke after total arch replacement for distal arch aneurysm. The operative approach we have described for distal arch aneurysm exhibits a low mortality rate and a low risk of perioperative stroke. Other cardiac operations routinely can be performed concomitantly with it in standard fashion. Distal arch aneurysm that does not involve a large segment of the descending thoracic aorta can thus be repaired with a low mortality rate and few cerebral complications through a midsternotomy.

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DISCUSSION

DR SARA SHUMWAY (Minneapolis, MN): I have just two quick questions. What do you consider a large area of involvement in the area of the subclavian artery, and also, how did you decide to cool to just 20° to 25°C for your circulatory arrest?

DR MINATOYA: You mean, not only distal arch but also including the transverse arch?

DR SHUMWAY: How do you decide whether it is a large area that is involving the descending thoracic?

DR MINATOYA: The point is a distal anastomosis; if the diseased area is included in the transverse arch, it does not matter. We can perform the operation in the same fashion. But the point of this presentation is we believe that kind of aneurysm could not be performed safely through a left thoracotomy.

As to the second question, the temperature was mainly about 20 degrees in this series, but actually we raised that to 25 to 28 degrees these days, and so far our result is not so different.

DR JOHN FEHRENBACHER (Indianapolis, IN): These are very impressive results. Would you describe in detail your technique for antegrade cerebral protection? Specifically, did you directly cannulate the arch vessels or did you rely on axillary cannulation?

DR MINATOYA: In most cases, as I mentioned in the presentation, we are using the right axillary cannulation, and as an aortic return we are using basically femoral cannulation or ascending cannulation, and we start axillary perfusion first, before ascending or femoral perfusion. After establishment of circulatory arrest, we put the cannulation on the second arch branch and third arch branch from inside. But at the time, we check the back flow from the arch branches.

DR D. CRAIG MILLER (Stanford, CA): I have faced this dilemma many times where you look at these distal arch or proximal descending aneurysms and wonder if it would be safer for the patient (mostly in terms of cerebral protection) and easier on you as well as the patient to go in from the front or the side using a thoracotomy; the correct answer is not obvious in all circumstances.

I rise to bring up something I have been teasing Dr Teruhisa Kazui about for a couple of years. I'm sure you know Dr Kazui over in Hamamatsu, who has shown us that he can even do total arch replacement for those with acute type A dissections with surprisingly good results in Japanese patients. My word of caution is that I do not believe this applies to large 90 to 100 kg lumberjacks from North America or Europe. Many of the patients we operate on in California tend to be larger and have much deeper chests; one has to very careful here in deciding which approach is best. In a thin Asian person, you can probably get down the descending thoracic aorta as far as the left main stem bronchus without too much trouble using a sternotomy, but I don't think this is true for large Western subjects.

Therefore, I am interested in the size and weight of your patients. What was the average (and range) of weight and body surface area of the patients you presented today? You may be a lot luckier than we are here in North America by operating on smaller Japanese people.

DR MINATOYA: I am sorry, I don't have precise data of the size and weight, but you are actually right. I am actually one of the biggest Japanese, and North American guys are much bigger than me, but the point of Dr Kazui's method is the standardization and the simplification. We actually do a lot of total arch replacement for these kind of patients and also total transverse aortic arch aneurysm patients, of course, for acute dissection patients. But we select every time this type of operation. So all staff, including residents, know how to do this operation. That is the point, I guess, of our good results.

DR JOSEPH BAVARIA (Philadelphia, PA): I rise to congratulate you on an incredible series. Isolated distal aortic arch aneurysms are usually very atherosclerotic, and Stanley Crawford taught us a few years back that these had very high morbidity and mortality rates. However, my observation is, for most of the CT scans that you showed in the presentation, our group would place endovascular thoracic aortic stent grafts in many of these distal aortic arch aneurysms. Your slides (CT scans) show normal middescending aortic diameters and enough of a distal arch neck proximally so that a thoracic aortic stent graft could be placed proximally, with or without a left subclavian artery transposition. So my first question is: Did you place any thoracic aortic stent grafts for these types of distal arch aneurysms? And if not, why not?

And my second comment is, I would like to propose, as we begin to write the history of thoracic aortic stent grafting for distal arch aneurysms, that this paper represent the benchmark in the stent graft community for open distal arch aneurysm repair. The stent graft operation will need to beat this number. These are very, very good results. Thank you very much.

DR MINATOYA: Thank you very much. Actually we don't do a lot of stent grafting in our institute. Stent grafting might be a good choice for this type of aneurysm, but I have no idea at this moment.

DR KIYOFUMI MORISHITA (Sapporo, Japan): You reported a high incidence of stroke using circulatory arrest through a left thoracotomy, and I performed this procedure with 0% stroke, though the number is 20 or so. And it seems to me that circulatory arrest reduced the incidence of stroke whatever approach, mid, right, or left thoracotomy. Why did you perform circulatory arrest through a left thoracotomy with a high incidence of stroke?

DR MINATOYA: Those data included older data, so these days we don't do circulatory arrest so frequently through a left thoracotomy. When we face the distal arch aneurysm extended to descending aorta, we do a two-stage operation. I mean, firstly, we do a total arch replacement with the elephant trunk, and secondly, we do a descending replacement with femoral-femoral perfusion. So we don't have to do circulatory arrest through a left thoracotomy.

DR REX STANBRIDGE (London, UK): You have a great experience with these two incision exposures, but I wonder why you do not consider using a transverse clamshell incision across the chest this way [illustrating a line drawn transversely across the lower border of the rib cage up toward both axillae], which gives extremely good exposure of the heart, the ascending aorta, the arch, the subclavian, the descending aorta right back down to the diaphragm and would eliminate some of the restrictions of the use of your procedure?

DR MINATOYA: I know there are lots of alternative incisions like you mentioned, maybe we have to try to do it. At this moment, we just need a midsternal incision, and we can see almost the distal arch perfectly, and if we need more, maybe we do, additional T incision. But we would like to try your incision too. Thank you very much.

Integrated Total Arch Replacement Using Selective Cerebral Perfusion: A 6-Year Experience

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Background. The purpose of this study was to evaluate the recent outcome of integrated total arch replacement using antegrade selective cerebral perfusion with right axillary artery perfusion.

Methods. Between 2000 and 2005, 305 patients underwent elective total arch replacement for arch or distal arch aneurysm using a Dacron (DuPont, Wilmington, DE) quadrifurcated prosthesis through a median sternotomy. There were 34 dissecting and 271 nondissecting aneurysms. Brain protection was standardized using antegrade selective cerebral perfusion with right axillary artery cannulation at 20° to 28°C. Risk factors for early mortality and neurologic complications were investigated using multivariate logistic regression analyses.

Results. The durations of hypothermic circulatory arrest, myocardial ischemia, selective cerebral perfusion, cardiopulmonary bypass, and surgery were 60.9 ± 16.8, 125.2 ± 39.3, 150.1 ± 39.0, 229.8 ± 91.4, and 466.4 ± 175.8 minutes, respectively. Seven patients died, for a 2.3% early mortality. Permanent neurologic dysfunction developed in 5 patients (1.6%), and temporary neurologic dysfunction in 20 (6.6%). The mid-term survival rate was 94.6% ± 1.5% at 3 years. On multivariate analyses, prolonged surgery was a risk factor for early mortality. Preoperative cerebral hypoperfusion was a significant determinant for temporary neurologic dysfunction and male gender for permanent neurologic dysfunction.

Conclusions. Integrated total arch replacement using antegrade selective cerebral perfusion with right axillary artery cannulation yields a favorable outcome with low mortality and cerebral morbidity rates.

(Ann Thorac Surg 2007;83:S805–10)

Total arch replacement (TAR) for arch-to-distal arch aneurysms still has surgically challenging aspects and features high mortality and morbidity [1, 2]. In particular, postoperative neurologic complications resulting in mortality or other morbidities remain prevalent, although great progress in brain protection has recently been achieved [1–7]. We have changed our technique of brain protection from retrograde cerebral perfusion (RCP) [5, 6, 8] with profound hypothermia to antegrade selective cerebral perfusion (SCP) [2–4] combined with right axillary artery cannulation [9]. In the present study, the recent outcome of integrated TAR using SCP with right axillary artery cannulation is reviewed, and relevant risk factors for early mortality and cerebral morbidity are examined.

Patients and Methods

Patients

A retrospective review was performed of 305 patients (238 men) undergoing elective TAR for arch or distal arch aneurysm between 2000 and 2005 in the National Cardiovascular Center, Osaka, Japan. Median patient age was 73 years (range, 52 to 87 years). There were 34 dissecting and 271 nondissecting aneurysms. The cause of the aneurysm was atherosclerotic in 289 patients, non-Marfan degenerative in 10 patients, Marfan in 2 patients, and aortitic (including Behçet disease) in 4 patients. Ten patients requiring reoperative surgery were included. Also included were 18 patients with extensive thoracic aortic aneurysm involving the arch; for them, two-stage surgery was performed with stage I TAR, followed by stage II descending aortic replacement in 12 patients and endoluminal stent grafting in 6 older patients with respiratory dysfunction. Institutional approval for this study was obtained, and each patient in the study gave informed consent to serve as a subject.

Surgical Techniques and Brain Protection

All aneurysms were approached through a median sternotomy and replaced using a quadrifurcated Dacron (DuPont, Wilmington, DE) prosthesis with open distal anastomosis.

1. Cardiopulmonary bypass (CPB) establishment with right axillary artery perfusion. In the last decade, we have refined surgical techniques, including strategies of CPB, brain protection, and lowest core temperature (Fig 1). Since 2000, right axillary artery perfusion has been routinely used in conjunction

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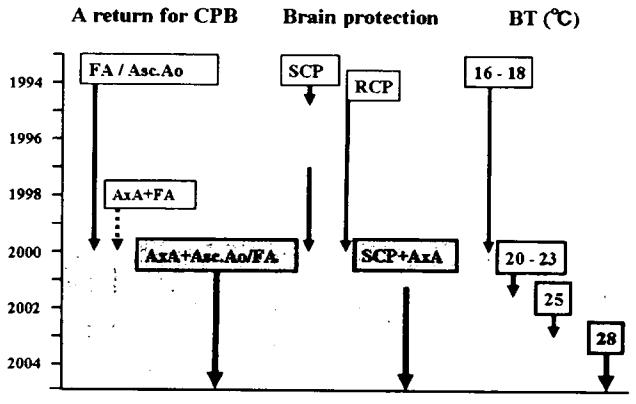

Fig 1. Refinement of cardiopulmonary bypass (CPB), brain protection, and body (core) temperature (BT). (FA = femoral artery; Asc.Ao = ascending aorta; AxA = axillary artery; SCP = antegrade selective cerebral perfusion; RCP = retrograde cerebral perfusion.)

with femoral artery or ascending aorta cannulation for CPB to prevent cerebral emboli caused by retrograde femoral artery perfusion and for quick and easy conversion to SCP (Fig 2) [9].

The right axillary artery is exposed through a 5-cm to 7-cm incision in the right armpit. After full heparinization, a 10F to 16F straight thin-walled cannula is inserted into the right axillary artery. Empirically, 12F cannulae produce up to 1500 mL/min flow for CPB. Right axillary artery perfusion is initiated to prevent cerebral emboli before femoral artery perfusion. Bicaval venous drainage with left ventricular venting is routine.

2. Brain protection using SCP with right axillary artery perfusion. Before 2000, RCP with deep hypothermia at 16° to 18°C was predominantly used for brain protection (Fig 1) [5, 6]. Since the beginning of 2000, we have routinely used the current method of SCP combined with right axillary artery perfusion [9]. After induction of hypothermic circulatory arrest (HCA), SCP through right axillary artery perfusion is commenced by clamping the brachiocephalic (innominate) artery. The transverse arch is opened, and a 12F SCP balloon-tipped cannula is inserted from within the aorta into the left common carotid artery.

   Between 2000 and 2002, SCP was instituted by right axillary artery and left common carotid artery perfusion, with the left subclavian artery clamped, at 20° to 23°C. During SCP, the bilateral superficial temporal artery pressures or the pressures of the balloon tips were monitored and maintained in the range of 30 to 50 mm Hg. Subsequently, SCP flows were about 10 mL/(kg · min), generated by a single roller pump separate from the systemic circulation (Fig 2). In 2003, left subclavian artery perfusion using another balloon-tip cannula was added, and the lowest core temperature was gradually increased to 25° or 28°C. SCP flow is also increased to maintain perfusion pressure between 50 and 70 mm Hg (800 to 1200 mL/min). Cardiac arrest is induced by antegrade and retrograde cardioplegia.
3. Stepwise distal anastomosis (Fig 3). Through the aneurysm, the proximal descending aorta distal to the aneurysm is transected completely to prevent phrenic and vagal nerve injury. Open distal anastomosis is performed during HCA of the lower half of the body. Before 2000, a quadrifurcated arch graft was directly anastomosed to the descending aorta with 3-0 or 4-0 polypropylene continuous suture. Since 2000, stepwise distal anastomosis has been used for an easy and secure anastomosis [10, 11] with routine right axillary artery perfusion.

In this original technique, an invaginated tube graft (7-cm to 12-cm long) composed of the main arch graft is initially inserted into the descending aorta. We have recently refined our stepwise technique to reinforce the anastomosis and prevent bleeding (Fig 3). In making the stepwise graft, 2 to 3 cm of the proximal end is left without invagination to reinforce the anastomosis from the inside, using a sandwich technique with a Teflon (DuPont, Wilmington, DE) felt strip. We call this the mini-elephant trunk technique. The proximal end of the graft is anastomosed to the descending aorta using an over-and-over running suture, with outside reinforcement with a strip of Teflon felt. After the anastomosis, the distal end of the inserted graft is extracted proximally. Debris is flushed from the descending aorta by femoral artery perfusion. The main arch graft is connected to this short interposed graft end using a 3-0 polypropylene running suture.

Systemic circulation is resumed using a branch of the arch graft. The left subclavian artery is initially reconstructed using a branch graft, and the patient is slowly rewarmed to 30°C. The proximal aortic anastomosis follows, above the sinotubular junction, using 4-0 polypropylene running suture. Coronary circulation is initiated by unclamping the aorta. The other two arch vessels are finally reconstructed with branch grafts.

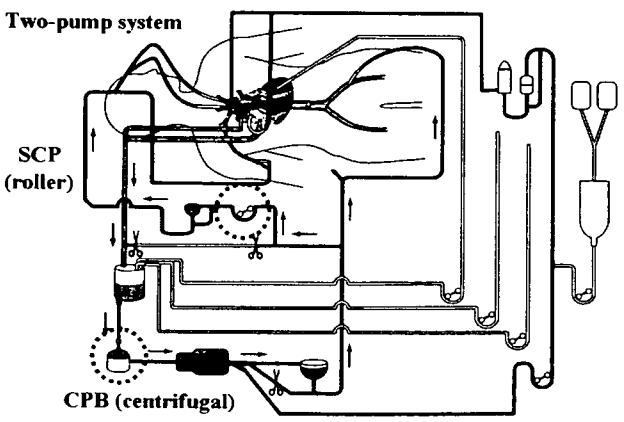

Fig 2. A two-pump system for cardiopulmonary bypass (CPB) and antegrade selective cerebral perfusion (SCP).

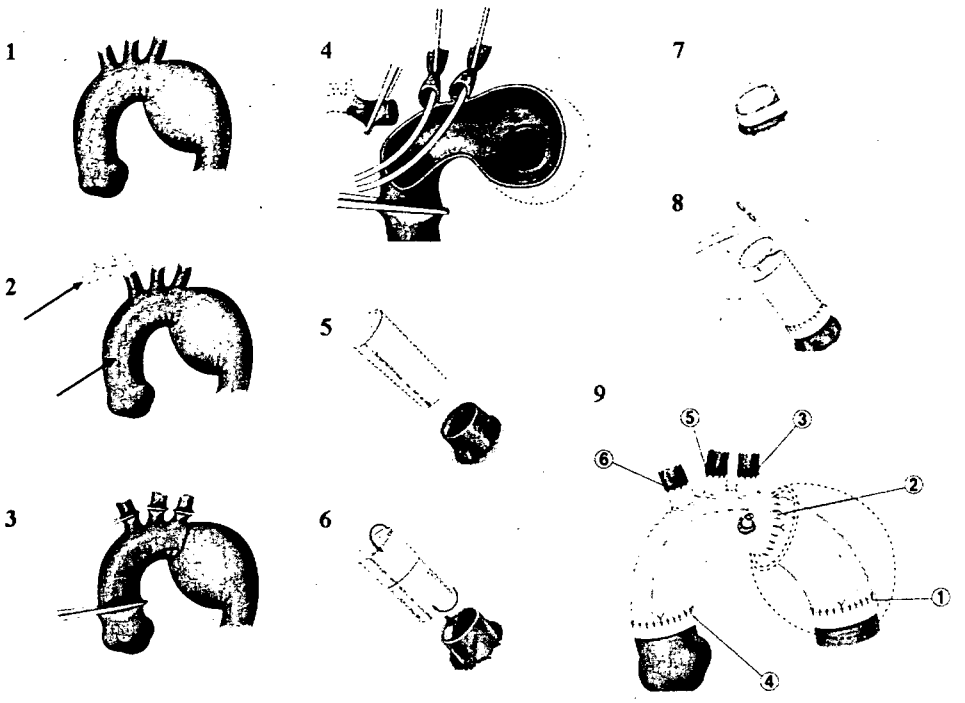

Fig 3. Integrated total arch replacement using antegrade selective cerebral perfusion with right axillary artery perfusion. (1) Arch or distal arch aneurysm. (2) Ascending aorta and right axillary artery cannulation for cardiopulmonary bypass. (3) Induction of hypothermic circulatory arrest with the arch vessels clamped. (4) Selective cerebral perfusion with right axillary artery, left common carotid artery, left subclavian artery perfusion. (5) Original stepwise technique. (6) Refined stepwise technique with mini-elephant trunk. (7) Distal anastomosis. (8) Graft-to-graft anastomosis. (9) Total arch replacement using quadrifurcated graft. Circled numbers show the turn of the anastomosis.

Definition of Neurologic Deficits and Other Variables

Permanent neurologic dysfunction (PND) was defined as the presence of permanent neurologic deficits either focal or global in nature, persisting at discharge. Transient neurologic dysfunction (TND) was defined as the occurrence of postoperative confusion, agitation, delirium, or prolonged obtundation [12]. Cerebral hypoperfusion was defined as the preoperative presence of more than 50% stenosis of the arch vessels or the intracranial vessels, or both, on echo or magnetic resonance angiography study, or hypoperfusion on acetazolamide-loading cerebral flow scintigraphy.

Data Collection and Statistical Analysis

Medical records were reviewed for clinical variables including preoperative status, intraoperative data, postoperative complications, and mid-term survival. Follow-up was 100% complete. The mean follow-up period was 31.0 ± 19.6 months. We retrospectively reviewed the overall outcome of TAR and investigated risk factors for hospital mortality and cerebral morbidity by multivariate logistic regression analyses.

Statistical analysis was done with SPSS software (SPSS Inc, Chicago, IL). Values are expressed as the mean ± standard deviation or medians (range), with values of $p < 0.05$ considered significant. Logistic regression was used to investigate risk factors for mortality and cerebral morbidity. Kaplan-Meier estimates were used to calculate survival rates.

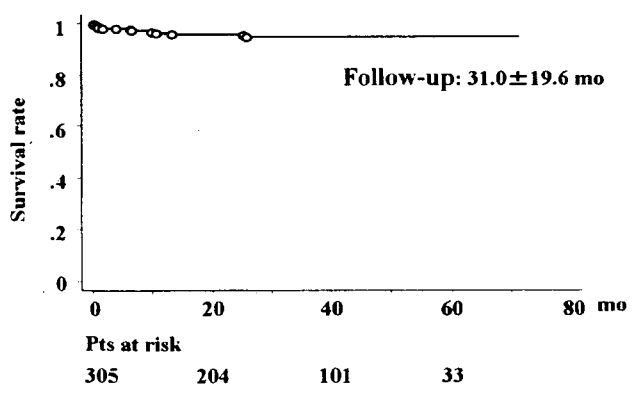

Fig 4. Mid-term survival (Kaplan-Meier estimates).

Results

The mean of the lowest nasopharyngeal temperature was 23.1° ± 3.7°C. The durations of hypothermic circulatory arrest for open distal anastomosis, myocardial ischemia, SCP, CPB, and surgery were 60.9 ± 16.8, 125.2 ± 39.3, 150.1 ± 39.0, 229.8 ± 91.4, and 466.4 ± 175.8 minutes, respectively. No patients died within 24 hours after surgery; however, 6 patients (1.9%) died within 30 days after surgery from low output syndrome in 1 patient, respiratory failure due to pulmonary bleeding in 1, sepsis (mediastinitis) in 2 [1] patients, and bowel necrosis in 2 patients. Four months after surgery, another 84-year-old patient died from sepsis caused by stent graft infection after a second procedure that followed TAR during the same hospitalization. Thus, there were 7 (2.3%) early deaths. PND developed in 5 (1.6%) patients and TND developed in 20 (6.6%). The mid-term survival rate was 94.6 ± 1.5% at 3 years (Fig 4).

On multivariate logistic regression analyses, prolonged surgery was the only risk factor for early mortality (odds ratio [OR], 1.011, $p = 0.027$). Risk factors for TND were

preoperative cerebral hypoperfusion (OR, 0.204, p = 0.014) and for PND, male gender (OR, 0.011, p = 0.017). Cerebral hypoperfusion (OR, 0.360, p = 0.049) and male gender (OR, 0.267, p = 0.030) were predictors of any neurologic complication, including both TND and PND.

Comment

Great advances in TAR commenced with the induction of RCP with profound HCA in 1993 [5, 6, 8]. This widely accepted adjunct improved overall outcome remarkably [12–16]. However, many patients still sustained TND, especially after prolonged HCA even with RCP [5, 6, 13–16], although Okita and colleagues [5] at our institute reported no difference. Reich and colleagues [17] reported that profound HCA exceeding 25 minutes and advanced age were associated with memory and fine motor deficits and prolonged hospital stay. The incidence and severity of TND correlates with poor performance on neuropsychologic testing, which predicts continued deficits in memory and motor function [18]. To decrease TND, we switched our strategy for brain protection from RCP to SCP, which is basically physiologic and has a longer cerebral safety margin. Since the beginning of 2000, we have consistently performed SCP for brain protection [9].

With SCP, however, atheromatous cerebral emboli remain a major concern. A variety of embolic phenomena are caused by systemic CPB perfusion through the ascending aorta across the arch aneurysm or by retrograde femoral artery perfusion. Arch vessel cannulation also carries a risk of cerebral embolization [17, 18]. For these reasons, an alternative perfusion pathway using the right axillary artery has been routinely used for both CPB and SCP [9].

The right axillary artery can easily be exposed and cannulated and is less atherosclerotic than the arch. Unfortunately, in Japanese patients, particularly small women, the axillary artery is too small to accept larger-size cannulae, so that additional cannulation through the femoral artery or the ascending aorta is necessary for systemic CPB flow. Femoral artery cannulation was previously performed. Retrograde femoral artery perfusion was useful not only for flushing out debris in the descending aorta but also to check for bleeding from the key distal anastomosis in the descending aorta.

The combination of right axillary artery and femoral artery perfusion was used for approximately 3 years, between 2000 and 2002, and we have previously reported its advantages [9]. With this combination, downstream flow through the right axillary artery can compete with retrograde femoral artery perfusion in the descending aorta and may prevent cerebral emboli. However, in the absence of atherosclerosis, ascending aorta cannulation, which yields antegrade CPB flow, has become our first choice rather than femoral artery perfusion [19].

We believe that right axillary artery cannulation is of use even with ascending aorta cannulation because the switch from systemic perfusion by way of CPB to SCP is easy, with no discontinuity, and cannulation-induced emboli from the brachiocephalic artery can be avoided. We have therefore been able to increase core temperature to 28°C [20]. If the ascending aorta is atherosclerotic, however, the femoral artery is chosen as an alternative site of cannulation.

We have thus continued integrated TAR using SCP with right axillary artery perfusion in the last 6 years. In this study, the early and mid-term outcomes were reviewed, with assessment of significant determinants for mortality and cerebral morbidity. Our integrated TAR yielded a satisfactory 2.3% early mortality rate, even with difficult surgery including a two-stage repair of extensive thoracic aortic aneurysms involving the arch, and even for elderly individuals with a median age of 73 years. The current outcome is comparable or superior to those described in previous reports [21–23].

Multivariate analysis demonstrated that only prolonged surgery was an independent determinant of early death. In contrast, Kazui and colleagues [3] reported that chronic renal failure, long CPB time (>300 minutes), participation in an early series, and shock were risk factors for death in their first study, and PND in a more recent study [4]. No other factors such as age, coexisting coronary artery disease, reoperative surgery, dissection, neurologic comorbidities, postoperative neurologic complications, or concomitant surgery including coronary artery bypass or root surgery were significant predictors of early mortality.

Preoperatively, 30.2% of the patients had coexisting coronary artery disease. The strategy for treatment of coronary artery disease associated with arch aneurysms is still controversial. Cardiologists tend to suggest safer catheter intervention before TAR because TAR has a high mortality risk and most patients who need TAR are old. Conversely, prompt performance of combined TAR with coronary artery bypass grafting (CABG) is recommended by surgeons because CABG is can be performed concomitantly with a low risk. In the present series, TAR and CABG were performed together in 62 patients, 3 of whom died, yielding a mortality rate of 4.8%. The causes of death were unrelated to coronary artery disease: bowel necrosis in 2 patients and mediastinitis in 1. We therefore believe that use of combined TAR with CABG is justified.

Ergin and colleagues [12, 18] reported that postoperative cerebral morbidity after TAR remains a major problem resulting in mortality and serious morbidity. The present study did not yield similar results. Multivariate analysis revealed preoperative cerebral hypoperfusion—including old cerebral infarction—to be a risk factor for TND, although it was not an independent predictor for PND. This finding was expected. TND is considered to be due to cerebral hypoperfusion during CPB, or HCA with selective or retrograde cerebral perfusion [12]. Conversely, most strokes are considered due to embolism originating from hematoma or atheroma in the aorta or in the arch vessels [12]. For patients with cerebral hypoperfusion before surgery, our strategies were modified to include higher CPB perfusion pressure (>60 mm Hg), more profound hypothermia (20° to 22°C), and higher SCP flow rates, with increases in SCP pressure of 20% to

30%. These refinements yielded good outcomes empirically.

On multivariate analysis, male gender was the only significant determinant of PND. As mentioned, the cause of PND is believed to be emboli caused by atheroma in the ascending aorta/aortic arch and in the arch vessels, particularly under well-established brain protection, whether in conjunction with RCP or SCP [12]. In the present series, 78.0% of the 305 patients were men, and all patients who sustained PND were men. Male gender is widely considered a risk factor for atherosclerotic change in all arteries. This finding was therefore also expected.

The degree of atheromatous change was not assessed in this study, although atherosclerotic aneurysm was evaluated as a potential risk factor. It is of interest that the Mt. Sinai group [7] assessed clot/atheroma as a risk factor for adverse outcome including cerebral complications; however, it is difficult to quantitatively evaluate the degree or amount of atheromatous lesions.

For patients with severe atheromatous lesions in the arch vessels or their orifices, adequate brain protection technique is still controversial. RCP has been criticized because prolonged HCA results in cerebral morbidity [5, 6, 13–15], whereas SCP theoretically has a longer cerebral safety margin [2–4]. However, SCP—requiring cannulation of the arch vessels—has the potential for cerebral embolism, which has been considered its worst shortcoming [8, 12].

In our strategy involving right axillary artery cannulation, we can avoid cannulation of the brachiocephalic artery, which sometimes exhibits atheromatous change. Fortunately, the left common carotid artery is generally less atheromatous, making its cannulation easy and safe. Cannulation of the left subclavian artery is sometimes dangerous because it often has the most severe atheromatous changes of the three arch vessels. In these situations, cannulation must be carefully performed, or patients must be cooled to below 22° to 23°C and the left subclavian artery left uncannulated.

Atheroma in the ascending aorta and the aortic arch is another potential source of emboli to the brain. Atheromatous change in the ascending aorta and the arch should be assessed by preoperative computed tomography or epiaortic echo examination, and ascending aortic cannulation for CPB should be carefully performed. The impact of right axillary artery perfusion on cerebral safety in TAR has been elucidated in this study.

In addition, since 2000 we have used a unique stepwise technique for easy and secure anastomosis [11, 12]. With this technique, distal anastomosis has become much easier, with good exposure of the anastomosis site even in difficult aneurysms extending distally. In TAR through a median approach, the distal anastomosis is a key aspect. One shortcoming of the stepwise anastomosis is the need for another graft-to-graft anastomosis, but this normally takes only 5 to 10 minutes. Another is the possibility of dislodgement of atheroma in the descending aorta. One patient who experienced bowel necrosis a few days after surgery required resection of the gut. However, we believe the stepwise anastomosis technique is useful and safe overall, particularly for aneurysms extending distally, and consequently will improve the overall outcome of integrated TAR with SCP and right axillary artery perfusion. The recent outcome of integrated TAR using SCP with right axillary artery cannulation was satisfactory, with low hospital mortality and cerebral morbidity rates.

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