in vivo *24*: 635-640 (2010)

Thrombogenicity of Sirolimus-eluting Stents and Bare Metal Stents: Evaluation in the Early Phase after Stent Implantation

THOMAS WALTER[1], KATJA S. REY[2], HANS PETER WENDEL[3], SEBASTIAN SZABO[4], TIM SUSELBECK[1], CARL-ERIK DEMPFLE[1], MARTIN BORGGREFE[1], STEFANIE SWOBODA[5], MARTIN E. BEYER[2] and HANS MARTIN HOFFMEISTER[4]

[1]*University Medical Centre Mannheim, First Department of Medicine, 68167 Mannheim, Germany;*
[2]*Klinik Kirchheim, Department of Internal Medicine II, 73230 Kirchheim, Germany;*
[3]*Clinic for Thoracic, Cardiac and Vascular Surgery, University of Tuebingen, 72076 Tuebingen, Germany;*
[4]*Staedtisches Klinikum Solingen, Department of Internal Medicine II, 42653 Solingen, Germany;*
[5]*Pharmacy Department of the University Hospital of Heidelberg, 69120 Heidelberg, Germany*

Abstract. *Background: Thrombogenicitiy of drug-eluting stents is a matter of controversial debate. The aim of this study was to evaluate the thrombogenicity of sirolimus-eluting stents (SES) compared to bare metal stents (BMS) in a standardised in vitro model. Materials and Methods: Nine SES and nine BMS were implanted in tubing loops and nine loops without stent served as controls. Initially and after 90 minutes of blood circulation in a modified chandler loop model, thrombin-antithrombin III complex (TAT), PMN-elastase, factor XIIa, SC5b-9, sP-selectin and platelet count were measured. Expression of CD62P, CD45/41 and PAC-1 on platelets were determined by flow cytometry. Results: After 90 minutes, platelet count decreased significantly in the loops with BMS and SES ($p<0.05$). Levels of TAT, PMN-elastase and SC5b-9 were significantly elevated after 90 minutes in all loops ($p<0.05$). sP-selectin significantly increased in the loops with BMS and SES after 90 minutes. No significant changes occurred in any flow cytometric data. Platelet count, sP-selectin, TAT, PMN-elastase, SC5b-9, CD62P, CD41/CD45 and PAC-1 showed no significant difference between BMS and SES. Conclusion: These data provide evidence that there is no difference in thrombogenicity of BMS and SES in the in vitro model.*

Correspondence to: Thomas Walter, MD, University Medical Centre Mannheim, First Department of Medicine (Cardiology), Theodor-Kutzer-Ufer 1-3, D-68167 Mannheim, Germany. Tel: +49 621383 3050, Fax: +49 62213832172, e-mail: thomas.walter.med@umm.de

Key Words: Drug-eluting stent, hemostasis, platelet activation, stent thrombosis.

Coronary stenting is routinely performed in more than 80% of percutaneous coronary intervention. Although the use of drug-eluting stents (DES) is associated with significantly lower rates of in-stent stenosis than bare metal stents (BMS), early and late stent thrombosis is still an unsolved problem (1-4). Sirolimus, a macrocyclic lactone that blocks cytokine-proliferation of T-lymphocytes, and similar drugs are used in DES (1). Although sirolimus reduces re-stenosis by inhibiting neointimal hyperplasia, it also delays the healing process (2). Incomplete endothelialisation of the stent struts is one of the reasons for late stent thrombosis (2, 3). The incidence of stent thrombosis after drug eluting stent (DES) implantation has been reported to range between 0 and 2.7% (2, 4). To reduce the rates of early and late stent thrombosis, a prolonged dual antiplatelet therapy with aspirin and clopidogrel is recommended (5). Premature discontinuation of antiplatelet therapy appears to predispose to stent thrombosis (3).

Beside the incomplete or late endothelialisation, other causes for stent thrombosis, such as hypersensitivity reactions associated with drug elution, or thrombogenic complications associated with other modulators of fibrointimal proliferation have been discussed (2, 6). Thrombocytopenia as a classical side-effect has been frequently associated with the use of sirolimus (7). Moreover, platelet-independent defects in haemostasis associated with sirolimus have been reported, such as impaired fibrin formation or impaired fibrinolysis (8). Sirolimus significantly potentiates agonist-induced platelet aggregation in a time- and dose-dependent manner (9). Therefore, the debate on whether the sirolimus-eluting stents (SES) are more thrombogenic compared with BMS is ongoing (10-17). Stent thrombosis seems to result from a series of complex interactions involving the presence of a thrombogenic surface and the activation of platelets or

coagulation proteins (18). Coating of a stent with a polymer and sirolimus may enhance thrombogenic properties of the stent, by activation of platelets and coagulation proteins.

The aim of this study was to evaluate the thrombogenicity of SES compared with BMS by investigating their effects on inflammation markers, haemostasis, complement activation and platelet activation. A standardised *in vitro* model was used to simulate the early phase after stent implantation.

Materials and Methods

The experiments were performed by using a modified Chandler loop system as described previously (18). The rotating loops result in passive blood circulation to avoid possible cell damage and coagulation activation typical of an actively pumping system. The rotation speed was 15 rpm which resulted in a flow velocity of 12.5 cm/s. Heparin-coated loops were used (Carmeda; Medtronic, Netherlands) to minimise the coagulation activating effects of the tubing surface. The total length of the closed tubing was 50 cm. The temperature was maintained at 37˚C by a water bath. Nine BMS (BX Velocity; Cordis, the Netherlands) and nine SES stents (Cypher; Cordis) were placed in polyvinyl chloride tubing loops. The total length of each stent was 23 mm with a diameter of 3.5 mm. Nine additional loops were kept plain to serve as controls.

Blood was obtained from nine volunteers (men, aged 22-35 years). All of them were apparently healthy non-smokers and claimed to not have taken any drugs in the 14 days before blood sampling. Blood was taken by venous puncture using 10 ml polypropylene sample tubes containing a final concentration of 1 IU of heparin (Liquemin N25000; Roche, Germany) per ml of blood. The blood of each donor was used for three loops (one loop without stent (Ctr), one loop with BMS (BMS) and one loop with SES (SES)).

Samples for analysis were drawn into 3 ml tubes containing citrate, 2.7 ml tubes containing EDTA, 2.7 ml tubes containing heparin (Sarstedt, Germany) and 4.5 ml tubes (Becton & Dickinson, Belgium) containing CTAD. Similar samples were drawn from the Chandler loops after 90 minutes of circulation. Measurement of platelet count and flow cytometry were performed immediately. The tubes containing citrate were centrifuged at 3000 ×*g* for 20 min. The separated plasma was then snap frozen and stored at –20˚C until analysis.

Platelet count was assessed by a cell counter (Axon Lab AG, Switzerland). Quantitative detection of serum concentrations of sP-selectin was performed using an ELISA technique employing a commercially available assay kit (R&D Systems, Germany). Determination of thrombin-antithrombin III complex (TAT) was performed using an enzyme-linked immunosorbent assay (Enzygnost TAT micro; Dade Behring, Germany). For determination of PMN-elastase, an enzyme immunoassay (Merck, Germany) was used. SC5b-9 was also determined with an enzyme immunoassay (Quidel, USA). Determination of factor XIIa was performed using an ELISA-test in sandwich technique (Axis-Shield, Scotland).

To visualise microscopic changes on the stent surfaces after blood contact, electron microscopic scanning was performed. One BMS and one SES was left without blood contact for scanning electron microscopy and two BMS and two SES were scanned after 90 min of blood circulation.

Surface expression of platelet receptors was determined by flow cytometry on a FACScan cytometer (Becton Dickinson [BD], Heidelberg, Germany) with labelled monoclonal antibodies. Heparinised blood was diluted with 980 μl of modified Tyrode's buffer (150 mM NaCl, 2.5 mM KCl, 12 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 1 mg/ml glucose). Fifty μl of this dilution (1:50) were put in Eppendorf tubes. The corresponding antibody was then added to each sample (5 μl fluorescein isothiocyanate (FITC)-labelled antibody against CD41 (Immunotech, Marseille, France), 5 μl phycoerythrin (PE)-labelled antibody against CD45 (BD), 10 μl FITC-labelled antibody against P-selectin (CD62P) (Immunotech), 2 μl FITC-labelled antibody against PAC-1 (BD) and incubated at room temperature for 30 min. After incubation, 500 μl of Cellfix (BD, Erembodegem, Belgium) was added for fixation. Flow cytometric measurement was performed within 6 h after fixation. A total of 10000 events were counted in each measurement. Objects positive for CD41-binding were distinguished regarding their forward scatter (size) properties into a region depicting platelets bound in aggregates and another region with single platelets. Fluorescence of CD45 antibodies was analysed on aggregate-bound platelets and leucocytes. Platelet expression of PAC-1 and CD62P was assayed by selecting the single platelet population. Single platelets were detected regarding their forward and sideward scatter characteristics and FITC fluorescence. Results are expressed as the percentage positivity (i.e. the percentage of platelets positive for the antibody)

Statistical analysis was performed by the statistical software package JMP (SAS Institute Inc., NC, USA). Data are presented as mean values±standard error of mean. Non-normally distributed data were converted to a logarithmic scale for normal distribution before statistical analysis. All comparisons were made using a multifactorial analysis of variance, followed by a Tukey-Kramer test for the comparison of all pairs. *p*-Values of <0.05 were regarded as significant.

Results

Soluble plasma markers. After 90 minutes, a trend towards lower platelet count was found in the loop without stent (Ctr). A significant decrease in platelet count was detected in the loop with BMS ($p<0.05$) and in the loop with SES ($p<0.05$) after 90 min of blood circulation, whereas no significant change was found between the two stent types. There was also a strong trend towards lower platelet counts in the loops with the stents compared with the control loop (Figure 1).

Significantly elevated TAT levels, PMN-elastase values and SC5b-9 levels were observed after 90 minutes of blood circulation in the control loop, the BMS and SES loop ($p<0.05$) compared to the initial values. The highest TAT level was seen in the BMS loop, while the highest SC5b-9 level was found in the SES loop. Between the loops, there was no significant difference concerning TAT, PMN-elastase, SC5b-9 and factor XIIa (Figure 1).

The assessed sP-selectin values after 90 min of blood circulation were significantly elevated in the BMS loop and the SES loop compared to the initial values. In the control loop, a trend towards elevated sP-selectin levels was found after 90 min. In both loops with stents, higher sP-selectin

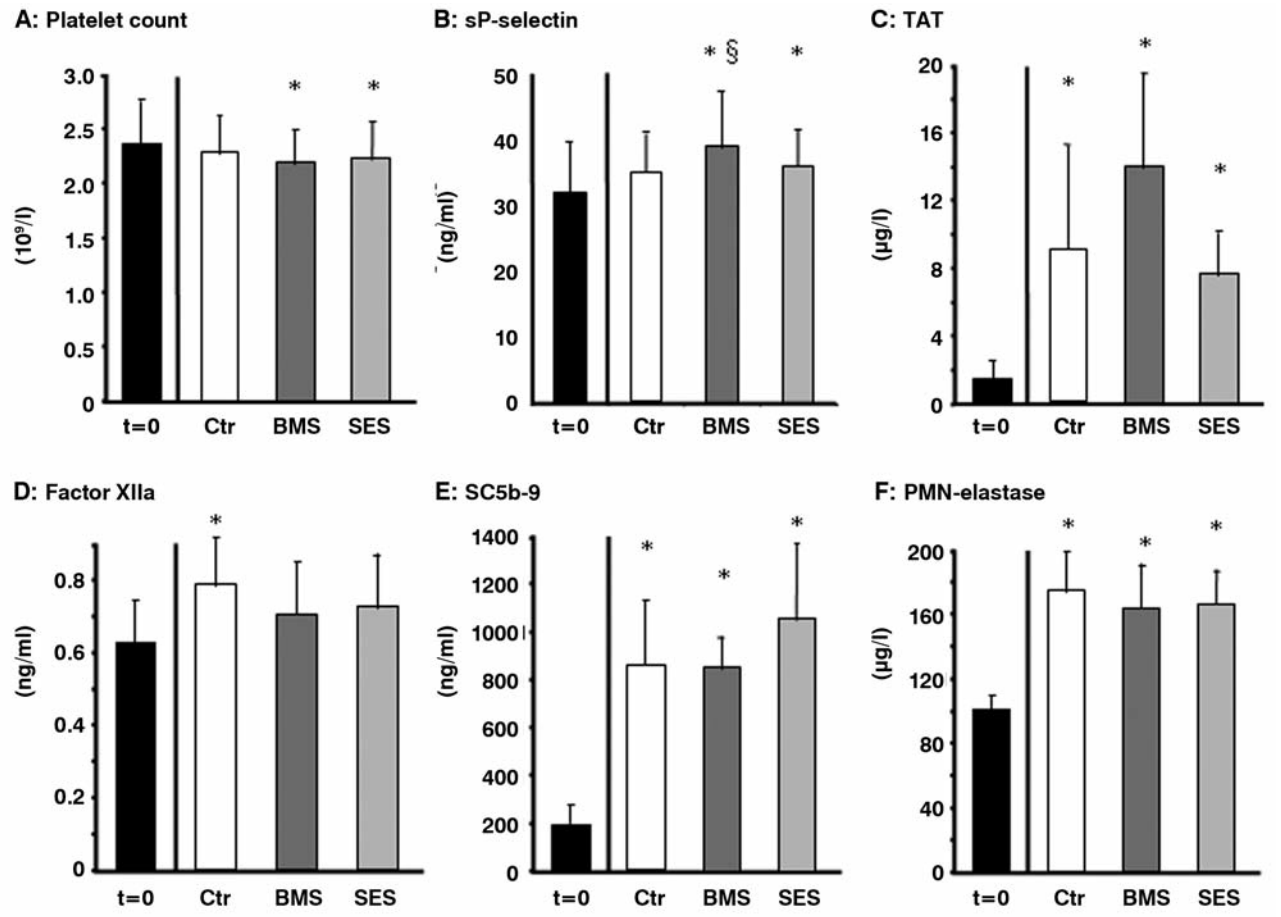

Figure 1. *Platelet count (A), sP-selectin (B), thrombin-antithrombin III complex (TAT) (C), factor XIIa (D), SC5b-9 (E) and PMN-elastase (F) at the beginning (t=0) and after 90 minutes of blood circulation in the loops without a stent (Ctr), a bare metal stent (BMS) and with a sirolimus-eluting stent (SES). Data are presented as means ± standard error of mean. *p<0.05 vs. t=0; §p<0.05 vs. Ctr.*

values were detected compared with the control loop, although only the difference between the control loop and the BMS loops was significant ($p<0.05$). No significant difference of sP-selectin values were detected between the loops with BMS and SES (Figure 1).

Cellular adhesion molecules on platelets. The expression of cellular adhesion molecules on platelets after blood circulation in the chandler loops is shown in Table I. The circulation of blood in the Chandler loop for 90 min resulted in no significant change in the expression of the cellular adhesion molecules CD62P and CD45/CD41 on platelets in all three loops. A trend towards a higher platelet expression of PAC-1 was found after 90 min compared to the beginning, but the results were not significantly different. The comparison of BMS with SES showed no significant differences regarding platelet expression of CD45/CD41, PAC-1 and CD62P after 90 min of blood circulation.

Scanning electron microscopy. Both stent types were covered with a polymer fibrin net and cellular deposition and multiple platelet aggregates after being exposed for 90 min to the circulating blood (Figure 2).

Discussion

Platelet and thrombin activation are important factors in the development of stent thrombosis (18). Patients with CHD often display an increased level of platelet activation even before coronary stenting (19). Increased activation of platelets is a consistent finding after coronary stent implantation (20). Combined therapy with aspirin and clopidogrel is recommended to inhibit platelet activity and prevent stent thrombosis. Extensive evaluations of thrombogenic properties of various stent types were performed by Tepe *et al*. (18). Beythien *et al*. (21) demonstrated that stent length has an effect on platelet activation. In the present experimental study,

Table I. *Platelet surface expression of P-selectin (CD62P), platelet-leukocyte aggregates (CD41/45) and PAC-1 (CD62) at the beginning and after 90 min of blood circulation in the loops without a stent (Ctr), with a bare-metal stent (BMS) and with a sirolimus-eluting stent (SES). Data are presented as the means±standard error of mean.*

	t = 0	Ctr	BMS	SES
P-Selectin (CD62P) [%]	56.9±1.6	54.0±1.3	54.4±1.6	54.6±1.4
PAC-1 (CD62) [%]	22.5±2.4	33.9±6.9	35.9±8.2	28.5±5.7
CD41/CD45 [%]	77±7.1	76±2.4	77±2.2	77±2.0

BMS: bare-metal stents, Ctr.: control loop/loops without a stent, SES: sirolimus-eluting stents.

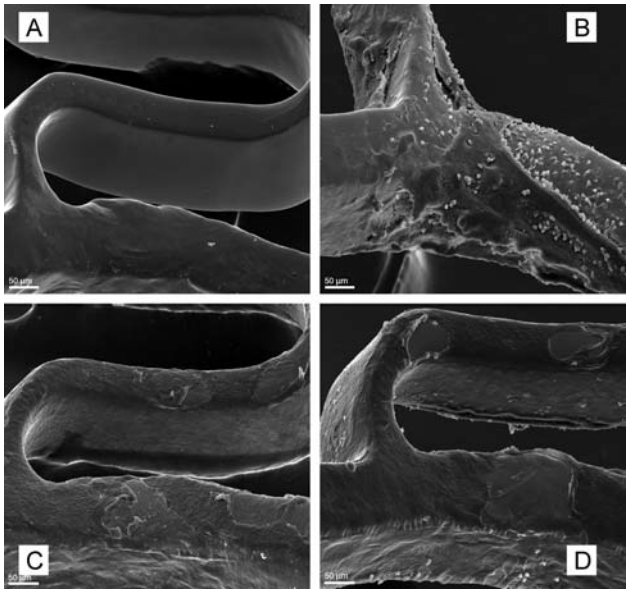

Figure 2. *SEM of a bare-metal stent before (A) and after (B) blood exposure, and of a sirolimus-eluting stent before (C) and after (D) blood exposure. Both stent types were covered with a polymer fibrin net and cellular deposition and multiple platelet aggregates after being exposed for 90 minutes to the circulating blood. (Bar=50 μm).*

a significant decrease of platelets was found in the Chandler loops with stents after 90 min of blood circulation. This result can be explained by platelet adhesion to the stent surface, also visualised by scanning electron microscopy, demonstrating the thrombogenicity of the stent surface. No significant difference in platelet count was detected between SES and the BMS. These data indicate that coating of the surface of the struts with a polymer plus impregnation with sirolimus has no effect on platelet adhesion in the early phase after stent implantation. Plasma levels of sP-selectin were significantly higher in the loops with SES and BMS than in the control loops. These data are in accordance with other clinical investigations, where increased sP-selectin levels were documented after primary stenting (22). Thus, stent placement leads to platelet activation with subsequent release of sP-selectin, without any difference between SES and BMS.

To assess the activation of the coagulation system, thrombin generation, measured as TAT complex, was determined. PMN-elastase release was measured to detect polymorphonuclear neutrophils activation. Factor XIIa and SC5b-9 were determined to assess contact activation and activation of the complement system. After 90 min of blood circulation, elevated levels of TAT, factor XIIa, PMN-elastase and SC5b-9 were found in all loops (control, SES and BMS). Even though heparin-coated polyvinyl chloride loops were used in the model and only a minimal background activation by using this loops was previously described (18), the present data showed activation of coagulation, complement system and neutrophils even in the control loop. There were no differences between SES, BMS and control loops. There was also no difference between the two stent types. Thus, coating of stents with a polymer and sirolimus has no effect on the coagulation system, complement activation and activation of neutrophils compared with BMS in the *in vitro* system of this study.

Flow cytometric analysis was used to demonstrate platelet activation and platelet-leukocyte interaction. In clinical trials, increased P-selectin surface expression predicts stent thrombosis (23). Coronary stent implantation with balloon-injury of the atherosclerotic plaque may lead to platelet activation due to intimal damage. In the *in vitro* system, without endothelial damage, no change in P-selectin expression, PAC-1 binding to activated GPIIb/IIIa receptors and platelet-leukocyte aggregates was detected. These data indicate that both stent types have similar actions on platelet activation and platelet-leukocyte interactions *in vitro*.

In conclusion, coronary stents are thrombogenic, and coating of the stents with sirolimus-impregnated polymers has no relevant effect on platelet binding and platelet activation in the early phase after stent placement. This emphasises the importance of combined antiplatelet therapy to be administered until complete endothelialisation of the stent surface has been achieved.

References

1 Laroia ST and Laroia AT: Drug-eluting stents. A review of the current literature. Cardiol Rev *12*: 37-43, 2004.

2 Karvouni E, Korovesis S and Katritsis DG: Very late thrombosis after implantation of sirolimus eluting stent. Heart *91*: e45, 2005.

3 Iakovou I, Schmidt T, Bonizzoni E, Ge L, Sangiorgi GM, Stankovic G, Airoldi, F, Chieffo A, Montorfano M, Carlino M, Michev I, Corvaja N, Briguori C, Gerckens U, Grube E and

Colombo A: Incidence, predictors, and outcome of thrombosis after successful implantation of drug-eluting stents. JAMA *293*: 2126-2130, 2005.
4 Kuchulakanti PK, Chu WW, Torguson R, Ohlmann P, Rha SW, Clavijo LC, Kim SW, Bui A, Gevorkian N, Xue Z, Smith K, Fournadjieva J, Suddath WO, Satler LF, Pichard AD, Kent KM and Waksman R: Correlates and long-term outcomes of angiographically proven stent thrombosis with sirolimus- and paclitaxel-eluting stents. Circulation *113*: 1108-1113, 2006.
5 Smith SC Jr., Feldman TE, Hirshfeld JW Jr., Jacobs AK, Kern MJ, King SB, III, Morrison DA, O'Neill WW, Schaff HV, Whitlow PL, Williams DO, Antman EM, Adams CD, Anderson JL, Faxon DP, Fuster V, Halperin JL, Hiratzka LF, Hunt SA, Nishimura R, Ornato JP, Page RL and Riegel B: ACC/AHA/SCAI 2005 Guideline Update for Percutaneous Coronary Intervention-summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines ACC/AHA/SCAI Writing Committee to Update the 2001 Guidelines for Percutaneous Coronary Intervention. Circulation *113*: 156-175, 2006.
6 Urban P, Gershlick AH, Guagliumi G, Guyon P, Lotan C, Schofer J, Seth A, Sousa JE, Wijns W, Berge C, Deme M and Stoll HP: Safety of coronary sirolimus-eluting stents in daily clinical practice: one-year follow-up of the e-Cypher registry. Circulation *113*: 1434-1441, 2006.
7 Buhaescu I, Izzedine H and Covic A: Sirolimus-challenging current perspectives. Ther Drug Monit *28*: 577-584, 2006.
8 Rampino T, Marasa M, Malvezzi PM, Soccio G, Roscini E, Gamba G, Noris P, Alessiani M and Dal CA: Platelet-Independent defect in hemostasis associated with sirolimus use. Transplant Proc *36*: 700-702, 2004.
9 Babinska A, Markell MS, Salifu MO, Akoad M, Ehrlich YH and Kornecki E: Enhancement of human platelet aggregation and secretion induced by rapamycin. Nephrol Dial Transplant *13*: 3153-3159, 1998.
10 Kastrati A, Mehilli J, Pache J, Kaiser C, Valgimigli M, Kelbaek H, Menichelli M, Sabate M, Suttorp MJ, Baumgart D, Seyfarth M, Pfisterer ME and Schomig A: Analysis of 14 trials comparing sirolimus-eluting stents with bare-metal stents. N Engl J Med *356*: 1030-1039, 2007.
11 Lagerqvist B, James SK, Stenestrand U, Lindback J, Nilsson T and Wallentin L: Long-term outcomes with drug-eluting stents *versus* bare metal stents in Sweden. N Engl J Med *356*: 1009-1019, 2007.
12 Stone GW, Moses JW, Ellis SG, Schofer J, Dawkins KD, Morice MC, Colombo A, Schampaert E, Grube E, Kirtane AJ, Cutlip DE, Fahy M, Pocock SJ, Mehran R and Leon MB: Safety and efficacy of sirolimus- and paclitaxel-eluting coronary stents. N Engl J Med *356*: 998-1008, 2007.
13 Serruys PW and Daemen J: Are drug-eluting stents associated with a higher rate of late thrombosis than bare metal stents? Late stent thrombosis: a nuisance in both bare metal and drug-eluting stents. Circulation *115*: 1433-1439, 2007.
14 Ellis SG, Colombo A, Grube E, Popma J, Koglin J, Dawkins KD and Stone GW: Incidence, timing, and correlates of stent thrombosis with the polymeric paclitaxel drug-eluting stent: a TAXUS II, IV, V, and VI meta-analysis of 3445 patients followed for up to 3 years. J Am Coll Cardiol *49*: 1043-1051, 2007.
15 Camenzind E, Steg PG and Wijns W: Stent thrombosis late after implantation of first-generation drug-eluting stents: a cause for concern. Circulation *115*: 1440-1455, 2007.
16 Mauri L, Hsieh WH, Massaro JM, Ho KK, D'Agostino R and Cutlip DE: Stent thrombosis in randomized clinical trials of drug-eluting stents. N Engl J Med *356*: 1020-1029, 2007.
17 Spaulding C, Daemen J, Boersma E, Cutlip DE and Serruys PW: A pooled analysis of data comparing sirolimus-eluting stents with bare-metal stents. N Engl J Med *356*: 989-997, 2007.
18 Tepe G, Wendel HP, Khorchidi S, Schmehl J, Wiskirchen J, Pusich B, Claussen CD and Duda SH: Thrombogenicity of various endovascular stent types: an *in vitro* evaluation. J Vasc Interv Radiol *13*: 1029-1035, 2002.
19 Serebruany VL, Cummings CC, Malinin AI, Steinhubl SR and Gurbel PA: Uniform platelet activation exists before coronary stent implantation despite aspirin therapy. Am Heart J *142*: 611-616, 2001.
20 Inoue T, Sohma R, Miyazaki T, Iwasaki Y, Yaguchi I and Morooka S: Comparison of activation process of platelets and neutrophils after coronary stent implantation *versus* balloon angioplasty for stable angina pectoris. Am J Cardiol *86*: 1057-1062, 2000.
21 Beythien C, Gutensohn K, Bau J, Hamm CW, Kuhnl P, Meinertz T and Terres W: Influence of stent length and heparin coating on platelet activation: a flow cytometric analysis in a pulsed floating model. Thromb Res *94*: 79-86, 1999.
22 Onohara T, Komori K, Inoguchi H and Yamamura S: Local blood serotonin and soluble P-selectin levels during percutaneous transluminal balloon angioplasty and primary stenting of the iliac artery. Surgery *131*: S256-S260, 2002.
23 Gawaz M, Neumann FJ, Ott I, May A, Rudiger S and Schomig A: Role of activation-dependent platelet membrane glycoproteins in development of subacute occlusive coronary stent thrombosis. Coron Artery Dis *8*: 121-128, 1997.

Received 13 April 2010
Revised 6 June 2010
Accepted 11 June 2010