Effects of clopidogrel, aspirin and combined therapy in a porcine ex vivo model of high-shear induced stent thrombosis


Cardiovascular Interventional Research Center, Division of Cardiology, Department of Medicine, The Burns and Allen Research Institute, Cedars-Sinai Medical Center, and UCLA School of Medicine, Los Angeles, CA, U.S.A; *Sanofi Recherche, Toulouse, France

Aims Use of ticlopidine in coronary stenting is limited by delayed onset of action. We studied the effects of clopidogrel, a rapidly acting analog of ticlopidine alone, and in combination with aspirin, in inhibiting stent thrombosis.

Methods Unpolished nitinol stents were deployed in a porcine ex vivo arteriovenous shunt and exposed to flowing arterial blood at a shear rate of approximately 1500 s⁻¹. Stent thrombus, platelet aggregation and bleeding times were measured at baseline and after treatment.

Results Intravenous clopidogrel produced a rapid (within 30 min) and dose-dependent inhibition of stent thrombosis, with 87% reduction at a dose of 10 mg kg⁻¹ (P <0·001).

Aspirin alone (10 mg kg⁻¹) was minimally effective (20% inhibition P >0·05) in inhibiting stent thrombosis. Combined treatment with clopidogrel and aspirin produced 95–98% inhibition of stent thrombosis, even at low doses of clopidogrel (2·5–5·0 mg kg⁻¹) (P <0·0001). At effective doses both clopidogrel and combined therapy produced significant prolongation of bleeding time (P <0·05) and inhibition of platelet aggregation (P <0·05).

Conclusion Clopidogrel, either alone or combined with aspirin, may have a potential role in preventing stent thrombosis in high-risk clinical situations. (Eur Heart J 1998; 10: 1538–1546)

Key Words: Stent, platelets, angioplasty, stent thrombosis.

Introduction

Coronary stents now account for 30–50% of coronary interventions in many centres[1]. This is in part related to the fact that stent thrombosis rates have been significantly reduced by technical and pharmacological changes in procedure protocol popularized over the last few years[2–5]. This notwithstanding, stent thrombosis still occurs in 1–3% of patients[2–4] and is often catastrophic[6–11]. Thrombosis is more common in small arteries and this has limited stent utilization in arteries less than 3 mm in diameter[6–8].

Ticlopidine, a thienopyridine derivative and a selective platelet adenosine diphosphate receptor antagonist has been demonstrated to reduce stent thrombosis as compared to warfarin when used in combination with aspirin and high pressure stent deployment techniques in arteries greater than 3 mm in diameter[12]. The efficacy of this approach, however, has not been tested in smaller vessels where propensity for thrombosis is greater due to high shear rates[12]. Though generally safe, ticlopidine is not without limitations. Non life-threatening side effects including skin rash and diarrhoea occur in 10–18% of patients[13]. Serious and occasionally fatal neutropaenia and aplastic anaemia have been reported in approximately 1–2% of patients[13,14]. Further, the onset of the drug’s antiplatelet effect is delayed, with peak effect seen only after 5 days[13].

Clopidogrel, a new platelet antagonist, like ticlopidine is a thienopyridine derivative that selectively blocks adenosine diphosphate induced platelet aggregation[15]. Clopidogrel, however, has a rapid onset of action and a better safety profile than ticlopidine[13]. As such, this drug may provide an alternative for platelet inhibition in patients undergoing stent placement for emergency indications, or in patients in whom stents are placed at positions with a high likelihood of
The purpose of this study was to investigate the efficacy of clopidogrel, aspirin and combined clopidogrel and aspirin in inhibiting high shear mediated stent thrombosis in a porcine arteriovenous shunt model.

Materials and methods

Coronary stents

The stents (n=75) tested were 7 mm-long slotted-tube geometry devices made from nickel-titanium alloy-nitinol (Advanced Coronary Technology, Menlo Park, CA, U.S.A.). Stents weighed 16±1 mg, had a strut thickness of 0.006 inch, and a silicon carbide grit blasted surface finish, known to be highly thrombogenic in this model[12]. All stents were expanded on a tapered mandrel to an outer diameter of 2.0 mm prior to deployment in the perfusion chamber.

Experimental model

All procedures were approved by the Institutional Animal Care and Use Committee and conformed to American Heart Association Guidelines for Animal Research. A previously described ex-vivo extracorporeal perfusion system[13] was adapted to study acute stent thrombosis. Experiments were performed in nine pigs weighing 23-29 kg. After overnight fasting, animals were sedated with phenobarbital (5 mg·kg⁻¹) and anaesthesia was maintained with 1% isoflurane following endotracheal intubation. The right carotid artery and jugular vein were isolated and cannulated with 8 French sheaths to establish an extracorporeal circuit. Arterial blood gases and pH were monitored periodically and maintained at normal levels by adjustment of the ventilation rate and tidal volume. Invasive arterial pressure, oxygen saturation, electrocardiogram and rectal temperature were continuously monitored. A thermostatically controlled blanket was used to maintain temperature at 37°C.

Venous blood was collected for baseline platelet aggregation, complete blood cell count and activated clotting time. Following this, the animals were given heparin sodium (10 units/kg bolus i.v.) to prevent occlusion of vascular catheters. At the conclusion of the experiment, animals were euthanized while under anaesthesia.

Figure 1 Diagram of the extracorporeal perfusion system used to study platelet-thrombus formation on stents. Expanded stents were mounted in the tubular perfusion chamber and exposed to flowing blood from the carotid arteries. Blood was returned to the jugular vein. Shear was regulated by varying the blood flow with the peristaltic pump, and calculated by the following formula: shear (s⁻¹)=4Q/(πR³).
1.0 × 2.5 cm in size, to obtain a watertight seal. The arterial cannula was connected to the inlet of the perfusion chamber and the outlet was connected to the venous catheter through a variable speed peristaltic pump (Masterflex, Cole-Palmer Instrument Co., Chicago, IL, U.S.A.). A transit time Doppler flow probe (Transonic System Inc., New York, NY, U.S.A.) was interposed in the circuit after the pump to document continuous blood flow through the circuit. The chamber and some tubing were placed in a water bath maintained at 37°C.

**Perfusion protocol**

The perfusion protocol is illustrated in Fig. 2. After a 60 min stabilization period, stents were mounted in the tubular chamber and perfused with Krebs solution for 60 s at 37°C. Using a switch valve to prevent stasis, blood was circulated through the system and flow regulated at 70 ml.min⁻¹ for 20 min. This flow rate generates a wall shear rate of 1486 s⁻¹ at the chamber surface and 2100 s⁻¹ at the stent surface. At the end of each perfusion period, Krebs buffer was circulated through the chamber for 30 s at 40 ml.min⁻¹ to wash off unattached cells and blood from the stent and the perfusion system. At the completion of each perfusion period, the stents were removed from the chamber, dried and weighed. The perfusion chamber and ex vivo system were perfused with normal saline for several minutes to clear any visible blood before mounting another stent. Digital images of stents were obtained at ×15 magnification in side and end-on views, using a video-microscope, PC frame grabber and image analysis software (Bioscan, Optimas Corporation, Bothell, WA, U.S.A.).

**Preparation and administration of drugs**

Clopidogrel was supplied by Sanofi Recherche (Toulouse, France) as dry powder. The drug was reconstituted in non-pyrogenic normal saline as 10 mg.ml⁻¹ solution, kept refrigerated at 4°C and reconstituted 10–20 min before administration. Parenteral aspirin (Bayer AG, Leverkusen, Germany) was dissolved in distilled water just prior to use as instructed by the manufacturer. All drugs were administered intravenously by bolus injection.

**Platelet aggregation assay**

Blood samples were collected in 3.8% trisodium citrate solution (9:1 v/v) 2 h after the administration of the drugs. Platelet-rich plasma (PRP) was obtained by centrifugation of the blood (500 × g, 10 min, 15°C) and the platelet count was adjusted to 10⁶ cells.ml⁻¹ in the final suspension using platelet-poor plasma. Platelet aggregation was measured by the turbidometric method of Born and Gross on a dual-channel Chrono-Log aggregometer[17]. Platelet-rich plasma was equilibrated at 37°C for 1 min under constant stirring (900 rpm) and aggregation was induced by adenosine diphosphate (2.5 μM), collagen (10 μg.ml⁻¹) or arachidonic acid.
The extent of aggregation was estimated by quantitatively measuring the maximum curve height above baseline level.

**Bleeding and activated clotting time**

Bleeding time was measured by making an incision on the ventral surface of the thigh with a number 11 surgical knife. The time between incision and cessation of bleeding was recorded as bleeding time. Activated clotting time was performed using a Hemochron 400 (International Technidyne Corporation, Edison, NJ, U.S.A.) machine in standard fashion[18].

**Statistical analysis**

Data are presented as mean ± standard deviation. Statistical difference between means was determined by single-factor ANOVA. If means were shown to be significantly different, intergroup post-hoc multiple comparisons by pairs were performed by the Tukey HSD test (Statistica, version 4.0). Probability values <0.05 were considered to indicate statistical significance.

**Results**

**Stent thrombosis**

Figures 3 and 4 demonstrate qualitatively the effects of the test agents on stent thrombosis. On control stents, a large amount of predominantly white thrombus formed which was occlusive within the stent. Clopidogrel produced mild inhibition at a dose of 2.5 mg . kg⁻¹, while at higher doses, more pronounced inhibition of stent thrombosis was seen. At doses of 10 mg . kg⁻¹ of clopidogrel, minimal fibrinous thrombotic material was seen predominantly at the edges of stent slots without encroachment into the lumen. Figure 4 shows an example of synergism between aspirin and clopidogrel. Combined treatment with aspirin and clopidogrel produced a dramatic reduction of stent thrombosis compared to aspirin or clopidogrel alone. Stents treated with aspirin (10 mg . kg⁻¹)+clopidogrel(5 mg . kg⁻¹) had virtually no detectable thrombus.

The quantitative effects of treatment with clopidogrel, aspirin and clopidogrel +aspirin on stent thrombosis are shown in Fig. 5. A diminution of clopidogrel produced a dose-dependent inhibition of thrombus weight. Stent thrombus weight was decreased by 86% at the highest dose of 10 mg . kg⁻¹ (from 24 ± 4 to 3 ± 2 mg, P <0.001). Aspirin produced minimal inhibition under experimental conditions (24 ± 4 to 20 ± 2 mg; P >0.05). Treatment with aspirin (10 mg . kg⁻¹)+clopidogrel (2.5 or 5 mg . kg⁻¹) was highly effective (95 and 98% inhibition respectively; P <0.001) compared to aspirin (19% reduction), or clopidogrel (7% and 56%

**Platelet studies**

The effects of the test agents on platelet aggregation induced by adenosine diphosphate, collagen and arachidonic acid are shown in Fig. 6. A denosine diphosphate-induced platelet aggregation was inhibited in a dose dependent manner after the administration of increasing doses of clopidogrel. Platelet aggregation induced by collagen was also inhibited but to a lesser extent. Under the same experimental conditions, clopidogrel did not significantly affect arachidonic acid-induced platelet aggregation. At doses of 10 mg . kg⁻¹, aspirin only slightly affected adenosine diphosphate and collagen-induced platelet aggregation, but when the two drugs were combined, aspirin strongly potentiated the anti-aggregating effect of clopidogrel. A significant synergistic effect was observed with regard to adenosine diphosphate- and collagen-induced platelet aggregation. Aspirin did not potentiate the effect of clopidogrel on arachidonic acid-induced platelet aggregation.
Haematological studies

The effects of study drugs on bleeding and activated clotting times are shown in Fig. 6. Clopidogrel produced a dose-dependent prolongation of bleeding time but had virtually no effect on activated clotting time. Bleeding time was prolonged from 137 ± 73 to 292 ± 130 s (n=6; P <0.05) at the highest dose of 10 mg · kg⁻¹. Aspirin had no significant effect on either bleeding or activated clotting time. Combined treatment (aspirin 10+clopidogrel 2.5 or 5 mg · kg⁻¹) significantly increased bleeding time (442 ± 115 and 720 ± 120 vs 137 ± 73 s for control).

There were no episodes of significant bleeding in any of the animals studied. Treatment with clopidogrel had no significant effects on platelets, white blood cell counts or haematocrit. No significant effects were noted on mean blood pressure and heart rate with any of the treatment regimens.

Discussion

This study uses a porcine arteriovenous shunt model to study the effects of clopidogrel and aspirin on high shear mediated stent thrombosis. Our principal finding is that clopidogrel, a thienopyridine derivative and selective blocker of adenosine diphosphate-mediated platelet aggregation inhibited stent thrombosis, whereas treatment with aspirin did not. Combined treatment with aspirin and clopidogrel was more effective than either agent alone, suggesting aspirin and clopidogrel have synergistic effects in inhibiting stent thrombosis under the experimental conditions tested. The antithrombotic effects of clopidogrel are rapid in onset, dose-dependent.
and are associated with significant inhibition of platelet aggregation and prolongation of bleeding time.

Clopidogrel is a powerful analog of ticlopidine with a similar mechanism of action. It is a potent inhibitor of adenosine diphosphate-induced platelet aggregation and blocks the amplification of platelet activation by released adenosine diphosphate[15,19–21]. Unlike aspirin, clopidogrel has no effects on thromboxane synthetase or cyclo-oxygenase[21]. It is inactive in vitro and must be administered in vivo and metabolized in liver to obtain full antiplatelet effects[22]. The anti-thrombotic effects of clopidogrel have been documented in the Folt’s model of porcine arterial thrombosis[21]. Clopidogrel has been shown to prevent cyclic flow variations[23], potentiate the thrombolytic effects of streptokinase[24] and reduce intimal hyperplasia after intimal injury[25] in several animal models. The antiplatelet effects of clopidogrel have been documented as early as 15–30 min after intravenous administration[21]. Further, the antithrombotic effects of this drug are rapidly reversible by administration of aprotinin[26]. As such, this drug may have an important role in interventional cardiology.

Animal model

The Badimon chamber used in this study has been extensively characterized to study the mechanisms of experimental thrombosis[16]. We have found this stent thrombosis model to be reproducible and stable after multiple perfusions over several hours. Scanning electron microscopy studies of stents perfused in this system have revealed the following steps in stent thrombosis: (a) coating of stent surface with protein (b) adhesion of platelets to stent surface followed by (c) formation of large platelet aggregates with entrapment of fibrin, leukocytes and red cells[27].

In previous studies, we have used this model to study the effects of surface characteristics, such as local shear[12], antiplatelet agents (including nitric oxide donors)[28], and glycoprotein receptor IIb/IIIa antibody[29] on stent thrombosis. This model also offers the advantage of changing the shear rates by changing the flow rates. In this study we specifically examined stents under conditions of high shear rates (1500 s⁻¹) to simulate the high-risk clinical situation of inadequate stent deployment and stenting in small diameter vessels. Although the relevance of this model to clinical stent thrombosis remains to be defined, its simplicity, reproducibility and capacity to enable a study of multiple stents and drugs types in the same experiment make it attractive when examining the mechanisms of stent thrombosis and the effects of various antiplatelet agents.

Mechanism of action, ineffectiveness of aspirin, synergism between clopidogrel and aspirin

Platelets are important in the genesis of stent thrombosis[27,30] with the formation of large platelet aggregates being crucial to the formation of occlusive stent thrombus[30]. Despite the moderate effectiveness of aspirin in reducing platelet aggregation in a test tube it was ineffective in reducing stent thrombosis under high shear. High shear induces platelet aggregation by stimulating a diacylglycerol-independent pathway of protein kinase C activation which is not affected by aspirin[31]. Adenosine diphosphate plays an important role in mediating platelet aggregation under high shear situations[32]. A recent study has shown that clopidogrel inhibits the adenosine diphosphate-mediated pathway that is necessary for full activation of GP IIb/IIIa and formation of stable platelet aggregates[33]. The inhibitory
effects of clopidogrel, an adenosine diphosphate receptor antagonist, on stent thrombosis correlate well with antiplatelet effects and prolongation of bleeding time. Clopidogrel has no anticoagulant effects, as assessed by activated clotting time, which was essentially unchanged. The superior efficacy of clopidogrel compared to aspirin can be attributed to more potent and flow-independent antiplatelet effects[15].

We have tested the effects of shear on the efficacy of aspirin in stent thrombosis under different shear rates. While aspirin was effective under low shear rates of 500 s$^{-1}$, it was ineffective under higher shear rates. At shear rates of 2000 s$^{-1}$, even higher doses of aspirin (20-40 mg . kg$^{-1}$) were not effective[34]. The limited efficacy of aspirin in inhibiting platelet mediated thrombosis under high shear conditions has also been demonstrated by other investigators[35].

Although the exact mechanism is not known, the synergy between aspirin and clopidogrel may be secondary to the different platelet activation pathways inhibited by these drugs. The combination of Thromboxane A$_2$ and adenosine diphosphate antagonism (attained in this study by aspirin and clopidogrel, respectively) has been shown to be highly effective in inhibiting platelet aggregation and cyclic flow variations in stenosed and endothelium-injured canine coronary arteries[36].

**Implications**

Our study establishes the efficacy of clopidogrel, a potent antiplatelet agent for inhibiting stent thrombosis under high-shear conditions. High-risk clinical situations such as small vessel stenting, inadequate stent deployment and the presence of thrombus are characterized by high shear. Clopidogrel may therefore be a useful antiplatelet strategy in these clinical situations. Currently, some operators are treating these patients with an intravenous glycoprotein IIb/IIIa receptor monoclonal antibody, 7E3[37], followed by oral ticlopidine. This approach is limited by the high cost of 7E3 and the frequent untoward effects of ticlopidine. Intravenous clopidogrel, followed by oral administration, together with aspirin may be an excellent alternative in some cases. Such a combination would provide continuous antiplatelet activity.

Clopidogrel has an excellent safety profile. CAPRIE was a randomized, blinded trial designed to assess the efficacy of clopidogrel (75 mg p.o. daily) vs aspirin (325 mg p.o. daily) in reducing the risk of a composite outcome cluster of ischaemic stroke, myocardial infarction, or vascular death in approximately 20 000 patients with atherosclerotic vascular disease[38]. Compared to aspirin, clopidogrel resulted in a small but significant relative-risk reduction of 9% in favour of clopidogrel. The safety profile of clopidogrel was better than aspirin in this study. Further, the incidence of significant neutropaenia with clopidogrel in this study was 0.1% compared to a reported incidence of 2–3% with ticlopidine in most series[13,14].

**Limitations**

Though this study supports the possibility of a clinical investigation of clopidogrel, it is subject to a number of limitations. This is an ex-vivo study which examined acute thrombus formation over 20 min. Although the peak incidence of stent thrombosis in humans is seen at 3–5 days, acute stent thrombosis (within 24 h) is occasionally observed and the role of acute platelet deposition in subacute stent thrombosis is not known.

Figure 7  Bar graph showing the effects of clopidogrel (clop), aspirin (asa) and clopidogrel+aspirin on platelet aggregation. Values are mean ± SD. *P<0.001 vs control and clopidogrel 2.5 mg . kg$^{-1}$; **P<0.001 vs clopidogrel 5 mg . kg$^{-1}$; ***P<0.001 vs aspirin 10 mg . kg$^{-1}$; n=9 for each group.
The study design focused on the platelet–stent interaction and not the coagulation cascade. The high-shear conditions used in this model generated large quantities of thrombus, a situation generally not encountered in large, adequately stented vessels. We tested high-shear conditions to simulate small vessel stenting and inadequate stent expansion. The ex-vivo model excludes underlying vascular injury or the effect of drugs on the vessel wall, both of which may potentially affect stent thrombosis.

**Conclusion**

Our study demonstrates a potent inhibitory effect of clopidogrel on stent thrombosis under high shear blood flow conditions. It also demonstrates synergy between aspirin and clopidogrel. Therefore, clopidogrel merits clinical investigation in coronary stenting especially for small diameter arteries, in emergency stent implantation and other circumstances where the risk for stent thrombosis is likely to be high.

Authors wish to thank Ivan Velasquez, M.D., Hao Zeng, M.D., Susan Schauer M.S., and Adrian Glenn for technical assistance and Juan H. Badimon, Ph.D., for generously providing the perfusion chamber.

**References**


