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Prevention of thrombosis and rethrombosis and enhancement of the thrombolytic actions of recombinant tissue-type plasminogen activator in the canine heart by DMP728, a glycoprotein IIb/IIIa antagonist

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1 We studied DMP728, a non-peptide glycoprotein (GP) IIb/IIIa receptor antagonist, for prevention of coronary artery thrombosis or rethrombosis in a chronic canine model subjected to arterial injury.

2 In protocol I, DMP728 (1.0 mg kg^{-1}, i.v., $n = 8$) or saline ($n = 8$) was administered and a 150 μA anodal current was applied to the intimal surface of the left circumflex coronary artery (LCX). Dogs were monitored for 6 h and again on each of 5 subsequent days.

3 *Ex vivo* platelet aggregation was inhibited but returned to baseline 1 day after drug administration. Thrombus weight was reduced (saline, 20.7 ± 5.0 mg; DMP728 1.7 ± 0.4 mg; $P < 0.05$), as was infarct size [saline, 27.5 ± 4.3; DMP728, 1.6 ± 0.7 (per cent left ventricle); $P < 0.05$]. All control animals died by day 3, while all but one of the treated dogs survived the entire protocol ($P < 0.05$).

4 In protocol II, an LCX thrombus was induced and thrombolytic therapy was initiated 30 min later. DMP728 (1.0 mg kg^{-1}, i.v., $n = 8$) or saline ($n = 8$) was administered 5 min after recombinant tissue-type plasminogen activator infusion had begun. The incidence of reocclusion was reduced by DMP728 (saline, 4/8; DMP728, 1/8). One day after thrombolysis, 7/8 DMP728-treated animals were alive compared with 1/8 in the control group ($P = 0.01$).

5 DMP728 inhibited *ex vivo* platelet aggregation, prevented primary and secondary occlusive thrombus formation, reduced thrombus weight and infarct size and increased survival in a chronic canine model of coronary artery thrombus formation. DMP728 is an effective anti-platelet intervention when used as the singular adjunctive agent in association with thrombolytic therapy.

Keywords: Fibrinogen receptor; anti-platelet agent; thrombolysis; integrin receptor; DMP728

Introduction

Pharmacological modulation of platelet reactivity is effective clinically in the secondary prevention of stroke, myocardial infarction and vascular death in patients with atherosclerosis exhibiting symptoms related to impaired perfusion of the cerebral, coronary or peripheral vasculature (Antiplatelet Trialists Collaboration, 1988; Harrison, 1990; Cairns *et al.*, 1992). When used as the sole adjunctive antithrombotic therapy, conventional platelet-modifying agents are less effective in preventing platelet-dependent thrombosis associated with mechanical interventions or thrombolytic therapy in the management of patients with acute arterial thrombotic occlusion (Harker, 1986; Schwartz *et al.*, 1988; Grines, 1992). In the latter instances, failure to prevent arterial reocclusion despite adjunctive therapy with aspirin and/or heparin suggests the persistence of a residual lesion capable of exerting an intense thrombogenic stimulus leading to the continued recruitment of platelets and activation of the coagulation cascade (Harker, 1987; Becker & Gore, 1991; Chesebro & Fuster, 1991).

The beneficial effects of thrombolytic therapy for myocardial infarction are well documented (GISSI Trial, 1986; ISIS-2 1988; TAMI Study Group, 1989; GUSTO Investigators, 1993). However, thrombolytic therapy with either recombinant tissue-type plasminogen activator (rt-PA) or streptokinase is associated with reocclusion rates of 5–30% and a rate of reinfarction of about 4% in the absence of adjunctive therapy (Cairns, 1990; Cairns *et al.*,; 1992). The stated percentages represent reocclusive events in patients who are receiving concomitant aspirin and/or heparin as adjunctive therapy. The relatively high incidence of reocclusion raises a question about the efficacy of heparin and aspirin for the prevention of reocclusion and suggests the need to develop more effectve pharmacological interventions to be used in conjunction with thrombolytic therapy and/or coronary angioplasty.

The present experimental study examines the *in vivo* antithrombotic effectiveness of DMP728 [cyclo(D-2-aminobutyrate-N-methyl-L-arginyl-glycyl-L-aspartyl- 3-aminomethylbenzoic acid) methanesulphonate; Figure 1], a glycoprotein (GP) IIb/IIIa antagonist with a high affinity and specificity for the human and the canine platelet GPIIb/IIIa receptor (Mousa *et al.*, 1994). The results in two experimental models demonstrate the efficacy of DMP728 as the sole adjunctive treatment for the prevention of either occlusive primary thrombus formation in response to severe vessel wall injury or secondary thrombus formation occurring after successful thrombolysis.

Methods

Animal investigation

The studies conform to the Position of the American Heart Association on Research Animal Use adopted 11 November 1984. The procedures were in accordance with the guidelines of the University of Michigan (Ann Arbor) University Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory

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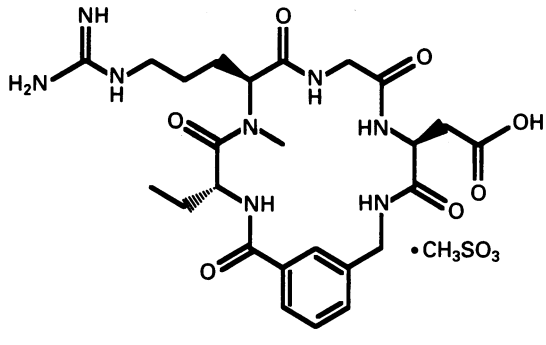

DMP728

Figure 1 Chemical structure of DMP728 [(cyclo(D-2-aminobutyrate-*N*-methyl-L-arginyl-glycyl-L-aspartyl-3-aminomethylbenzoic acid) methanesulphonate]. The molecular weight is 656.7 and its water solubility exceeds 150 mg ml^{-1}. DMP728 is stable in solution at pH 7.4 and 4.5 when at room temperature. The purity of the research sample used in this study was certified as being >99.0%.

Animal Medicine. The University of Michigan is accredited by the American Association of Accreditation of Laboratory Animal Care, and the animal care and use programme conforms to the standards in '*The Guide for Care and Use of Laboratory Animals*', Department of Health, Education and Welfare Publication no. NIH 78-23.

Model of coronary occlusion

The experimental model used in this investigation is a modification of one developed by our laboratory for the study of experimentally induced coronary artery thrombosis. The experimental procedure results in the formation of a platelet-rich intravascular thrombus at the site of an electrolytically induced endothelial lesion in proximity to a distal arterial stenosis. In each of the models studied, occlusive coronary artery thrombosis developed in response to vascular injury resulting from application of an anodal current to the intimal surface of the left circumflex coronary artery. The model facilitated the evaluation of DMP728 for the prevention of primary occlusive arterial thrombosis as well as prevention of secondary thrombosis (rethrombosis) after administration of rt-PA under conditions in which the antiplatelet agent was the only adjunctive drug used in conjunction with the thrombolytic agent. Neither heparin nor aspirin was employed at any time in the experimental protocols present in this report.

Surgical preparation and experimental protocol

The surgical procedures described in this study were conducted using aseptic techniques. Male mongrel dogs weighing 14–20 kg were anaesthetized with sodium pentobarbital (30 mg kg^{-1}, i.v.), intubated and ventilated with room air and positive pressure at a stroke volume of 30 ml kg^{-1} and a frequency of 12 breaths min^{-1} (Harvard Apparatus, South Natick, MA, U.S.A.). Cannulae were placed in the left carotid artery for monitoring arterial blood pressure (Statham P23 pressure transducer, Gould, Oxnard, CA, U.S.A.) and in the right jugular vein for obtaining blood samples. The jugular venous cannula was flushed thoroughly with 0.9% sodium/chloride solution for injection after drug administration or after obtaining blood samples. The heart was exposed by a left thoracotomy through the fifth intercostal space and was suspended in a pericardial cradle. A 2–3 cm segment of the left circumflex coronary artery was isolated from surrounding tissue by blunt dissection. The artery was instrumented with a Doppler flow probe (Model 100, Triton Technology, San Diego, CA, U.S.A.), and indwelling electrode, and a ligature stenosis formed by tying a suture around an 18 gauge needle and the left circumflex coronary artery and then withdrawing the hypodermic needle. The intracoronary electrode consisted of a 30 gauge, Teflon-insulated, silver-coated, copper wire attached to the tip of a 25 gauge hypodermic needle. The wire and needle tip assembly were inserted through the wall of the left circumflex coronary artery and positioned in a manner that allowed the uninsulated region of the electrode to remain in contact with the intimal surface of the wall of the vessel. Proper positioning of the electrode in the coronary artery was confirmed by visual inspection at the end of each experiment. The external portion of the stimulating electrode and the flow probe wires were secured to the myocardium with a suture.

The intravascular electrode was connected to the positive pole (anode) of a dual-channel stimulator (Grass S88 stimulator and a Grass Constant Current Unit, Model CCU1A, Grass Instrument, Quincy, MA, U.S.A.). The cathode was connected to a distant subcutaneous site. Application of an anodal d.c. current to the intimal surface of the circumflex coronary artery results in a deep, thrombogenic, vascular lesion (Bates *et al.*, 1992). The current delivered to the circumflex coronary artery was monitored continuously with an ammeter and maintained at 150 μA.

Recordings of blood pressure, limb lead II electrocardiogram and mean and phasic blood flow velocity from the left circumflex coronary artery were obtained on a model 7 polygraph recorder (Grass Instrument). The electrocardiogram was examined for evidence of ST-segment alterations coinciding with the fluctuations in coronary artery blood flow velocity.

Protocol 1: prevention of coronary artery thrombus formation

The protocol for studies designed to determine the efficacy of DMP728 for the prevention of primary occlusive coronary artery thrombosis is shown in Figure 2a. The experimental protcol was initiated after completion of the surgical instrumentation and closure of the thoracotomy incision. The previously instrumented animals were maintained under anaesthesia, as described above. The animals were allocated to receive either saline or DMP728 1.0 mg kg^{-1}, i.v. intravenously over a period of 5 min. Fifteen minutes later, the anodal current was applied (150 μA) to the intimal surface of the left circumflex coronary artery for a maximum period of 3 h or was terminated 10 min after blood flow velocity in the coronary vessel remained stable at zero flow velocity to verify having achieved formation of a stable occlusive thrombus. Blood pressure, heart rate, coronary artery blood flow velocity and *ex vivo* platelet aggregation were monitored over the initial 6 h experimental period. The animals were returned to their housing facilities in the Unit for Laboratory Medicine upon completion of the protocol on each of the days. On the subsequent 5 days, surviving dogs were returned to the laboratory. The animals were allowed to rest in quiet surroundings without the use of anaesthesia or sedation. Recordings of coronary artery blood flow velocity were obtained to confirm the presence or absence of flow in the left circumflex coronary artery and the limb lead II electrocardiogram was monitored for electrocardiographic changes suggestive of ischaemic injury. Blood was withdrawn for cell counts and aggregation studies. The dogs were returned to the holding rooms each day after completion of the daily recordings and data acquisition.

Survivors were euthanized on the sixth study day. After spontaneous death or euthanasia, the chest was opened and the heart was removed. The left circumflex coronary artery was dissected free as far as possible and opened longitudinally. The intracoronary position of the electrode was verified and the thrombus was removed and weighed on an analytical balance. The heart was sectioned from apex to base in 1.0 cm-thick sections that were incubated, without agitation, in triphenyltetrazolium chloride for 5 min at 37°C.

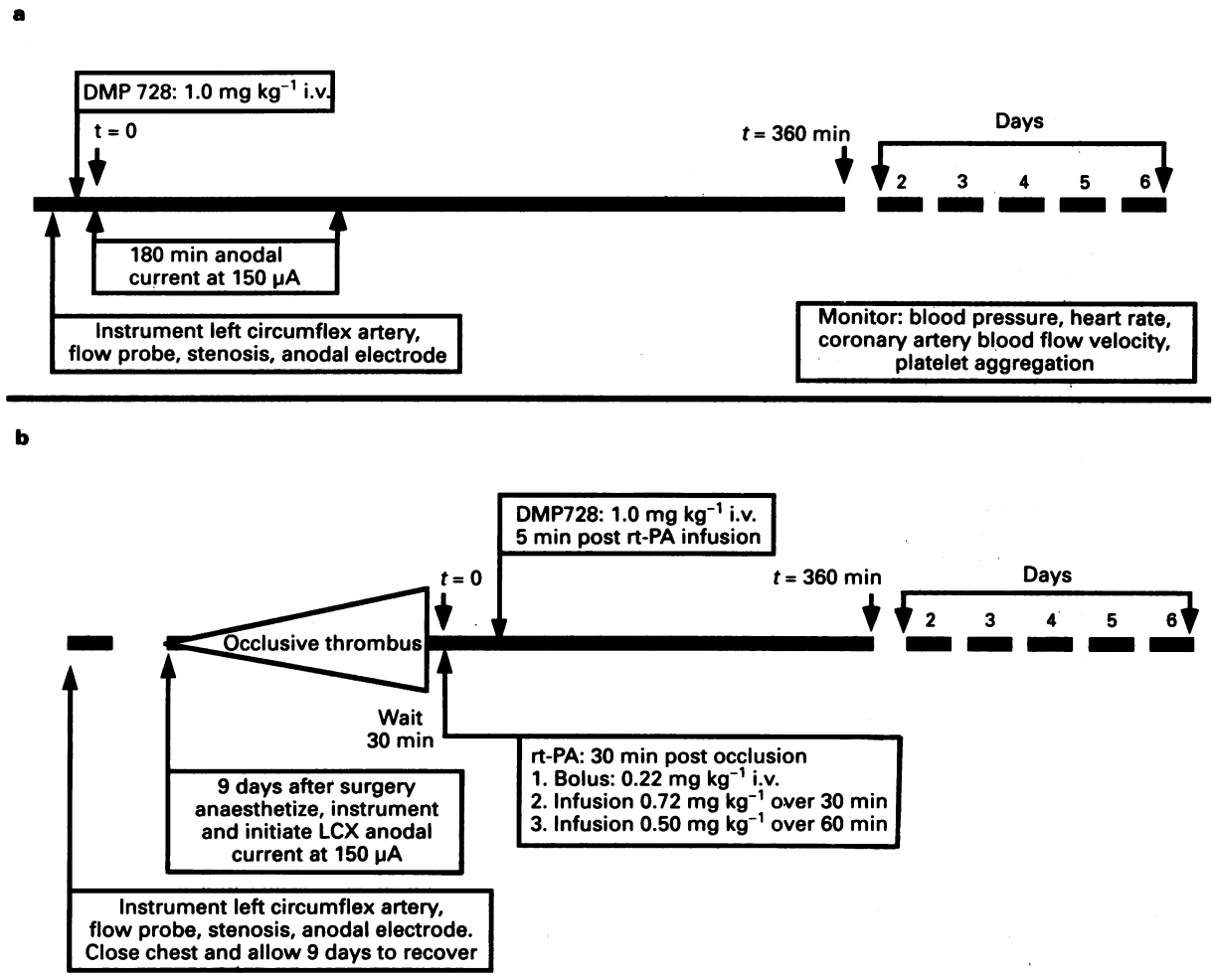

Figure 2 (a) Diagrammatic representation of the experimental protocol used to determine the ability of DMP728 to prevent primary coronary artery thrombosis in response to electrolytic current-induced injury to the vessel wall. Heart rate, coronary artery blood flow velocity, whole blood cell counts and platelet aggregation were monitored throughout the study. The period of the study is represented in the figures with the heavy dark line for day 1 and the broken dark lines representing days 2–6. DMP728 or saline was administered on day 1 before the initiation of anodal current application to the left circumflex coronary artery (LCX). (b) A protocol diagram for studies conducted to determine the ability of DMP728 to prevent secondary thrombus formation (rethrombosis) after rt-PA-induced thrombolysis of a preformed occlusive thrombus in the left circumflex coronary artery (LCX). The LCX was instrumented with a stimulating electrode, a flow probe and a critical stenosis. The dogs were allowed 9 days to recover from surgery. Dogs were returned to the laboratory and a thrombus was formed in the LCX. The coronary thrombus was allowed to age for 30 min and thrombolytic therapy with rt-PA was begun. Five mintues after the start of the graded rt-PA infusion, either DMP728 or saline was administered as an adjunctive agent. The dogs were monitored for 6 h after the start of thrombolytic therapy and then daily for 1 h on each of the five subsequent days. Heart rate, coronary artery blood flow velocity, whole blood cell counts and platelet aggregation were monitored throughout the study.

The transverse ventricular sections were weighed and traced onto clear acetate sheets. The red pigmented tissue containing the precipitated formazan complex was considered to represent viable tissue, whereas tissue that remained pallid was considered to be irreversibly injured or infarcted. The demarcated areas were scanned on a flatbed scanner and digitized using a Macintosh IIci microcomputer (Apple Computer, Cupertino, CA, U.S.A.) and appropriate software (MacDraft, Innovative Data Design, Concord, CA, U.S.A.). Infarct size was expressed as a percentage of total ventricular mass.

Protocol 2: prevention of rethrombosis after lysis

The protocol for studies designed to determine the efficacy of DMP728 for the prevention of rethrombosis is shown in Figure 2b. Animals were instrumented as described previously and were allowed 9 days to recover from the surgical procedure to ensure adequate wound healing. The animals were returned to the laboratory and anaesthetized with intravenous sodium pentobarbital (30 mg kg^{-1}, i.v.). A cannula was placed into the left jugular vein for obtaining blood samples and the injury current (150 μA) was applied to the left circumflex coronary artery for a maximum period of 3 h or was terminated 10 min after blood flow in the vessel had remained stable at zero velocity to certify formation of a stable occlusive thrombus. The thrombus was allowed to age for 30 min before initiating thrombolytic therapy. The thrombolytic agent, recombinant tissue plasminogen activator (rt-PA), was administered as a graded intravenous infusion according to the following regimen: 0.22 mg kg^{-1}, i.v. bolus, followed by 0.72 mg kg^{-1}, i.v. infused over 30 min and, finally, 0.50 mg kg^{-1}, i.v. infused over 90 min. The total intravenous dose of rt-PA was 1.44 mg kg^{-1}. DMP728 (1.0 mg kg^{-1}, i.v.) was administered as a slow intravenous injection given over 5 min and commencing 5 min after beginning the rt-PA infusion. Reperfusion was defined as the restoration of blood flow velocity to 20% of baseline values. Blood pressure, heart rate and coronary artery blood flow velocity were monitored for the remainder of the 6 h observation period, after which the animals were placed in their housing facilities until returned to the laboratory on each of the next 5 days.

Haematological measurements

Blood (20 ml) was withdrawn for platelet studies from the jugular cannula into a plastic syringe containing 3.2% sodium citrate as the anticoagulant [1:10 citrate–blood (v/v)]. Blood was taken for platelet aggregation and whole blood cell counts at baseline and 60, 240 and 360 min after the administration of DMP728 and again on each subsequent day of the protocol. The platelet count was determined with an H-10 cell counter (Texas International Laboratories, Houston, TX, U.S.A.). Platelet-rich plasma (PRP), the supernatant present after centrifugation of anticoagulated whole blood at 1000 r.p.m. for 5 min (140 g), was diluted with platelet-poor plasma (PPP) to achieve a platelet count of 200 000 mm^{-3}. PPP was prepared after the PRP was removed by centrifuging the remaining blood at 2000 *g* for 10 min and discarding the bottom cellular layer. *Ex vivo* platelet aggregation was measured by established spectrophotometric methods with a four-channel aggregometer (BioData-PAP-4, BioData Corp., Hatboro, PA, U.S.A.) by recording the increase in light transmission through a stirred suspension of PRP maintained at 37°C. Aggregation was induced with arachidonic acid (0.65 mM and 0.325 mM) and ADP (20 μM and 5 μM). A subaggregatory dose of adrenaline (550 nM) was used to prime the platelets before stimulation (Romson *et al.*, 1980). Values were expressed as percentage of aggregation, representing the percentage of light transmission standardized to PRP and PPP samples yielding 0% and 100% light transmission respectively.

Inclusion criteria

Animals to be included in the final protocol satisfied the following pre-established criteria: (a) a circulating platelet count of not less than 100,000 mm^{-3}; b) demonstrated ability for platelets to aggregate in response to arachidonic acid before administration of DMP728; and (c) absence of heart worms upon final post-mortem examination.

Drugs

DMP728 used in this study was provided by Du Pont Merck Pharmaceutical Company, Wilmington, DE, U.S.A. The recombinant tissue plasminogen activator, rt-PA (Activase), was purchased from the University of Michigan Hospital Pharmacy of the University of Michigan Medical Center, Ann Arbor, MI, U.S.A. All other chemicals used in this study were obtained from Sigma (St Louis, MO, U.S.A.)

Statistical analysis

The data are expressed as mean ± s.e.mean. *Ex vivo* platelet aggregation in response to arachidonic acid and to ADP was assessed before and after DMP728. The data were analysed by either paired or group analysis using Student's *t* test when applicable; differences were considered significant if $P<0.05$.

Results

Protocol 1: prevention of primary coronary artery thrombus formation

A total of 16 dogs was entered into protocol 1. Each of the 16 animals met the criteria for inclusion in the protocol. Eight of the dogs were assigned to each of the treatment groups, either saline of DMP728. Blood pressure, heart rate and coronary artery flow velocity were not different between groups at baseline. The administration of DMP728 had no effect on either mean arterial blood pressure or heart rate.

Circulating blood cells The effect of DMP728 (1 mg kg^{-1}, i.v.) on whole blood cell counts, haemoglobin and haematocrit are shown in Table 1. No changes were noted in red blood cell count or haematocrit over the 6 days of the study. Over the acute post-operative period, white blood cell counts increased on days 3–6 out of the range of normal laboratory values, but did not become statistically different from baseline values. Haemoglobin values were decreased on days 5 and 6 and platelet counts were significantly increased 6 days after the administration of DMP728. The importance of these changes are unclear at this time. None of the animals showed evidence of infection related to the surgical procedure or abnormal values in body temperature.

Platelet aggregation studies *Ex vivo* platelet aggregation in response to adenosine diphosphate (ADP) and arachidonic acid was determined at baseline and again at 60, 240 and 360 min after DMP728 administration. *Ex vivo* platelet aggregation determinations were repeated on the subsequent days 2 through 6 (Figure 3). Aggregation to each of the agonists was inhibited significantly on day 1 among those animals given DMP728, but remained unaltered from baseline responses in the saline-treated controls. *Ex vivo* platelet aggregation responses to arachidonic acid and ADP were consistent in the surviving control animals on each of the subsequent days. Among the animals treated with DMP728, *ex vivo* platelet aggregation returned to baseline within the first 24 h and remained consistent over the next 5 days in the surviving animals.

Thrombus formation and vessel patency Coronary artery blood flow velocity was monitored daily with the use of a chronically implanted Doppler flow probe on the left circumflex coronary artery. The data are presented graphically in Figures 4a and 4b. Blood flow velocity in the saline-treated group (Figure 4a) declined to zero within the 180 min period of anodal current application and was accompanied by char-

Table 1 Effects of DMP728 (1 mg kg^{-1}, i.v.) on whole blood cell counts in the dog

Time after DMP728	*Red cell count* ($\times 10^6$ mm^{-3})	*White cell count* ($\times 10^3$ mm^{-3})	*Platelet count* ($\times 10^3$ mm^{-3})	*Haematocrit* (%)	*Heamoglobin* (g dl)
Baseline	6.0 ± 0.6	16.4 ± 2.3	215 ± 22	34.4 ± 3.2	17.2 ± 2.9
60 min	5.8 ± 0.3	17.3 ± 2.3	242 ± 25	34.8 ± 2.6	14.8 ± 2.7
240 min	6.0 ± 0.4	14.9 ± 1.7	227 ± 19	34.5 ± 2.0	15.5 ± 2.8
360 min	6.1 ± 0.4	16.3 ± 2.2	228 ± 24	34.0 ± 2.7	15.0 ± 2.9
Day 2	5.7 ± 0.4	16.2 ± 2.5	220 ± 25	33.4 ± 2.3	15.1 ± 3.1
Day 3	6.1 ± 0.3	19.2 ± 2.9†	304 ± 46	36.9 ± 2.2	13.3 ± 2.1
Day 4	6.3 ± 0.5	19.4 ± 3.2†	265 ± 43	36.2 ± 3.2	13.4 ± 2.3
Day 5	6.3 ± 0.3	25.6 ± 4.4†	286 ± 39	35.9 ± 1.9	11.4 ± 0.5†
Day 6	6.0 ± 0.5	21.3 ± 3.4†	313 ± 39*	36.4 ± 3.6	11.5 ± 0.8†

Each value represents the mean ± s.e.mean, $n = 8$ in each group; *$P<0.05$ *vs* baseline values; †signifies out of normal range, but not statistically different from baseline values. Normal ranges (canine): red cell count 4.4–6.4, white cell count 5.8–17.8 platelet count 200–300, haematocrit 26.0–42.0, haemoglobin 12.6–24.6.

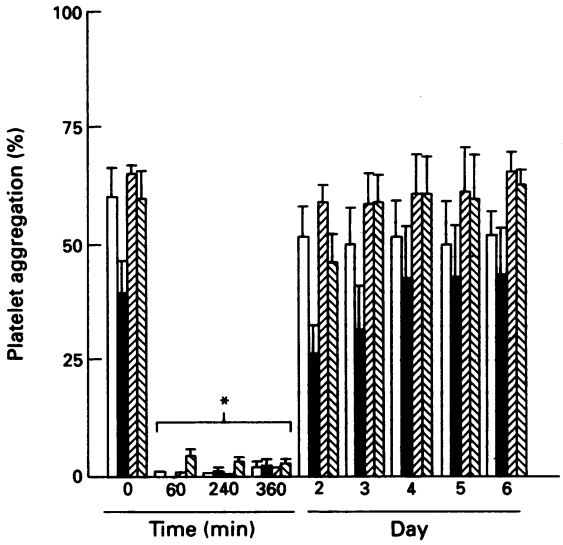

Figure 3 Adenosine diphosphate (ADP)-and arachonic acid-induced *ex vivo* platelet aggregations (mean ± s.e.mean; $n = 8$) from animals that received DMP728 (1.0 mg kg^{-1} i.v.). Platelet aggregation studies were performed at baseline and again at 60, 240 and 360 min after DMP728 administration. Aggregations also were measured on subsequent days 2–6. *$P<0.05$ *vs* baseline. □, ADP, 20 μM; ■, ADP, 5 μM; ▨, arachidonic acid, 0.650 mM; ▩, arachidonic acid, 0.32 M.

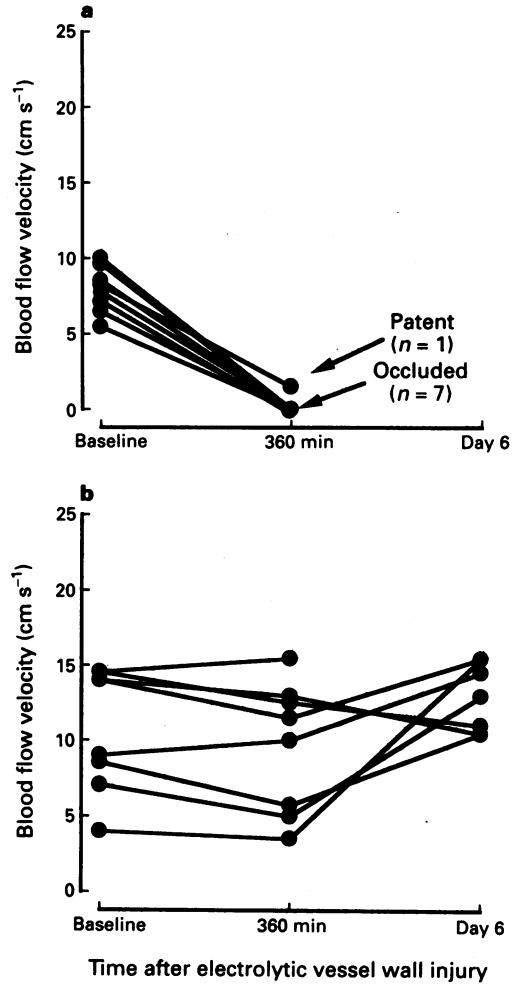

Figure 4 (a) The blood flow velocity measured in left circumflex coronary arteries in animals that received saline before application of the anodal current (180 min, 150 μA) to the intimal surface of the artery. Values are shown for each animal at baseline and 360 min after application of the electrolytic current and induction of vessel wall injury. Occlusive thrombus formation developed in seven of eight dogs before the end of the 360 min protocol on day 1. None of the control dogs survived on the day 6 time point. (b) Coronary artery blood flow velocity for dogs given DMP728 (1 mg kg^{-1}, i.v.) before the induction of vessel wall injury. Platelet inhibition with DMP728 limited thrombus formation and prevented the subsequent reduction in blood flow. Flow was maintained during the next 5 days in all but one dog, which died suddenly in the early hours of day 5.

acteristic changes in the lead II electrocardiogram showing initial depression and then persistent elevation in ST segment. In seven of the eight saline-treated control animals, the left circumflex coronary artery remained occluded for the remainder of the day, with one animal showing slight, but measurable, blood flow velocity. By day 6, all control animals had died, suddenly and without warning.

The administration of the platelet GPIIb/IIIa receptor antagonist, DMP728, limited the extent of intracoronary thrombus formation and prevented the subsequent reduction in coronary artery blood flow velocity as well as the electrocardiographic changes suggestive of regional myocardial ischaemia and/or injury. In seven of the eight animals assigned to receive DMP728, coronary artery blood flow velocity was present on each of the subsequent 5 days. The one remaining DMP728-treated animal died suddenly and unexpectedly during the evening of day 5 after being returned to its holding facilities.

The patency status of individual animals over the 6 day experimental period is summarized in Figure 5 (a and b). The open bars signify that the relevant coronary artery is patent. The hatched bars represent thrombotic occlusion of the coronary artery as determined by the absence of blood flow velocity and the presence of initial ST-segment changes and evolving Q-waves on the subsequent days of observation. The absence of a bar or lack of representation in the chart indicates that the animal has expired. Anodal current vessel wall injury resulted in complete thrombotic occlusion of the left circumflex coronary artery in each of eight animals in the control saline treatment group on day 1 of the experimental protocol. The mean time to occlusion was 90.4 ± 13.9 min in the saline-treated dogs. In contrast, those animals receiving DMP728 maintained patent coronary arteries throughout the initial 6 h period of observation. Each of the animals treated with DMP728 was alive on day 2 of the protocol as compared with three of eight (38%) animals in the saline-treated group. Among the survivors in the control group, one animal had a measurable blood flow velocity on day 2, but exhibited electrocardiographic signs of myocardial infarction. The remaining two animals had persistent thrombotic occlusion accompanied by electrocardiographic signs of myocardial injury and/or infarction. Only one of the saline-treated animals was alive on day 3, but by day 4 had expired suddenly. In contrast, each of the animals in the DMP728 group had patent coronary arteries on each of the subsequent days. One dog died suddenly between day 4 and 5, and this was the only death in the DMP728-treated animals. Survival over the course of the 6 day protocol was 88% for the DMP728 treatment group, which different significantly from the control group ($P<0.05$, Fisher's exact).

Thrombus weight Thrombus weight was reduced significantly in the group of animals treated with DMP728 when compared with the thrombi removed from the control animals (DMP728 = 1.7 ± 0.4 mg and controls = 20.7 ± 5.0 mg). The thrombi were removed either after spontaneous death of the animal or at the time of euthanasia on the sixth day. The data with respect to thrombus weights should be interpreted with caution, however, because the age of the individual thrombi differs from animal to animal and the contribution of the endogenous fibrinolytic system to further

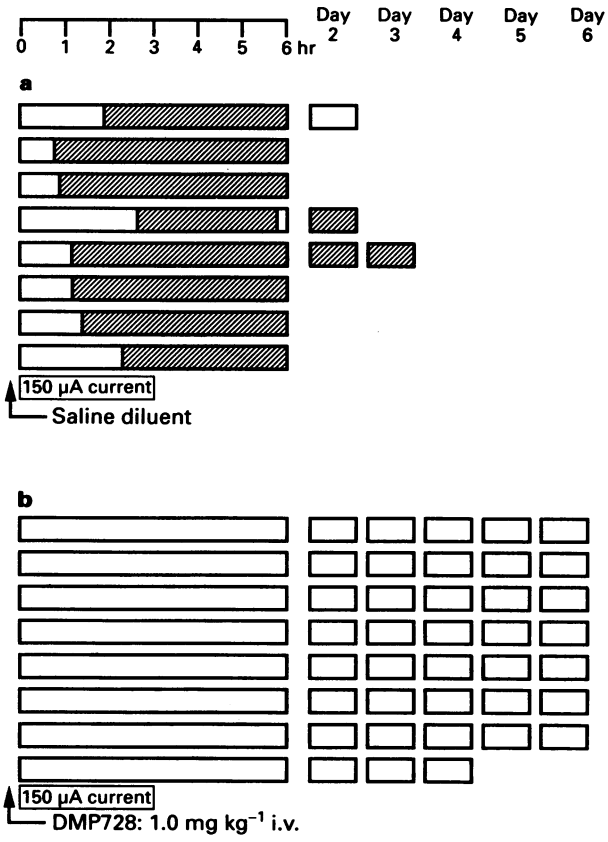

Figure 5 Diagram representing the patency status of left circumflex coronary arteries during the experimental protcol. Each horizontal bar represents one dog over 36 min on day 1; individual boxes signify patency status on days 2–6. (a) Dogs in the control group given saline. The animals shown in (b) received DMP728 (1.0 mg kg^{-1}, i.v.) before the start of anodal current application. Open or white bars represent patent arteries; hatched bars signify thrombotic occlusion; the absence of a bar indicates that the dog has died.

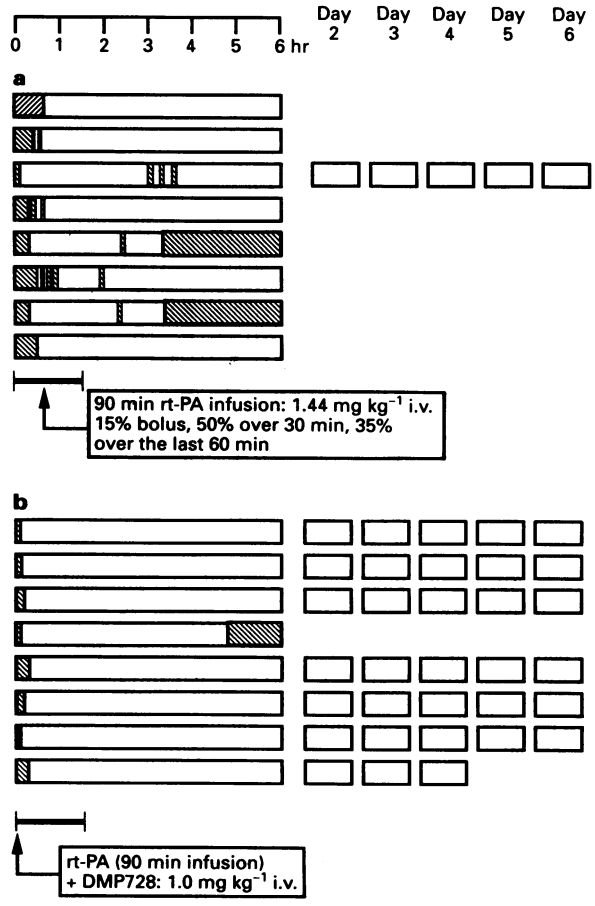

Figure 6 Diagram representing the patency status of left circumflex coronary arteries during the experimental period. Each horizontal bar represents one dog over 360 min on day 1 with zero time equal to the start of rt-PA infusion. Individual boxes indicate the patency status on days 2–6. (a) Dogs receiving rt-PA + saline. The animals shown in (b) received DMP728 (1.0 mg kg^{-1}, i.v.) as adjunctive therapy with rt-PA. Open or white bars represent patent arteries; hatched bars signify thrombotic occlusion; the absence of a bar indicates that the animal had expired.

modification of the residual thrombus is uncertain. The thrombi removed from dogs receiving DMP728 were obtained on the sixth day with the exception of one animal which died suddenly on the fourth day of the protocol.

Infarct size Each of the eight dogs in the control group exhibited evidence of extensive irreversible myocardial injury as determined by the tetrazolium method. Evidence of myocardial infarction was absent in three of the eight DMP728-treated animals, and overall infarct size was reduced significantly compared with the control group. Infarct size expressed as a percentage of the left ventricle was $1.7 \pm 0.8\%$ in the DMP728-treated group *vs* $27.5 \pm 4.5\%$ in the control group. The observations correlate with the results reported for thrombus weight and the incidence of mortality. Infarct size was expressed as a percentage of the total left ventricle. Therefore, caution should be used when interpreting the results on infarct size since determination of neither collateral blood flow nor area at risk was made owing to the limitations imposed as a result of the experimental protocol.

Protocol 2: prevention of rethrombosis after rt-PA-induced thrombolysis

A total of 17 dogs was used in this arm of the study. Eight of the dogs were assigned to each of the treatment groups, either saline or DMP728. One animal from the control group was excluded owing to the presence of heart worms upon post-mortem examination. Blood pressure, heart rate and coronary artery blood flow velocity were not different between groups at baseline. The administration of DMP728 had no effect on either mean arterial blood pressure or heart rate.

Platelet aggregation studies *Ex vivo* platelet aggregation in response to adenosine diphosphate (ADP) or arachidonic acid was determined at baseline and again at 30 and 300 min after DMP728 or saline administration. Aggregations were repeated on the subsequent days 2–6. Aggregation to each agonist was prevented on day 1 among the animals receiving DMP728. As in protocol 1, *ex vivo* platelet aggregation responses returned to baseline values within 24 h and remained consistent over the subsequent study days. The *ex vivo* platelet aggregation responses in PRP from the saline-treated animals did not differ from baseline at any time point in the study.

Thrombosis/thrombolysis/rethrombosis The patency status of the left circumflex coronary artery of individual animals in each group over the 6 day experimental period is represented in Figure 6 (a and b). As before, open bars signify that the coronary artery is patent. The hatched bars represent thrombotic occlusion of the coronary artery as indicated by the absence of a Doppler flow signal and the presence of electrocardiographic ST-segment changes or evolution of Q-

waves indicative of myocardial ischaemia or cell death respectively. The absence of a bar signifies that the animal expired suddenly and unexpectedly. All vessels became occluded in response to anodal current application and all arteries became patent with a restoration of blood flow after administration of rt-PA (1.44 mg kg^{-1}, i.v.) Reocclusions, characterized by oscillatory patterns of blood flow, of varying durations occurred in six of the eight saline-treated control animals. In contrast, oscillatory blood flow did not occur in the drug-treated animals and occlusive rethrombosis developed in only one of the dogs receiving DMP728. On day 2, only one of eight saline-treated animals had survived, whereas seven of eight dogs in the DMP728-treated group ($P<0.05$, Fisher's exact) were alive. Of the remaining animals in the DMP728-treated group, all but one survived to the end of the sixth day. One animal died suddenly between day 4 and day 5.

Time to thrombolysis The time to thrombolysis, defined as the restoration of coronary artery blood flow velocity to 20% of the baseline, was determined in each of the animals. Treatment with DMP728 during the administration of rt-PA was associated with a decrease in the time to reperfusion when compared with the group of animals receiving rt-PA plus saline (rt-PA + DMP728 (1.0 mg kg^{-1}, i.v. = 9.9 ± 2.2 min *vs* rt-PA + saline = 18.4 ± 2.3 min; $P<0.05$).

Residual thrombus size GPIIb/IIIa receptor antagonism with DMP728 showed a trend towards reduced residual thrombus weight. The thrombi were removed either after spontaneous death of the animal or at the time of euthanasia on day 6. Thrombus weights in the DMP728 group were smaller, but the difference was not significant (saline = 7.1 ± 1.8 mg; DMP728 = 2.8 ± 1.0 mg, P = NS). The comparison of thrombus weights, however, should be viewed with caution when interpreting data from these measurements owing to the varying time points at which the thrombi were recovered relative to the time of thrombus formation.

Infarct size Myocardial infarct size, expressed as a percentage of left ventricular mass averaged, 17.9 ± 2.9% in the controls *vs* 6.6 ± 1.7% in the DMP728-treated group ($P<0.05$). Adjunctive therapy with DMP728 was associated with a reduction in the extent of irreversible myocardial damage as compared with animals in the saline-treated control group.

Discussion

Interruption in the continuity of the endothelial surface of a normal blood vessel initiates the primary haemostatic response to injury invoking platelet activation accompanied by a localized vasoconstriction (Marcus, 1991) and cyclic flow variations in stenosed and endothelium-injured vessels mediated by platelet aggregates (Yao *et al.*, 1993). Endothelial cell injury leads to the exposure of subendothelial collagen fibrils that serve as an agonist for platelet activation and release of additional agonists including adensoine diphosphate, thromboxane and serotinin (Kroll & Schafer, 1989). The recruitment of additional platelets to the primary layer of adherent platelets forms the haemostatic plug. Concomitant with the activation and adhesion of platelets there is initiation of the coagulation cascade and generation of thrombin at the site of vessel injury. Thrombin is the catalyst for conversion of fibrinogen to fibrin. In addition, thrombin is a potent agonist for platelet activation (Marcus & Safier, 1993). The response to the agonistic potential of a deep vascular lesion differs from that occurring in response to normal haemostasis in injured, but otherwise healthy, vessels. Pathological or occlusive arterial thrombi that form in response to a severe arterial lesion may have the morphological features of a haemostatic plug, but involve far more platelet adhesion, activation, recruitment and consolidation (Marcus & Safier, 1993). Development of pathological arterial thrombi is the result of multiple factors required for induction, assembly and stabilization of the occlusive mass at the site of extensive vessel wall injury. The difficulty in preventing occlusive arterial lesions may be the result of the multifactorial nature of the arterial thrombotic process.

A further complication arises from the fact that thrombolytic agents activate platelets (Fitzgerald *et al.*, 1988a,b) as a result thrombin activation (Fitzgerald *et al.*, 1988b) or the effects of plasmin generation (Guccione *et al.*, 1985; Schafer *et al.*, 1986). The outcome of thrombolytic therapy is determined, in part, by the relative influences of the local proaggregatory and antiaggregatory factors exerted upon the circulating platelets and the residual vascular lesion. The circulating platelets have an important role in rethrombosis owing to their ability to be activated by the residual thrombus as well as by local thrombin and plasmin generation in response to lytic therapy (Guccione *et al.*, 1985; Schafer *et al.*, 1986; Fitzgerald *et al.*, 1988a). The final common pathway in the formation of a platelet aggregate is the cross-linking of adjacent activated platelets by binding of fibrinogen to the platelet GPIIb/IIIa receptor complex (referred to in the integrin nomenclature, as αIIbβ3) (Phillips *et al.*, 1987; Gatter *et al.*, 1988; Keiffer & Phillips, 1990; Kieffer *et al.*, 1992). Theoretically, the prevention of occlusive arterial lesions may be achived by modulating the reactivity of the blood platelet by inhibiting interaction of the GPIIb/IIIa receptor with its ligand.

GPIIb/IIIa is the most abundant of the platelet integrins and is found only on platelets and cells of megakaryoblastic potential. GPIIb/IIIa becomes a receptor for a number of soluble adhesive proteins (fibrinogen, von Willebrand factor, vitronectin, fibronectin) via interaction with the Arg-Gly-Asp (RGD) recognition sequence (Ginsbger *et al.*, 1988a,b).

There is compelling evidence that adhesive proteins binding to the GPIIb/IIIa complex and that platelet aggregates are integral components of thrombus formation *in vivo* (Coller, 1990; 1992). The GPIIb/IIIa complex provides a logical site for pharmacological interventions directed towards an inhibition of platelet aggregation and arterial thrombus formation. Several RGD-containing peptides, either synthetic or derived from snake venoms (disintegrins), are known to block fibrinogen binding and to prevent the formation of platelet thrombi (Cadroy *et al.*, 1989; Dennis *et al.*, 1990; Gould *et al.*, 1990; Tschopp *et al.*, 1993). Furthermore, blockade of the GPIIb/IIIa receptor with monoclonal antibodies has proved to be antiaggregatory in experimental (Mickelson *et al.*, 1990a,b; Bates *et al.*, 1991; Rote *et al.*, 1994a) and clinical studies (Coller, 1990; 1992; Gold *et al.*, 1990; Kleiman *et al.*, 1993). More recently, peptidomimetic and non-peptide RGD mimetics have been developed in an effort to achieve greater antithrombotic potency and selectivity at the site of the GPIIb/IIIa receptor (Kouns *et al.*, 1992; Peerlinck *et al.*, 1993; Rote *et al.*, 1993; Roux *et al.*, 1993). The present study provides experimental findings with DMP728, a non-peptidomimetic antagonist of the platelet GPIIb/IIIa integrin receptor. The intravenous administration of a single 1.0 mg kg^{-1}, dose of DMP728 prevented primary thrombus formation in response to deep arterial wall injury as well as rethrombosis after successful thrombolysis in a chronic canine model. The comparison between the control and drug-treated animals subjected to primary thrombosis or rethrombosis demonstrates the importance of modulating platelet reactivity by virtue of inhibiting the platelet GPIIb/IIIa receptor.

The first experimental protocol was designed to determine if DMP728 could prevent occlusive thrombus formation in response to severe arterial wall injury (primary thrombosis). The study was initiated in the instrumented, closed-chest, anaesthetized dog in which electrolytic injury of the left circumflex coronary artery was induced in either control saline-treated animals or in animals pretreated with DMP728 administered as a single intravenous dose of 1.0 mg kg^{-1}. The

dose of DMP728 was selected on the basis of previous studies in which it was found to prevent rethrombosis in a canine model of arterial thrombosis and thrombolysis (Mousa *et al.*, 1994; Rote *et al.*, 1994a,b). The animals were monitored continuously for 6 h on the first day of the protocol and on each of the subsequent 5 days. The results indicate that DMP728 prevented primary thrombus formation. The protective effect persisted for the duration of the protocol despite the presence of a deep arterial wall lesion and the return to control values of *ex vivo* platelet reactivity within 24 h after drug administration. The results in the saline-treated control are in marked contrast. Each of the control animals developed an occlusive thrombus in response to electrolytic injury of the arterial wall. The occurrence of sudden death due to ventricular fibrillation within the first 24 h was more common in the control group than in the DMP728-treated animals. Overall survival at the end of the 6 day protocol was improved significantly in the DMP728-treated animals as compared with the saline-treated controls. The observed long-term beneficial effects of DMP728 were obtained despite the fact that platelet reactivity, as assessed by *ex vivo* aggregation, was not inhibited beyond the first 24 h after drug administration. Thus, thrombus formation did not develop over the subsequent 5 days in the DMP728-treated animals, suggesting that the injured vessel wall had lost its potential as a thrombogenic site. As would be expected, thrombus weight and, in particular, infarct size were reduced significantly in animals receiving DMP728 as compared with the saline-treated control group.

The results of the present study are in accord with our earlier observations (Bates *et al.*, 1992) demonstrating that temporary, but complete, inhibition of the platelet GPIIb/IIIa receptor for several hours after deep arterial wall injury significantly reduced later intravascular thrombus formation and thrombotic occlusion. The long-term prevention of primary thrombus formation by selective inhibition of the GPIIb/IIIa receptor in a chronic model of severe vessel wall injury illustrates the important role of the platelet glycoprotein receptor as a mediator of thrombus formation. Temporary inhibition of the GPIIb/IIIa receptor prevents thrombus formation in contrast to merely delaying the development of the occlusive lesion. Inhibition of platelet deposition for 8 h with dipyridamole or prostacyclin after percutaneous balloon de-endothelization rendered rabbit aortae non-reactive to circulating blood platelets (Groves *et al.*, 1986). *In vivo* studies using a reperfusion model demonstrated that the vessel wall lost the ability to initiate thrombus formation within 40 min after endothelial injury, thus emphasizing that platelet deposition and thrombus formation are time-limited phenomena (Baumgartner, 1973). The existence of a specific time window in which the injured vessel wall remains thrombogenic is an important consideration in designing appropriate dosing regimens and schedules for prevention of arterial occlusive events, as might develop after invasive procedures likely to be associated with vessel wall damage and increased thrombogenicity at the site of injury. The time-limited duration of the 'window of thrombogenicity' may provide a reasonable explanation for why a single dose of DMP728 was able to prevent primary occlusive thrombus formation. Whether or not the same 'window of thrombogenicity' will apply to clinical situations of coronary artery thrombolysis or coronary angioplasty remains to be determined, especially in light of recent information suggesting the need for prolonged platelet inhibition by the chimeric 7E3-Fab antibody after thrombolytic therapy (Kleiman *et al.*, 1993).

The second phase of our study evaluated the ability of DMP728 to prevent rethrombosis after successful thrombolysis in a chronic canine model. Thrombolysis and recanalization of the affected vessel was achieved readily in all animals after the intravenous administration of rt-PA. As observed in previous studies (Mickelson *et al.*, 1990a), using the same experimental procedure, thrombolysis with rt-PA, in the absence of adjunctive therapy, was accompanied by repetitive episodes of oscillatory variations in coronary artery blood flow. The erratic flow pattern was characterized by periods of total cessation of blood flow followed by periods of reactive hyperaemia, eventually culminating in sudden arrhythmic death or total thrombotic occlusion within the first 24 h. In the present study, only one of eight saline-treated control animals undergoing thrombolysis maintained a patent circumflex coronary artery and survived the subsequent 5 days of the study protocol. Seven of the eight DMP728-treated animals maintained patent vessels and survival was improved significantly as compared with the control group.

The present study was not designed to assess the influence of DMP728 upon rt-PA-induced thrombolysis. It became apparent, however, that the administration of the GPIIb/IIIa receptor antagonist 5 min after completion of the rt-PA infusion, but before recanalization had occurred, significantly decreased the time needed to achieve thrombolysis. Therefore, the treatment regimen consisting of rt-PA followed by the intravenous administration of 1.0 mg kg^{-1} of DMP728 accelerated recanalization of the occluded vessel and significantly reduced both the incidence of reocclusion and death. Furthermore, the extent of irreversible myocardial tissue injury was reduced significantly in the animals treated with DMP728. In addition to being involved in thrombus formation, aggregating platelets can initiate intense vasoconstriction after the activation and release of potent vasoactive mediators (Saldeen *et al.*, 1993; Yao *et al.*, 1993). Infarct size expansion is related to the extent of platelet accumulation in myocardium that has undergone reperfusion in the presence of a critical stenosis (Rousseau *et al.*, 1993). The beneficial effect on the salvage of myocardial tissue, after inhibition of the GPIIb/IIIa receptor, may derive, in part, from the fact that coronary artery blood flow is maintained and not compromised by repetitive episodes of oscillatory blood flow commonly observed after thrombolysis in the absence of adjunctive anti-platelet therapy.

A recent study (Kohmura *et al.*, 1993) assessed the effect of an initial single intravenous bolus injection of chimeric 7E3-Fab followed by repeated bolus injections of rt-PA on reperfusion and reocclusion in heparinized baboons with a femoral arterial thrombosis with superimposed high-grade stenosis. The presence of the platelet GPIIb/IIIa receptor antagonist, chimeric 7E3-Fab, decreased the time to recanalization and sustained femoral artery blood flow in contrast to control animals receiving saline or aspirin as the adjunctive treatment. In addition, it has been reported that concomitant systemic therapeutic heparin anticoagulation is required for the maximal effect of 7E3 (Yasuda *et al.*, 1990). A major difference between the previous study (Kohmura *et al.*, 1993) and the present investigation is that we did not use heparin. Despite the absence of heparin in the present study, DMP728 was capable of decreasing the time to clot lysis. Whether or not heparin is necessary for attaining beneficial effects with other inhibitors of the platelet glycoprotein IIb/IIIa receptor requires further investigation. With the exception of enhancing the apparent time to clot lysis, DMP728 provided beneficial effects identical to those observed in a previous study (Mickelson *et al.*, 1990a) in which 7E3 $F(ab')_2$ was administered after rt-PA in a chronic canine model of coronary artery thrombosis and thrombolysis. As in the present study, 7E3 $F(ab')_2$ administration resulted in a preservation of vessel patency and abolition of repetitive oscillatory flow in the severely injured circumflex coronary artery. The present results complement the recent observations (Mousa *et al.*, 1994) and provide additional insight into the efficacy of DMP728 as an antithrombotic agent in a chronic model of vessel wall injury. DMP728 in a dose of 1.0 mg kg^{-1} prevented both primary and secondary thrombus formation when used as the sole anti-platelet agent and was fully effective in the absence of heparin or antithrombins. The dose of DMP728 was the same as that used by Mousa *et al.*

(1994), who reported that the drug's duration of action and efficacy in preventing thrombus formation was decreased at lower doses. A similar dose–response relationship with respect to the prevention of primary and secondary thrombosis was reported by Rote *et al.* (1994).

The ability of inhibitors of the platelet glycoprotein IIb/IIIa receptor to maintain vessel patency when used as the sole adjunctive agent in combination with thrombolytic therapy provides compelling evidence that the platelet plays a major role in rethrombosis. Platelets are activated during thrombolysis (Coller, 1990), and the residual thrombus along with the stenotic lesion present a thrombogenic substrate for recruitment of new platelets and fibrin deposition leading to rethrombosis. Thrombolysis therefore, is accompanied by a dynamic interaction in which local lytic and thrombogenic activity occur simultaneously. Inhibition of the platelet GPIIb/IIIa receptor shifts the equilibrium so that lytic activity predominates, thereby maintaining blood flow and permitting time for the residual thrombus and injured vessel to become non-thrombogenic.

Critique of the experimental model

The original electrically induced coronary artery thrombosis model in the canine heart employed alternating current delivered to the vascular lumen through a catheter electrode positioned in the coronary artery under fluoroscopic control (Salazar, 1961). The experimental model used in this study was described previously (Romson *et al.*, 1980). Since it was first described, the model has been used by several investigators to examine a variety of anti-platelet interventions for the prevention of primary thrombus formation (Fitzgerald *et al.*, 1986; Mickelson *et al.*, 1990b; Rote *et al.*, 1993) or prevention of rethrombosis after successful thrombolysis (Mickelson *et al.*, 1990a; Rote *et al.*, 1993; 1994a; Saldeen *et al.*, 1993; Tschopp *et al.*, 1993). The arterial wall lesion and subsequent formation of an occlusive thrombus result from direct application of an anodal direct current to the endothelial surface of the vessel, leading to exposure of proaggregatory subendothelial elements (Mickelson *et al.*, 1990b; Bates *et al.*, 1992). The resulting thrombus consists of a platelet–fibrin mass adherent to the vessel wall at the site of intimal injury (Romson *et al.*, 1980) and possess the morphological features of acutely formed thrombi found in patients with acute myocardial infarction (Falk, 1985). The cellular composition of the arterial thrombus is an important consideration in the interpretation of data derived from experimental studies. Erythrocyte-rich coronary arterial thrombi are consistently lysed after an intravenous infusion of rt-PA, whereas platelet-rich thrombi are relatively resistant to lysis even in the presence of high doses of a thrombolytic agent (Fitzgerald *et al.*, 1988a). It is recognized, however, that the experimental model lacks the atherosclerotic lesions that are prominent in most, if not all, diseased human arteries in which thrombus formation occurs. The inability to replicate the entire human clinical pathophysiological state may suggest that prevention of rethrombosis in a diseased human artery with atherosclerosis may not be prevented to the same degree as in the experimental animal.

An important feature of the experimental model is that it permits an extended period of observation in contrast to an acute animal model. During the subsequent days, one can evaluate the efficacy of a selective intervention with respect to preventing thrombus formation and determine the duration for maintaining inhibition of the GPIIb/IIIa receptor during which time the thrombogenic focus undergoes resolution and becomes non-thrombogenic. A minor limitation in the chronic animal model is the inability to evaluate bleeding times by the Simplate method owing to the humane concerns for the welfare of the experimental animal.

Summary

DMP728 is among the first of a new series of non-peptide, low molecular weight compounds designed to inhibit the platelet GPIIb/IIIa receptor. The present study, conducted in the chronically instrumented canine, demonstrates that DMP 728 is effective in preventing occlusive arterial thrombosis in response to severe vessel wall injury and to prevent rethrombosis after successful thrombolytic therapy. In both instances, DMP728 was administerd once as an intravenous dose of 1.0 mg kg^{-1}. DMP728, as the singular adjunctive agent, provided prolonged protection in terms of maintaining vessel patency and reducing mortality. Although the platelet GPIIb/IIIa receptor may appear to be a logical target site for therapeutic intervention in thromboembolic disease, the safety and efficacy of this class of agent will be determined by the outcome of well-designed clinical trials in man. The recently reported clinical results (Kleiman *et al.*, 1993) with a platelet GPIIb/IIIa receptor antagonist (monoclonal antibody 7E3-Fab) administered after thrombolytic therapy suggest that inhibition of the platelet integrin receptor has therapeutic potential. DMP728 provides an alternative intervention by which the concept can be evaluated further in both basic and clinical studies.

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