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Cardioprotective actions of oligotide, a single stranded polydeoxyribonucleotide complex, in myocardial ischaemia and reperfusion injury

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1 The efficacy of oligotide, a single stranded polydeoxyribonucleotide complex, was examined in a feline model of myocardial ischaemia (MI: 90 min) and reperfusion (R: 270 min). Oligotide (15 mg kg^{-1} bolus) was administered intravenously 80 min after occlusion of the left anterior descending (LAD) coronary artery (i.e., 10 min prior to R) and continued for an additional 280 min (10 mg kg^{-1} h^{-1} infusion).

2 Oligotide-treated cats showed significantly smaller myocardial necroses and lower cardiac myeloperoxidase activities (significantly lower neutrophil infiltration) in the necrotic zone as compared to MI+R cats receiving only vehicle.

3 LAD coronary arteries isolated from MI+R cats exhibited a significant endothelial dysfunction (i.e., reduced endothelium-dependent relaxation), and significantly increased adherence of polymorphonuclear neutrophils (PMNs) *ex vivo*. However, oligotide significantly preserved endothelial function and attenuated PMN adherence in ischaemic LAD coronary arteries.

4 Oligotide attenuated P-selectin expression on thrombin-stimulated platelets as well as PMN adherence to thrombin-stimulated coronary endothelium. Immunohistochemical examination *in vivo* revealed that oligotide treatment also significantly inhibited coronary endothelial P-selectin expression after 90 min MI and 20 min R.

5 Oligotide exerted a significant cardioprotection in MI+R injury. The mechanism appears to be related to attenuation of PMN-endothelial interaction and eventual infiltration into the ischaemic myocardium.

Keywords: Myocardial reperfusion injury; myeloperoxidase activity; endothelium-derived relaxing factor; neutrophil adhesion; polydeoxyribonucleotide; P-selectin

Introduction

Coronary artery reperfusion is the most effective treatment for patients with acute myocardial infarction; however, reperfusion itself often results in enhanced tissue damage, a phenomenon called reperfusion injury (Braunwald & Kloner, 1985; Farb *et al.*, 1993). Adhesion of polymorphonuclear leukocytes (PMNs) to the vascular endothelium and subsequent accumulation of PMNs into the ischaemic myocardium are believed to play an important role in MI+R injury (Engler *et al.*, 1986; Entman *et al.*, 1991). In this regard, inhibition of neutrophil-endothelial interaction after reperfusion (e.g., by monoclonal antibodies directed against cell adhesion molecules, or by a putative soluble ligand such as sialyl Lewis^x oligosaccharide) significantly attenuates MI+R injury (Simpson *et al.*, 1988; Ma *et al.*, 1991; Weyrich *et al.*, 1993; Buerke *et al.*, 1994).

Oligotide is low-molecular weight fraction of defibrotide and is a complex of single-stranded polydeoxyribonucleotides isolated by a controlled depolymerization of bovine lung DNA and comprises a cluster of chains of different length and base sequences (Bianchi *et al.*, 1993; Lanzarotti *et al.*, 1993; Skurk *et al.*, 1995). The chain length of oligotide is distributed in a Gaussian-like fashion, with a purine/pyramidine nucleotide molar ratio of 1.0 ± 0.2 (Lanzarotti *et al.*, 1993). The molecular weight of oligotide is 8 ± 2 kDa, while that of defibrotide is 20-30 kDa. Recently, defibrotide has been found to exert cytoprotective actions in acute inflammatory disorders (Niada *et al.*, 1986; Lefer *et al.*, 1990; Palmer & Goa, 1993). Interestingly, difibrotide has been shown to inhibit both activation and accumulation of PMNs in MI+R-induced tissue injury (DiPerri *et al.*, 1987; Lefer *et al.*, 1990). We therefore reasoned that these properties of oligotide could attenuate MI+R-induced myocardial tissue injury; however no study has been performed.

Accordingly, the main purposes of the present study were (a) to investigate whether oligotide has a cardioprotective action in MI+R injury *in vivo*, and (b) to elaborate the mechanism(s) by which oligotide may achieve cardioprotective effects with special reference to the PMN-endothelial interactions.

Methods

Myocardial ischaemia and reperfusion in cats

Adult male cats (2.5-3.9 kg) were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} body weight, i.v.). An intratracheal cannula was inserted, and cats were placed on intermittent positive-pressure ventilation. A polyethylene catheter was inserted into the right femoral artery for measurement of mean arterial blood pressure. Additional polyethylene catheters were inserted into the right external jugular vein for infusion of oligotide or its vehicle, and into the right femoral vein for additional infusion of anaesthetic. A midsternal thoracotomy was performed, and the pericardium was opened to place a 3-0 silk ligature around the left anterior descending (LAD) coronary artery 8 to 10 mm from its origin. Standard lead II of the scalar ECG was used to measure ST-segment elevation and heart rate. The ECG and mean arterial blood pressure were continuously monitored and recorded on a Grass Instrument model 7 oscillographic recorder (Quincy, MA, U.S.A.) every 20 min.

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Experimental protocol

Animals were allowed to stabilize for 30 min after surgery, and baseline recordings of ECG and mean arterial blood pressure were made. Myocardial ischaemia was produced by tightening the silk ligature previously placed around the LAD to completely occlude the vessel. This was designated as time zero. After 90 min of ischaemia, the LAD slip knot was untied, and the ischaemic myocardium was reperfused for 270 min, resulting in a total experimental period of 6 h. Ten minutes prior to reperfusion, 15 mg kg^{-1} of oligotide (Crinos Biol. Res. Labs. Villa Guardia (CO) Italy) or its vehicle (2 ml of Krebs-Henseleit solution; K-H solution) was injected as a bolus intravenously. This was followed by an infusion of 10 mg kg^{-1} h^{-1} of oligotide or 1.34 ml h^{-1} of K-H solution (vehicle) which continued until the end of the protocol. In preliminary experiments, we tested a half dose of oligotide (i.e., 7.5 mg kg^{-1} bolus injection followed by 5 mg^{-1} kg^{-1} h^{-1} infusion) in three cats in the same experimental protocol. This concentration produced only partial cardioprotective effects, thus, we employed 15 mg kg^{-1} bolus plus 10 mg kg^{-1} h^{-1} infusion of oligotide for the entire experimental protocol. The animals were randomly divided into 3 groups: (a) sham MI + R cats treated with oligotide which were subjected to all surgical manipulations except that the ligature around the LAD was not tightened ($n=5$); (b) MI + R given K-H solution as a vehicle ($n=8$); and (c) MI + R treated with oligotide ($n=7$).

Blood sampling and CK measurement

Arterial blood samples (2 ml) were drawn every hour during the experimental protocol. Each blood sample was collected in tubes containing 200 iu of heparin sodium. After counting white blood cells in a haemocytometer (Unopette; Fisher Scientific, Pittsburgh, PA, U.S.A.), blood was centrifuged at 2500 *g* at 4°C for 20 min. The plasma was drawn off and analyzed spectrophotometrically for creatine kinase (CK) activity according to the method of Rosalki (1967). Plasma protein concentration was measured by the biuret method (Gornall *et al.*, 1949).

Determination of myocardial necrosis

Myocardial necrosis was analyzed by a double staining method (i.e., Evans blue and nitroblue tetrazolium) as reported previously (Ma *et al.*, 1991). At the end of the protocol, the LAD was again occluded and Evans blue was rapidly injected into the left ventricle. Thus, the area-at-risk (AAR) was determined by negative staining of Evans blue. The heart was isolated in warmed oxygenated K-H solution consisting of (in mmol l^{-1}): NaCl 118, KCl 4.75, $CaCl_2$ 2.54, KH_2PO_4 1.19, $MgSO_4$ 1.19, $NaHCO_3$ 12.5 and glucose 10.0. After isolating the left circumflex (LCX) and the LAD coronary arteries, the left ventricle was sliced and the unstained AAR portion was separated from the Evans blue-stained area-not-at-risk (ANAR) portion. Only the AAR myocardium was sliced and further incubated in 0.1% nitroblue tetrazolium at 37°C for 7 min. Nitroblue tetrazolium forms a blue formazan colour in the presence of intact coenzymes and dehydrogenases and thus stains only non-necrotic tissue (Klein *et al.*, 1981). Finally nitroblue tetrazolium-negative portion (necrotic tissue) was separated from the nitroblue tetrazolium-positive portion (i.e. non-necrotic tissue). Three portions of the left ventricular myocardium (i.e., ANAR, non-necrotic AAR, and necrotic AAR) were then weighed, and the results were expressed as the AAR as a percentage of the total left ventricular mass, and the area of necrosis calculated as a percentage of either the AAR of the total left ventricular mass. Aliquots of these three regions of myocardium were stored at −70°C for later assay of myeloperoxidase activity.

In three additional cats receiving vehicle, the above procedures were repeated except that half of the AAR was incubated with oligotide to rule out the possibility that oligotide directly altered the staining properties of the nitroblue tetrazolium. The area of necrotic tissue calculated as a percentage of the AAR was 38.4±6.2% in the control AAR samples and was 36.5±4.7% in the AAR samples incubated with oligotide (1 mg ml^{-1}) indicating that oligotide had no artifactual effect on nitroblue tetrazolium staining.

Determination of tissue myeloperoxidase activity

Myeloperoxidase is virtually exclusively present in neutrophils; thus, cardiac myeloperoxidase activity represents the extent of PMN infiltration. The enzyme activity was determined by the method of Bradley *et al.* (1985) as modified by Mullane *et al.* (1985). The myocardium was homogenized in 50 mmol l^{-1} potassium phosphate buffer at pH 6.0 containing 0.5% hexadecyltrimethyl ammonium bromide (Sigma) with a Polytron (PCU-2) homogenizer for 30 s at 7000 r.p.m. Homogenates were centrifuged at 15,000 *g* at 4°C for 30 min. The supernatants were then collected and reacted with 0.167 mg ml^{-1} of *o*-dianisidine dihydrochloride (Sigma) and 0.0005% H_2O_2 in 50 mmol l^{-1} phosphate buffer at pH 6.0. The change in absorbance was measured spectrophotometrically at 460 nm. One unit of myeloperoxidase activity was defined as that quantity of enzyme hydrolyzing 1 mmol of hydrogen peroxide min^{-1} at 25°C. The assays were performed without knowledge of the origin of the samples.

Isolated coronary artery ring studies

After finishing the 6 h experimental protocol, hearts were rapidly excised and immersed in warmed (37°C) K-H solution, and both LAD and left circumflex (LCX) coronary arteries were isolated with ceramic microscissors. Adherent connective tissue and fat were dissected free from the vessels and cleaned with special care not to injure the endothelial surface. The vessels were cut into rings of 2 to 3 mm in length which were then mounted on stainless steel hooks, suspended in organ baths, and connected to FT-03 force displacement transducers (Grass Instrument Co., Quincy, MA) to record isometric force. The baths were filled with 10 ml K-H solution and aerated with a gas mixture of 95% O_2 + 5% CO_2 at 37°C. Coronary rings were initially stretched to give a resting force of 0.5 g and equilibrated for 60 min. During this period, the K-H solution was replaced every 20 min. After equilibration, the rings were exposed to 100 nM U-46619 (Biomol, Plymouth Meeting, PA, U.S.A.), a thromboxane A_2-mimetic, to generate 0.5 g of preload. Once a stable contraction was obtained, an endothelium-dependent vasodilator, acetylcholine (0.1, 1, 10 and 100 nmol l^{-1}) (Sigma) was added to the bath. After the response stabilized, the rings were washed and allowed to equilibrate to baseline again. The procedure was repeated twice with another endothelium-dependent vasodilator calcium ionophore A-23187 (1, 10, 100 and 1000 nmol l^{-1}) (Calbiochem, La Jolla, CA, U.S.A.) and then with an endothelium-independent dilator acidified sodium nitrite (0.1, 1, 10, and 100 μmol l^{-1}; $NaNO_2$).

Determination of PMN adherence to ischaemic-reperfused coronary endothelium ex vivo

Autologous PMNs were isolated from arterial blood (20 ml) freshly drawn before surgery by the method of Lafrado & Olsen (1986) as modified by Weyrich *et al.* (1993). Isolated PMNs were >95% viable by trypan blue exclusion and were >95% pure. Isolated autologous PMNs were then labelled with a fluorescent PKH2-GL dye (Sigma, St. Louis, MO, U.S.A) according to the method of Yuan & Fleming (1990). Two ml of PBS with 10% platelet-poor plasma was added to stop the labelling reaction. Cells were then centrifuged at 400 *g* for 10 min at room temperature. The supernatant was removed, and the cells were resuspended in PBS and then recounted. This isolating and labelling procedure yields cat PMNs possessing normal morphology and function.

Ischaemic-reperfused LAD (non ischaemic LAD in sham) and control LCX coronary arteries were isolated at the end of the protocol as described above and cut into segments. Coronary segments were then placed in round cell culture dishes containing 3 ml of oxygenated K-H buffer at 37°C. Autologous labelled PMNs (400,000 PMNs ml^{-1}) were then gently added to the dishes and allowed to incubate for 20 min in a metabolic shaker bath at 37°C. After incubation, the segments were removed and number of PMNs adhered to coronary endothelial surface was counted using epifluorescence microscopy (Nikon, Garden City, NY) as previously reported (Ma *et al.*, 1991). The number of PMNs was counted in duplicate and reported as PMNs per square millimeter of the endothelial surface.

Effect of oligotide on unstimulated PMN adherence to thrombin-stimulated coronary endothelium in vitro

To clarify further the effects of oligotide on PMN-endothelial interaction, in five additional cats, peripheral blood (100 ml) was collected from the femoral artery after anaesthesia, and anticoagulated with citrate-phosphate-dextrose solution (Sigma). PMNs were later isolated and labelled. Hearts were immediately removed and placed in oxygenated K-H buffer. Both the LAD and LCX coronary arteries were removed and cut into segments 2 to 3 mm in length, and placed into a cell culture dish for adherence assay as described above. Then, graded concentrations of oligotide (0.1, 0.25, 0.5 and 1 mg ml^{-1}) or its vehicle (K-H solution) were added to the cell culture dishes and incubated for 10 min. To stimulate endothelial cells, the coronary segments were incubated for 10 min with 2 u ml^{-1} of thrombin (Sigma). This concentration of thrombin has been demonstrated to facilitate P-selectin-mediated PMN-endothelial interaction (Weyrich *et al.*, 1993; Murohara *et al.*, 1994). After this incubation, the coronary segments were removed and placed in fresh K-H solution. Labelled autologous PMNs (400,000 PMNs ml^{-1}) were then added to coronary segments. After 20 min of incubation with PMNs, the coronary segments were removed and adherent PMNs were counted by epifluorescence microscopy as described above.

Immunohistochemistry

To assess the effect of *in vivo* oligotide administration on the coronary endothelial expression of P-selectin, 3 additional cats given oligotide (15 mg kg^{-1} bolus plus 10 mg kg^{-1} h^{-1} infusion) and 3 cats given vehicle were exposed to 90 min of MI followed by 20 min of R. This time course has been shown to upregulate P-selectin significantly on coronary endothelium (Weyrich *et al.*, 1993). The hearts were subsequently removed, and prepared for immunohistochemical analysis of P-selectin using an immunoperoxidase method as described by Beckstead *et al.* (1986) as modified by Weyrich *et al.* (1993).

The percentage of immunolabelled post-capillary venules was assessed in random tissue sections as an index of the extent of upregulation of endothelial P-selectin. The total number of venules (50 to 100 vessels/section) for each tissue section was analyzed using 100 separate fields with a viewing radius of 500 μm. The number of immunostained vessels (i.e., peroxidase reaction occurring along any part of the endothelium) was counted for each field. The number of stained vessels was divided by the total number of vessels examined and was expressed as the percentage of stained vessels.

Effect of oligotide on P-selectin expression on the stimulated platelets in vitro: *flow cytometric analysis*

The effect of oligotide on rapid P-selectin expression on freshly isolated cat platelets after stimulation was determined by flow cytometric analysis. Briefly, peripheral arterial blood was collected from the femoral artery of three pentobarbitone-anaesthetized cats and was anticoagulated with sodium citrate. The blood was placed in round-bottom polycarbonate centrifuge tubes (Nalge) and PRP was obtained by centrifuging the blood at 400 *g* for 20 min as described above. The PRP was removed and recentrifuged at 2500 *g* at room temperature to form a platelet-rich pellet. This platelet pellet was washed twice in calcium-free Tyrode solution containing 0.2% bovine serum albumin (BSA). The final cell pellet was resuspended in the K-H solution. Aliquots of platelet suspension were pre-incubated with different concentrations of oligotide (i.e., 0.1, 0.5 and 1.0 mg ml^{-1}) of its vehicle (K-H solution) at 37°C for 5 min, followed by incubation with thrombin (2 u ml^{-1}), and the mixture was incubated at 37°C for additional 10 min without stirring. Subsequently, platelets were fixed with an equal volume of 2% paraformaldehyde in PBS at pH 7.2, washed twice with PBS containing 0.2% BSA. The platelet suspensions were treated with human block IgG (4.0 mg ml^{-1}, Sigma) and then anti-P-selectin monoclonal antibody PB1.3 (40 μg ml^{-1}) was added to the platelet suspensions. The platelets were incubated at 4°C for 60 min and then washed in Dulbecco's PBS with 0.2% BSA to remove any excess of the primary antibodies. F(ab')2 fragments of a goat anti-mouse IgG-phycoerythrin conjugate (Tago, Inc., Burlingame, CA, U.S.A.) was used as the secondary antibody reagent at a 1:100 dilution and the cells were placed at 4°C for 30 min. The stained platelets were washed and fixed in 1.0% paraformaldehyde, and immediately analyzed on a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, U.S.A.).

Statistical analysis

All values in the text, table and figures are presented as means $\pm$ standard errors of the mean of n independent experiments. All data were subjected to analysis of variance followed by the Fisher's correction for *post-hoc t* test analysis. Probabilities of 0.05 or less were considered to be statistically significant.

Results

Cardiac electrophysiological and haemodynamic changes

In five cats subjected to sham ischaemia, intravenous administration of oligotide had no detectable effect on any of the measured haemodynamics or electrocardiographic parameters during a 6 h experimental period. Furthermore, there were no significant differences in any of these variables observed between oligotide and vehicle-treated MI + R cats. In all cats subjected to coronary artery occlusion the ST-segment became elevated within 10 min of LAD occlusion peaking 40 min after coronary occlusion. After reperfusion, the ST-segment markedly declined. There was no significant difference in peak ST-segment elevation between MI + R cats given either vehicle or oligotide (0.25 ± 0.08 vs. 0.24 ± 0.9 mV), indicating that the degree of ischaemic insult was comparable in these two groups of cats. Immediately after coronary occlusion, the mean arterial blood pressure decreased significantly in all MI + R cats. However, there were no significant differences between these two MI + R groups at any of the pressure-rate (Figure 1) or heart rate readings, suggesting that oligotide did not exert any significant effect either on systemic haemodynamics or on myocardial oxygen demand.

Effect of oligotide on ischaemia reperfusion-induced myocardial injury

Figure 2 summarizes the changes in plasma CK activity as a marker of myocardial necrosis in all three groups of cats. Modest increases in plasma CK activity were seen in the sham-operated control group. However, marked increases in CK activity were observed in cats subjected to MI + R receiving only the vehicle. In contrast, the plasma CK activities of the oligotide treated ischaemic group were significantly lower than

vehicle group at each hour beyond 4 h ($P<0.05$). Thus, oligotide treatment effectively attenuated the increase in plasma CK activity after MI + R.

In the two MI + R groups, we also measured anatomically the necrotic ischaemic heart, and the amount of necrotic myocardium was expressed as a percentage of both the area-at-risk or of the total left ventricular mass. There was no significant difference in the total areas-at-risk index to total left ventricle between two MI + R groups, indicating that the severity of ischaemia was comparable in both MI + R groups (Figure 3). However, the necrotic area expressed either as a percentage of the area-at-risk or of the total left ventricular mass was significantly lower ($P<0.01$ and $P<0.05$, respectively) in cats treated with oligotide compared to cats receiving only the vehicle, indicating that oligotide significantly attenuated myocardial necrosis occurring after MI + R (Figure 3).

Prevention of PMN accumulation in ischaemic myocardial tissue by oligotide: analysis by cardiac myeloperoxidase activity

We measured cardiac myeloperoxidase activity as a marker for neutrophil accumulation. Very low cardiac myeloperoxidase activities were observed in the sham-operated control cats in all portions of the myocardium. In the non-ischaemic myocardium (i.e., area-not-at-risk), myeloperoxidase activity was also very low in the two MI + R groups, indicating that few neutrophils accumulated in the non-ischaemic portion of myocardium (Figure 4). However, MI + R cats receiving vehicle exhibited a marked increase in myeloperoxidase activity in necrotic myocardium. In contrast, oligotide-treated MI + R cats exhibited a significantly lower myeloperoxidase activity in necrotic myocardial tissue (Figure 4). In the ischaemic but non-necrotic area, moderately attenuated myeloperoxidase activity was observed in cats treated with oligotide. These results indicate that infiltration of neutrophils to necrotic myocardium was significantly attenuated by oligotide infusion.

Depletion of circulating neutrophils *per se* could result in a significant cardioprotection after ischaemia reperfusion leading to a reduced PMN accumulation in the ischaemic myocardium (Romson *et al.*, 1983). In order to evaluate this possibility, we also examined the effect of oligotide infusion on the number of circulating PMNs. As shown in Table 1, there was no significant difference in circulating PMN count between the two groups of MI + R cats. Thus, oligotide did not induce a leukopaenia which could have contributed to the cardioprotection.

Effects of in vivo *treatment of oligotide on coronary endothelial function after ischaemia and reperfusion*

Figure 5 summarizes the vasorelaxant responses to acetylcholine, A-23187, and acidified $NaNO_2$ in ischaemic re-

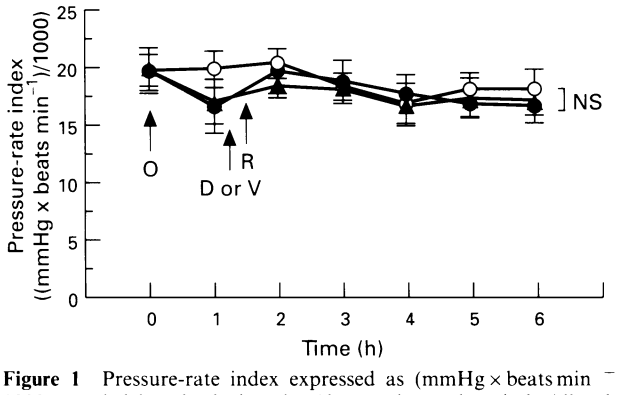

Figure 1 Pressure-rate index expressed as (mmHg × beats min $^{-1}$)/1000 sampled hourly during the 6 h experimental period. All values are means ± s.e.mean; (○) sham MI/R + oligotide group ($n=5$); (●) MI + R treated with vehicle group ($n=8$) and (▲) MI + R treated with oligotide group ($n=7$). O = occlusion, R = reperfusion, D or V = drug (oligotide) or vehicle.

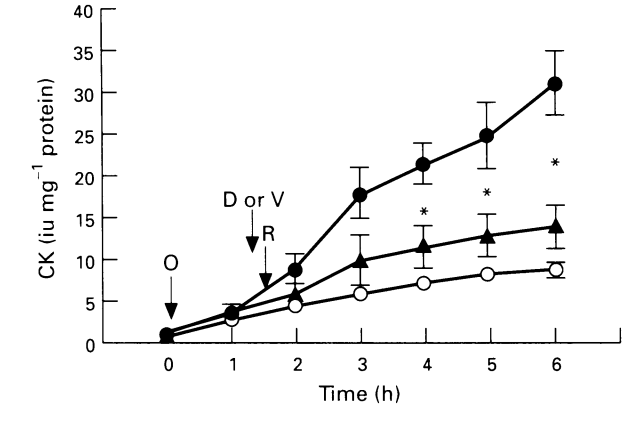

Figure 2 Plasma creatine kinase (CK) activity expressed as International units (i.u.) mg^{-1} protein sampled hourly during 6 h experimental period. All values are means ± s.e.mean; (○) sham MI + R + oligotide group ($n=5$); (●) MI + R treated with vehicle group ($n=8$), (▲) MI + R treated with oligotide group ($n=7$). Significant increases of CK (*$P<0.05$) occurred in the MI + R treated with vehicle compared to the MI + R treated with oligotide group at 4 h and thereafter. O = occlusion, R = reperfusion, D or V = drug (oligotide) or vehicle.

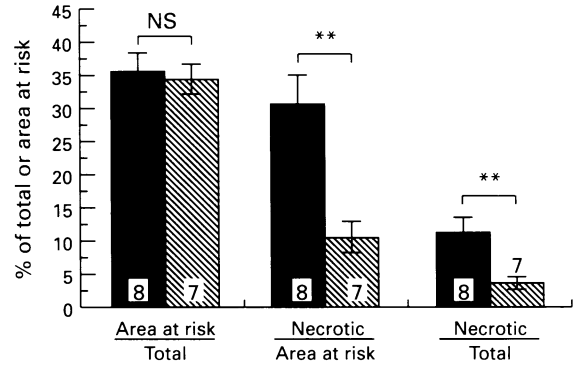

Figure 3 Tissue wet weight of area-at-risk as a percentage of the total left ventricular wet weight and of the necrotic tissue either as a percentage of area-at-risk and of the total left ventricle for the two MI + R groups. Solid columns, MI + R treated with vehicle; hatched columns, MI + R treated with oligotide. Columns show mean ± s.e.mean; numbers in the columns are numbers of animals examined. **$P<0.01$.

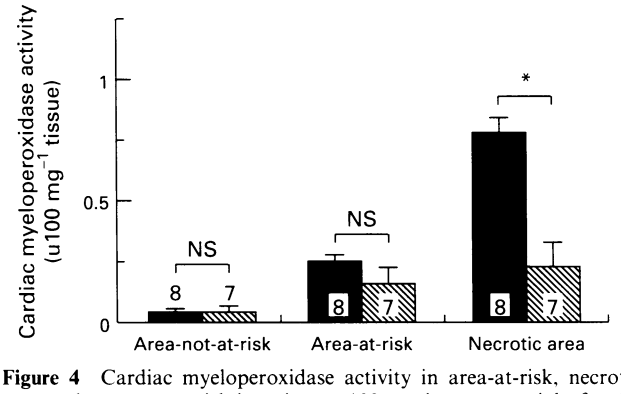

Figure 4 Cardiac myeloperoxidase activity in area-at-risk, necrotic area and area-not-at-risk in units per 100 mg tissue wet weight for the two MI + R groups. Solid columns, MI + R treated with vehicle; hatched columns, MI + R treated with oligotide. Columns are means ± s.e.mean; numbers in the columns are numbers of animals examined. *$P<0.05$.

Table 1 Circulating leukocyte counts

Time (h)	*0*	*1.5 End of ischaemia*	*2*	*4*	*6*
MI/R + vehicle	12.8 ± 0.8	12.1 ± 1.4	10.8 ± 1.0	11.8 ± 0.9	11.9 ± 3.0
MI/R + oligotide	14.3 ± 1.8	13.1 ± 2.9	11.5 ± 1.2	14.0 ± 1.8	14.5 ± 2.3

There is no significant difference between the two myocardial ischaemia plus reperfusion (MI/R) groups at any time points during the experimental period. Data are expressed as mean WBCs × 1000 μl^{-1} ± s.e.mean MI/R + vehicle ($n = 8$), MI/R + oligotide ($n = 7$).

perfused LAD and control non-ischaemic LCX coronary artery rings in three groups. The responses of LAD coronary artery rings to the endothelial-dependent vasodilators, acetylcholine and A-23187, were markedly attenuated in cats treated with vehicle. These endothelium-dependent relaxations were significantly preserved in the oligotide-treated MI + R cats as compared to vehicle. Relaxation to acidified $NaNO_2$ was preserved in ischaemic reperfused LAD coronary artery rings obtained from all three groups. The non-ischaemic LCX coronary artery rings, whether obtained from control non-ischaemic or from ischaemic cats, showed full relaxation to all three vasodilators. Thus, there were no significant differences in vasorelaxant response to any of the vasodilators studied in non-ischaemic LCX coronary artery rings.

The direct vascular effects of oligotide were also examined in ischaemic LAD and non-ischaemic LCX coronary artery rings *in vitro* since a direct coronary dilator effect could be cardioprotective in the ischaemic heart. In isolated coronary artery rings, oligotide did not significantly alter basal tone of ischaemic LAD rings. The increase in force to 1 mg ml^{-1} of oligotide was 0.04 ± 0.02 and 0.05 ± 0.03 grams after 60 min incubation in non-ischaemic LCX and ischaemic LAD coronary artery rings, respectively. Moreover, 1 mg ml^{-1} of oligotide did not exert any detectable vasorelaxation or vasocontraction responses in either ischaemic LAD or control LCX coronary artery rings following precontraction with U-46619, a thromboxane-mimetic.

Effects of in vivo *administration of oligotide on PMN adherence to ischaemic-reperfused coronary endothelium* ex vivo

When autologous labelled PMNs were added alone to non-ischaemic-reperfused control LCX coronary segments and incubated for 20 min, few neutrophils adhered to the endothelial surface (Figure 6). However, when PMNs were added to ischaemic-reperfused LAD coronary segments isolated 270 min after reperfusion from cats treated with vehicle, a dramatic increase in PMN adherence was observed (Figure 6). In contrast, when labelled PMNs were added to ischaemic-reperfused LAD coronary segments isolated from the cats treated with oligotide, the number of PMNs adhering to the coronary endothelium was significantly attenuated as compared to vehicle-treated cats (Figure 6). Thus, oligotide significantly blocked adherence of PMNs to ischaemic LAD coronary endothelium 270 min after reperfusion *ex vivo*.

Effects of oligotide on PMN adherence to thrombin-stimulated coronary endothelium in non-ischaemic control cats in vitro

Since oligotide significantly preserved endothelial function and since endothelial injury is critically related to PMN-endothelial interaction, we further examined whether oligotide has any specific effects on vascular adhesion molecule expression. P-selectin is one of the important adhesion molecules which facilitate early PMN-endothelial interaction during inflammation. Because inflammatory stimuli such as thrombin induce expression of P-selectin on the endothelial cell surface, we stimulated the coronary endothelium with 2 u ml^{-1} thrombin

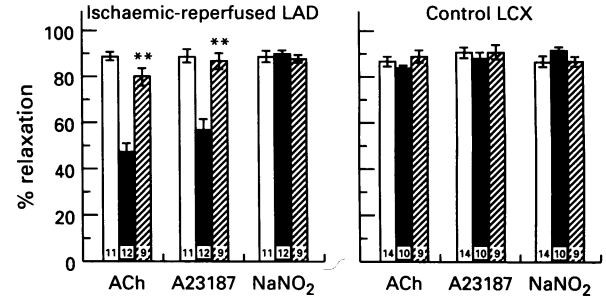

Figure 5 Summary of responses of ischaemic-reperfused left anterior descending (LAD) coronary artery rings and non-ischaemic left circumflex (LCX) coronary artery rings to the three vasodilators: 100 nmol l^{-1} acetylcholine (ACh), 1 μmol l^{-1} A-23187, and 100 μmol l^{-1} acidified $NaNO_2$. Open columns, sham MI treated with oligotide; solid columns, MI + R treated with vehicle; hatched columns, MI + R treated with oligotide; 11 LAD and 14 LCX; 12 LAD and 10 LCX; and 9 LAD and LCX coronary rings were obtained from 5 sham + oligotide, 6 MI + vehicle and 6 MI + oligotide cats, respectively. Columns are means ± s.e.mean. **$P < 0.01$ vs. MI + R treated with vehicle.

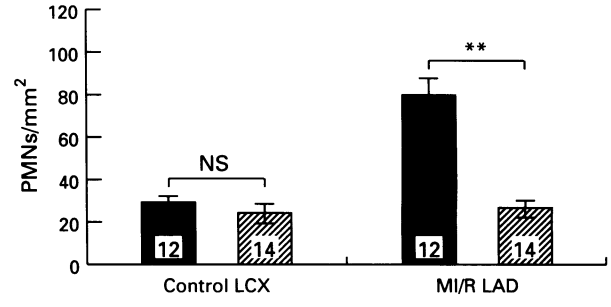

Figure 6 Effects of oligotide treatment on unstimulated PMN adherence to nonischaemic control left circumflex (LCX) coronary endothelium and ischaemic reperfused (MI + R) left anterior descending (LAD) coronary endothelium. Data are expressed as numbers of adhered PMNs per square millimetre. Solid columns, MI + R treated with vehicle, hatched columns, MI + R treated with oligotide. Twelve LAD and LCX, and 14 LAD and LCX coronary rings were obtained from 6 MI + vehicle cats and 6 MI + oligotide cats, respectively. Columns are means ± s.e.mean, and numbers within columns are numbers of coronary segments examined. **$P < 0.01$.

for 10 min. Figure 7 summarizes the results obtained from 6 to 10 coronary segments prepared in this manner. Adherence of labelled PMNs to autologous coronary endothelium was significantly increased after thrombin stimulation as compared to non-stimulated control coronary endothelium ($P < 0.01$). Pretreatment with oligotide (0.25 to 1 mg ml^{-1}) before thrombin stimulation significantly attenuated PMN adherence of the stimulated coronary endothelium in a concentration-dependent manner. At a concentration of 1 mg ml^{-1}, a maximal inhibitory effect was obtained (about 80% inhibition from control values), which was not significantly different from the

PMN adherence observed in control unstimulated coronary endothelium.

Effect of in vivo *oligotide treatment on coronary endothelial P-selectin expression after ischaemia reperfusion: immunohistochemical analysis*

Coronary endothelial expression of P-selectin was detected immunohistochemically by employing a monoclonal antibody (i.e., PB 1.3) coupled with a sensitive avidin-biotin immunoperoxidase procedure. The immunohistochemical results are illustrated in Figure 8. Nonischaemic control tissue (area-not-at-risk) demonstrated very little cytoplasmic immunostaining only in a few endothelial cells lining coronary venules (Figure 8a). The percentage of positively staining venules was consistently low (less than 5%). Negative control immunohistochemical preparations, in which the primary antibody (PB 1.3) was replaced by non-immune serum, did not label endothelial cells at all. On the other hand, endothelial cell P-selectin was evident in untreated ischaemic reperfused heart tissue (Figure 8b), the percentage staining positively being $58 \pm 4\%$. This P-selectin expression on the endothelial cells in ischaemic reperfused myocardial tissue was significantly attenuated by *in vivo* administration of oligotide (Figure 8c), resulting in $34 \pm 7\%$ positive staining of venules.

Effect of oligotide on P-selectin expression on thrombin-stimulated cat platelets in vitro*: flow cytometric analysis*

Cat platelets incubated with only the secondary antibody exhibited $27 \pm 2\%$ of positive staining with a mean channel fluorescence value of 9 ± 2 ($n=3$). In contrast, addition of thrombin (2 u ml^{-1}) to platelets significantly ($P<0.05$) increased the percentage of positive cells to $77 \pm 2\%$ with a mean channel fluorescence value of 65 ± 9 ($n=4$). Preincubation of platelets with oligotide (0.1, 0.5 and 1.0 mg ml^{-1}) before addition of thrombin markedly decreased the percentage of positive cells to $43 \pm 3\%$, $52 \pm 5\%$ and $39 \pm 3\%$ ($n=3$ each group; $P<0.05$ in oligotide 1.0 mg ml^{-1} vs. thrombin stimulated platelets), respectively. The mean channel fluorescence also decreased to 38 ± 1, 26 ± 6 and 27 ± 5 ($n=3$ each group; $P<0.05$ in oligotide 0.5 and 1.0 mg ml^{-1} vs. thrombin stimulated group), respectively. A representative series of histograms of fluorescence-labelled platelets analyzed by flow cytometry is shown in Figure 9.

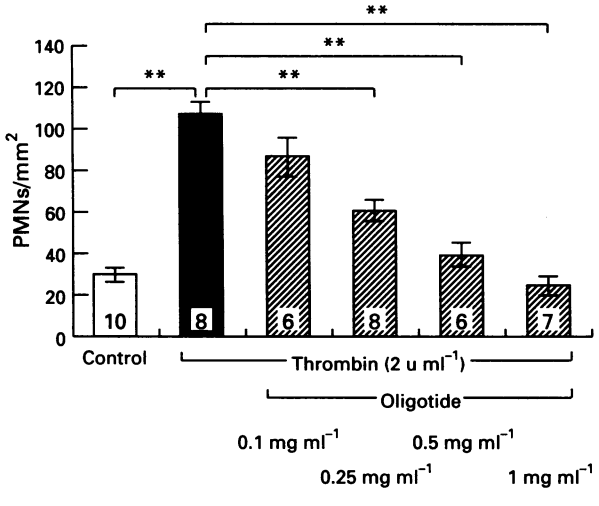

Figure 7 Concentration-response relation of *in vitro* oligotide treatment on inhibition of unstimulated autologous PMN adherence to thrombin (2 u ml^{-1})-stimulated coronary endothelium. Stimulation with thrombin significantly increased PMN adherence to coronary endothelium, which was concentration-dependently inhibited by oligotide. Data are expressed as numbers of adhered PMNs per square millimetre. Columns are means ± s.e.mean, and numbers at the base of the columns are numbers of coronary segments examined. $^{**}P<0.01$.

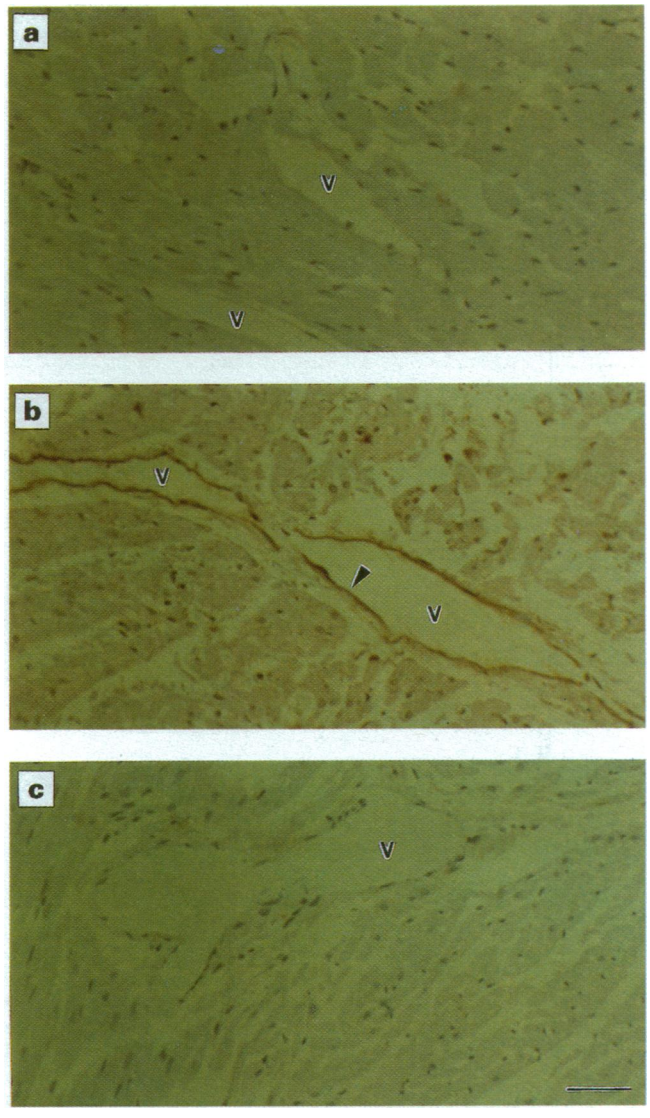

Figure 8 Representative photomicrographs of immunohistochemical staining of myocardial tissue incubated with anti-P-selectin monoclonal antibody (PB 1.3) and labelled with peroxidase substrate solution. Brown reaction product is present at sites of P-selectin antigen localization (*arrow heads*). (a) Myocardium of area-not-at-risk (control area not exposed to ischaemia) in a vehicle-treated cat. (b) Myocardium of area-at-risk (area exposed to 90 min of ischaemia followed by 20 min of reperfusion) in a vehicle-treated cat. (c) Myocardium of area-at-risk (area exposed to 90 min of ischaemia followed by 20 min of reperfusion) in a oligotide-treated cat. Bar in (c) represents 50 μm and magnification is the same in all three panels.

Discussion

Oligotide is a newly developed single-stranded polydeoxyribonucleotide complex, and was extracted by controlled depolymerization from bovine DNA. It represents a cluster of single-stranded DNA of different lengths and base sequences with a mean molecular weight of 8 ± 2 kDa (Lanzarotti *et al.*, 1993; Skurk *et al.*, 1995). A polydeoxyribonucleotide-derived drug such as defibrotide has been shown to elicit protective actions against ischaemic tissue injury (Thiemermann *et al.*, 1985; Niada *et al.*, 1986). Defibrotide preserves or enhances the release of the anti-aggregatory eicosanoid, prostacyclin (Palmer & Goa, 1993), maintains endothelial release of nitric oxide

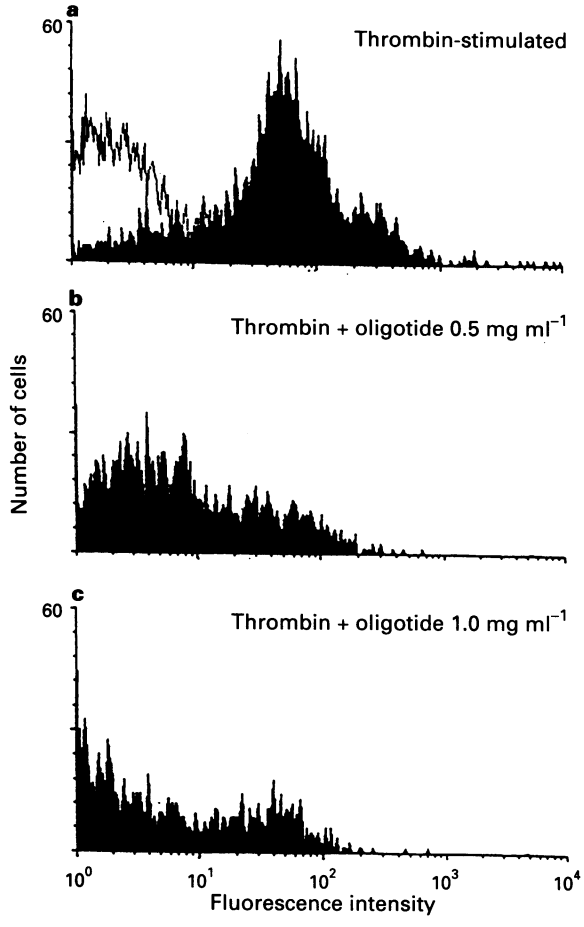

Figure 9 Representative fluorescence histograms of thrombin-stimulated cat platelets incubated with anti-P-selectin monoclonal antibody (PB 1.3) and labelled with phycoerythrin conjugated secondary antibody. Thrombin (2 u ml^{-1}) markedly increased the number of P-selectin positive cells as well as the mean channel fluorescence. Preincubation of oligotide (0.5 or 1 mg ml^{-1}) with platelets attenuated both the number of cells positive for P-selectin and the mean channel fluorescence after stimulation with thrombin. The control histogram in (a) represents omission of the primary antibody.

(Lefer *et al.*, 1990) and exerts profibrinolytic activity by liberating tPA (Klöcking, 1992). However, it is unknown whether oligotide has similar beneficial actions against acute inflammatory tissue damage such as MI + R injury.

The present study clearly demonstrates a cardioprotective action of *in vivo* administration of oligotide in a feline model of MI (90 min) and R (270 min). First, despite comparable degrees of ischaemic insult (i.e., similar extent of AAR and ST-segment elevation), oligotide significantly reduced the left ventricular necrotic area by 70% of that observed in cats receiving the vehicle. Secondly, plasma CK activity, another index of cardiac necrosis, was significantly lower in cats treated with oligotide than vehicle. These results suggest that *in vivo* oligotide treatment significantly preserved the ischaemic myocardium and prevented necrosis after MI + R. Since there is little collateral circulation in cats (Greve *et al.*, 1989), and since oligotide did not alter the pressure-rate index or coronary vascular tone, it is less likely that oligotide protected myocardium by either increasing coronary blood flow (i.e., oxygen supply) or decreasing afterload (i.e., cardiac oxygen demand) (Gobel *et al.*, 1978). Romson *et al.* (1983) have indicated that depletion of circulating leukocytes exerts cardioprotection after MI + R due to reduced PMN accumulation in the ischaemic myocardium. However, this possibility is less likely since the total number of circulating leukocytes was not significantly different between vehicle- and oligotide-treated MI + R cats (Table 1).

Substantial evidence has suggested that activated PMNs play a significant role in MI + R injury (Romson *et al.*, 1983; Engler *et al.*, 1986; Lefer *et al.*, 1991). Activated PMNs release oxygen radicals, proteases, and cytokines such as tumour necrosis factor alpha. Superoxide released from PMNs have been shown to inactivate nitric oxide (Rubanyi & Vanhoutte, 1987) and induce vasoconstriction (Murohara *et al.*, 1993, 1994) and disrupt cellular membranes through lipid peroxidation. In addition, PMN aggregates may participate in microvascular plugging leading to the 'no-reflow' phenomenon (Kloner *et al.*, 1974; Engler *et al.*, 1986). Serine proteases such as elastase and cathepsin G released from PMNs cause endothelial and matrix protein disruption, thus enhancing MI + R injury (Murohara *et al.*, 1995). Tissue PMN infiltration is mediated by sequential steps of PMN-endothelial interaction, in which a series of adhesion molecules on both endothelium and PMNs play an important role (Butcher, 1991). We have recently demonstrated that monoclonal antibodies directed against adhesion molecules such as P-selectin or CD18 significantly attenuated PMN-endothelial adherence, PMN infiltration and myocardial necrosis after MI + R *in vivo* (Simpson *et al.*, 1988; Ma *et al.*, 1991; Weyrich *et al.*, 1993). In the present study, *ex vivo* adherence of autologous PMNs to the ischaemic-reperfused LAD coronary endothelium was significantly attenuated by oligotide as compared to the vehicle. Furthermore, myeloperoxidase activity in the necrotic myocardium, a marker of neutrophil infiltration (Mullane *et al.* 1985), was significantly lower in MI + R cats treated with oligotide. Therefore, it is likely that oligotide attenuated reperfusion-induced myocardial injury by inhibiting PMN-endothelial interaction and subsequent extravasation.

Thus, we further examined the mechanism of oligotide-mediated inhibition of PMN-endothelial interaction. P-selectin is an important adhesion molecule which supports PMN rolling on the endothelium after MI + R (Weyrich *et al.*, 1993). P-selectin is rapidly translocated from intracellular stores to the cell surface of the endothelium and platelet after stimulation with thrombin, histamine and oxygen free radicals (Lorant *et al.*, 1991; Patel *et al.*, 1991). Superoxide anions and thrombin generated after MI + R may greatly facilitate P-selectin expression on the endothelium (Weyrich *et al.*, 1993). In our immunohistochemical study, we confirmed P-selectin expression on venular endothelium shortly after reprefusion (90 min MI and 20 min R). Recently, a polydeoxyribonucleotide-based drug such as defibrotide has been reported to inhibit superoxide production in leukocytes (Cirillo *et al.*, 1991), to act as a thrombin receptor antagonist via aptamer sequence (e.g., 5'-GGTTGGATTGGTTGG-3') (Bracht & Schrör, 1994), and to release prostacyclin (Hohlfeld *et al.*, 1993). We reasoned that these properties of oligonucleotide drug may inhibit P-selectin expression by an anti-thrombin or by anti-superoxide mechanisms, and by prostacyclin (Rösen *et al.*, 1994). We thus examined the effects of oligotide on (a) PMN adherence to thrombin-stimulated coronary endothelium *in vitro*, (b) P-selectin expression on thrombin-stimulated platelets *in vitro*, and (c) immunohistochemical localization of P-selectin on coronary endothelium after 90 min MI and 20 min R. Thrombin has been shown to promote P-selectin-mediated PMN adherence to the coronary endothelium (Weyrich *et al.*, 1993; Murohara *et al*, 1994). In the present study, oligotide significantly attenuated PMN adherence to thrombin-stimulated coronary endothelium in a concentration-dependent manner *in vitro*. Secondly, our flow cytometric analysis clearly showed that oligotide inhibits P-selectin expression on thrombin-stimulated cat platelets. The concentrations of oligotide used in our *in vitro* studies were calculated and set from the dose we used *in vivo* experiments and approximate total blood volume of the cats. Furthermore, immunohistochemical study clearly showed that *in vivo* administration of oligotide significantly attenuated P-selectin expression on the venular endothelium 20 min after R or the MI (90 min). This time course has been shown to up-regulate maximally P-selectin on coronary endothelium after MI + R

(Weyrich *et al.*, 1993, 1995). Taken together, this *in vivo* and *in vitro* evidence clearly suggests that oligotide inhibits P-selectin expression on both endothelium and platelets. This may be one of the key mechanisms by which oligotide inhibits PMN-endothelial interaction thus attenuates myocardial necrosis.

Although oligotide attenuated P-selectin expression, it may also potentially influence expression of other adhesion molecules. Recently, Masini *et al.* (1995) demonstrated that defibrotide increases nitric oxide (NO) release during MI + R. Since NO has been shown to inhibit endothelial ICAM-1 expression (Lefer *et al.*, 1993a), oligotide could inhibit ICAM-1 expression during MI + R and attenuate MI + R injury although this possibility remains to be determined.

Another important finding in the present study is that oligotide markedly preserved endothelial function (i.e., release of EDRF) in ischaemic-reperfused LAD coronary arteries. Endothelial dysfunction, occurring shortly after reperfusion (Tsao *et al.*, 1990; Lefer *et al.*, 1991) is an important trigger of PMN adherence to the endothelium (Kubes *et al.*, 1991; Ma *et al.*, 1993). EDRF-nitric oxide is produced from L-arginine by a constitutive nitric oxide synthase (cNOS) (Palmer *et al.*, 1988). Nitric oxide produces vascular smooth muscle relaxation and inhibits platelet aggregation and neutrophil adherence to the endothelium (Furchgott & Zawadzki, 1980; Furlong *et al.*, 1987; McCall *et al.*, 1988). Because of these biological effects, nitric oxide donors have been shown to protect against MI + R injury (Lefer *et al.*, 1993b). In the present study, MI + R induced marked endothelial dysfunction (i.e., reduced endothelium-dependent relaxation to acetylcholine and A-23187). In contrast, *in vivo* oligotide treatment significantly prevented endothelial dysfunction *ex vivo*. This may be another important mechanism of cardioprotection afforded by oligotide. There are several possible mechanisms by which oligotide protected endothelial function. Since endothelial injury after reperfusion is significantly mediated by oxygen free radicals or proteases released from activated PMNs (Tsao *et al.*, 1990; Inauen *et al.*, 1993), it is likely that oligotide attenuated endothelial dysfunction by inhibiting PMN adherence to endothelium. Secondly, defibrotide has been shown to inhibit direct Ca^{2+}-dependent neutrophil activation and subsequent release of oxygen free radicals (DiPerri *et al.*, 1987; Cirillo *et al.*, 1991). If oligotide also had these activities, it might protect endothelial function by inhibition of PMN release of superoxide radicals. More precise mechanisms, by which oligotide affects leukocyte-endothelial interaction are currently under investigation in our laboratory.

It is known that another polydeoxyribonucleotide, defibrotide, stimulates endogenous prostacyclin which ameliorates MI + R injury (Hohlfeld *et al.*, 1993). Although it is unknown whether oligotide can also stimulate prostacyclin production, possible involvement of prostacyclin in oligotide-mediated inhibition of P-selectin expression (Rösen *et al.*, 1994) and cardioprotection (Lefer *et al.*, 1978) needs to be examined further.

In conclusion, we have demonstrated that *in vivo* administration of oligotide, a newly developed single stranded polydeoxyribonucleotide complex, protects against MI + R injury in cats. The mechanism of this cardioprotection appears to be related to inhibition of PMN-endothelial interaction and accumulation of the PMNs in the necrotic myocardium, and preservation of endothelial function in the coronary vasculature. Interestingly, oligotide markedly attenuates P-selectin expression on endothelium and platelets. Polydeoxyribonucleotides may be a new category of beneficial agents against acute ischaemic heart diseases. Further studies are needed to clarify the precise molecular mechanism or action of these substances.

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References

BECKSTEAD, J.H., STENBERG, P.E., MCEVER, R.P., SHUMAN, M.A. & BAINTON, D.F. (1986). Immunohistochemical localization of membrane and α-granule proteins in human megakaryocytes: application of plastic-embedded bone marrow biopsy specimens. *Blood*, **67**, 285–293.

BIANCHI, G., BARONE, D., LANZAROTTI, E., TETTAMANTI, R., PORTA, R., MOLTRASIO, D., CEDRO, A., SALVETTI, L., MANTOVANI, M. & PRINO, G. (1993). Defibrotide, a single-stranded polydeoxyribonucleotide acting as an adenosine receptor agonist. *Eur. J. Pharmacol.*, **238**, 327–334.

BRACHT, F. & SCHRÖR, K. (1994). Isolation and identification of aptamers from defibrotide that act as thrombin antagonists in vitro. *Biochem. Biophys. Res. Commun.*, **200**, 933–937.

BRADLEY, P.P., PRIEBAT, D.S., CHRISTENSEN, R.D. & ROTHSTEIN, G.R. (1985). Measurement of cutaneous inflammation: Estimation of neutrophil content with and enzyme marker. *J. Clin. Invest.*, **76**, 1713–1719.

BRAUNWALD, E & KLONER, R.A. (1985). Myocardial reperfusion: a double edged sword. *J. Clin. Invest.*, **76**, 1713–1719.

BUERKE, M., WEYRICH, A.S., ZHENG, Z., GAETA, F.C.A., FORREST, M.J. & LEFER, A.M. (1994). Sialyl $Lewis^X$-containing oligosaccharide attenuates myocardial reperfusion injury in cats. *J. Clin. Invest.*, **93**, 1140–1148.

BUTCHER, E.C. (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell*, **67**, 1033–1036.

CIRILLO, F., MARGAGLIONE, M., VECCHIONE, G., AMES, P.R.J., COPPOLA, A., GRANDONE, E., CERBONE, A.M., MARELLI, C. & DI MINNO, G. (1991). In vitro inhibition by defibrotide of monocyte superoxide anion generation: A possible mechanism for the antithrombotic effect of a polydeoxyribonucleotide-derived drug. *Haemostasis*, **21**, 98–105.

DIPERRI, T., LAGHI PASINI, F., CAPECCHI, P.L., CECCATELLI, L., PASQUI, A.L. & ORRICO, A. (1987). Defibrotide in vitro inhibits neutrophil activation by a Ca^{++}-involving mechanism. *Int. J. Tissue React.*, **9**, 399–406.

ENGLER, R.L., DAHLGREN, M.D., MORRIS, D.D., PATERSON, M.A. & SCHMID-SCHÖNBEIN, A.W. (1986). Role of leucocytes in response to acute myocardial ischemia and reflow in dogs. *Am. J. Physiol.*, **251**, H314–H323.

ENTMAN, M.L., MICHAEL, L., ROSSEN, R.D., DREYER, W.J., ANDERSON, D.C., TAYLOR, A.A. & SMITH, C.W. (1991). Inflammation in the course of early myocardial ischaemia. *FASEB J.*, **5**, 2529–2537.

FARB, A., KOLODGIE, F.D., JENKINS, M. & VIRMANI, R. (1993). Myocardial infarct extension during reperfusion after coronary artery occlusion: pathologic evidence. *J. Am. Coll. Cardiol.*, **21**, 1245–1253.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

FURLONG, B., HENDERSON, A.H., LEWIS, M.J. & SMITH, J.A. (1987). Endothelium-derived relaxing factor inhibits in vitro platelet aggregation. *Br. J. Pharmacol.*, **90**, 687–692.

GOBEL, F.L., NORDSTROM, L.A., NELSON, R.R., JORGENSON, C.R. & WANG, Y. (1978). The rate-pressure product as an index of myocardial oxygen consumption during exercise in patients with angina pectoris. *Circulation*, **57**, 549–556.

GORNALL, A.G., BARDOWILL, C.T. & DAVID, M.M. (1949). Determination of serum protein by means of the biuret method. *J. Biol. Chem.*, **77**, 741–766.

GREVE, G., ROTEVATIN, S. & STANGELAND, L. (1989). Morphological changes across the border zone of cat hearts subjected to regional ischaemia. *Virchows Arch. A Pathol. Anat.*, **415**, 323–333.

HOHLFELD, T., STROBACH, T. & SCHRÖR, K. (1993). Stimulation of endogenous prostacyclin protects the reperfused pig myocardium from ischemic injury. *J. Pharmacol. Exp. Ther.*, **264**, 397–405.

INAUEN, W., GRANGER, D.N., MEININGER, C.J., SCHELLING, M.E., GRANGER, H.J. & KVIETYS, P.R. (1993). Anoxia-reoxygenation-induced, neutrophil-mediated endothelial cell injury: role of elastase. *Am. J. Physiol.*, **259**, H925–H931.

KLEIN, H.H., PUSCHMANN, S., SCHAPER, J. & SCHAPER, W. (1981). The mechanism of the tetrazolium reaction in identifying experimental myocardial infarction. *Virchow's Arch.*, **393**, 287–297.

KLÖCKING, H.P. (1992). Acute t-PA release by defibrotide. *Thromb. Res.*, **66**, 779–785.

KLONER, R.A., GANOTE, C.E. & JENNINGS, R.B. (1974). The 'no reflow' phenomenon after temporary coronary occlusion in the dog. *J. Clin. Invest.*, **54**, 1496–1508.

KUBES, P., SUZUKI, M. & GRANGER, D.N. (1991). Nitric oxide: An endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4651–4655.

LAFRADO, L.J. & OLSEN, R.G. (1986). Demonstration of depressed polymorphonuclear leukocyte function in non-viremic FeLV-infected cats. *Cancer Invest.*, **4**, 297–300.

LANZAROTTI, E., PORTA, R., MANTOVANI, M., CEDRO, A., PRINO, G. & MOLTRASIO, D. (1993). New oligodeoxyribonucleotides having anti-ischemic activity and methods or preparation thereof. *Eur. Patent Publication number* 0 558 833 A2.

LEFER, A.M., AOKI, N. & MULLOY, D. (1990). Coronary endothelium-protective effects of defibrotide in ischemia and reperfusion. *Naunyn-Schmied. Arch. Pharmacol.*, **341**, 246–250.

LEFER, A.M., OGLETREE, M.L., SMITH, J.B., SILVER, M.J., NICOLAU, K.C., BARNETTE, W.E. & GASIC, G.P. (1978). Prostacyclin: a potentially valuable agent for preserving myocardial tissues in acute myocardial ischaemia. *Science*, **200**, 52–54.

LEFER, A.M., TSAO, P.S., LEFER, D.J. & MA, X.-L. (1991). Role of endothelial dysfunction in the pathogenesis of reperfusion injury following myocardial ischemia. *FASEB J.*, **5**, 2029–2034.

LEFER, D.J., KLUNK, D.A., LUTTY, G.A., MERGES, C., SCHLEIMER, R.P., BOCHNER, B.S. & ZWEIER, J.L. (1993a). Nitric oxide (NO) donors reduce basal ICAM-1 expression on human aortic endothelial cells (HAECs). *Circulation*, **88**, I-565.

LEFER, D.J., NAKANISHI, K., JOHNSTON, W.E. & VINTEN-JOHANSEN, J. (1993b). Antineutrophil and myocardial protecting actions of a novel nitric oxide donor after acute myocardial ischemia and reperfusion in dogs. *Circulation*, **88**, 2337–2350.

LORANT, D.E., PATEL, K.D., MCINTYRE, T.M., MCEVER, R.P., PRESCOTT, S.M. & ZIMMERMAN, G.A. (1991). Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *J. Cell Biol.*, **115**, 223–234.

MA, X.-L., TSAO, P.S. & LEFER, A.M. (1991). Antibody to CD-18 exerts endothelial and cardioprotective effects in myocardial ischemia and reperfusion. *J. Clin. Invest.*, **88**, 1237–1243.

MA, X.-L., WEYRICH, A.S., LEFER, D.J. & LEFER, A.M. (1993). Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. *Circ. Res.*, **72**, 403–412.

MASINI, E., LUPINI, M., MUGNAI, L., RASPANTI, S. & MANNAIONI, P.F. (1995). Polydeoxyribonucleotides and nitric oxide release from guinea-pig hearts during ischaemia and reperfusion. *Br. J. Pharmacol.*, **115**, 628–635.

MCCALL, T., WHITTLE, B.J.R., BOUGHTON-SMITH, N.K. & MONCADA, S. (1988). Inhibition of fMLP-induced aggregation of rabbit neutrophils by nitric oxide. *Br. J. Pharmacol.* **85**, 517P.

MULLANE, K.M., KRAEMER, R. & SMITH, B. (1985). Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J. Pharmacol. Methods*, **14**, 157–167.

MUROHARA, T., BUERKE, M. & LEFER, A.M. (1994). Polymorphonuclear leukocyte-induced vasoconstriction and endothelial dysfunction: role of selectins. *Arterioscler. Thromb.*, **14**, 1509–1519.

MUROHARA, T., GUO, J.-P. & LEFER, A.M. (1995). Cardioprotection by a novel recombinant serine protease inhibitor in myocardial ischemia and reperfusion injury. *J. Pharmacol. Exp. Ther.*, **274**, 1246–1253.

MUROHARA, T., KUGIYAMA, K., SUGIYAMA, S., OHGUSHI, M. & YASUE, H. (1993). Activated human polymorphonuclear leukocytes elicit endothelium-dependent contraction in isolated pig coronary arteries. *J. Cardiovasc. Pharmacol.*, **21**, 760–766.

NIADA, R., PESCADORE, R., PORTA, R., MANTOVANI, M. & PRINO, G. (1986). Defibrotide is antithrombotic and thrombolytic against rabbit venous thrombosis. *Haemostasis.*, **16**, (Suppl. 1), 3.

PALMER, K.J. & GOA, K.L. (1993). Defibrotide A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in vascular disorders. *Drugs*, **45**, 259–294.

PALMER, R.M.J., ASHTON, D.S. & MONCADA, S. (1988). Vascular endothelial cells synthesize nitric oxide from L-arginine. *Nature*, **333**, 664–666.

PATEL, K.D., ZIMMERMAN, G.A., PRESCOTT, S.M., MCEVER, R.P. & MCINTYRE, T.M. (1991). Oxygen radicals induce human endothelial cells to express GMP-140 and bind neutrophils. *J. Cell. Biol.*, **112**, 749–759.

ROMSON, J.L., HOOK, B.G.F., KUNKEL, S.L., ABRAMS, G.D., SCHORK, A.S. & LUCCHESI, B.R. (1983). Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation*, **67**, 1016–1023.

ROSALKI, S.B. (1967). Improved procedure for creatine phosphokinase determination. *J. Lab. Clin. Med.*, **69**, 696–705.

RÖSEN, P., SCHWIPPERT, P., KAUFMAN, B. & TSCHÖPE, D. (1994). Expression of adhesion molecules on the surface of activated platelets is diminished by PGI_2-analogues and an NO (EDRF)-donor: a comparison between platelets of healthy subjects and diabetic subjects. *Platelets*, **11**, 42–57.

RUBANYI, G.M. & VANHOUTTE, P.M. (1987). Oxygen-derived radicals, endothelial dysfunction and responsiveness of vascular smooth muscle. *Am. J. Physiol.*, **250**, H815–H821.

SIMPSON, P.J., TODD III, R.F., FANTONE, J.C., MICKELSON, J.K., GRIFFIN, J.D. & LUCCHESI, B.R. (1988). Reduction of experimental canine myocardial reperfusion injury by a monoclonal antibody (anti-Mol, anti-CD18) that inhibits leukocyte adhesion. *J. Clin. Invest.*, **81**,624–629.

SKURK, C., NUSS, C. & LEFER, A.M. (1995). Beneficial effects of oligotide, a novel oligodeoxyribonucleotide, in murine traumatic shock. *Shock*, **1**, 13–20.

THIEMERMANN, C., LÖBEL, P. & SCHRÖR, K. (1985). Usefulness of defibrotide in protecting ischemic myocardium from early reperfusion damage. *Am. J. Cardiol.*, **56**, 978–982.

TSAO, P.S., AOKI, N., LEFER, D.J., JOHNSON III, G. & LEFER, A.M. (1990). Time course of endothelial dysfunction and myocardial injury during myocardial ischemia and reperfusion in the cat. *Circulation*, **82**, 1402–1412.

WEYRICH, A.S., BUERKE, M., ALBERTINE, K.H. & LEFER, A.M. (1995). Time course of coronary vascular endothelial adhesion molecule expression during reperfusion of ischemic feline myocardium. *J. Leukoc. Biol.*, **57**, 45–55.

WEYRICH, A.S., MA, X.-L., LEFER, D.J., ALBERTINE, K.H. & LEFER, A.M. (1993). In vivo neutralization of P-selectin protects feline heart and endothelium in myocardial ischemia and reperfusion injury. *J. Clin. Invest.*, **91**, 2620–2629.

YUAN, Y. & FLEMING, B.P.A. (1990). Method for isolation and fluorescent labeling of rat neutrophils for intravital microvascular studies. *Microvasc. Res.*, **40**, 218–229.

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