A new model for evaluation of thrombosis and ischaemia/reperfusion injury

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Background: The purpose of the present study was to describe infarct size and platelet accumulation when reperfusion injury was combined with a thrombogenic lesion in the coronary artery. The left anterior descending artery was damaged in 11 pigs and subsequently occluded proximal to the lesion for 50 min, followed by 4 h of reperfusion.

Results: The infarct size/area at risk was 40 (35-63)%. Infarct size correlated with troponin-T-3 h (p=0.85, p<0.002), but not with creatine kinase-3 h. Platelet aggregation decreased by 34% (p<0.01) at 15 min of reperfusion, but returned to baseline. Platelet accumulation in the left ventricle was significantly higher in the area at risk (194 (157-206)%) compared to the right ventricle (137 (120-142)%; p<0.05). Conclusion: A decreased platelet reactivity and increased accumulation of platelets in the area at risk indicates that activated platelets become entrapped in the myocardium. Troponin-T was a better marker of myocardial damage than creatine kinase in this in vivo model with pigs.

Key words: Animals; infarct size; ischaemic markers; platelets; thrombosis.

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Early recanalisation of the coronary artery in acute myocardial infarction (MI) is associated with improved survival (1). Reperfusion ameliorates hypoxia and is an absolute prerequisite for the survival of the ischaemic myocardium. However, it has been proposed that reestablishment of blood flow may at the same time have deleterious effects due to a process termed reperfusion injury (2). This has led to numerous experimental studies to obtain a better understanding of the pathophysiological processes involved and several pharmacological strategies have been investigated to find a therapy to limit reperfusion injury (3).

Experimental models have generally been used to look at the specific effects of reperfusion injury, including that on the myocardium at the cellular level and the inflammatory response following a simple mechanical occlusion of the vessel. These models have the advantage that reperfusion time is well-defined and blood flow is restored completely as soon as the occlusion is removed. However, angiographic studies have shown intermittent coronary occlusion in the acute phase of myocardial infarction and during thrombolytic therapy (4), which may lead to repetitive ischaemic episodes and induction of reperfusion injury on several occasions. Reoc-
occlusion of the coronary artery is generally caused by a highly thrombogenic area in the artery, most often by a ruptured plaque, stimulating thrombus formation (5). Also of importance is the activation of platelets originating from exposure of collagen in the vessel wall and subsequent thrombus formation by thrombin activation (6), increased adrenaline and thromboxane A2 levels during the acute phase (7), increased shear stress in the stenotic parts of the coronary circulation (8), and the platelet-activating effect of the thrombolytic agents given (9, 10). The importance of platelets is clearly documented by the beneficial effect of aspirin on mortality in patients with myocardial infarction (11). Our aim was to describe a new in vivo model to study ischaemia/reperfusion injury in which a thrombogenic area is introduced in the nutrient artery. A pig model was chosen for several reasons: the heart anatomy, and especially the coronary distribution, in pigs shows several similarities with the human coronary circulation; the pig is characterised by a relative absence of preexisting collateral blood flow; the size of the heart is large enough to produce regional ischaemia and the left anterior descending artery (LAD) calibre is sufficient to produce localised thrombogenic damage.

METHODS

Model description

Eleven pigs (40–51 kg) were anaesthetised with fentanyl 0.3 mg and propofol 150 mg, intubated, and mechanically ventilated (4.5 L/min) with a mixture of atmospheric air and O2. Anaesthesia was maintained with an infusion of fentanyl 0.3 mg/h, propofol 8–10 mg/kg/h, and pancuronium 3 mg/h. Serial blood gas measurements were performed to maintain a physiological level of oxygenation and ventilation. All animals received amiodarone, 150 mg prophylactically, to reduce the rate of arrhythmia during reperfusion. Animals were not anticoagulated or heparinised. Temperature was kept between 36.5–38.0°C using a heated blanket. The principles stated in the Danish law on animal experiments were followed.

After exposure of the common carotid arteries, two 8F introducer sheaths (Fast-Cath, Cordis, Roden, The Netherlands) were inserted for blood pressure measurements and blood sampling. A 6F introducer sheath (Fast-Cath, Cordis, Roden, The Netherlands) was placed in the internal jugular vein for infusion of anaesthetics. Access to the heart was obtained by median sternotomy, and the heart was suspended in a pericardial cradle. A 15–20 mm length of the artery was exposed and isolated in the midportion of the LAD.

Thrombogenic lesion

A medial injury, resulting in exposure of adventitial tissue to the lumen, was created by external application of two serrated clamps, followed by twisting of the artery by moving one clamp clockwise and the other clamp counterclockwise. This was done without inducing any perivascular haematoma (Fig. 1). Prior to the experiment a series of pilot studies were carried out (n=4) where in vivo thrombus formation was visualised by transilluminating the LAD from beneath, as described previously (12). A growing thrombus shedding emboli could be seen inside the vessel lumen after the thrombogenic injury. The vessels were cut out 30 min after reperfusion and a combined red and white thrombus was found within the lumen. However, due to the movement of the heart a major concern was whether the transilluminator would intermittently cause obstruction of the blood flow in the artery; the transilluminator was therefore not applied during the final experiment.

The LAD was occluded by a vessel clamp just proximal to the thrombogenic lesion. Occlusion was maintained for 50 min and after that the vessel clamp was released and the artery was allowed to reperfuse. At 4 h reperfusion the LAD was once again occluded by placing a suture around the isolated part of the LAD and the corresponding vein. Immediately after occlusion the heart was perfusion stained with 20 ml Na-fluorescein to determine the area at risk (unstained). The dye was injected into the left atrium, ventricular fibrillation was induced with a 9-V battery, and the heart was excised 10–15 s after injection (15). Following exsiccation the heart was immediately placed in ice-cold water, the suture around LAD was loosened, and the veins in the area at risk were quickly emptied. The right and left ventricle were stored at −20°C until later analysis.

In order to evaluate the nature of the thrombogenic lesion the isolated part of the LAD was cut out at the end of the experiment and placed in formaldehyde/sodium phosphate 4% w/v. Vessel specimens were cut out in longitudinal sections, prepared for light microscopy, and stained with trichrome staining.

Infarct size/area at risk

The heart was cut into 5 mm slices, perpendicular to the septum from the apex to the base, with an electrical meat slicer, until the entire slice was stained with Na-fluorescein, indicating the area above the occlusion of LAD. All slices were weighed. The area at risk was marked with a glowing needle on each slice under a Wood's lamp. Viable myocardium was stained bright red by incubating the slices in 1% TTC (2,3,5 triphenyltetrazolium chloride; Sigma, St. Louis, MO, USA) for 15 min at 37°C. All slices were video-
taped both before and after staining using a CCD camera (JAI Protec 2040, JAI, Japan) and a video machine (Sony SVO 9500 MMP, Tokyo Japan), and stored on videotapes (Fuji-Pro SE-180N, Fuji Magnetics GmbH, Kleve, Germany) for later analysis.

For each slice, area at risk (AR), area not at risk (ANR), and infarct size (IS) were assessed by computer planimetry using an IBM personal computer and the mass/ratio of area at risk/left ventricular mass and infarct size/area at risk (IS/AR) were calculated.

**Platelets**

Indium-labelled platelets were produced from 100 ml blood drawn from a central venous line into acid citrate dextrose (ACD) anticoagulant and gently rotated. The blood was centrifuged at 1,000 rpm for 10 min to isolate platelets in plasma. Platelet-rich plasma was centrifuged at 2,200 rpm for 10 min and the platelet-poor plasma (PPP) was removed for later resuspension. The platelet pellet was gently resuspended in 1.5 ml plasma and incubated with a mixture of 111-Indium-tropolone for 10 min. After incubation, 5 ml PPP was added and the sample was centrifuged at 2,200 rpm for 10 min to remove unbound Indium. After the second centrifugation, the platelets were resuspended in 5 ml PPP. The labelling efficiency was 98±0.02%. The radioactive solution was injected back into the pigs 30 min prior to harvesting of the heart.

Blood samples for platelet aggregation were drawn from the arterial sheath and anticoagulated with hirudin (0.1 mg/ml final concentration) at baseline and 15 min, 1 h, and 3 h after reperfusion. Platelet-rich plasma (PRP) was prepared by centrifugation at 180×g for 10 min at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation at 2,500×g for 10 min. Platelet aggregation was measured in a single-channel aggregometer (Model 560 vs, Chronolog, Haverton) using the turbimetric method described by Born (14). Platelet aggregation was induced with fixed concentrations of collagen (0.2 mg/ml) (Sigma, St. Louis, MO, USA) and the aggregation response was recorded during the following 5 min.

**Platelet accumulation**

After the left ventricle had been stained with TTC, the slices were divided into three parts based on staining characteristics. The area at risk was cut out following the glowing needle marks, and within this area the necrotic tissue (white-yellowish) was separated from the viable tissue (stained red). Representative samples of the central part of the right ventricle were obtained. The tissue was placed in pre-weighed scintillation vials in four separate groups: a) area at risk-necrotic, b) area at risk-viable, c) area not at risk-left ventricle, and d) right ventricle.

The vials with tissue were weighed before radioactivity counting. Quantitative counting of the tissue radioactivity was done on a Packard gamma counter. The window was set at 350-500 keV for 111-Indium, and counts were corrected for background. Counts/g tissue were calculated and the radioactivity was expressed as percentage of the area not at risk in the left ventricle (ANR=100%).

**Tissue oedema**

The tissue specimens were freeze dried in a Heto-sicc (Heto, Birkerod, Denmark) for 24 h. The water content was estimated from the difference in weight before and after the tissue had been dried.

**Ischaemic markers**

Blood samples were drawn into heparinised Venoject® tubes (Terumo Europe, Leuven, Belgium) at baseline and 30 min, 1 h, 3 h of reperfusion. Samples were immediately centrifuged at 3,000 rpm, 4°C, for 15 min, and plasma was stored at -20°C for later analysis. Plasma levels of creatine kinase were measured using a Johnson & Johnson, Vitros-250. Troponin-T was measured with Enzymun-Test Troponin-T:1556428 (Boehringer, Mannheim, Germany).

**Statistics**

All variables are given as median values and range (min-max). Comparison between two groups was done using Student's t-test or paired-rank sum test if data were not normally distributed. With more than two groups, data were analysed using one-way ANOVA. The relationship between infarct size and ischaemic markers was explored using Spearman's correlation coefficient. A p value <0.05 was considered significant.

**RESULTS**

**Animal survival**

Eleven animals were included in the protocol, but three died due to irreversible ventricular fibrillation during reperfusion, despite prophylactic treatment with amiodarone.

**Infarct size and platelet accumulation**

A thrombogenic lesion was produced in the midportion of the LAD, which resulted in disruption of the media and protrusion of the highly thrombogenic adventitia into the vessel lumen (Fig. 1). The clamp squeeze resulted in disruption of the lamina externa, leaving a gap in the area between the media and the adventitia. After 4 h reperfusion, thrombus material, mainly platelets were seen in the area with ex-
Two serrated forceps were placed next to each other on the LAD, and the artery was twisted by moving the forceps clockwise and counterclockwise. This resulted in a thrombogenic lesion with disruption of the media and exposure of the adventitia to the lumen. Thrombus material was often seen in close contact with the exposed adventitia (Masson-trichrome staining).

The LAD was occluded just proximal to the lesion, whereby an area including the septum and part of the anterior wall was rendered ischaemic. The area at risk amounted to 22 (18–30)% of the left ventricle in the present model. After 50 min ischaemia and 4 h reperfusion an infarct was found in all hearts. Following removal of the mechanical occlusion, the blood flow in the LAD was restored within a few minutes. However, intermittent occlusion of the artery was often observed, especially within the first 30 min of reperfusion. The combination of an I/R injury with a thrombogenic lesion resulted in a median infarct size/area at risk of 40 (24–69)%.

Necrosis was primarily found in the septum and in the endocardial part of the anterior wall of the ventricle.

The ischaemic burden at the cellular level was estimated by measuring creatine kinase (CK) and troponin-T (TNT). A significant increase was also observed for TNT, and with respect to this parameter the increase was more than 50 fold. A significant correlation was observed between TNT-3 h and infarct size (p=0.85; p<0.002), but not between CK-3 h and infarct size (Fig. 2).

Platelet aggregation decreased significantly from 31 (16–62)% to 21 (9–40)% 15 min after reperfusion (p<0.01), but returned to baseline within the following 3 h (Table 1). The decrease in platelet aggregation may reflect a consumption of hyperreactive platelets as $^{111}$Indium-labelled platelets were observed to accumulate in the area at risk. The relative accumulation of platelets was almost twice as high in the area at risk compared to the area not at risk. Not surprising the highest accumulation was observed in the necrotic parts of the heart (Table 2).

A rough estimate of oedema in the tissue was obtained by measuring water content in tissue.
TABLE 1. Ischaemic markers and platelet aggregation Data are given as median and range

|                      | Baseline | t=15 min | t=1 h | t=3 h | ANOVA p-value |
|----------------------|----------|----------|-------|-------|---------------|
| Creatinine kinase, U/L| 802 (522-1103) | 848 (570-1444) | 1295 (617-2437) | 1732 (715-3246) | <0.001 |
| Troponin-T, µg/L     | 0.03 (0.02-0.05) | 0.07 (0.03-0.56) | 0.82 (0.07-2.69) | 1.71 (0.09-5.9) | <0.01 |
| Platelet aggregation, % | 31 (16-62) | *21 (9-40) | 25 (18-31) | 25 (17-45) | NS |

* p<0.05 compared to baseline.

TABLE 2. Platelet accumulation and water content in the myocardium Data are given as median and range

|                      | AR-vital | AR-necrotic | ANR | RV |
|----------------------|----------|-------------|-----|----|
| 111Indium platelets, % of ANR | t150 (133-312) | t212 (137-359) | 100 | 137 (94-185) |
| Water content, % of total weight | 78.1 (77.2-80.5) | 80.2 (78.0-82.2) | 78.4 (77.8-79.3) | 80.0 (79.3-82.0) |

† AAR-total: *1 94 (135-288); * p=0.01 compared to RV.

samples. The tissue was freeze dried and the water content was found to be approximately 78-80% of the total weight irrespective of the sampling area (Table 2).

DISCUSSION

Coronary reperfusion is absolutely fundamental in the treatment of acute ischaemic syndromes in order to reestablish nutrition and oxygen supply to the ischaemic myocardium. However, the return of oxygenated blood to the ischaemic myocardium has been associated with several deleterious effects collectively known as reperfusion injury (2). Ischaemia-reperfusion injury involves an incompletely understood cascade of events that include the effects of oxygen free radicals, complement activation, vasoactive, proteolytic, and chemotactic factors (3).

Angiographic studies have shown intermittent coronary occlusion in the early phase of acute myocardial infarction and during thrombolytic therapy, demonstrating the dynamic character of thrombus formation and thrombus resolution/embolisation (4). Little is known about the effect of repetitive ischaemia/reperfusion periods, but it is conceivable that the reperfusion-associated platelet activation may contribute to continuous thrombosis and coronary reocclusion. Oxygen free radicals are known to activate platelets, probably through phospholipase-induced arachidonic acid release and thromboxane A2 formation. Furthermore, oxygen free radicals may impair the synthesis and release of nitric oxide and prosta-

cyclin, as a result of endothelial damage, leading to augmented platelet reactivity during reperfusion (15).

Complete reperfusion (TIMI grade 3 flow) is known to reduce mortality and improve myocardial function, but is achieved in only one third to one half of the patients following various thrombolytic therapies (16). These numbers underline the importance of efficient adjunctive therapies to limit the rate of reocclusion and to reduce platelet activation during reperfusion of the ischaemic myocardium. This model provides the opportunity to evaluate the effect of various pharmacological interventions on platelet reactivity and infarct size following reperfusion injury in a set-up which bears a close resemblance to the clinical events observed in patients with myocardial infarction.

A reduced ex vivo platelet aggregation has been observed in patients with myocardial infarction within the first 2 days after the onset of symptoms (17). A possible explanation for the hypoaggregability may be consumption of active platelets in the circulation of the reperfused myocardium. Platelet aggregates have been demonstrated in small intramyocardial vessels in more than 40% of patients dying from sudden cardiac death after the onset of unstable angina (18). Aggregates contained platelets with only minimal amounts of fibrin and the aggregated platelets were almost exclusively confined to the myocardium immediately downstream of a coronary artery containing a ruptured plaque with a superimposed mural thrombus (18). A significant decrease in platelet aggregation was observed 15 min after reperfusion of the LAD.
in the present study. Furthermore, an almost two-fold accumulation of platelet was found in the area at risk, the segment of the myocardium immediately downstream of the thrombogenic artery. In the area at risk, maximal platelet accumulation was found in the necrotic part, indicating that platelets take part in the developing infarct.

In order to evaluate cellular damage during reperfusion, CK and TNT were measured in the present study. The most frequently used laboratory markers of acute MI are CK and the isoenzyme CK-MB (19). However, skeletal muscle injury can also result in increased levels of these enzymes, thus challenging the cardiospecificity of these markers. The identification of cardiac TNT has provided a more sensitive and specific marker of minor cardiac injury (20, 21). The release in serum of both CK and TNT was assessed at baseline and during reperfusion. Both markers increased significantly and reached maximum levels 3 h after the LAD was reopened. At 3 h reperfusion the CK activity was more than twice the baseline level and there was a more than 50-fold increase in TNT concentration. TNT was significantly correlated with infarct size, which was not the case with the enzyme activity of CK, indicating that TNT is a much more sensitivity marker of cellular damage than CK. This is in good agreement with clinical studies, where TNT has proved to be a much more sensitive and specific marker of cardiac damage during myocardial infarction (22).

Experimental data have shown that TNT can be used as an ischaemic maker in rats and in dogs (23, 24), but to our knowledge this is the first study in pigs which demonstrates that TNT is a useful and sensitive method for measuring myocardial damage.

Water content in the myocardium was measured in the present study as a rough measure of inflammation, but no significant changes were demonstrated between the area at risk and the remaining regions of the heart. This observation does not rule out that an inflammatory reaction takes place, but it may simply reflect that capillary leakage has not yet manifested itself at this early stage of reperfusion.

In conclusion, the present model describes infarct size and platelet accumulation after a combined thrombogenic and ischaemic injury. There is a closer resemblance to the clinical situation where reperfusion injury is complicated by repetitive reclosures of the coronary artery. Serial measurements of TNT can be used to monitor the development of myocyte damage in pigs. This model offers the advantage of evaluating the combined antithrombotic and myocardial protective effect of various pharmacological interventions.

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