

Attenuation of myocardial reperfusion injury in pigs by Mirococept, a membrane-targeted complement inhibitor derived from human CR1

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Abstract

Objectives: Membrane-targeted application of complement inhibitors may ameliorate ischemia/reperfusion (I/R) injury by directly targeting damaged cells. We investigated whether Mirococept, a membrane-targeted, myristoylated peptidyl construct derived from complement receptor 1 (CR1) could attenuate I/R injury following acute myocardial infarction in pigs.

Methods: In a closed-chest pig model of acute myocardial infarction, Mirococept, the non-tailed derivative APT154, or vehicle was administered intracoronarily into the area at risk 5 min pre-reperfusion. Infarct size, cardiac function and inflammatory status were evaluated.

Results: Mirococept targeted damaged vasculature and myocardium, significantly decreasing infarct size compared to vehicle, whereas APT154 had no effect. Cardioprotection correlated with reduced serum troponin I and was paralleled by attenuated local myocardial complement deposition and tissue factor expression. Myocardial apoptosis (TUNEL-positivity) was also reduced with the use of Mirococept. Local modulation of the pro-inflammatory and pro-coagulant phenotype translated to improved left ventricular end-diastolic pressure, ejection fraction and regional wall motion post-reperfusion.

Conclusions: Local modification of a pro-inflammatory and pro-coagulant environment after regional I/R injury by site-specific application of a membrane-targeted complement regulatory protein may offer novel possibilities and insights into potential treatment strategies of reperfusion-induced injury.

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Abbreviations: AAR, area at risk; ANR, area not at risk; aPTT, activated partial thromboplastin time; CH50, (classical pathway) complement activity; CR1, complement receptor 1; EC, endothelial cell; EF, ejection fraction; ELISA, enzyme-linked immunosorbent assay; ET-1, endothelin-1; FS, fractional shortening; HR, heart rate; I/R, ischemia/reperfusion; LAD, left anterior descending artery; LVEDP, left ventricular end-diastolic pressure; LVM, left ventricular mass; MAP, mean arterial pressure; MI, myocardial infarction; MPO, myeloperoxidase; NIT, necrotic ischemic tissue; TF, tissue factor; TTC, triphenyl tetrazolium chloride; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; VIT, vital ischemic tissue.

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1. Introduction

Reperfusion following ischemia is known to induce a pro-inflammatory reaction, involving activation of the classical, alternative and lectin complement pathways [1,2]. The importance of each of the three different complement pathways may, in part, depend on the organ affected. Furthermore, the injured organ itself up-regulates the production of complement components, which contribute to reperfusion damage [3].

In vivo, membrane-bound regulators of complement, including membrane cofactor protein (MCP, CD46), decay-

accelerating factor (DAF, CD55), protectin (CD59) as well as complement receptor type 1 (CR1, CD35) protect against unwanted, harmful complement activation [4]. CR1 influences the classical and alternative pathway by inhibiting the C3/C5 convertases, acting as a cofactor for factor-I mediated cleavage of C4b/C3b and for the subsequent cleavage of iC3b to C3c and C3dg complement fragments [4]. Furthermore, CR1 is up-regulated on human vascular endothelial cells following hypoxic stress and mediates immune complex processing [5]. These properties render it ideal to exploit therapeutically in complement-mediated disease states. In a soluble recombinant form, it has been used in several studies to successfully treat I/R- and cardiopulmonary bypass-associated injury [6–8]. Local, directed cytoprotection and complement inhibition with CR1, modified to bind to (damaged) cell membranes, holds out the prospect of improved efficacy through increasing the effective concentration of the complement regulatory pharmacophore at the site where it is needed.

In this report we describe the use of Mirococept [9], an engineered fragment of soluble (s)CR1, which, through coupling to a reagent comprising a peptide motif and a lipophilic tail [9,10], enables tethering of the inhibitor to cell surfaces. We hypothesized that since pro-inflammatory complement components are formed and assembled at the surface of damaged cells, intracoronary infusion of Mirococept to damaged endothelium and myocardium may reduce I/R injury in a pig model of acute myocardial infarction.

2. Materials and methods

Care and use of animals in the present study were in compliance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23, revised 1996) as well as Swiss National guidelines.

2.1. Materials

Mirococept is a soluble, recombinant protein derived from soluble complement receptor 1 (sCR1) modified with membrane-binding properties through a myristoylated peptide sequence. The membrane-binding moiety was designed for posttranslational chemical attachment to the short consensus repeats 1–3 [9,10] and is based on the myristoyl electrostatic switch structural feature of intracellular proteins [11]. Membrane localization of Mirococept is rapid (complete *in vitro* within 2 min at 25 °C) and the dissociation half-life averages several hours. Radiolabelling experiments suggest that the modified protein is retained 5–10 times longer than the unmodified form (see below). Further details on construction and structure of Mirococept can be found in the publication of Pratt et al. [12] and elsewhere [10,13,14]. The recombinant non-tailed CR1 fragment APT154 possesses identical chemical and complement inhibitory properties as Mirococept, but is untagged and therefore does not contain the membrane-binding moiety. A vehicle control was used

which contains all but the active Mirococept or APT154, respectively. All substances were produced and provided by Inflazyme (Inflazyme Pharmaceuticals Ltd., Richmond, Canada).

2.2. Experimental model

Eighteen large white pigs (30±2 kg) were premedicated with intramuscular ketamine (20 mg/kg) and xylazine (2 mg/kg), followed by intravenous midazolam (0.5 mg/kg) and atropine (0.05 mg/kg), intubated and mechanically ventilated with a Draeger respirator (O₂/N₂O 1:3, Isoflurane 1–1.5 vol.%). Central venous and arterial lines were introduced and a single bolus of unfractionated heparin (2500 IU) was administered intravenously. Baseline values were recorded during a 30-minutes stabilization period.

The left anterior descending artery (LAD) was occluded with a semi compliant over-the-wire percutaneous coronary intervention catheter (Concerto, Occam, the Netherlands, balloon length 10 mm, diameter 2.5 mm to 3 mm) just distally of the first diagonal branch. The balloon was inflated under angiographic control to completely occlude the vessel (Encore™ 26 inflation device, Boston Scientific, Ireland) for 60 min.

Ischemia was followed by two hours of reperfusion. Five minutes prior to reperfusion, 10 ml of Mirococept (1.5 mg/ml; corresponding to 0.5 mg/kg [22 nmol/kg]; the dose and application scheme i.e. one-time application were chosen according to previous experience and studies [12,15], *n*=6), APT154 (non-tailed control, 1.37 mg/ml [equimolar to Mirococept], *n*=6) or vehicle control (*n*=6) were slowly injected intracoronarily, through the catheter tip, into the area at risk. Thereafter the balloon was deflated to allow for two hours of reperfusion. Following reperfusion the balloon was re-inflated, 60 ml Evan's Blue injected intravenously and the animals sacrificed by intravenous potassium chloride. The heart was excised for further analysis.

ECG and invasive arterial pressure were recorded throughout with a Hewlett-Packard CMS patient monitor. Ejection fraction (EF) was determined angiographically and calculated using the area-length-method according to Dodge [16]. Fractional shortening (FS) was also determined angiographically and was assessed in three regions (apical, medial and basal). The geometric mean of the three regional values was used for further analyses.

All experimenters were blinded with regard to treatment regimen. Randomization of the animals into the three groups was done using a randomization code with a random number generator (SAS, version 9.1.2, SAS Institute Inc., Cary, NC, USA), prior to the begin of the series (Mirococept=0, APT154=1, vehicle controls=2). The 10 ml samples (Mirococept, APT154 or vehicle solution) were prepared according to the randomization output and stored at –80 °C until use. Randomization and sample preparation was performed by an independent laboratory technician. Prior to pre-medication of the animals, the corresponding vial was allocated

to the pig (sequential number of vial = sequential number of the pig). All 18 consecutively enlisted pigs were treated according to the above-described protocol. No animal was excluded from or dropped out during the experiment. Sample size ($n=6$) was determined in advance, estimated from previous work [12,17] and experience and not by formal sample size calculations.

In case of ventricular fibrillation a biphasic defibrillator (150 J) was used for cardioconversion.

2.3. Infarct size

The area at risk (AAR) was determined by re-occlusion of the LAD and intravenous injection of 60 ml Evan's Blue (2% wt/vol solution) at the end of the experiment. Tissue stained blue was defined as the area not at risk. The left ventricle was cut perpendicular to the septum into 3 mm slices. Incubation with triphenyl tetrazolium chloride (TTC, Sigma, pH 7.4, 1% in phosphate buffered saline) for 20 min at 37 °C was used to stain viable myocardium (vital ischemic tissue, VIT) from the area at risk bright red (formazan complex in presence of active dehydrogenases) whereas infarcted tissue (necrotic ischemic tissue, NIT) remains unstained. All three tissue sections were dissected from each other and weighed. The area at risk was defined as the sum of necrotic and vital ischemic tissue. Data were expressed as area at risk as percent of LVM (AAR/LVM) and necrotic ischemic tissue as percent of the area at risk (NIT/AAR).

2.4. Complement and coagulation

Venous blood samples were collected (EDTA plasma/serum), kept on ice until centrifugation (1750 x g for 10 min at 4 °C) and stored at -80 °C until further analysis.

C3a and C5a were measured by sandwich ELISA as previously described [18]. In brief, microtiter plates were coated with a monoclonal antibody (IgG2b) against porcine C3a/C3a(desArg) or rabbit anti-mouse IgG (Dako, Glostrup, Denmark) followed by a monoclonal antibody (IgG1) against porcine C5a. After washing with PBS-Tween, plasma samples were incubated at a 1:50 (C3a ELISA) or 1:2 (C5a ELISA) dilution in PBS/Tween/EDTA for 2 h. Biotinylated monoclonal anti-C3/C3a antibody or rabbit anti-C5a, followed by streptavidin-alkaline phosphatase conjugate (Amersham Pharmacia Biotech, Bucks, UK) and 4-nitrophenyl phosphate substrate (Sigma) were used to detect bound C3a and C5a (all non-commercial antibodies were obtained from the Georg-August University, Goettingen — Sciencebridge GmbH, Germany).

Classical pathway complement activity was determined by standard a CH50 assay with antibody-coated sheep erythrocytes [19].

Activated partial thromboplastin time (aPTT) was measured using Dade Actin FS reagent in a standard coagulation assay as a global parameter for the coagulation system.

2.5. Troponin I

Troponin I was measured by liquid suspension array technique using the Luminex fluorescent bead platform. Mouse anti-human troponin I (Abcam Ltd, clone 19C7), biotinylated using the EZ-Link NHS-PEO Solid Phase Biotinylation Kit mini spin columns from Pierce (Pierce, Rockford, IL, USA), was used as the detection antibody and mouse anti-human troponin I, cross-reactive with porcine troponin I (Abcam Ltd., Cambridge, UK, clone 16A11), was used as the capture antibody. Covalent coupling of the capture antibody to the region-specific microspheres was performed using the Bio-Plex Amine Coupling Kit (Bio-Rad). Analysis was done with the Bio-Plex system and Bio-Plex Manager version 4.0 software (Bio-Rad) with five-parametric curve fitting. Assay conditions were derived from those of commercial tests.

2.6. Histology and immunostaining

Tissue samples from the area not at risk as well as necrotic and vital ischemic tissue were fixed in 4% buffered formaldehyde, paraffin-embedded, cut into 3 µm sections and stained with hematoxylin-eosin for histological evaluation. Neutrophil tissue numbers were determined by myeloperoxidase (MPO) staining on additional tissue sections. Antigen-retrieval was performed with target retrieval solution (Dako, Glostrup, Denmark) and demasking at 96 °C for 30 min. Following peroxidase block (Dako) MPO was detected using a polyclonal antibody to MPO (Dako) and visualized with a horseradish peroxidase-labeled secondary antibody and EnVision+ System (Dako). Neutrophil numbers were quantified in 10 randomly selected high-power viewing fields from the tissue samples of each experiment. The intravascular/interstitial ratio was calculated for each section and experiment and averaged for all experiments in each of the three groups.

For complement and tissue factor detection, 5 µm sections were cut from all snap frozen tissue samples, air-dried, acetone fixed, hydrated and labeled using a two-step indirect immunofluorescence technique. The following antibodies were used: rabbit anti-human C1q, C3b/c and C4b/c (Dako); goat anti-human C6 (Quidel, for detection of the membrane attack complex). Cross-reactivity with the respective porcine antigens was verified. Secondary antibodies were goat anti-rabbit IgG(H+L)-FITC (Southern Biotechnology Associated) and rabbit anti-goat Ig-FITC (Dako). Immunohistochemical staining for tissue factor (TF) with polyclonal rabbit anti-human TF antibody was carried out as reported previously [20].

For every individual experiment four samples per area (area not at risk, ischemic viable and necrotic areas) were graded according to the following scoring system: 0: no staining, 1: minimal focal or diffuse staining, 2: moderately strong focal or diffuse staining, 3: extensive focal or diffuse staining.

Tissue binding of Mirococept was visualized on acetone-fixed cryosections using Cy3-labeled 3E10 antibody (mouse anti-human sCR1-3 monoclonal antibody, provided by Inflazyme).

2.7. Quantification of myocyte apoptosis by TUNEL

Myocyte apoptosis was detected on paraffin-embedded sections in high-power fields by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL, Chemicon Inc., Millipore, Billerica, MA, USA). TUNEL-positive nuclei that were not definitively of myocyte origin were excluded from counting. The extent of apoptosis was expressed by normalizing the results to the total number of myocyte nuclei per high-power field.

2.8. Statistics

Data from triphenyl tetrazolium chloride staining (AAR/LVM in % and NIT/AAR in %, primary endpoint) were compared between the three groups by use of one-way-analysis of variance (ANOVA). Results for all other parameters (secondary endpoints) were compared by use of two-way repeated-measures ANOVA with time and treatment as fixed factors. Subject number was included as random. Effects of interest were overall between-group differences and interaction between group and time. The assumption of the sphericity of our data was analyzed by Mauchly's test and corrections were made where appropriate (Greenhouse-Geisser). In case of a significant p -value in the overall ANOVA with respect to

group*time interaction, post-hoc analyses were performed. Holm's procedure was used in post-hoc pair-wise comparisons to control for type I error. For all analyses p -values were two-sided and differences were considered to be statistically significant with a p -value of <0.05 . SAS Version 9.1.2 (SAS Institute Inc., Cary, NC, USA) and SPSS Version 12.0.1 (SPSS Inc., Chicago, IL, USA) software were used for all analyses. Data, unless otherwise specified, are presented as average \pm standard deviation in text and figures.

3. Results

3.1. Tissue and plasma levels of Mirococept

Bound Mirococept was found mainly in the area at risk, both in viable and necrotic ischemic tissue, on the inner lining of small and large blood vessels and in the myocardium, and to a lesser extent in non-ischemic myocardial tissue (Fig. 1). Staining for APT154 essentially revealed no tissue positivity. No staining was observed at distant sites (lung, kidney, liver). In an ELISA for Mirococept, small amounts were detected in circulation 5 min after reperfusion (3.4 ± 0.8 $\mu\text{g/ml}$). Plasma levels decreased after 1 h and 2 h reperfusion (1.8 ± 0.5 $\mu\text{g/ml}$, 0.8 ± 0.2 $\mu\text{g/ml}$ respectively, results not shown).

3.2. Infarct size

The myocardial area at risk (AAR) as percentage of left ventricular mass (LVM) was not different in the three groups:

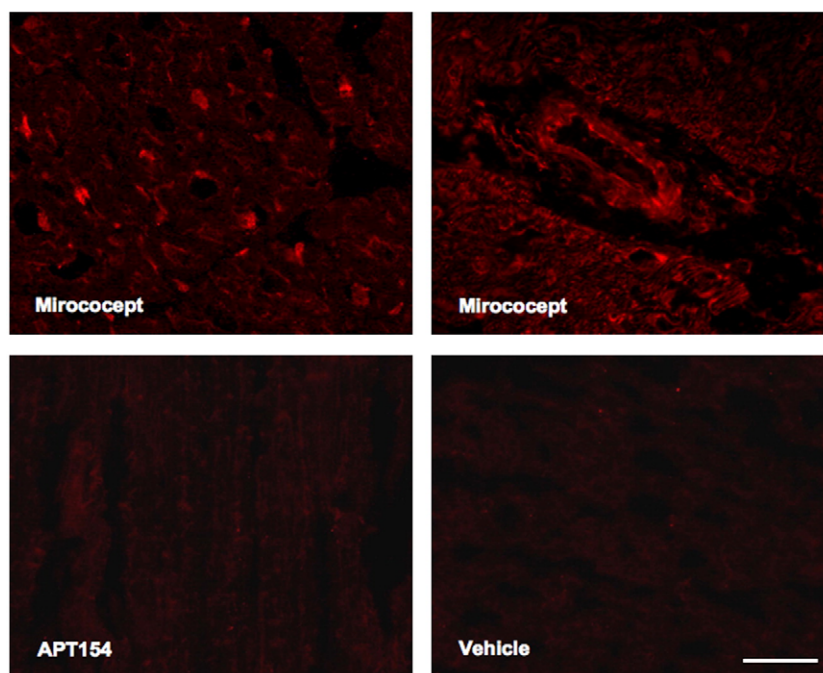


Fig. 1. Staining for Mirococept using Cy3-labeled 3E10 antibody (mouse anti-human sCR1-3 monoclonal antibody). Specific deposition of Mirococept is detected in damaged vessels and myocardium in Mirococept-treated animals. No staining is detected in representative samples from APT154- or vehicle-treated animals. Scale bar: 50 μm .

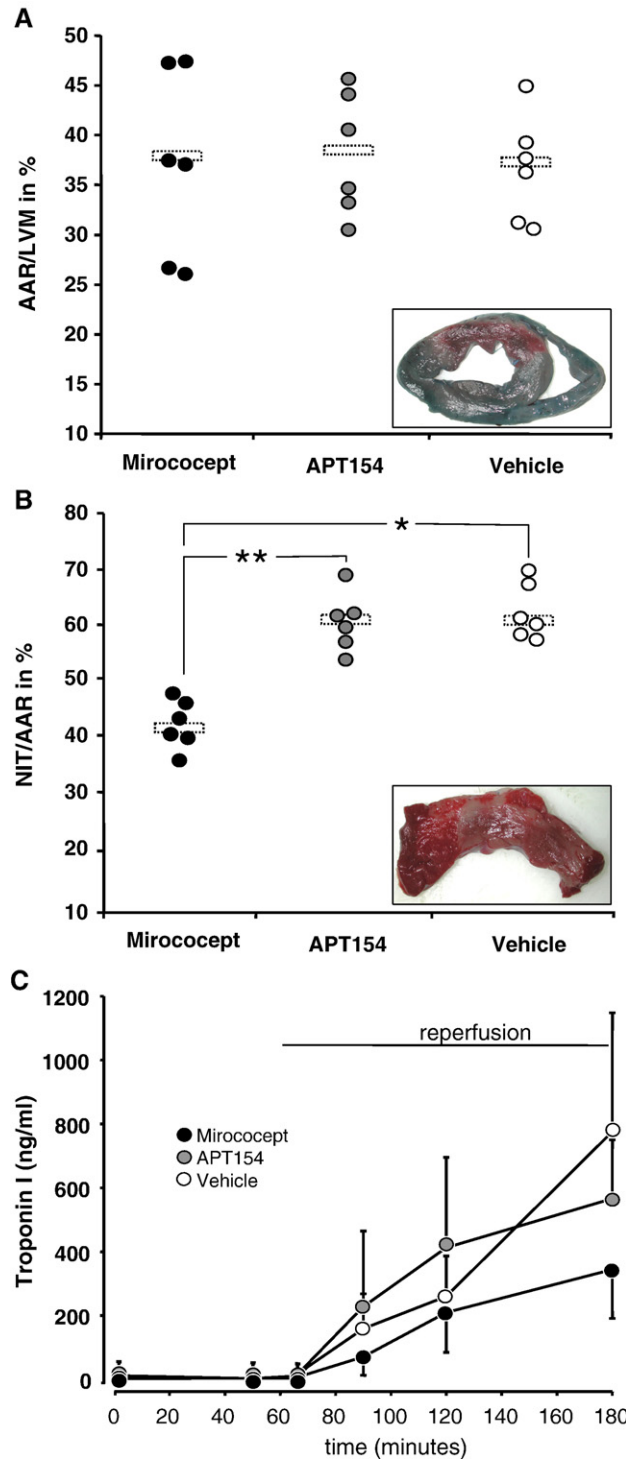


Fig. 2. Ratio of area at risk (AAR) to left ventricular mass (LVM), as detected by Evan’s Blue staining (A). Ratio of necrotic ischemic tissue (NIT) to AAR as detected by triphenyl tetrazolium chloride (TTC) staining (B). Individual values and means (bars) are shown. Comparable AAR in all groups ($p=ns$ between groups). NIT as percent of AAR: $p=0.0003$ (*) Mirococept vs. controls, $p=0.0001$ (**) Mirococept vs. APT154, $p=0.880$ APT154 vs. controls. Representative pictures of the respective staining are shown as inserts (in A: whole section of left and right ventricle, in B: magnified image of Evan’s Blue negative section after TTC staining). Levels of troponin I as measured in EDTA plasma samples by sandwich immunoassay (C). Results are presented as mean±standard deviation.

The measured mean values±standard deviations were $37.1 \pm 9.4\%$, in the Mirococept animals, $38.3 \pm 6.5\%$ in the APT154 animals, and $36.5 \pm 4.8\%$ in the vehicle controls ($p=0.968$ Mirococept vs. APT154; $p=0.886$ Mirococept vs. vehicle

controls, $p=0.900$ APT154 vs. vehicle controls). The corresponding wet weights in grams were 33.7 ± 6.7 g, 34.7 ± 8.5 g and 29.5 ± 3.3 g, respectively. However, infarcted tissue (necrotic ischemic tissue, NIT) as percentage of AAR was

significantly reduced from $61.9 \pm 6.3\%$ in vehicle controls to $42.5 \pm 4.0\%$ in the Mirococept treated animals ($p < 0.001$). Administration of APT154 did not reduce infarct size, which, at $60.8 \pm 5.1\%$ of the AAR, was not significantly different to vehicle controls ($p = 0.880$). Wet weights of NIT were 14.4 ± 3.3 g in the Mirococept animals, 21.0 ± 4.8 g in the APT154 group, and 18.1 ± 1.6 g in the vehicle controls (Fig. 2A and B).

3.3. Plasma troponin I

Plasma troponin I increased in all groups following reperfusion from similar baseline- (average for all groups 6 ± 8 ng/ml) and ischemia-levels to 776 ± 372 ng/ml in the

vehicle controls, 566 ± 246 ng/ml in APT154 animals, and 344 ± 153 ng/ml in Mirococept treated animals after 2 h reperfusion ($p = 0.888$ for overall group-time interaction, Fig. 2C).

3.4. Hemodynamics

Heart rate did not differ significantly amongst the three groups throughout the experimental procedure ($p = 0.888$ for overall group-time interaction, Fig. 3A).

Baseline mean arterial pressures (MAP) as well as pressures during the experiments were also not significantly different in the three groups. ($p = 0.888$ for overall group-time interaction, Fig. 3B).

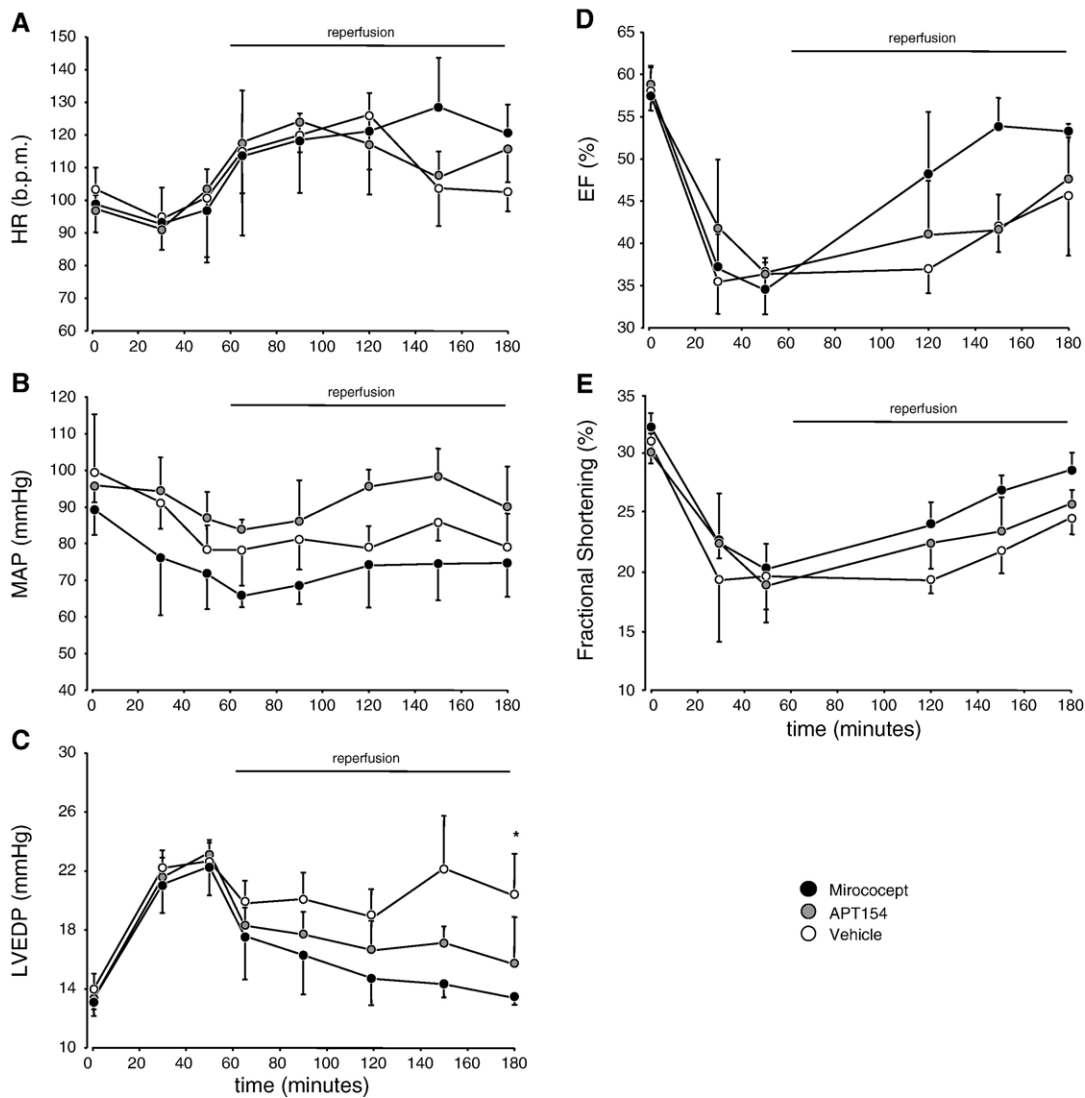


Fig. 3. Heart rate (HR, A), mean arterial pressure (MAP, B), left ventricular end-diastolic pressure (LVEDP, C), ejection fraction (EF, D) and regional wall motility (fractional shortening, FS, E; geometric means of basal, medial and apical values) recorded throughout the experiments. HR and MAP: $p = ns$ between all groups throughout. LVEDP: $p = 0.033$ (*) for Mirococept vs. vehicle controls during reperfusion. EF: $p = 0.048$ for overall group-time interaction during ischemia, $p = 0.081$ for Mirococept vs. vehicle controls, $p = 0.096$ for Mirococept vs. APT154 during reperfusion. Fractional shortening: $p = 0.032$ for overall group-time interaction during ischemia, $p = 0.078$ for Mirococept vs. vehicle controls, $p = 0.098$ for Mirococept vs. APT154 during reperfusion. Data are mean \pm standard deviations.

Left ventricular end-diastolic pressure (LVEDP) rapidly and significantly increased in all three groups during ischemia ($p=0.009$ for overall group-time interaction). Following reperfusion, LVEDP decreased in all groups, most markedly in the Mirococept treated group. LVEDP was significantly lower in the Mirococept group as compared to vehicle controls ($p=0.033$). Other between group differences (Mirococept vs. APT154 and APT154 vs. vehicle controls) were not significant (Fig. 3C).

Ejection fraction significantly decreased in all three groups during LAD occlusion from similar baseline values (overall group-time interaction $p=0.048$). Recovery during reperfusion was more rapid in the Mirococept group. Post-hoc analyses for between group differences showed $p=0.081$ for Mirococept vs. vehicle controls, $p=0.096$ for Mirococept vs. APT154 and $p=0.678$ for APT154 vs. vehicle controls (Fig. 3D).

Basal and medial, but particularly apical (not shown separately) regional wall motility (fractional shortening, FS) was impaired in all three groups during ischemia as compared to baseline, and only showed some recovery towards the end of the reperfusion period, with a tendency towards better recovery in the Mirococept group as compared to vehicle controls and the APT154 animals (overall group-time interaction $p=0.032$, post-hoc analysis: $p=0.078$ for Mirococept vs. vehicle controls, $p=0.098$ for Mirococept vs. APT154 and $p=0.323$ for APT154 vs. vehicle controls, Fig. 3E).

The need for defibrillation for ventricular fibrillation was not significantly different in the three groups (2 animals per group).

3.5. Histology and neutrophil numbers

Reperfusion damage was evident in the necrotic myocardium of all three groups, comprising contraction bands and coagulation necrosis. Gross hemorrhage was observed in two animals of each group. Microscopic hemorrhagic infarction was detected in all groups ($n=2$ for Mirococept and APT154, $n=3$ for vehicle controls). Focal minimal signs of ischemia (wavy fibers) were noted in all three groups in the viable ischemic tissue. Samples from non-ischemic myocardium showed normal histological findings.

The total numbers and intravascular/interstitial ratios of neutrophils in tissue samples are shown in Table 1. Mirococept significantly prevented neutrophil extravasation, particularly in vital ischemic tissue (ratio 1.27 ± 0.35 , $p=0.021$ vs. vehicle controls). Ratios in the area not at risk and in necrotic ischemic tissue were not significantly different between the three groups. Neutrophil numbers in non-ischemic and necrotic tissue from all three groups did not differ significantly. However, treatment with Mirococept significantly reduced MPO positive cells within the vital ischemic tissue as compared to sections from APT154 and vehicle control animals ($p=0.031$).

Table 1

Representation of intravascular/interstitial ratio and total number of tissue neutrophils in myocardial samples

Group	ANR	VIT	NIT
Mirococept	0.84 ± 0.34 (5±1)	1.27 ± 0.35 (10±4)*	0.37 ± 0.08 (16±3)
APT154	0.80 ± 0.21 (4±1)	1.06 ± 0.26 (10±4)**	0.35 ± 0.06 (14±2)
Vehicle controls	0.81 ± 0.22 (4±1)	0.77 ± 0.24 (16±4)	0.29 ± 0.07 (14±2)

Four samples per area and experiment were examined and an average calculated from these. * denotes $p=0.021$ Mirococept vs. vehicle controls (neutrophil ratio) and $p=0.040$ Mirococept vs. vehicle controls (neutrophil numbers); ** denotes $p=0.043$ APT154 vs. vehicle controls (neutrophil numbers). ANR: area not at risk, VIT: vital ischemic tissue, NIT: necrotic ischemic tissue.

3.6. Complement deposition and soluble complement parameters

Fig. 4 summarizes the data of complement deposition and soluble complement parameters. Samples from all experiments were stained and graded: 0: no staining, 1: minimal focal or diffuse staining, 2: moderately strong focal or diffuse staining, 3: extensive focal or diffuse staining. Normal tissue samples from healthy pigs (not shown) and from myocardium not at risk served as controls. Mirococept markedly reduced complement deposition (C1q, C4b/c, C3b/c, and membrane attack complex) in necrotic ischemic tissue as compared with vehicle controls and APT154 treated animals. Essentially no, or only minimal, complement staining was observed in myocardium of healthy pigs or in myocardium not at risk of all three treatment groups (Fig. 4A and B).

Levels of the anaphylatoxins C3a and C5a, expressed as percentage of baseline, did not vary greatly throughout the experiment and were not significantly different between the three groups after 2 h reperfusion ($p=0.422$ Mirococept vs. vehicle controls, $p=0.277$ Mirococept vs. APT154, $p=0.395$ APT154 vs. vehicle controls for C3a; $p=0.317$ Mirococept vs. vehicle controls, $p=0.162$ Mirococept vs. APT154, $p=0.243$ APT154 vs. vehicle controls for C5a, Fig. 4C).

CH50 tests were performed to evaluate systemic complement activity of the classical pathway. CH50 values were calculated as % lysis, where lysis of red blood cells in water was set to 100%. Baseline CH50 values were not significantly different in the groups and values remained largely unaffected in both the APT154 and vehicle groups. Mirococept significantly inhibited the classical pathway of complement, as apparent CH50 values dropped and remained significantly lower after 2 h reperfusion ($14\pm 2\%$ at 5 min reperfusion, $25\pm 5\%$ at 2 h reperfusion; $p=4.3\times 10^{-7}$ for overall group-time interaction. Post-hoc analysis at 120 min reperfusion: $p=0.0018$ Mirococept vs. vehicle controls, $p=0.0012$ Mirococept vs. APT154, $p=0.332$ APT154 vs. vehicle controls, results not shown).

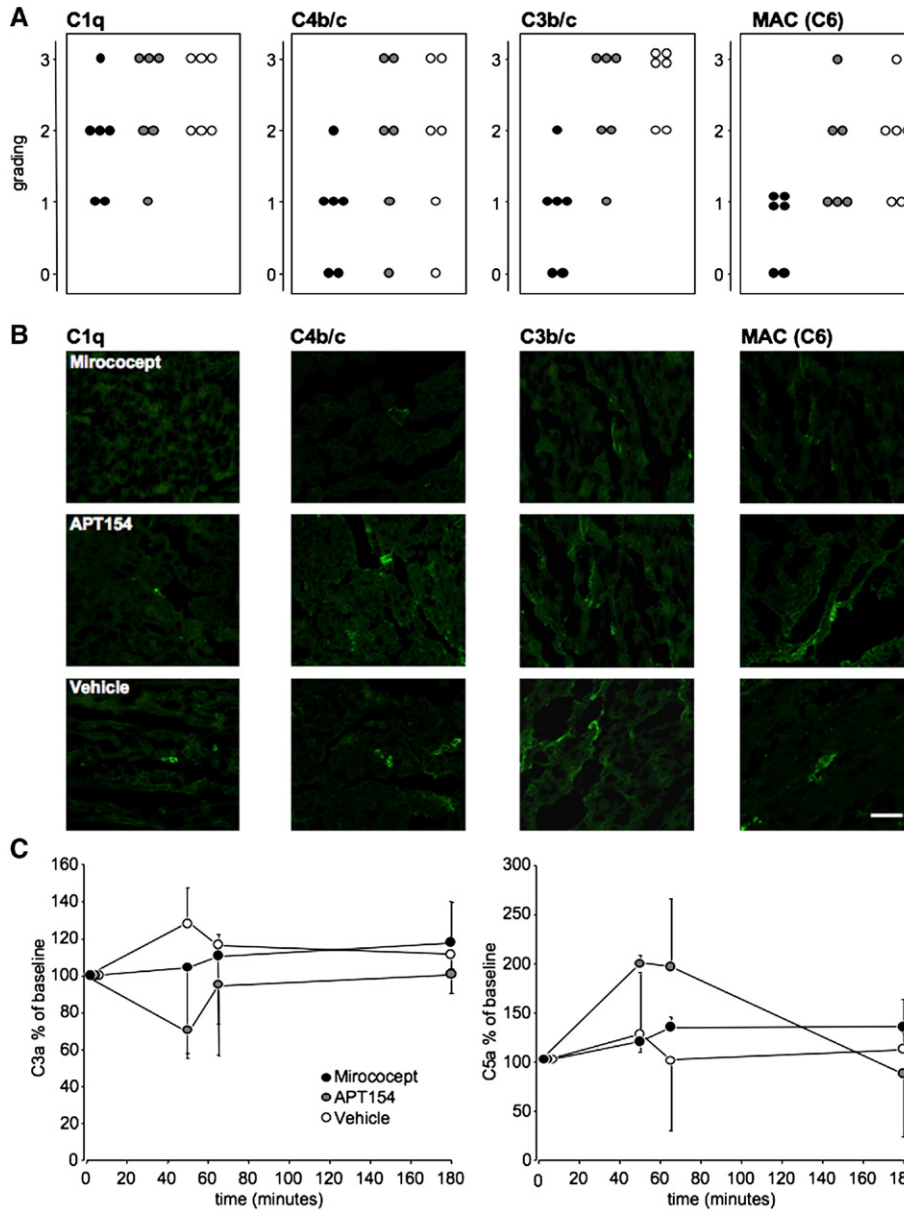


Fig. 4. Grading for complement tissue deposition of C1q, C3b/c, C4b/c and membrane attack complex (detected as C6), as well as ELISA for C3a and C5a in plasma. Four samples per area and experiment were graded with respect to intensity for complement staining. Each point represents the average of all evaluations for one experiment. 0=essentially no staining, 1=minimal focal or diffuse staining, 2=moderately strong focal or diffuse staining 3=extensive diffuse staining. Results for staining in the necrotic ischemic tissue (NIT) are shown for Mirococept- (black circles), APT164-treated animals (grey circles) as well as vehicle controls (white circles, A). Representative samples from the NIT of all three experiments for complement staining are visualized (B). Scale bar: 50 μ m. Plasma C3a and C5a levels are presented as a percentage of baseline (set to 100%). Systemic C3a and C5a generation was not influenced by treatment with Mirococept (C).

3.7. Tissue factor expression and aPTT

Compared to vehicle controls, Mirococept treatment reduced vascular TF expression in necrotic ischemic tissue. Upregulation of TF in the vehicle controls as well as APT154 treated animals within the injured vasculature was associated with the infarcted areas. Some weak TF staining was noted in vessels obtained from the area not at risk and vital ischemic tissue, with no differences between the groups. Minimal expression of TF was observed within native cardiac vasculature of healthy swine (Fig. 5).

Values for activated partial thromboplastin time (aPTT) did not differ significantly at baseline or any other time between the groups ($p=0.875$ for overall group-time interaction, $p=0.698$ for overall between group differences, not shown).

3.8. TUNEL staining

Fig. 6 illustrates TUNEL-positive myocytes at high-power magnification from representative experimental samples. Although a large fraction of myocytes were TUNEL-positive

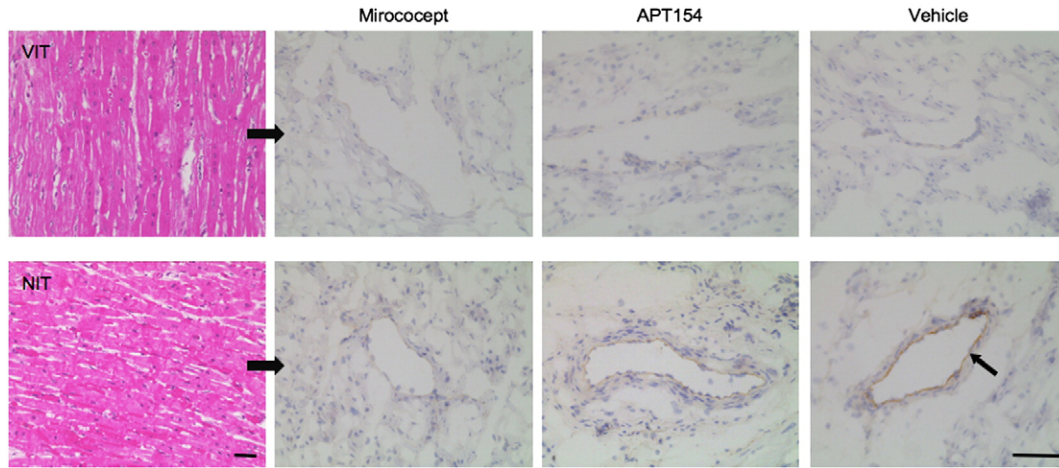


Fig. 5. Staining for pro-coagulant tissue factor in vital, ischemic tissue (VIT) and necrotic, ischemic tissue (NIT) sections. Positivity is revealed as brown staining (black arrow). Representative sections from each of the three groups are shown. Scale bar: 50 μ m.

within the ischemic necrotic area, the proportion of positive cells was significantly greater in samples from vehicle controls (69 ± 8) and from the APT154 (63 ± 7) group as compared to the Mirococept (37 ± 4) treated animals ($p = 1.0 \times 10^{-6}$ and $p = 1.6 \times 10^{-6}$, respectively). Within the non-ischemic and vital ischemic area, the numbers of TUNEL-positive myocytes were few and not significantly different between the three groups (not shown).

4. Discussion

Our data show that site-specific application of the membrane-targeted recombinant complement inhibitor Mirococept to ischemic myocardium significantly prevented I/R injury and in part ameliorated cardiac function in the early reperfusion period. Cardioprotection was associated with binding of Mirococept to ischemic tissue and was not

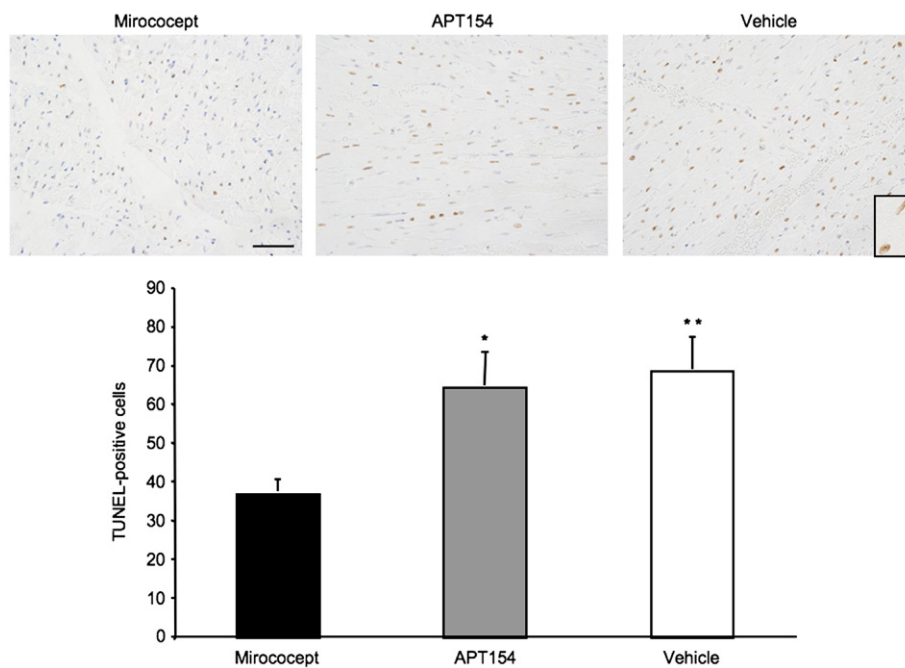


Fig. 6. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) of myocardial tissue (necrotic ischemic tissue, NIT). TUNEL-positive myocytes stain brown. Representative sections from each of the three groups are shown. Inlay with magnification of two TUNEL-positive myocytes. Scale bar: 100 μ m. Mirococept vs. APT154: $p = 1.6 \times 10^{-6}$ (*), Mirococept vs. vehicle: $p = 1.0 \times 10^{-6}$ (**).

observed with the use of the untagged, non-membrane binding form (APT154).

As organs injured following ischemia and reperfusion locally up-regulate complement [3], it may be advantageous to target protective agents to the actual site of injury. Indeed, previous work by Horstick et al. has documented the efficacy of local, intracoronary C1-inhibitor in reducing myocardial damage in a porcine infarction model [21]. Furthermore, although the therapeutic success of systemic complement inhibition, for example using soluble CR1, has been well documented [6,22–24], strategies chiefly, but not necessarily exclusively, targeting the organ locally may provide several advantages over systemic application. Undesired binding to non-target organs, particularly excretory organs such as the kidney, may largely be avoided and lower doses may be required to achieve the same effect, avoiding complications potentially associated with systemic complement inhibition. Indeed, in our study, Mirococept was effective at a dose of 0.5 mg/kg, as compared to studies using soluble CR1 in pigs, where up to 10 mg/kg were used [25]. However, as the results reveal, Mirococept's beneficial effect was not entirely complete for all investigated aspects. Possibly an intermediate dose (between 0.5 and 10 mg/kg) may be more effective for local application strategies. Although such dosage considerations may be of less importance where short-term complement inhibition is needed, they may well prove significant for long-term therapies. Clearly however, pronounced systemic inflammation in addition to local I/R injury, such as occurs during cardiopulmonary bypass surgery, may require systemic complement inhibition in addition to site-targeted cytoprotection. Also, it remains to be investigated whether concurrent low-dose systemic inhibition may provide additional benefits.

Strategies to target complement inhibitors to cell membranes have included linking these to sialyl Lewis-x moieties [26]. Other strategies include taking advantage of the fact that endothelial damage causes shedding of the glycocalyx [27] to reveal a pro-inflammatory and pro-coagulant surface. We previously demonstrated that the complement inhibitor [28–30] and glycosaminoglycan analog low molecular weight dextran sulfate selectively binds to endothelial cells denuded of their protective glycocalyx layer *in vitro* [30] and protects from I/R injury in acute myocardial infarction in pigs following intracoronary administration [17]. The current data with Mirococept confirm the possibility of achieving a similar degree of protection, albeit using a substance with different cell membrane-tethering properties.

Unlike the myristoyl-tailed Mirococept, non-tagged APT154 was not detected in myocardial tissue samples and did not provide cardioprotection; this, despite infusion of an equivalent molar dose and local, intracoronary application. We attribute the failure of APT154 to protect from I/R injury to the fact that the infused agent was not targeted to bind to the site of damage and, once systemically in circulation, did not suffice to offer protection. Results from the CH50 test, which was used to evaluate systemic complement inhibition, would

indeed suggest that the APT154 in circulation did not systemically affect complement activity. Results with Mirococept imply that the applied dose not only sufficed to locally bind to and inhibit tissue complement activation, but also affected complement activity systemically. This may, in part, represent an *in vitro* phenomenon [10], whereby small amounts of unbound, quasi-surplus Mirococept, added *in vitro* to antibody-sensitized sheep erythrocytes, were enough to bind to the comparatively few erythrocytes and substantially inhibit complement. However, these results may also reflect a certain amount of true systemic complement inhibition *in vivo*. Whether this is due to shedding of initially bound Mirococept into the blood stream or represents excess, unbound Mirococept, washed out of the coronary circulation without initial local binding to the vasculature, remains to be investigated. Nonetheless, the peak amounts detected *in vivo* suggest that most of the injected Mirococept was bound locally. Indeed, no staining for Mirococept was detectable in distant organs (including liver and kidneys).

The mechanism by which Mirococept exerts its cardioprotective effects may, in part, be deduced from the known mode of action of native CR1, as Mirococept retains enough activity of CR1 necessary for breakdown of complexed complement fragment C3b within C3 convertases [14] and hence for an inhibitory effect on generation of the anaphylatoxins C3a and C5a and the membrane attack complex. Indeed, all of the complement components studied, from C1q and C4b/c of the classical complement pathway to C3b/c, and C6 of the membrane attack complex, were reduced in myocardial tissue samples from Mirococept-treated animals. Reduction of anaphylatoxins has been described in vena cordis magna blood with the use of C1-inhibitor. Systemic C3a and C5a levels however did not vary greatly and were not influenced by Mirococept administration. This may be attributed to the minimal amounts of systemically-available unbound Mirococept and its largely restricted local action. C3b and membrane attack complex are associated with promotion of leukocyte activation via upregulation of adhesion molecules [31]. Compared to vehicle controls and APT154, Mirococept significantly reduced total neutrophil numbers in the viable ischemic tissue and decreased the extravasation of neutrophils into this area. sCR1 and sialyl Lewis-x glycosylated sCR1 have been shown to reduce neutrophil accumulation in a model of stroke [32]. However, maximal tissue neutrophil accumulation is not observed following only two hours of reperfusion and non-complement-dependent mechanisms of leukocyte recruitment may also be of importance in this setting. As a certain reduction in neutrophil extravasation was also observed in the APT154 group, it remains to be verified if Mirococept's protective effects are, at least in part, attributable to modification of neutrophil extravasation, particularly in later stages of more pronounced tissue extravasation.

This endothelial protective effect translated into reduced tissue factor (TF) expression in Mirococept treated animals,

the induction of which critically contributes to I/R damage. While Mirococept does not influence coagulation systemically (no effect on aPTT), attenuating intravascular thrombosis and inflammation by limiting endothelial TF expression may prove fundamental in reducing I/R damage [33].

Myocardial ischemia has also been shown to induce apoptosis in animal models and humans [34,35]. Treatment with Mirococept not only significantly reduced myocardial necrosis but also I/R-induced apoptosis. This reduction may be related to the observed reduced terminal complement complex deposition in the ischemic myocardium, which has been shown to play an important role not only in I/R-induced necrosis but also apoptosis [36].

4.1. Limitations

TTC staining, though under debate for determining infarct size following short reperfusion times, was used here, as its staining validity for 120 min reperfusion in pigs has previously been confirmed, where viable TTC positive islets within TTC negative necrotic tissue correlated with electron microscopy findings [37]. Isoflurane, known to have a preconditioning effect [38], may have contributed to a certain degree of cardioprotection. However, this effect would be equal in all groups and would not explain the benefits observed with the use of Mirococept.

Although Mirococept provides protection in the critical initial phase of reperfusion, further studies are required to validate whether a one-time application of Mirococept offers extended protection from complement-mediated damage. In particular, functional analysis, including regional wall motility, would also need to be evaluated at a later stage, as myocardial stunning, a likely significant finding in all groups in the early post-reperfusion phase, may hamper initial investigations and skew early results.

5. Conclusions

In summary, we have demonstrated the feasibility of modifying the local pro-inflammatory and pro-coagulant environment encountered following regional myocardial ischemia and reperfusion by targeting a complement regulatory protein to the site of injury. This protection was associated with a single dose application of a molecule previously proven safe for use in humans. The concept of targeted cytoprotection of ischemic tissue pre-reperfusion using substances such as Mirococept, may offer an attractive therapeutic option in treating reperfusion injury and warrants further investigation.

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