Influencing the macro- and microcirculatory complications of nonocclusive mesenteric ischemia by complement C5a inhibitor treatments

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1. INTRODUCTION

1.1. Acute mesenteric ischemia

Acute mesenteric ischemia (AMI) is a serious, life-threatening condition with an approximately 50-70% mortality rate (Acosta et al, 2010). Transient decreases of the mesenteric blood flow may cause only minimal mucosal lesions, but if prolonged, splanchnic ischemia leads inevitably to transmural bowel necrosis and perforation (van den Heijkant et al, 2013). Besides, it is also recognized that inadequate intestinal blood supply can lead to secondary inflammatory response, which reduces the integrity of the gastrointestinal wall in the long run (Sun et al, 2015).

AMI is most often classified as occlusive or nonocclusive. The main causes of occlusive AMI are embolus or thrombus of the superior mesenteric artery (SMA) and mesenteric venous thrombosis. Nonocclusive mesenteric ischemia (NOMI), however, evolves in the absence of apparent anatomical obstruction of the mesenteric circulation in a variety of low flow states. Although NOMI may account for 20-30% of AMI cases and different degrees of NOMI occur in many patients with unstable conditions of systemic circulation, including hemorrhagic shock (Toung et al, 2000), burn injury (Muschitz et al, 2015), pancreatitis (Tsuji et al, 2011) and the postoperative phase of cardiac and aortic surgery (Goleanu et al, 2014), it often remains undiagnosed (Trompeter et al, 2002).

The mortality rate of NOMI is constantly high (more than 50%), which highlights the need for prompt diagnosis and adequate treatment (Björck et al, 2010). Treatment is more likely to be effective, if started early after the onset of signs and symptoms, because the mortality increases exponentially after 6-8 hours (Klar et al, 2012). While the success rate of surgical treatments of occlusive AMI improved during the last decades, this has remained poor for NOMI (Schoots et al, 2004).

1.2. The pathomechanism of NOMI

Almost any condition that can induce circulatory shock may precipitate NOMI. In circulatory shock the blood flow of the vital organs is damaged, which can be initially compensated by the centralization of circulation. During these processes, the blood supply of the heart, the brain and the adrenal glands are supported, but the hypoxic organs (visceral organs, kidneys) will suffer an irreversible damage. The shock can be divided into four main groups: cardiogenic, distributive, hypovolemic and obstructive. In cardiogenic shock, the intravascular volume is appropriate, but there is a decreased cardiac output (CO) caused by the failure of the heart to pump effectively. In distributive shock, the peripheral vascular
resistance is reduced to a large extent, while hypovolemic shock is induced by the reduction of circulating blood volume. Obstructive shock is due to obstruction of the heart or major blood vessels.

Information on the pathogenesis of NOMI is rather scarce, but it is assumed that the potentially life-threatening structural damage of the mucosa is associated with excessive and sustained splanchnic vasoconstriction (Trompeter et al, 2002). The major causes are tension pneumothorax, constrictive pericarditis or pericardial tamponade (Seferović et al, 2006). The increased afterload may subsequently worsen the hypoperfusion of peripheral organs and results in an increased oxygen demand of the myocardium. The deficit in the delivery of intestinal blood provokes mesenteric vasoconstriction (Williams et al, 1980). Splanchnic hypoxia causes the activation of several mechanisms of endogenous vasoconstrictors including the endothelin-1 (ET-1) system (Weitzberg et al, 1991). High-mobility group box protein-1 (HMGB-1), released passively by necrotic and damaged cells, has been recently identified as an important signal for leukocyte recruitment (Scaffidi et al, 2002). Moreover, the activation of the complement system was described as well (Soop et al, 2004).

Importantly, the reoxygenation can generate a more severe tissue injury than ischemia alone (Yasue et al, 1988). During the reperfusion phase, the mesenteric blood flow decreases, the mesenteric vascular resistance increases, and the accumulation and free radical production capacity of the leukocytes rises (Wolfárd et al, 1999). The elevated level of reactive oxygen species (ROS) plays a central role in this phase of ischemia/reperfusion (IR) process (Boros et al, 1991). Other endogenous compounds such as platelet-activating factor (Carter et al, 1996), ET-1 (Wolfárd et al, 1999), histamine (Kaszaki et al, 1994), TNF-α (Yao et al, 1994) and interleukins (Tamion et al, 1997) play also an important role in the final tissue damage. More importantly, the IR can activate the immune system, including the complement system via antigen-independent inflammatory pathways (Riedemann et al, 2003).

As a consequence of hypoxia and reoxygenation, significant amount of anaphylatoxin C5a may be produced (Granger et al, 2003). The C5a fragment, a biologically active side-product of the complement cascade, can induce smooth muscle contraction, chemotaxis, and the activation of neutrophils (with the release of ROS and cytokines (Sacks et al, 1978)), which can further exacerbate the process of inflammation (Ehrengruber et al, 1994).

A relatively new solution is the administration of acetyl-peptide-A (AcPepA). The acetylated N-terminal alanine (synthesized and purified (>95% purity) by Biologica, Nagoya, Japan) containing PepA (ASGAPAPGPAGPLRPMD) is an antisense homology box-derived peptide, which is capable of binding directly to C5a in its 37–53 amino acid region. It is
important to note that the binding between the peptide and the C5a is not an antigen-antibody interaction. AcPepA has proved to be highly effective in pilot studies about endotoxin shock (Fujita et al, 2004; Okada et al, 2011).

1.3. Animal models of NOMI

The consequences of experimental NOMI were investigated in several large animal models. In these cases NOMI is most frequently induced by pericardial tamponade (PT). In these studies the short-term consequences of NOMI can be followed, nevertheless, the long-term macro- and microcirculatory consequences of NOMI-induced changes are still unknown.

In large animal PT models the tamponade is induced by filling of the pericardial sack with various fluids such as blood, saline or dextran. Previously, experimental PT models have been developed by our research group as well. In these studies, PT is reproducibly leads to a decline in CO and a significant increase in heart rate (HR). After relief from the tamponade, a significantly lower mean arterial pressure (MAP) and long-lasting impairment of the venous return are observed, while the CO and HR return to normal levels. Briefly, the deterioration of macrohemodynamics after PT is fairly well described, but the splanchnic microcirculatory and inflammatory changes and the long time consequences of NOMI are still unexplored (Érces et al, 2013).

2. MAIN GOALS

- We hypothesized that experimental PT offers an opportunity to study the pathophysiology of NOMI. Our primary aim was to characterize the acute effects of PT on the intestinal microcirculatory alterations and inflammatory response in a large animal model.

- We hypothesized that the inhibition of production of complement component C5a would provide a plausible way to influence the potentially detrimental consequences of NOMI. The aim was to investigate the acute effects of complement C5a inhibitor treatment on microcirculatory and inflammatory complications caused by NOMI.

- Our next aim was to develop a reliable rodent model of NOMI of extramesenteric origin and thus to investigate the major components of local and systemic circulatory reactions. We hypothesized that partial aorta occlusion (PAO) in rodents leads to an established low-flow state in the splanchnic area, and this would provide a way to examine the pathophysiology of NOMI. Using this new model we decided to
characterize the macro- and microcirculatory changes and inflammatory response 24 hours after the initial insult.

- Our final goal was to investigate the effects of AcPepA treatment on macro- and microcirculatory changes and inflammatory response induced by NOMI so as to provide relevant information on the consequences of C5a inhibitor therapy in a clinically relevant time frame.

3. MATERIALS AND METHODS

3.1. Experimental protocol

The experiments were performed according to the National Institutes of Health guidelines and EU directive 2010/63 for the protection of animals used for scientific purposes and the study was approved by the Ethical Committee for the Protection of Animals in Scientific Research at the University of Szeged (number of approval: V/148/2013).

In Study I, the surgical interventions were carried out in minipigs during continuous infusion of propofol. After endotracheal intubation, the animals were mechanically ventilated. The right femoral artery and jugular vein were cannulated for the measurement of MAP and CO by thermodilution method and for fluid or drug administration, respectively. After a midline abdominal incision, an ultrasonic flow probe was placed around the exposed SMA to measure the mesenteric blood flow. The animals were monitored continuously, arterial blood gases were checked regularly.

The animals were randomly allocated to one of the three experimental groups. Group 1 (n = 6) served as sham-operated control, with the same time frame and sampling as in groups 2 (n=7), and 3 (n=6), but without the induction of a cardiac tamponade. Left lateral thoracotomy was performed in all groups, and in the cardiac tamponade groups, a cannula was fixed in the pericardial cavity. PT was induced for 60 minutes by intrapericardial administration of colloid solution, while the MAP was maintained between 40 and 45 mmHg. After the end of the PT the animals were monitored for 180 minutes. Group 3 was treated by AcPepA after the 45th minute of cardiac tamponade. Peripheral blood samples were taken at baseline, after 75 and 150 minutes, and at the end of the observation period (240 minutes) to detect the levels of HMGB-1, big-endothelin (big-ET), and superoxide production in whole blood. Biopsies were taken from the tissue of small intestine at the end of the experiments for measurement of myeloperoxidase (MPO) activity.

In Study II, after intraperitoneal (ip) sodium pentobarbital anesthesia, the rats were placed in a supine position on heating pads. Tracheostomy was performed to facilitate
spontaneous breathing, and the right jugular vein was cannulated for drug administration and Ringer-lactate infusion. The left carotid artery and the left femoral artery were cannulated for MAP and HR measurements. A catheter equipped with thermistor-tip was positioned into the ascending aorta through the right carotid artery for CO measurements, using thermodilution technique. After a midline abdominal incision, a silicone catheter with tourniquet was positioned around the origin of the SMA. An ultrasonic flow-probe was placed around the SMA to measure the flow in SMA. Parameters of macrohemodynamics (MAP, CO, flow in SMA) were recorded after a 30-minute recovery period. Hemodynamic measurements were taken at baseline, in 30 minute after the induction of PAO, before the relief of PAO, at 90 and 120 minutes.

The animals were randomly divided into two groups. Group 1 (n=7) underwent a 1 hour partial occlusion of the abdominal aorta induced by controlled tightening of the tourniquet. The goal was to keep MAP in the femoral artery continuously between 30 and 40 mmHg. MAP, CO and SMA flow were recorded at baseline, in 30 minute after the induction of PAO, before the relief of PAO, at 90 and 120 minutes. Differences in MAP between the femoral (MAPF) and the carotid arteries (MAPC), i.e. above and under the site of PAO, were registered. Group 2 (n=6) served as sham-operated control.

In Study III. The rats were placed in a supine position on heating pads and the left femoral artery was cannulated for recording of MAP and HR. A silicone catheter with tourniquet was positioned around the vessel with same protocol as in Study II. The baseline variables were determined during a 30-minute control period.

Group 1 (n=8) served as the sham-operated control group, while in group 2 (n=8) and group 3 (n=9), PAO was induced for 1 hour by controlled tightening of the tourniquet. The goal was to maintain MAP in the femoral artery continuously between 30 and 40 mmHg. In group 3 the injection of AcPepA into the tail vein started 15 minute prior to the end of PAO. The animals were observed for 90 minutes, the beginning of PAO denotes 0 min. MAP and HR were recorded 4 times. In each group, intravital videomicroscopy was utilized in the baseline condition to examine the microcirculation of the serosa of the ileum 5 cm proximally from the cecum. After the observation period, the femoral catheter was carefully removed, the wounds were closed and the animals recovered.

24 hours after surgery the animals were reanesthetized, and the right jugular vein and the left common carotid artery were cannulated for drug administration, as well as MAP and HR measurements. A catheter with a thermistor tip was positioned into the ascending aorta through the right common carotid artery for CO measurements. The abdominal wound was
reopened and an ultrasonic flow-probe was placed around the exposed SMA to measure the flow of the mesenteric artery. Parameters of macrohemodynamics were recorded after a 30-minute recovery period and intravitral videomicroscopy was performed to examine the microcirculation of the ileal mucosa. Through another incision, fluorescence confocal laser scanning endomicroscopy (CLSEM) was used for in vivo histological investigation. At the end of the experiments, blood samples were taken for determination of TNF-α, HMGB-1 and ET-1 levels in plasma. Tissue samples were taken from the ileum for conventional histological examinations and detection of leukocyte accumulation.

3.2. Intravital videomicroscopy of the microcirculation

Intravital orthogonal polarization spectral imaging was used for non-invasive visualization of the mucosal microcirculation of the small intestine. A ×10 objective was placed on the surface of the serosa of the ascending colon, and microscopic images were filed. Quantitative assessment of the microcirculatory parameters was accomplished off-line by frame-to-frame analysis of the videotaped images. Changes in red blood cell velocity (RBCV, μm/s) in the postcapillary venules were determined in three separate fields by means of computer-assisted image analysis.

3.3. Measurements of HMGB-1, big-ET-1, histamine, TNF-α and ET-1 in plasma

The plasma concentration of HMGB-1 and big-ET (protein precursor of ET-1 consisting of 38 amino acids) and ET-1 were measured with a commercially available kit. Plasma histamine concentrations were determined with a commercially available enzyme-linked immunosorbent assay (ELISA). Plasma TNF-α concentration was evaluated in duplicate by means of a commercially available enzyme-linked immunosorbent assay.

3.4. MPO activity, superoxide production in whole blood

The activity of MPO, a marker of neutrophil activation, was determined in ileal biopsy samples according to the method of Kuebler et al. For the measurements of superoxide production in whole blood, the chemiluminometric method described by Zimmermann et al. was used.

3.5. Histological investigation of leukocyte infiltration in tissues

Full-thickness ileal biopsies obtained at the end of the experiments were analysed in each group. The tissue was fixed in 6% buffered formalin, embedded in paraffin, cut into 4-
µm-thick sections and stained with hematoxylin and eosin. The infiltration of leukocytes was detected and the number of leukocytes was counted in at least 20 fields of view at an original magnification of 400x.

3.6. In vivo detection of structural and microvascular damage of the mucosa

In the rodent PAO model, the extent of microvascular damage of the terminal ileum was evaluated by fluorescence confocal laser scanning endomicroscopy (CLSEM) developed for in vivo histology. Records were taken on day 2 for the observation of the effects of C5a inhibitor treatment. The mucosal surface of the terminal ileum was surgically exposed and laid flat for examination. The microvascular structure was recorded after the iv administration of fluorescein isothiocyanate-dextran dye. The injury of mucosal architecture was examined following topical application of the fluorescent dye acriflavin. Non-overlapping fields of active areas of PAO were compared to the samples of AcPepA-treated or control groups by using a semiquantitative scoring system.

3.7. Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows; Jandel Scientific, Erkrath, Germany). Friedman test with repeated measures analysis of variance on ranks was applied within groups. Time-dependent differences from the baseline were assessed by Dunn’s method for each group, and differences between groups were analyzed with Kruskal-Wallis one-way analysis of variance on ranks, followed by Dunn’s method for pairwise multiple comparison. Differences between groups in the Study II were analysed by Mann-Whitney test, followed by Dunn's method for pairwise multiple comparison. In the figures, median values and 25th and 75th percentiles are given; p values of less than 0.05 were considered to be significant.

4. RESULTS

4.1. Consequences of the experimental PT

A significant decrease in SMA flow indicated redistribution of the mesenteric circulation during the tamponade. After the removal of the pericardial fluid, the SMA flow returned to the control values.

A heterogeneous, oscillating microcirculation was present in the small intestinal mucosa in all groups. The RBCV during the fast flow periods showed no change in the sham-operated group, but at the end of the cardiac tamponade, a significant decrease from the
baseline was observed in the group where cardiac tamponade was not used. The duration of the slow flow period in the cardiac tamponade group increased significantly at 240 min.

The big-ET levels in plasma increased significantly four- to five-fold in the non-treated group after cardiac tamponade. The plasma level of HMGB-1 was elevated significantly after the compression of the heart. The level of MPO activity was significantly higher in the tissue samples of the small intestine of the PT group, indicating the increased accumulation of neutrophils. In the PT group, increased superoxide production was perceptible in the blood at the beginning of the post-tamponade phase.

4.2. AcPepA treatment in experimental PT

Administration of AcPepA 45 minute after cardiac tamponade resulted in a significant elevation of SMA flow in the post-tamponade period. Simultaneously, by the end of the post-tamponade phase, the microcirculation was significantly increased in the AcPepA-treated group. The duration of the slow flow period in the cardiac tamponade group increased significantly at 240 minute, but it was reduced in the treated group.

After AcPepA administration, the characteristic biochemical changes following the cardiac tamponade were significantly different. The AcPepA treatment reduced the concentrations of big-ET and HMGB-1 in the plasma. The amount of oxygen free radicals that were formed was also reduced by the treatment, and the MPO activity decreased as well.

4.3. Consequences of the experimental PAO

During PAO MAP was maintained between 30-40 mmHg. The CO started to decrease after 30 minute of PAO and reached a significant difference compared to the sham-operated group and to the baseline values by the end of PAO. MAP increased significantly in 30 minute after the relief from PAO and then returned gradually to the control values by the end of the observation period. The CO started to increase after the PAO and no significant differences could be detected during the post-occlusion period. The SMA flow decreased significantly during the PAO and remained significantly lower compared to the baseline values as well as values of sham-operation until the end of the experiments.

24 hours after the PAO, MAP did not differ between the groups or the baseline values of day 1. The surgical interventions caused an increase in HR by day 2.

In the PAO group, the evaluation by CLSEM technique demonstrated significant damage of tissue and vascular structure in contrast with the sham-operated group.
The concentrations of inflammatory mediators TNF-α and HMGB1 increased significantly 24 hours following the ischemic insult. The level of ET-1 was also elevated on the day after the PAO. On the second day of the experiments, significant leukocyte accumulation was observed in the PAO group as compared to the sham-operated animals.

4.4. AcPepA treatment in experimental PAO

The HR was significantly higher in the PAO group compared to the group of sham-operated animals, while after AcPepA treatment, the HR did not increase significantly in respect of the values of the sham-operated group. The SMA flow in the treated group remained at the control level, while in the non-treated group it was significantly higher in comparison with the sham-operated group. The elevated CO decreased significantly following C5a inhibitor treatment on day 2. On day 2, both the serosal and mucosal RBCV were significantly low in comparison with the sham-operated group. AcPepA treatment resulted in a significantly higher RBCV.

Administration of the C5a inhibitor AcPepA decreased the mucosal damage and significantly influenced both the changes in the microvascular structure and in the epithelial morphology of the small intestine.

The result of our examination demonstrated that the C5a inhibitor treatment decreased the level of these inflammatory mediators and ET-1 significantly. The administration of AcPepA significantly reduced also the leukocytes infiltrating the wall of the small intestine.

5. DISCUSSION

We have characterized the acute hemodynamic consequences of NOMI in a large animal model. In this porcine study, the post-tamponade period was characterized by decreased MAP and significantly elevated HR. These acute hemodynamic alterations were accompanied by definite signs of inflammatory activation, with the release of vasoactive and proinflammatory mediators. The deterioration of the systemic circulation was followed by simultaneous impairment of the microcirculation in the splanchnic area. These changes were accompanied by an increase in local MPO activity, a quantitative marker of ROS-producing neutrophils in the plasma and various tissues (Malle et al, 2007; Vollmar et al, 2011).

After the relief from the cardiac tamponade, the MAP decreased, whereas the CO remained compensated, and there were no significant differences if compared to the control group. This was achieved by the elevated HR, which refers to the increased strain of heart.
muscle. Following AcPepA treatment, the MAP was elevated compared to the control level and the CO was also maintained. Thus, the most pronounced differences between the compensating mechanisms of treated and non-treated tamponade groups were the restored CO and the lower HR. This seems to be especially crucial if we consider the fact that the circulation in the heart muscle is supplied during the diastolic phase, and it nearly stops during the systole. If the HR is increased, the length of the systole does not change, while the diastole is shortened. A higher frequency results in less time for oxygen delivery to the cardiac muscle cells, and therefore a lower HR and maintained CO provide better oxygenation for the heart. These changes might be the consequences of reduced ET release after AcPepA treatment. Indeed, it has been shown that non-selective ET-receptor antagonism increases the CO and decreases the peripheral resistance in patients with congestive heart failure (Lüscher et al.). The share of the splanchnic area from the reduced CO was diminished during the tamponade, but the SMA flow, which reflects the blood supply of the small intestine and colon, was restored thereafter, and no differences were observed compared to the sham-operated group. Nevertheless, in the AcPepA-treated group, the SMA flow was significantly elevated at the beginning of the post-tamponade phase, and gradually returned to the control level by the end of the experiments. This early flow elevation may be attributable to the relative lack of vasoconstrictor ET-1 effects, because it has been demonstrated that ET-A receptor inhibition is able to improve the splanchnic circulation in similar cases (Wolfárd et al, 1999).

We have designed an animal model, in which the main clinical features of NOMI are reproducible. Key elements are persistently decreased SMA flow after an extramesenteric insult and the reduced invasiveness, which makes the long-term observations possible. In this context we have obtained conclusive evidences for the significantly diminished SMA blood flow after partial occlusion of the subdiaphragmatic aorta, which persisted during the 24 hour post-occlusion period, despite the compensated CO and MAP. It should be noted that the late hemodynamic changes were characteristic to a systemic inflammatory response syndrome. Although MAP was in the normal range, the HR and CO was significantly increased, as part of the compensatory mechanism. During the early phase of systemic inflammation, the oxygen demand is increased due to the high metabolic rate. In order to meet this need, oxygen delivery is increased mostly by means of the elevation of CO. Nevertheless, the oxygenation of the cells cannot be improved without a properly functioning microcirculation, and a deterioration of capillary perfusion will prevent the oxygen consumption despite the seemingly increased delivery. In both models microcirculation of the small intestine was
significantly impaired as shown by the decreased RBCV at mucosal surfaces. This means that microcirculatory damage was present in the gastrointestinal wall notwithstanding the normal or increased SMA flow (Tao et al, 1995).

In the groups treated by AcPepA, the RBCV in the small intestine was significantly higher and the improved microcirculation was present without an increase in CO and SMA flow which may refer to the better efficacy of oxygen extraction.

The decreased ET-1 level after AcPepA treatment may have contributed to the improvement of the microcirculation in the small intestine, and the altered profile of proinflammatory cytokines may also imply a reduced inflammatory response as a consequence of C5a inhibitor therapy. More importantly, the excessive release of ET-1 enhances leukocyte activation in the postcapillary venules of the small intestine which may further increase the local tissue damage.

Both in the porcine model (following the PT) and in the rat model (2nd day) increased circulatory HMGB-1 levels were measured, which were reduced by AcPepA treatment. These findings correlate to the similar results of continuous AcPepA treatment in a endotoxin shock model in primates (Okada et al, 2011). These results can be explained by the involvement of the C5L2 receptor, a high-affinity C5a receptor, which was earlier thought to be a non-signaling, scavenger receptor of C5a as it is incapable of G-protein coupling. Recent findings, however, have proved that C5L2 has a more important role in mediating inflammatory responses of the innate immune system (Wang et al, 1999). The survival rate in C5L2 knockout mice increased after cecal ligation and puncture–induced sepsis in relation to the survival rate in wild-type mice (Rittirsch et al, 2008).

In conclusion, these results present the emergence of NOMI among the acute circulatory complications of cardiac tamponade, and the potential role of C5a antagonism in reducing signals that are important components in the development of a secondary splanchnic microcirculatory disturbance. We have also characterized some of the potentially detrimental circulatory and proinflammatory consequences of nonocclusive mesenteric hypoperfusion in a novel rodent model. In this experimental setup, a single iv dose of complement C5a antagonist AcPepA compound was suitable for improving the local circulatory changes and it reduced the secondary mucosal damage in a relatively wide time frame, at least 24 hours after the insult.
6. SUMMARY OF NEW FINDINGS

- Experimental pericardial tamponade causes systemic inflammation and diminished microcirculation in the splanchnic area, and this provides opportunity to study the pathophysiology of NOMI in a large animal model.
- Complement C5a antagonist treatment with AcPepA improves the splanchnic microcirculation and reduces the acute inflammatory consequences of pericardial tamponade, thus complement activation can play a role in the short-term microcirculatory disturbances of NOMI.
- We have developed and characterized a new rodent model of partial aorta occlusion, and adapted it to the investigation of the long-term hemodynamic and inflammatory consequences of experimental NOMI in a clinically relevant time frame.
- Experimental NOMI diminishes the microcirculation within the intestinal wall and causes inflammatory activation and secondary mucosal damage. The complement C5a antagonist AcPepA treatment is competent to improve the microcirculation and reduces the inflammatory activation, which suggests that complement activation plays a central role also in the late, potentially damaging consequences of NOMI.
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