J Gastrointest Surg (2007) 11:439–444
DOI 10.1007/s11605-007-0128-9

Platelet Function in Acute Experimental Pancreatitis

Thilo Hackert · Dagmar Pfeil · Werner Hartwig · Stefan Fritz · Lutz Schneider · Martha-Maria Gebhard · Markus W. Büchler · Jens Werner

Published online: 6 March 2007

Abstract Acute pancreatitis (AP) is characterized by disturbances of pancreatic microcirculation. It remains unclear whether platelets contribute to these perfusion disturbances. The aim of our study was to investigate platelet activation and function in experimental AP. Acute pancreatitis was induced in rats: (1) control (n=18; Ringer's solution), (2) mild AP (n=18; cerulein), and (3) severe AP (n=18; glycodeoxycholic acid (GDOC) + cerulein). After 12 h, intravital microscopy was performed. Rhodamine-stained platelets were used to investigate velocity and endothelial adhesion in capillaries and venules. In addition, erythrocyte velocity and leukocyte adhesion were evaluated. Serum amylase, thromboxane A2, and histology were evaluated after 24 h in additional animals of each group. Results showed that 24 h after cerulein application, histology exhibited a mild AP, whereas GDOC induced severe necrotizing AP. Intravital microscopy showed significantly more platelet–endothelium interaction, reduced erythrocyte velocity, and increased leukocyte adherence in animals with AP compared to control animals. Thromboxane levels were significantly elevated in all AP animals and correlated with the extent of platelet activation and severity of AP. In conclusion, platelet activation plays an important role in acute, especially necrotizing, pancreatitis. Mainly temporary platelet–endothelium interaction is observed during mild AP, whereas severe AP is characterized by firm adhesion with consecutive coagulatory activation and perfusion failure.

Keywords Acute pancreatitis · Platelets · Leukocytes · Endothelium interaction · Microcirculation · Coagulation

Introduction

Acute pancreatitis (AP) is characterized by an inflammatory affection of the exocrine pancreatic tissue and disturbances of pancreatic microcirculation.[1] Depending on the severity of AP, irreversible perfusion failure with consecutive tissue hypoxia and necrosis can complicate the course of the disease and trigger systemic inflammatory and septic complications.[2] The pathophysiology of AP has been investigated with regard to microcirculatory changes in several studies.[1–5] Attention was paid especially to erythrocyte flow patterns, leukocyte–endothelium interaction, and rheological approaches to improve perfusion and inhibit irreversible tissue damage.[1,3–5] Leukocyte–endothelium interaction as an early event of the inflammatory response has been characterized as a key step in the pathophysiology of AP.[6] Besides, activation of the humoral coagulation cascade plays an important role in the development of microcirculatory disorders in AP.[7,8] However, the role of platelets as the cellular elements of hemostasis that can functionally link inflammatory cells and humoral coagulation factors has not been investigated.

The aim of this study was to investigate platelet activation and function in experimental AP.

Parts of the results of this study were presented at the congress of the German Surgical Society, Berlin (May 2004), the Digestive Disease Week (May 2004), and the Annual Meeting of the American Pancreatic Association, Chicago (November 2004).

T. Hackert (✉) · D. Pfeil · W. Hartwig · S. Fritz · L. Schneider · M. W. Büchler · J. Werner
Department of Surgery, University of Heidelberg,
INF 110, 69120 Heidelberg, Germany
e-mail: Thilo_Hackert@med.uni-heidelberg.de

M.-M. Gebhard
Department of Experimental Surgery, University of Heidelberg,
Im Neuenheimer Feld 365,
69120 Heidelberg, Germany

Materials and Methods

Animals The experiments were performed in 54 male Wistar rats weighing 270 to 335 g. Animals were fasted overnight with free access to water before the experiments. Care was provided in accordance with the guidelines published in the "Guide for Care and Use of Laboratory Animals" (National Institutes of Health, publication no. 85-23, 1985). Surgical anesthesia was induced with intraperitoneal injection of pentobarbital (25 mg/kg) and intramuscular injection of ketamine (40 mg/kg) for the procedures of catheter placement and induction of pancreatitis. Anesthesia during intravital microscopy was induced by intravenous injection of pentobarbital (10 mg/kg). Polyethylene catheters (inner diameter 0.5 mm) were placed in the right jugular vein and left carotid artery, tunneled subcutaneously to the suprascapular area, and brought out through a steel tether that allowed the animals' free movement and access to water during the experiments.

Monitoring blood samples Mean arterial pressure and heart rate were monitored during intravital microscopy by an electromechanical pressure transducer (Baxter Uniflow, Baxter Healthcare Cooperation, Deerfield, IL, USA). Arterial blood samples for determination of serum amylase were obtained before (baseline) and 24 h after (end point) pancreatitis was induced. Serum amylase was determined by standard laboratory methods (Hitachi automatic analyzer, Boehringer Mannheim, Germany).

Animal models Animals were divided into three groups. In each group, pancreatic microcirculation was evaluated in 12 animals by intravital microscopy, and morphological changes were assessed in six animals by histology. In the control group, animals underwent sham operation and received Ringer's solution only. Acute pancreatitis of graded severity was induced in the other groups either as mild or severe AP. Mild AP was induced by intraarterial infusion of cerulein (5 $\mu g\ kg^{-1}\ h^{-1}$) for over 6 h. Cerulein was reconstituted in saline solution, and infusion volume was 4 ml/kg/h. Severe necrotizing pancreatitis was induced by infusion of bile salt (glycodeoxycholic acid [GDOC] 2.5 mM/l) into the pancreatic duct in combination with intraarterial infusion of cerulein (5 $\mu g\ kg^{-1}\ h^{-1}$) for over 6 h as described by Schmidt et al.[9] in detail. Bile-salt infusion into the pancreatic duct was performed in a volume- (1.2 ml/kg), time- (5 min), and pressure- (30 mmHg) controlled manner.

In each of the models, animals received saline solution during the observation period (0.9%, 4 ml $kg^{-1}\ h^{-1}$). Intravital microscopy was performed 12 h after the induction of pancreatic injury, and histological changes and blood samples were assessed 24 h after the infusions were started.

Platelet preparation One milliliter of whole blood was withdrawn before intravital microscopy. Platelets were separated and stained according to the method originally described by Massberg et al.[10] Briefly, platelets were stained by rhodamine 6G and separated by 2 cycles of centrifugation under the addition of prostacyclin. After suspending and washing the separated platelets, blood cell count was performed to calculate the number of platelets per microliter and to rule out animal-specific differences in the number of platelets. Platelets were then reinjected, and intravital microscopy was performed.

Intravital microscopy The abdomen was reopened, and the pancreas was carefully exteriorized in a horizontal position through the midline incision after the animal was placed on the right side. The duodenal loop with the head of the pancreas was carefully fixed on an anatomically designed stage in a temperature-controlled (37°C) Ringer's bath. Afterward, intravital microscopy was performed as described below. The animals were killed after the completion of intravital microscopy by a pentobarbital overdose.

Erythrocyte and leukocyte assessment A 0.5 ml/kg of erythrocytes (hematocrit 50%) labeled with fluorescein isothiocyanate (FITC) as described before[11] was applied intravenously. In addition, 1 ml/kg of rhodamine-6G solution was applied intravenously to label leukocytes in vivo.[12] Intravital microscopy was performed after an equilibration period of 15 min using a fluorescent microscope (Leitz, Wetzlar, Germany) with a 20-fold water immersion objective. An epi-illuminescent xenon lamp with an excitation filter of 450–490 nm was used for visualization of FITC-labeled erythrocytes and an excitation filter of 540–630 nm for rhodamine-labeled leukocytes.

Platelet assessment After platelet reinjection, intravital microscopy was performed by an epi-illuminescent xenon lamp with an excitation filter of 540–630 nm.

Off-line analysis Images were transferred to a monitor and simultaneously recorded on a videotape recorder. In each animal, five capillary fields of the exocrine pancreas and five postcapillary venules (20–40 μm) were recorded for 1 min. Off-line analysis was performed using a specially designed computer program (Capimage, Dr. Zeintl, Heidelberg, Germany). Erythrocyte velocity and platelet velocity were determined for 10 cells in each capillary field and venule. Additionally, temporarily (rolling) and permanently (sticking) adherent leukocytes and platelets were determined in pancreatic venules and capillary fields. Rolling cells were defined as cells with less than 66% of red blood cell velocity, whereas sticking cells are those that were adherent to the vessel wall for the whole observation period.[13]

Table 1 Serum Parameters, Wet–Dry Ratio, and Histopathology

	Control	Mild AP	Severe AP
Serum parameters			
Amylase (U/l)	586±116	27,200±4,012*	27,317±3,220*
Thromboxane A2 [pg/50 µl]	15.3±10.3	47.8±12.1*	61.9±15.8*
Wet–dry ratio	2.87±0.79	6.96±0.95*	4.77±0.70
Histopathology			
Inflammation	0.25±0.42	1.31±0.08*	1.95±0.17*†
Necrosis	0.08±0.20	1.10±0.11*	1.70±0.23*†

*$p<0.05$ vs control group
†$p<0.05$ vs mild acute pancreatitis

Edema A portion of pancreatic tissue was trimmed of fat and weighed. Pancreatic water content was determined by the ratio of the initial weight (wet weight) of the pancreas to its weight after incubation at 60°C for 72 h (dry weight).

Histology The pancreas was immediately removed after killing and was fixed in 4% buffered formalin solution. It was then embedded in paraffin, cut, and stained with hematoxylin eosin. Histopathological evaluation was performed in a blinded fashion. For quantification of edema, inflammation, and necrosis, a modification of the scoring system originally described by Schmidt et al.[9] was used, ranging from 0 to 3 (no pathological changes to severe injury).

Assessment of thromboxane A2 Thromboxane A2 was measured in frozen serum by commercially available enzyme-linked immunosorbent assay (University of Freiburg, Germany).

Statistical analysis Results are shown as mean±SEM. Student's *t* test was used when the data had a normal distribution, whereas Kruskal–Wallis and Mann–Whitney tests were utilized when the distribution was not normal. Statistical significance was accepted at the 5% level ($p<0.05$).

Results

Serum amylase Twelve hours after the induction of AP, serum amylase increased significantly compared to control animals. Hyperamylasemia was comparable in both mild and severe AP indicating the presence of pancreatic cell damage. However, amylase was not a marker for the extent of tissue damage or disease severity (Table 1).

Serum thromboxane A2 Thromboxane A2, as a marker of platelet activation, showed significantly higher levels in both AP groups compared to control animals after 24 h. Thromboxane liberation correlated with severity of AP, with the highest levels being present in animals with necrotizing AP (Table 1).

Intravital microscopy Erythrocyte velocity decreased significantly in mild as well as severe AP in both capillaries and venules compared to control animals. Platelets showed comparable flow features. Flow velocity decreased under both AP conditions, with a highly significant decrease in severe AP in venules and capillaries (Table 2). These changes were paralleled by increased interaction between leukocytes and endothelium (Table 2). Platelet adhesion in capillaries and venules increased significantly in both mild and severe AP (Figs. 1 and 2). Reversible adhesion (rolling platelets) were comparable during both forms of AP, whereas the increase in irreversible adhesion (sticking platelets) depended on the severity of AP and showed peak platelet–endothelium adherence in necrotizing AP (Figs. 1 and 2).

Tissue edema (wet/dry ratio) Supramaximal cerulein stimulation induced a significant increase in pancreatic water content compared to control animals. In contrast, there was only a slight increase in tissue edema after GDOC treatment (Table 1).

Histopathology Control animals showed no histopathological changes after sham operation and 24 h infusion therapy. Histopathology of mild AP was characterized by significant edema formation, inflammatory tissue infiltration, and

Table 2 Results of the Intravital Microscopy

Intravital microscopy	Control	Mild AP	Severe AP
Erythrocyte velocity (capillary) (mm/s)	0.65/0.02	0.42/0.01*	0.36/0.01*
Erythrocyte velocity (venule) (mm/s)	0.93/0.11	0.77/0.17	0.58/0.10*†
Platelet velocity (capillary) (mm/s)	0.54±0.04	0.35±0.03*	0.29±0.03*
Platelet velocity (venule) (mm/s)	0.67±0.05	0.63±0.02	0.53±0.05*
Rolling leukocytes (capillary)	1.3±0.2	4.5±1.4*	9.0±1.7*†
Rolling leukocytes (venule)	1.3±0.2	14.8±1.2*	18.9±1.9*
Sticking leukocytes (capillary)	1.1±0.3	10.2±1.8*	7.2±0.7*
Sticking leukocytes (venule)	0.7±0.1	5.6±0.9*	13.5±2.0*†

*$p<0.05$ vs control group
†$p<0.05$ vs mild acute pancreatitis

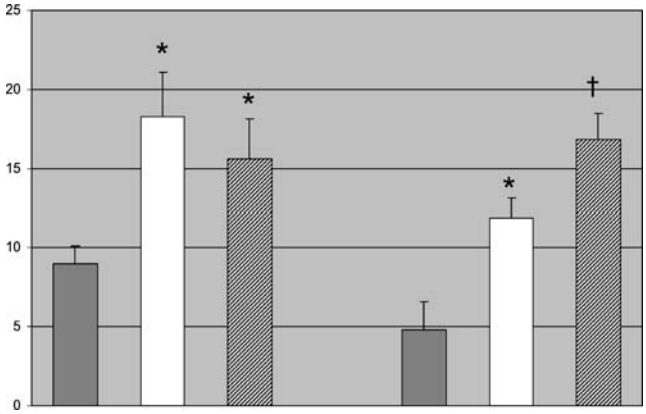

Figure 1 Intravital microscopy, capillary platelet adhesion (one per field). Control group (*gray*), mild acute pancreatitis (*white*), and severe acute pancreatitis (*striped*). Reversible platelet adhesion in mild and severe acute pancreatitis (*left columns*); irreversible platelet adhesion (*right columns*). *$p<0.05$ vs control group, †$p<0.05$ vs mild acute pancreatitis.

acinar cell necrosis. In severe AP, the changes regarding inflammation and necrosis were significantly more pronounced (Table 1).

Discussion

In the present study, we have investigated platelet function in experimental models of AP. We chose two animal models to induce a mild edematous or a severe necrotizing course of AP. Both models are established, well characterized, and have been used in numerous studies.[9,14,15] The induction of AP in these models results in a standardized grade of tissue damage, either mild or severe, with very little variance within each group. Therefore, the use of these models allows us to rule out the significant influence of preparatory or other methodological problems on the comparability of the results.

Analysis of platelet function by intravital microscopy has been established and standardized for examination of liver and small bowel perfusion by Massberg et al.[10] We have modified this method to investigate the pancreas.[15] In the present study, we could demonstrate that this method is not only suitable for the examination of healthy pancreas but also for the detailed analysis of pancreatic microcirculation in mild and severe AP.

Acute pancreatitis is characterized by an impairment of microcirculation due to an activation of inflammatory cells with a consecutive increase of leukocyte–endothelium interaction. These pathophysiological events mediate an inflammatory tissue infiltration, edema, and hemorrhagic lesions. While the inflammatory response is well investigated, the platelet function and the role of the coagulation cascade have not yet been investigated in detail.

It is well known that the inhibition of certain coagulatory steps, e.g., by applying hemodiluting or anticoagulatory substances, improves the outcome of AP.[16,17] Coagulation and hemostasis comprise two interacting pathways: humoral coagulatory factors leading to the activation of fibrinogen as the final step of the coagulation cascade and cellular factors, which are represented by activated platelets. Different mechanisms of platelet interaction are responsible for their physiological function, namely, interactions with endothelium, leukocytes, and humoral coagulatory and inflammatory proteins.[18,19]

In the present study, we could demonstrate that the platelet–endothelium interaction increases during AP and correlates with the degree of its severity. Comparable to leukocyte–endothelium interaction, temporary and permanent adhesions of platelets to the vessel wall were evident in our experiments. This correlates with the activation patterns that have been observed in vivo in ischemia models of the liver and the pancreas,[15,20] as well as in vitro.[21] Therefore, it seems likely that these activation patterns reflect the severity of the pancreatic affection, leading to reversible adhesion in mild AP and irreversible adhesion in more severe organ affection. Especially, the firm adhesion of platelets contributes to microcirculatory disturbances and may induce perfusion failure and tissue necrosis in the progression to severe AP. The significantly elevated thromboxane levels correlate well with platelet activation and microcirculatory failure observed during intravital microscopy. The increase in serum thromboxane elucidates one mechanism of our results as it executes a direct platelet stimulation and leads to the conversion of "resting" to "activated" platelets with the consecutive adhesive action. Furthermore, thromboxane does not only activate platelets but also acts as a complex pathophysio-

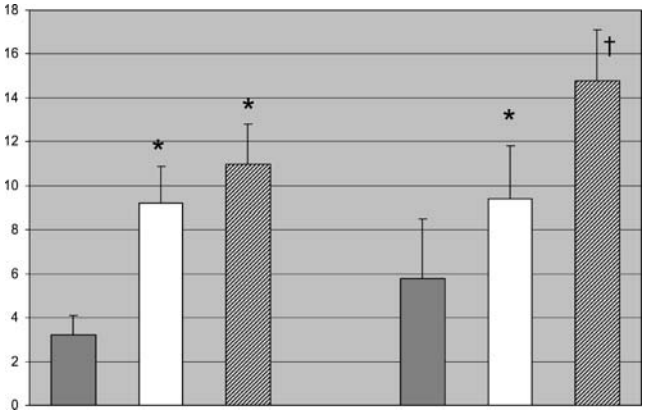

Figure 2 Intravital microscopy, venular platelet adhesion (one per 100 μm). Control group (*gray*), mild acute pancreatitis (*white*), and severe acute pancreatitis (*striped*). Reversible platelet adhesion in mild and severe acute pancreatitis (*left columns*); irreversible platelet adhesion (*right columns*). *$p<0.05$ vs control group, †$p<0.05$ vs mild acute pancreatitis.

logical mediator with multiple other targets. Its effects include leukocyte activation, upregulation of proinflammatory cytokines, and strong vasoconstricting effects. These are mediated via phosphatidylcholine and phosphatidylcholine-specific phospholipase-C pathway leading to a tonic contraction in smooth muscles and upregulating other vasoactive substances.[22,23] Especially, this vasoconstrictor mechanism may additionally contribute to perfusion failure in the course of AP as observed in our study. How far platelet inhibition itself could be an approach to attenuate the course of AP experimentally or clinically is hypothetical but should certainly be addressed to in further studies. Possible aims could be adhesion molecules such as selectins or platelet receptors and also synthesis of thromboxane and prostaglandins.

Platelet activation was accompanied by leukocyte activation in the present study. An interaction between these two cell types has been demonstrated by the different authors in the past.[24–26] Among others, P-selectin seems to be one of the most important adhesion molecules, which links the inflammatory and procoagulatory cascades and has the potency to activate leukocytes and platelets as the cellular elements of either pathway.[18,19,25,26] Besides their adherence to endothelial cells, activated platelets form stable aggregates with leukocytes. This results in a combined inflammatory and coagulatory contribution to thrombus formation and is also mediated by P-selectin and beta-integrins.[27–29] Especially, the formation of microthrombotic vessel occlusion with microcirculatory perfusion failure and consequent ischemia, hypoxia, and tissue necrosis was reflected by the intravital microscopic results in the present study.

Conclusion

The results of the present study show that activation and adhesion of platelets play an important role during AP. Platelet–endothelium and platelet–leukocyte interactions as well as thromboxane liberation show a correlation with the severity of experimental AP and seem to be of distinct importance in the progression from mild to severe necrotizing AP. A possible therapeutic use of these pathophysiological events should be evaluated in further studies.

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