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The role of vascular adhesion molecules PECAM-1 (CD 31), VCAM-1 (CD 106), E-Selectin (CD62E) and P-Selectin (CD62P) in severe porcine pancreatitis

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Summary. Inflammatory cytokines have been shown to mediate organ damage by their action on vascular endothelia and leukocytes, in part by upregulating the expression of adhesion molecules, which in turn convey transmigration of leukocytes into tissue.

The upregulation and activation of vascular cell adhesion molecules on the endothelial cells avail firm leukocyte adhesion to the vascular endothelium and enhance their transmigration and consecutive tissue injury. The aim of this study was to evaluate the expresion of vascular adhesion molecules CD 31 (PECAM-1), CD 106 (VCAM-1), CD 62E (E-Selectin) and CD 62P (P-Selectin) in the pancreas and distant organs of pigs suffering from acute necrotizing pancreatitis (AP).

AP was induced in 13 pigs by a combination of intravenous cerulein and intraductal glycodeoxycholic acid. For immunostaining of vascular adhesion molecules slides of porcine pancreas, lung, kidney and liver tissue were stained with monoclonal antibodies (Ab) against PECAM-1-1, VCAM-1 E- and P-SELECTIN.

The endothelial cell expression of CD 31 (PECAM-1), CD 106 (VCAM), CD 62E (E-Selectin) and CD 62P (P-SELECTIN) in severe porcine pancreatitis is detectable and upregulation is partly significantly.

Key words: Acute porcine pancreatitis, Vascular adhesion molecules, PECAM-1, VCAM-1, E-Selectin, P-Selectin

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Introduction

Mortality due to severe acute pancreatitis most often results from multiorgan dysfunction syndrome (MODS) occurring early after the onset of severe acute pancreatitis. Due to systemic inflammatory response syndrome and multiorgan failure, mortality ranges from 0 to 50 percent (Mutinga et al., 2000; Gloor et al., 2001).

Adhesion molecules are multifunctional cell surface proteins that mediate both cell-cell and cell-matrix interactions. In addition to their role in the preservation of tissue structure and integrity, these proteins participate in active cellular processes. For example, adhesion molecule-mediated interactions are critical for the development of many different inflammatory and immune responses, including leukocyte emigration from the vasculature into tissue following an inflammatory stimulus (Cronstein and Weissmann, 1993a; Cronstein, 1994b; Okpala, 2006; Singh et al., 2006). A large number of adhesion molecules have now been described, and many can be grouped into families based on similarities in structure and function, including the selectin, integrin, cadherin, CD44, and immunoglobulin superfamilies.

The inflammation is characterized histologically by accumulation of leukocytes at the inflamed site due to the directional migration of circulating leukocytes. Migration out of the vasculature is first initiated by contact between the leukocyte and the inflamed vascular endothelium. Experimental data indicate that both the vascular endothelium and the leukocytes actively mediate this adhesive interaction (Cronstein and Weissmann, 1993b; Springer, 1994; Middleton et al., 2002). The selectin family of adhesion molecules consists of three structurally-similar proteins: E-Selectin,

P-Selectin, and L-Selectin. Together, these proteins facilitate leukocyte rolling, the initial low-affinity step in leukocyte recruitment from the vascular lumen to the extravascular tissue (Kansas, 1996). Maximal expression of P-and E-Selectin were observed 48 hours after the initial stimulus (Lundberg et al., 2000e). Continued inflammatory stimuli induce higher affinity interactions mediated by members of the immunoglobulin superfamily ICAM-1(CD54), VCAM-1(CD106) and PECAM-1 (CD31).

Multiple, small animal studies have shown that severe pancreatitis is associated with an upregulation of these vascular adhesion molecules in the pancreas, as well as in distant organs (Folch et al., 1999; Frossard et al., 1999; Lundberg et al., 2000d; Zhao et al., 2005c). Efforts to block these mediators were partly able to reduce tissue damage and improve outcome in experimental models of AP (Inoue et al., 1996b; Werner et al., 1999a; Bhatia et al., 2000a; Eibl et al., 2002c). Although there is still no animal model identical to human AP, extensive research has greatly improved in terms of experimental AP-models. In vivo experimental research is limited to small animal models since very few large animal models have been evaluated and published so far.

To converge human conditions with reliable and predictive parameters, large animal models are valuable. To our knowledge the most suitable rat model of necrotizing AP with corresponding morphological changes and a genuine mortality rate is the Boston model (Schmidt et al., 1992). Therefore, we transferred it into a porcine model and found similar outcomes (Freitag et al., 2006). Since organ failure is a crucial step in severe pancreatitis the aim of this study was to evaluate the expresion of vascular adhesion molecules CD 31 (PECAM-1), CD 106 (VCAM-1), CD 62E (E-Selectin) and CD 62P (P-SELECTIN) in pancreas and distant organs of pigs suffering from acute necrotizing pancreatitis.

Materials and methods

After approval of the Ethics Committee of the Hamburg Federal Board of Veterinary Medicine and Animal Care, 13 pigs (German hybrid program; body weight: 25-38 kg) were used in the study. After 24 h of fasting with free access to water and 20% glucose up to 12 h before induction, animals were premedicated with intramuscular (i.m.) administration of 10 mg/kg Ketamine (Ketanest™ , Atarost, Twistingen, Germany), 4 mg/kg Azaperone (Stresnil™ , Janssen-Cilag, Neuss, Germany), and 0.015 mg/kg atropine sulfate (Atropin™ , B. Braun, Melsungen, Germany). Anesthesia was induced intravenously with 0.5 mg/kg midazolam (Dormicum™ , Janssen-Cilag, Neuss, Germany) and 0.05 mg/kg fentanyl (Fentanyl-Janssen™ , Janssen-Cilag). The animals were intubated. Balanced anesthesia was maintained by 0.05 mg/kg/h fentanyl and isoflurane (1-1.5 vol%) (Forene™ , Abbott, Wiesbaden, Germany).

Induction of AP

The abdominal cavity was opened though an upper abdominal laparotomy. The pancreas and duodenum were mobilized. The pancreatic duct was cannulated (Vaso. x 0.8 mm, B. Braun, Melsungen, Germany). AP was initiated by intraductal pressure and time-controlled infusion of 0.4 ml/kg glycodeoxycholic acid (GDOC) (10 mmol/l, pH 8, Sigma, Steinheim, Germany) at a perfusion pressure of 25 mm Hg over 10 min. This was paralleled by continuous intravenous infusion of cerulein at 5 μg/kg/h (Takus™, Pharmacia & Upjohn, Erlangen,Germany) for 1 h. In addition, 3 animals were sham operated. The pancreatic duct was cannulated, but no AP was induced. Development of severe pancreatitis was observed over a 6 hour period while the animals were under anaesthesia. Morphologic and systemic characteristics of severe pancreatitis were monitored. After 6 hours the animals recovered from anesthesia and were closely observed by a veterinarian with access to water, food, and analgetics. Immediately after natural death pancreatic, lung, kidney, and liver tissue was rapidly excised and snap frozen in liquid nitrogen. Tissue slides were stained with the primary monoclonal anti-pig antibody.

Antibodies

E-Selectin (attenuation:1:100, catalogue number CBL 180, Chemicon International Inc., CA, USA) and PECAM-1 (attenuation:1:100, catalogue number MAB 1393, Chemicon International Inc., CA, USA) were purchased.

VCAM-1 and P-Selectin were produced and provided by Professor Dorian O Haskard, Hammersmith Hospital, London W12 ONN, United Kingdom.

In a second step, incubation with a secondary fluorescein isothiocyanate-conjugated anti-murine immunoglobulin (Fluorescein AntiMouse IgG, Vectorlabs, Burlingame, California, USA) at an 1:200 attenuation was performed.

Fluorescent microscopy of postcapillary venules was processed and captured with a digital camera (Figs. 1-4). Evaluation of expression of the FITC-stained vascular adhesion molecules was determined by image analysis according to the method described by Tang and Hendricks (Tang and Hendricks, 1996). To evaluate the relative fluorescence intensity, the mean brightness value of the vessel was determined using Photoshop 6.0 (Adobe Systems, San Jose, CA, USA) with a set 900 pixel square area. Nine vessels of each organ were analyzed from these values, and the mean ± SE of the fluorescence intensity for each vessel was estimated.

Statistics

Values were expressed as the mean ± SE. Statistical significance was determined using the Mann-Whitney-U-test. A value of $p<0.05$ was considered to be

statistically significant. Evaluation was carried out by using SPSS 11.5.1 software (SPSS, Chicago, IL, USA).

Results

All animals survived anesthesia and surgery. The mean survival time of AP pigs was 51.9±55.7 hours. Sham-operated animals were sacrificed after 48 hours.

Histology showed severe pancreatitis with organ destruction in all animals dying during the observational period, while sham-operated animals had no signs of severe pancreatitis.

PECAM-1 (Fig. 5)

Compared to sham-operated animals the expression

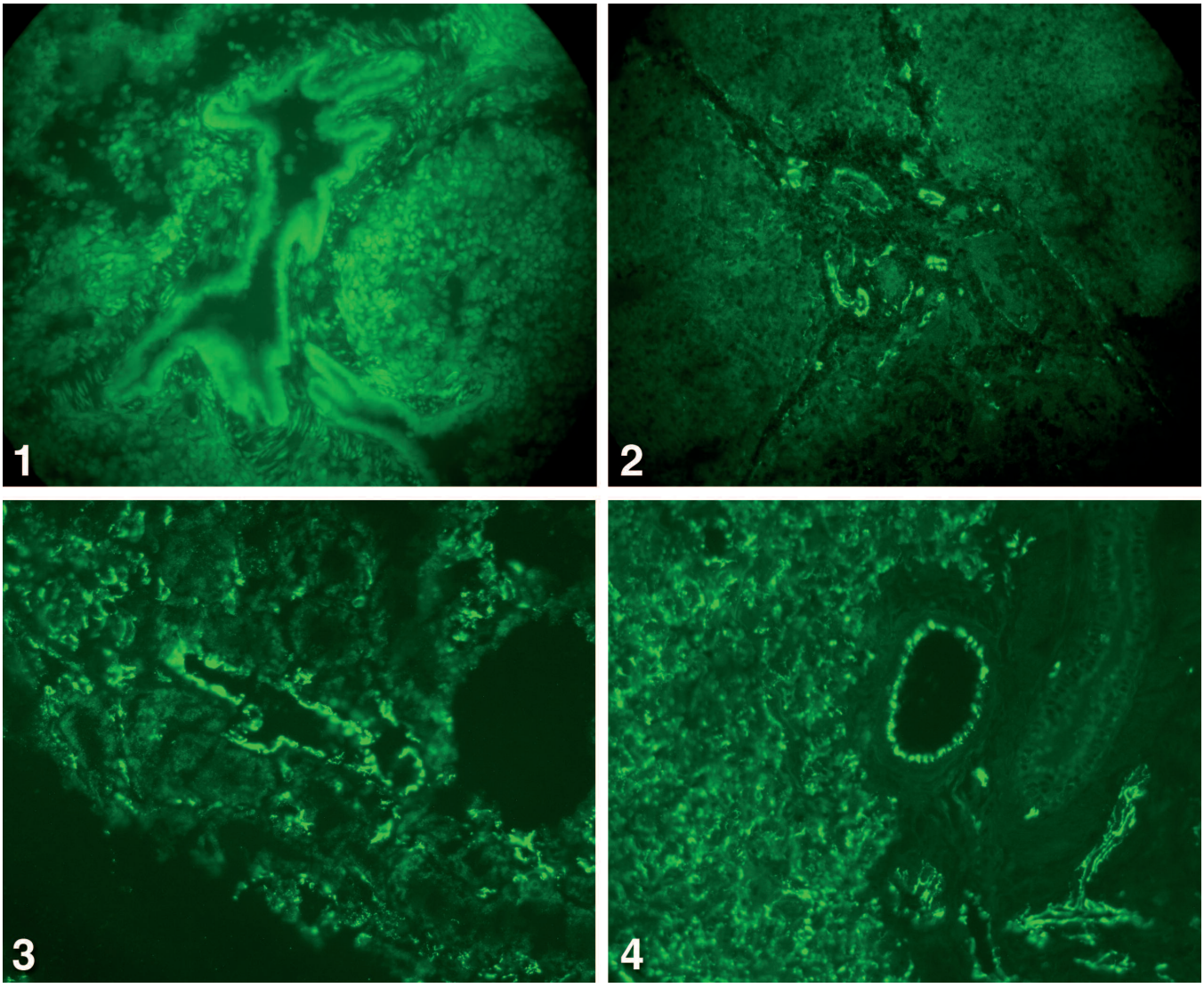

Fig. 1. Fluorescent microscopy of vascular lung CD 31(PECAM-1) expression in porcine pancreatitis (display alternation = 400). Relative fluorescence intensity with a brightness value of 118,8. x 200

Fig. 2. Fluorescent microscopy of vascular liver CD 31(PECAM-1) expression in porcine pancreatitis (display alternation = 200). Relative fluorescence intensity with a brightness value of 79,8. x 200

Fig. 3. Fluorescent microscopy of vascular kidney - CD 31(PECAM-1) expression in porcine pancreatitis (display alternation = 200). Relative fluorescence intensity with a brightness value of 82,6. x 200

Fig. 4. Fluorescent microscopy of pancreatic vascular CD 31(PECAM-1) expression in porcine pancreatitis (display alternation = 200). Relative fluorescence intensity with a brightness value of 77,6. x 200

of PECAM-1 was significantly higher in liver (p=0.024), kidney (p=0.048) and pancreatic tissue (p=0.048). Expression of PECAM-1 in lung tissue was also upregulated, but not significantly (p=0.286).

VCAM-1 (Fig. 6)

CD106 expression was significantly upregulated in kidney tissue (p=0,048), in pancreatic (p=0,229), liver

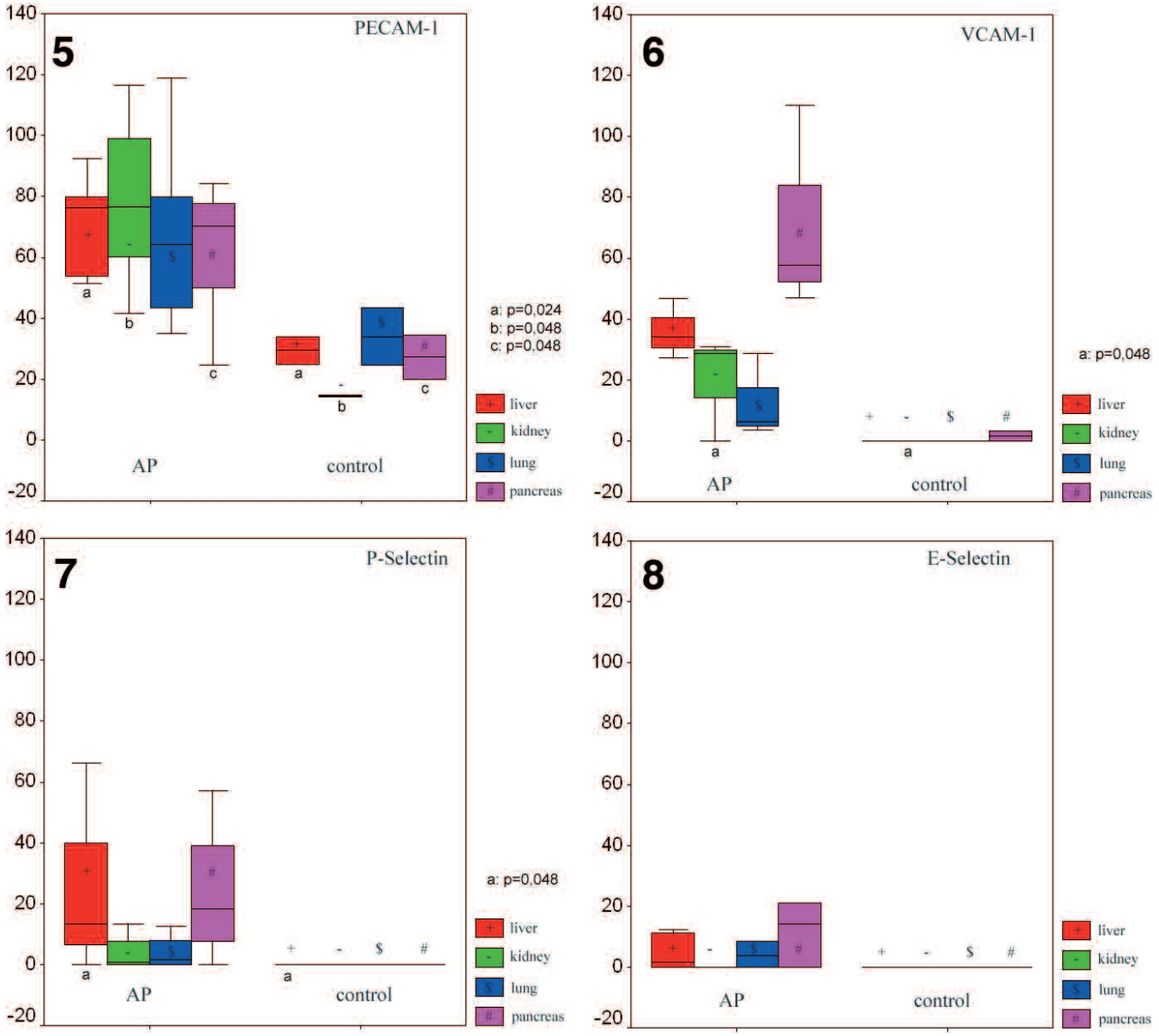

Fig 5. Boxplots diagram of PECAM-1 expression in porcine pancreatitis (AP) and controls. Y-axis shows the brightness value of the relative fluorescence intensity.

Fig. 6. Boxplots diagram of VCAM-1 expression in porcine pancreatitis (AP) and controls. Y-axis shows the brightness value of the relative fluorescence intensity.

Fig. 7. Boxplots diagram of P-Selectin expression in porcine pancreatitis (AP) and controls. Y-axis shows the brightness value of the relative fluorescence intensity.

Fig. 8. Boxplots diagram of E-Selectin expression in porcine pancreatitis (AP) and controls. Y-axis shows the brightness value of the relative fluorescence intensity.

tissue (p=0.167). In lung tissue CD106 expression was upregulated, but not significantly (p=0.095).

P-Selectin (Fig. 7)

P-Selectin expression was detectable in pancreatic (p=0.143), kidney (p=0.167), liver (p=0.048) and lung (p=0.143) tissue. However, only significant differences were found in liver tissue.

E-Selectin (Fig. 8)

Similar to the P-Selectin group, CD62E expression in liver (p=0.262), kidney (p=0.714), lung (p=0.429) and pancreatic tissue (p=0.167) is detectable but not significantly upregulated.

Discussion

Severe acute pancreatitis has been characterized as an acute inflammatory process of the pancreas, with variable involvement of other regional tissue or remote organ systems (Bradley, 1993). The clinical presentation varies from mild abdominal discomfort to multiple organ failure and death.

It is not clear why some individuals only suffer from mild pancreatitis, while others go on to develop severe systemic complications and the necrotizing form of the disease.

Various chemical mediators, including cytokines, lead to the accumulation of leukocytes not only in the pancreatic tissue but also in distant organs where they are believed to contribute to organ dysfunction (Bassi et al., 1994; Bhatia et al., 2000; Eibl et al., 2002).

Vascular adhesion molecules are closely involved in the pathogenesis of acute severe pancreatitis and its systemic complications (Doerschuk et al., 1996; Inoue et al., 1996; Folch et al., 1999; Lundberg et al., 2000; Nakae et al., 2001; Zhao et al., 2005). Blocking cytokines and adhesion molecules in small animal models led to a stable microcirculation, less tissue damage (Gross et al., 1993; Denham et al., 1997; Eibl et al., 2002; Callicutt et al., 2003; Wittel et al., 2004) and reduction of mortality (Werner et al., 1999).

These basic research data have been almost solely orientated on small non survival animal models (Rattner, 1996). Extensive research has underlined the physiological relevance of experimental AP models. The choice of the animal model should depend on the objective of the study. In this setting, we revert to the Boston model, an established model of duct infusion to induce severe pancreatitis (Freitag et al., 2006).

By intraductal infusion of glycodeoxycholic acid followed by intravenous caerulein application, hemorrhagic pancreatitis is induced (Schmidt et al., 1992). It is considered to have a high degree of similarity to human AP in terms of local histological changes, spectrum of microorganisms in the tissue, pancreatitis-assiciated MOF and delayed mortality (Foitzik et al., 2000).

Because of their comparability to human proportions, large animal models are more reliable in simulating clinical reality. However due to high costs, ethical objections, and extravagance they still remain rare.

To our knowledge this is the first study to evaluate a high bandwidth of adhesion molecules in the pancreas and distant organs within a porcine model of severe pancreatitis, using the gold standard (Kaifi et al., 2000; Saint et al., 2002; Tang and Hendricks, 1996) to generate a semiquantitative image of adhesion molecules in inflamed tissue.

Upregulation of vascular adhesion molecules such as P and E- Selectin in early stage (Subramaniam et al., 1995) and VCAM and PECAM after continued inflammatory stimuli is essential for regulation of leukocyte traffiking from blood into organ tissue (Malik and Lo, 1996). From small animal models we know that a peak of P- and E-Selectin expression in lung tissue is dectectable at 48 hours. Progressive histopathologic damage occurs from 48 to 72 hours (Lundberg et al., 2000).

In addition, several other research groups investigated the role of adhesion molecules in pancreatitis-induced lung injury (Folch et al., 1999; Frossard et al., 1999; Lundberg et al., 2000; Zhao et al., 2005) Some were able to demonstrate a direct correlation of increased expression and gravity of tissue damage (Frossard et al., 1999).

Besides pancreatitis, other diseases, such as rheumatoid arthritis, express adhesion molecules on the vascular endothelium of inflammed tissue (Cronstein, 1994). Blockade of integrin-immunoglobulin superfamily interactions retarded the development of experimental rabbit arthritis (Jasin et al., 1992). In 23 patients with rheumatoid arthritis the admission of a monoclonal antibody against ICAM-1 resulted in clinical improvement at follow-up periods of one and two months (Kavanaugh et al., 1994).

Nakae et al. were able to show that TNF-alpha stimulates the expression of soluble adhesion molecules sVCAM and sICAM. Patients` plasma levels of TNF-alpha and soluble adhesion molecules from patients suffering fom severe pancreatitis reflected the severity of the disease (Nakae et al., 2001).

In our setting, early stage adhesion molecules were detectable but not significantly upregulated. Since the mean time of analysis was 52 hours past onset of the disease, significant P- and E- Selectin expression could have already been turned down. However, stable molecules such as PECAM and VCAM showed significant expressions in pancreas, liver and kidney tissue.

PECAM and VCAM expression in lung tissue was stringent, but not significantly enhanced. One explanation could be pulmonary necrosis due to progressive tissue destruction.

Fundamentally we were able to produce a reliable

model for evaluation of adhesion molecules in severe pancreatitis. Similar to small animal models we were able to prove a significant upregulation of stable adhesion molecules in the pancreas and distant organs. With this study we provide a foundation for further investigations of AP complications in large animal models.

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