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An experimental model of hemolysis-induced acute pancreatitis

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Abstract

The literature indicates that acute pancreatitis is a complication of massive hemolysis with a prevalence of about 20%. We describe an experimental model of hemolysis-induced acute pancreatitis. Hemolytic anemia was induced in rats by a single *ip* injection of 60 mg/kg of 20 mg/ml acetylphenylhydrazine (APH) in 20% (v/v) ethanol on the first experimental day (day 0). One hundred and fifty Wistar albino rats weighing 180-200 g were divided into three groups of 50 animals each: groups 1, 2 and 3 were injected *ip* with APH, 20% ethanol, and physiological saline, respectively. Ten rats from each group were sacrificed on study days 1, 2, 3, 4 and 5. Serum amylase, lipase levels and pancreatic tissue tumor necrosis factor-α (TNF-α) and platelet-activating factor (PAF) contents were determined and a histological examination of the pancreas was performed. No hemolysis or pancreatitis was observed in any of the rats in groups 2 and 3. In group 1, massive hemolysis was observed in 35 (70%) of 50 rats, moderate hemolysis in seven (14%), and no hemolysis in eight (16%). Thirty-three of 35 (94.2%) rats with massive hemolysis had hyperamylasemia, and 29 of these rats (82.8%) had histologically proven pancreatitis. The most severe pancreatitis occurred on day 3, as demonstrated by histology. Tissue TNF-α and PAF levels were statistically higher in group 1 than in groups 2 and 3. Acute massive hemolysis induced acute pancreatitis, as indicated by histology, in almost 80% of cases. Hemolysis may induce acute pancreatitis by triggering the release of proinflammatory and immunoregulatory cytokines.

Key words
- Hemolysis
- Pancreatitis model
- Experimental pancreatitis
- Etiology
- Pathology

Introduction

In clinical practice, the common etiologies of acute pancreatitis are biliary tract disease and ethanol abuse (1). Acute pancreatitis is also associated with multiple rare causes such as drugs, infections, hyperparathyroidism, hyperlipidemia, shock, trauma, and pregnancy (1). After we diagnosed acute pancreatitis due to massive hemolysis in a patient with glucose-6-phosphate dehydrogenase deficiency, we reviewed the related literature and found some case reports (2,3). We had clinical and literature evidence that acute pancreatitis is a complication of massive hemolysis with a prevalence of about 20% (2-5). A decade ago, Druml et al. (2) suggested that back pain, which may occur in patients with a transfusion reaction, might be caused by hemolysis-induced acute pancreatitis. We now speculate that pancreatitis seen in patients after heart surgery is associ-

ated with the mechanical hemolysis that occurs during extracorporeal circulation.

The important role of inflammatory cytokines in the pathogenesis of acute pancreatitis is well known (6-8). The mechanisms responsible for the initiation of the inflammatory response and release of cytokines are not certain but the inflammatory response is clearly responsible for the mortality and morbidity associated with this disease (6). In hemolysis-induced acute pancreatitis, the source of cytokines cannot be the erythrocytes since they have no nucleus to synthesize them. The mechanism must be different in this particular model. In the literature, heme released from heme proteins has been shown to promote a systemic inflammatory response and organ failure (9,10). Even though the association between excessive heme in the circulation and cytokine release has been reported, there are no data in the literature about the mechanism of hemolysis-induced acute pancreatitis.

The first and correct way to define the association of massive hemolysis with the development of acute pancreatitis is to study it in an experimental model. Our literature review did not reveal any report of an experimental model of hemolysis-induced acute pancreatitis, although it has been well established in individual case reports that massive acute hemolysis is a causative event for the occurrence of acute pancreatitis.

Our objective in the present study was to establish the first experimental model of hemolysis-induced acute pancreatitis and to assess the factors that trigger acute pancreatitis in this model.

Material and Methods

Hemolysis induction in rats

A previously defined drug-induced hemolysis model was used in this study to produce acute massive hemolysis (11). Hemolytic anemia was induced in rats by a single intraperitoneal (*ip*) injection of 60 mg/kg of 20 mg/ml acetylphenylhydrazine (APH) dissolved in 20% (v/v) ethanol on the first experimental day (day 0). The presence of hemolysis was determined by hemoglobin, hematocrit, reticulocyte count and serum lactate dehydrogenase levels. Acute massive hemolysis has been described as a fall in hematocrit greater than 12% in 12 h and a decrease in hematocrit of less than 12% has been accepted as moderate hemolysis.

One hundred and fifty Wistar albino rats weighing 180-200 g were divided into three groups of 50 animals each. Group 1 received APH *ip*, group 2 was injected *ip* with 20% ethanol, which was the solvent for APH, and group 3 received physiological saline *ip*. All groups were treated with an equal volume of the different agents. Ten rats from each group were sacrificed on study days 1, 2, 3, 4 and 5. All groups were assessed and compared for the occurrence of hemolysis and/or pancreatitis. The data obtained were used to determine the optimum day of sacrifice.

Sampling

Blood was first taken from the heart of the rat by the percutaneous route under anesthesia, and measurements were performed for hemoglobin, hematocrit, reticulocyte, lactate dehydrogenase, amylase, and lipase on the same day. At the end of the experiment, blood was taken from the heart of the rats by the percutaneous route under anesthesia and the rats were sacrificed. These blood samples were assayed for the same parameters as determined in the initial measurements. Plasma amylase and lipase activities were determined by a standard method for automated analysis.

The pancreatic tissues were gently washed with cold phosphate-buffered saline (PBS). For the determination of tumor necrosis factor-α (TNF-α) and platelet-activating factor (PAF), the pancreatic tissue was immediately homogenized in 2 ml ice-cold PBS for

3 min at 24,500 cycles/min (Ultra Turrax Homogenizer) and supernatants were obtained by centrifugation at 1800 rpm for 10 min. Aliquots of the homogenate were assayed for total protein by the method of Lowry et al. (12), modified. TNF-α and PAF were extracted from the samples using Sep-Pak C18 (Waters MO, Milford, CT, USA) solid-phase extraction columns. Extracted samples were dried in a vacuum speed evaporator (Heto-1 Speed Vacuum Concentration, Copenhagen, Denmark) under liquid nitrogen. Dried samples were dissolved in 20 µl high-performance liquid chromatography (HPLC) solvent and kept at -70ºC. They were then purified by RP-HPLC and collected on the basis of their retention times, and again dried. TNF-α and PAF were measured quantitatively using an immunological assay system with monoclonal antibodies (TRK940 Amersham Pharmacia Biotech, Buckinghamshire, UK) and a Biotrack assay (TRK910 Amersham) according to manufacturer instructions with a beta-liquid scintillation counter (TRI CARB-1600 TR, LSA-Packard, Camberra Company, Meriden, CT, USA). TNF-α is reported as pg/mg tissue protein and PAF is reported as ng/mg tissue protein in the homogenate supernatants of the tissue.

Examination of the pancreas

Histological evaluation was performed according to Schoenberg et al. (13). Tissue samples removed from the caput, corpus and cauda of the pancreas were fixed in formalin for 24 h, paraffin-embedded, and stained with hematoxylin and eosin. Light microscopy slides were examined and graded by a pathologist (G. Yuce) with experience in experimental pancreatitis who was unaware of the previous treatment.

Statistical analysis

The data are reported as mean (± SEM) values. The histological data were evaluated for statistical significance using the Student *t*-test for independent means. Differences in hemoglobin, hematocrit, lactate dehydrogenase, amylase, lipase, TNF-α and PAF were analyzed pairwise by the Mann-Whitney test and were reevaluated by the Student *t*-test. P values of <0.05 were considered to be significant. Linear regression and correlation analysis were used to test the correlation between the degree of hemolysis and histological severity of pancreatitis, with $r>0.20$ and $P<0.05$ being considered significant. The negativity or positivity of *r* showed the direction of correlation.

Results

No hemolysis or pancreatitis was observed in any of the control rats in groups 2 (20% ethanol) and 3 (saline). In group 1, massive hemolysis was observed in 35 (70%) of 50 rats, moderate hemolysis in seven (14%), and no hemolysis in eight (16%). The hematocrit level of group 1 rats was decreased from 49.4 ± 8.1 to 33.2 ± 9.7% after the first 12 h. Serum lactate dehydrogenase levels were increased only in group 1. Also, reticulocytosis was seen only in this group.

Thirty-three of 35 (94.2%) rats with massive hemolysis had hyperamylasemia (more than ten times the normal amount) and 29 of these rats (82.8%) had histologically identifiable pancreatitis. The most severe pancreatitis occurred on day 3 and was demonstrable by histology. Figure 1A and B shows some histological features. On day 5 (Figure 1C), however, lymphocytic infiltration was the prominent finding, indicating the occurrence of a chronic condition. Table 1 shows the histological scores of group 1 (APH) as a function of days. There was no statistical correlation between the degree of hemolysis and histological severity of the pancreatitis for group 1 ($r<0.20$, $P>0.05$).

Pancreatic tissue TNF-α levels were significantly higher in group 1 (APH in 20%,

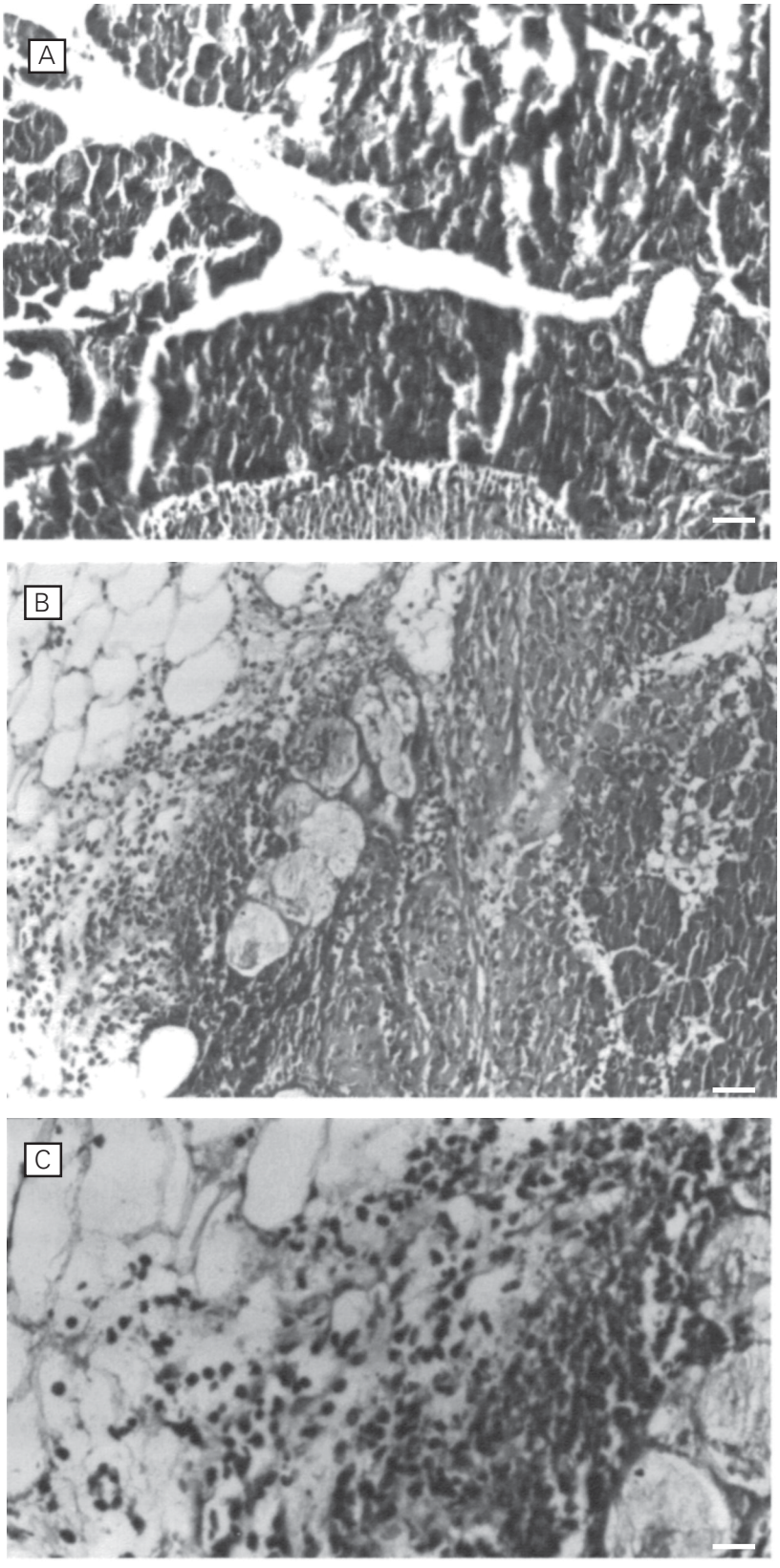

Figure 1. Hemolysis-induced pancreatitis. *A*, Normal pancreas histology in a rat without pancreatitis. *B*, Enzymatic fatty necrosis and inflammation in hemolysis-induced pancreatitis. HE, 220X. *C*, Enzymatic fatty necrosis and polymorphonuclear leukocyte infiltration in acute pancreatitis. HE, 440X. Bars in panels A and B = 45.5 µm; bar in panel C = 22.7 µm.

v/v, ethanol) than in groups 2 (20%, v/v, ethanol) and 3 (saline) ($P = 0.02$) but there was no statistically significant difference in TNF-α levels with time within the same group ($P>0.05$). This indicates that time after hemolysis did not affect TNF-α levels but the presence or absence of inflammation did (Table 2, Figure 2). Tissue PAF levels were higher in group 1 than in the other groups ($P = 0.001$, Table 3). As observed for TNF levels, PAF levels did not show significant differences between subgroups within the same group ($P>0.05$) (data not shown).

Discussion

The present study revealed that acute massive hemolysis induced acute pancreatitis in approximately 80% of rats, but not in all. Pancreatitis was not observed in animals without massive hemolysis, even if they had suffered acute hemolysis to some extent. Pancreatic tissue TNF-α and PAF levels were higher in animals with pancreatitis. However, there was no correlation between the duration of this increase after hemolysis and the time when pancreatitis occurred. This suggests that the only factor affecting the cytokine levels was the presence or absence of pancreatic inflammation.

Heme released from hemoglobin after episodes of vascular hemolysis has been shown to trigger the inflammatory process (9,14,15). Normally, heme-binding plasma proteins, such as hemopexin, can efficiently remove most of the heme produced intravascularly (16), and this prevents heme-induced damage. Increased hemolysis can lead to very high levels of free heme, which can exceed the binding capacity of hemopexin. It was

shown that heme can promote neutrophil migration *in vivo*, suggesting a direct proinflammatory effect of this blood component (9). Heme toxicity may arise in environments where there is pronounced hemolysis, at sites exposed to high erythrocyte shear forces or turbulent blood flow (9).

Leukocyte migration into tissues is the hallmark of all types of inflammatory responses. The inflammatory process can be amplified by the neutrophils themselves through the production of arachidonic acid-derived bioactive lipids such as leukotriene B4, cytokines (i.e., interferon-γ, IL-1, TNF-α), or chemokines (IL-8, growth-related oncogene-α). In experiments with rats, an intrathoracic injection of hemin induced an acute inflammatory reaction characterized by edema formation and intense accumulation of neutrophils in the pleural cavities (9). The stimulation of neutrophil migration *in vivo* and *in vitro* suggests that free heme has the potential to serve as an endogenous chemoattractant. The authors showed that hemin triggers the oxidative burst and promotes actin polymerization in human polymorphonuclear neutrophils, indicating that hemin is a potent neutrophil activator (9).

The expression of proinflammatory and immunoregulatory cytokines rapidly increases after hemorrhage (17,18) although the specific agent responsible for the onset of these proinflammatory responses remains unknown. The precise mechanisms underlying the pathogenesis of acute pancreatitis remain unclear (7,19). It has been suggested that the influx and accumulation of inflammatory cells into the pancreas and proinflammatory cytokines play an important role (7,20-22). The systemic inflammatory response syndrome is induced by proinflammatory cytokines, such as TNF-α and IL-6. The roles of proinflammatory cytokines in the early pathogenic mechanism of acute pancreatitis have been well established (8,23). It was recently shown that heme can boost the expression of α2-macroglobulin and has a possible role in the expression of positive acute-phase proteins (18).

Druml et al. (2) showed that activation of intravascular coagulation occurs in the pres-

Table 1. Effect of acetylphenylhydrazine on pancreas histology in a model of hemolysis-induced acute pancreatitis in rats.

Day of sacrifice	Mean ± SEM (N = 50)
1	1.8 ± 1.4
2	2.2 ± 1.6
3	3.4 ± 1.5
4	2.6 ± 1.1
5	1.2 ± 1.1

The scale is 1 to 5, with 5 indicating extensive damage.

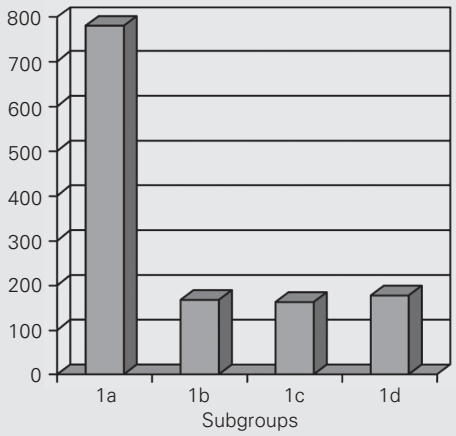

Figure 2. Tissue levels of TNF-α in the subgroups of group 1 (N = 50). Data are reported as means in pg/mg tissue protein. Subgroups: *1a*, massive hemolysis + pancreatitis (N = 29); *1b*, massive hemolysis, no pancreatitis (N = 6); *1c*, moderate hemolysis (N = 7); *1d*, no hemolysis (N = 8).

Table 2. Pancreatic levels of TNF-α after hemolysis-induced pancreatitis.

Day of sacrifice	Group 1 (APH)	Group 2 (EtOH)	Group 3 (PS)	P value
1	701.3 ± 226.5	161.5 ± 64.2	214.2 ± 59.1	$P_1 = 0.002$ $P_2 = 0.002$ $P_3 > 0.05$
2	847.5 ± 253.8	197.3 ± 73.8	ND	$P_1 = 0.002$
3	717.8 ± 189.0	ND	ND	–
4	727.7 ± 255.9	ND	ND	–
5	698.2 ± 197.3	174.1 ± 69.2	200.3 ± 46.2	$P_1 = 0.002$ $P_2 = 0.002$ $P_3 > 0.05$

Data are reported as means ± SEM in pg/mg tissue protein for 50 rats in each group. APH: acetylphenylhydrazine; EtOH: 20% ethanol; PS: physiological saline; ND: not determined. P_1 = group 1 *vs* group 2; P_2 = group 1 *vs* group 3; P_3 = group 2 *vs* group 3 (Mann-Whitney test and reevaluated by Student *t*-test).

ence of massive hemolysis. Clearance of fibrinogen-fibrin split products was reduced during hemolysis because of the blockade of the reticuloendothelial system by cell detritus which, in a vicious cycle, promoted further coagulation system activation (24). Both the accumulation of activated coagulation factors and decreased reticuloendothelial clearance maintained the formation of microthrombi and associated microvascular disturbances, resulting in the functional impairment of several organs (25). Pancreatic microvascular control is a complex and incompletely understood physiological process (26). Blood flow in the pancreas is altered by a large number of endogenous and exogenous factors. In acute pancreatitis reduction in blood flow and alterations of microvascular integrity resulting in impaired tissue oxygenation play an important role in the initiation and progression of the disease (7,26-28). Inhibition of endothelium-derived relaxing factor by free heme may cause a local vasoconstriction of the pancreatic microvascular structure (29). Hemoglobin degradation products may accelerate oxygen-free-radical formation and cell injury (30). The examination of the vascular structure of rats in our study did not show any microthrombi or vascular pathology (Figure 1C). However, there is evidence that ischemia may be an initiating factor of pancreatic microcirculatory injury in acute pancreatitis (31-34).

Table 3. Pancreatic levels of platelet-activating factor.

Day of sacrifice	Group 1 (APH)	Group 2 (EtOH)	Group 3 (PS)	P value
1	72.5 ± 8.20	11.9 ± 2.3	10.5 ± 2.2	$P_1 = 0.001$ $P_2 = 0.001$ $P_3 > 0.05$
2	87.2 ± 12.3	17.2 ± 5.9	ND	$P_1 = 0.001$
3	78.0 ± 11.1	ND	ND	–
4	69.5 ± 5.90	ND	ND	–
5	98.8 ± 17.4	11.1 ± 3.2	14.3 ± 6.7	$P_1 = 0.001$ $P_2 = 0.001$ $P_3 > 0.05$

Data are reported as means ± SEM in ng/mg tissue protein for 50 rats in each group. APH: acetylphenylhydrazine; EtOH: 20% ethanol; PS: physiological saline; ND: not determined. P_1 = group 1 *vs* group 2; P_2 = group 1 *vs* group 3; P_3 = group 2 *vs* group 3 (Mann-Whitney test and reevaluated by Student *t*-test).

Tissue PAF and TNF-α levels were increased in rats with acute pancreatitis but not in rats with massive hemolysis but without acute pancreatitis. This result may lead us to speculate that if heme-induced cytokine release exceeds a certain threshold a systemic effect may be present and hemolysis-induced acute pancreatitis occurs. After acute pancreatitis is induced, the inflammation of pancreatic tissue itself also becomes a source of high levels of cytokines. Therefore, it is not possible from our data to conclude if the release of cytokines is the cause or the consequence of acute pancreatitis. We sacrificed the rats on five different days from the beginning of the experiment to determine the perfect hemolysis model of experimental acute pancreatitis. We concluded that there were no major differences between days but day 3 was the most appropriate day, with the highest histological score. The histological changes in animals with hemolysis-induced acute pancreatitis were similar to those of other etiologies, indicating that pancreatic inflammation occurs in the same manner regardless of the etiology or mechanism.

The present study was carried out to characterize a model rather than as a pathophysiological trial. A model for the assessment of hemolysis-induced acute pancreatitis should enable us to identify some of the mechanisms involved in acute pancreatitis. It may also build a bridge between acute pancreatitis and multiple organ failure, making it easier to understand the underlying pathophysiology. Our experimental trial is being continued to discover if heme-induced cytokine release, coagulation factors, oxygen free radicals and endothelial-derived relaxing factors have any role in this model of acute pancreatitis, and if pancreatic inflammation can be decreased by anticoagulant agents or other antagonists.

The data reported in the literature and in the present study are consistent with the

view that heme has the potential to act as a signaling molecule involved in the triggering of the inflammatory processes associated with massive hemolysis. Hemolysis itself may induce acute pancreatitis by all or some pathways of neutrophil activation and chemoattraction, such as oxidative burst, direct proinflammatory effect, microcirculatory disturbance and increased expression of proinflammatory and immunoregulatory cytokines.

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