ICAM-1 and CD11b/CD18 Expression During Acute Pancreatitis Induced by Bile-Pancreatic Duct Obstruction: Effect of *N*-Acetylcysteine

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Different molecules are involved in the recruitment of leukocytes during inflammation. The aim was to investigate (i) the contribution of acinar cells to the overall production of ICAM-1 and (ii) the kinetics of leukocyte CD11b/CD18 expression during acute pancreatitis (AP) induced by bile-pancreatic duct obstruction (BPDO) to evaluate the contribution of both molecules to leukocyte homing. The role of reactive oxygen species (ROS) as mediators in the expression of ICAM-1 and CD11b/CD18 was examined by using *N*-acetylcysteine (NAC) as an antioxidant treatment. By mechanisms resistant to NAC treatment, acinar cells were able to produce ICAM-1 at first onset of AP; other cell sources contribute to maintaining increased ICAM-1 plasma levels during AP. By contrast, CD11b/CD18 was overexpressed in leukocytes in the course of AP by oxidant-dependent mechanisms. Since NAC treatment reduced neutrophil infiltration in the pancreas, we conclude that CD11b/CD18 overexpression is required for leukocyte recruitment; however, other adhesion molecules in addition to ICAM-1 seem to contribute to leukocyte homing during BPDO-induced AP. Exp Biol Med 232:737–743, 2007

Key words: acute pancreatitis; adhesion molecules; β_2-integrins; CD11b/CD18; ICAM-1; reactive oxygen species

Introduction

Acute pancreatitis (AP) is an autodigestive disease initiated by premature activation of digestive enzymes (1, 2) resulting in cellular damage that triggers a complex cascade of inflammatory events mediated by acinar cells, themselves (3–6), and activated leukocytes (7–9). Infiltration of leukocytes has commonly been found in the pancreas and lung during AP. It requires the development of a sequence of mechanisms (rolling, adhesion, and transmigration) in which a large number of adhesion molecules and inflammatory mediators are involved (10, 11).

Intercellular adhesion molecule-1 (ICAM-1) has been shown to play a key role in the course of AP (10, 12). It is a glycoprotein, mainly expressed on the surface of endothelial cells, which acts as counter-receptor for lymphocyte function-associated antigen 1 (LFA-1) and Mac-1 (CD11b/CD18), a β_2-integrin required for the firm adhesion of leukocytes to vascular endothelium (13, 14) and immediate transmigration into injured areas. Increased ICAM-1 levels have been found in the serum of patients with AP (15) and in the pancreas of rats with AP induced by taurocholate (16) and caerulein (17). ICAM-1 is upregulated during inflammation (11, 18) and redox-sensitive mechanisms, such as activation of the transcription factor NF-κB, are involved in regulating ICAM-1 gene expression (19, 20). Since reactive oxygen species (ROS) are overproduced in acinar cells during acute pancreatitis (21), they could become a cell source of adhesion molecules, as they have been shown to be for other inflammatory mediators (3–6).

This study was designed to investigate the contribution of acinar cells to the overall production of ICAM-1 as well as the kinetic of CD11b/CD18 expression in leukocytes in the course of acute pancreatitis, with the aim of evaluating the dynamic role of leukocyte adhesion receptors and endothelial cell counter-receptors involved in the regulation of leukocyte homing. The role of ROS as mediators in the expression of both types of molecules during AP was examined using *N*-acetylcysteine (NAC) as antioxidant treatment.

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Materials and Methods

Reagents. NAC, amino acid mixture, bovine serum albumin (BSA), collagenase type XI, soybean trypsin inhibitor (STI), *N*-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid) (HEPES), glutamine, streptomycin and penicillin solution, hexadecyltrimethylammonium bromide (HDTAB), 3,3′,5′5-tetramethylbenzidine (TMB) liquid substrate system**,** and buprenorphine were supplied by Sigma Chemical Co. (Madrid, Spain). Pharmingen (San Diego, CA, USA) supplied FACS lysis solution and the following anti-rat monoclonal antibodies (MoAb): fluorescein isothiocyanate (FITC)-labeled CD11b/CD18, phycoerythrin/cyanin 5 (PE/Cy5)-labeled CD45, isotype matched mouse IgG_1 κ-PE, and phycoerythrin (PE)-labeled ICAM-1. R&D Systems (Minneapolis, MN) supplied the enzyme-linked immunosorbent assay (ELISA) kit for ICAM-1. Medium 199 (Gibco, Paisley, Scotland), RPMI 1640, calf foetal serum (BioWhittaker, Walkersville, MD) and agarose (Iberlabo, Madrid, Spain) were also used. All other standard analytical grade laboratory reagents were obtained from Merck (Madrid, Spain).

Animals. Male Wistar rats (250–300 g) were housed individually in cages and maintained at 22° ± 1°C using a 12:12-hr light:dark cycle. The animals were fasted overnight before the experiment but were allowed free access to water. All experiments were performed in accordance with European Community guidelines on ethical animal research (86/609/EEC). The study was approved by the Institutional Animal Care and Use Committee of the University of Salamanca (Spain).

Animal Model of Pancreatitis and Treatment. Animals were randomly divided into three groups, each containing four subgroups. Group 1 contained rats with AP induced by bile-pancreatic duct obstruction (BPDO). For this, under anesthesia with 2%–3% isofluorane (Forane; Abbott Laboratories, Madrid, Spain), the common bile-pancreatic duct was ligated at the distal part, close to its exit to the duodenum. Afterwards, the abdominal wall was closed in a double layer and the animals were returned to their cages with free access to water and food. Group 2 contained sham-operated rats, subjected to the surgical procedure but without ligation, and Group 3 rats received 50 mg/kg of NAC by intraperitoneal injection 1 hr before and 1 hr after ligation of the bile-pancreatic duct. Postoperative analgesia was maintained in all animals by intramuscular injections of buprenorphine (0.2 mg/kg/8 hr). In all three groups, studies were carried out at the same periods: 3, 6, 12, and 24 hrs after surgery.

At the different BPDO times, animals were reanaesthetized with sodium pentobarbital (30 mg/kg). Blood samples were taken by cardiac puncture and divided into two parts for leukocyte CD11b/CD18 immunophenotyping and plasma-based measurement of soluble ICAM-1 (sICAM-1) levels. Pancreata were dissected and freed from fat and lymph nodes for measurement of ICAM-1 expression (mRNA and protein) in isolated acinar cells, myeloperoxidase (MPO) activity in pancreatic tissue, and histological studies.

Isolation of Acinar Cells. Acinar cells were isolated by digestion with collagenase in a previously oxygenated solution composed of (in m*M*) 25 HEPES (pH 7.4), 110 NaCl, 5 KCl, 1 $CaCl_2$ 14 D-glucose, 2 L-glutamine as well as 2% (w/v) BSA, 0.01% (w/v) STI, and 2% (v/v) amino acids mixture as previously described (5). In order to avoid the RNA acinar cell degradation, the isolation procedure of acinar cells used in RT-PCR analysis was carried out in an RNase-free environment and was shortened as follows: pancreata were digested by incubation with collagenase at 20°C for 8 mins with vigorous shaking. After two washes with PBS and following gentle pipetting through tips of decreasing diameter (from 3 mm down to 1 mm), cells were filtered through a sterile double layer of muslin gauze and centrifuged at 700 *g* for 4 mins at 4°C. Then the cell pellet was resuspended in PBS and centrifuged twice at 500 *g* for 3 mins at 4°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). To detect ICAM-1 mRNA, RT-PCR was performed on acinar cells immediately after isolation. Total RNA was extracted from the cells using the RNA easy kit treated with amplification-grade DNase 1 (Quiagen, Valencia, Spain) according to the manufacturer's instructions. Purity of RNA was verified by ethidium bromide staining on 1% agarose gels. cDNA synthesis was performed using combined Titan One tube reaction kit (Roche Applied Science, Penzberg, Germany) in the presence of 0.5 μg total RNA and 0.2 μ*M* ICAM-1 primers (sense: 5′-GGGTTGGAGA CTAACTGGATGA-3′, antisense: 5′-GGATCGAGCTCCACTCGCTC-3′, product size: 182 base pairs). Oligonucleotide primers for β-actin (sense: 5′-CACGGCATTGTAACCAACTG-3′, antisense: 5′-TCTCAGCTGTGGTGGTGAAG-3′, product size: 400 base pairs) were used as internal control. RT-PCR was performed following procedures: 50°C for 30 mins, 1 cycle; 94°C for 5 mins, 1 cycle; 94°C for 30 secs, 56°C for 30 secs, 68°C for 45 secs, 25 cycles; 68°C for 7 mins, 1 cycle. The amplified RT-PCR products were separated on a 2% agarose gel stained with ethidium bromide and densitometrically quantified with a Gel Doc 1000/2000 image analysis system (BioRad) using QuantityOne software. The ICAM-1 and β-actin RT-PCR products were run together on the same gel in order to normalize the band densities to the β-actin band and the results were expressed as a ratio.

Flow Cytometry ICAM-1 Analysis. Immediately after isolation, 100 μl of acinar cells (10^6 cells) were incubated with PE-labeled ICAM-1 MoAb in the dark at room temperature for 15 mins. Unbound antiserum was removed by washing twice in phosphate-buffered saline (PBS, 0.1 *M*, pH 7.4) and then immediately acinar cells were analyzed by flow cytometry using a three-color FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose CA, USA) equipped with a doublet

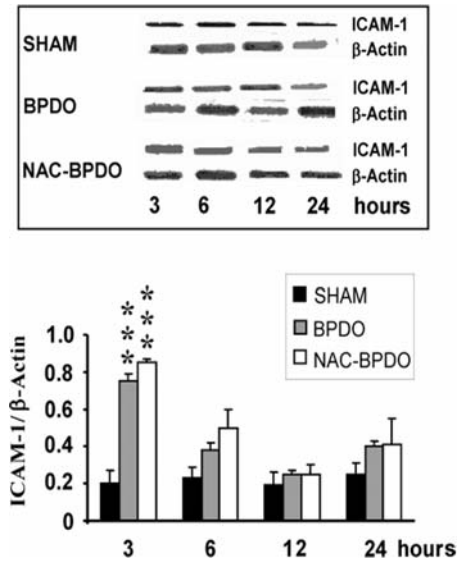

Figure 1. ICAM-1 mRNA expression in pancreatic acinar cells. A representative RT-PCR and the mean values ± SEM of acinar ICAM-1 mRNA expression (measured as ICAM-1/β-actin ratio). Number of animals per group and experimental period: 5. ANOVA followed by Scheffé test showed significant differences vs. sham-operated animals. (*** $P < 0.001$).

discrimination module. In order to assess the identity of acinar cells, parallel labeling was carried out with FITC-anti-trypsinogen antiserum as previously described (22) and analysis and histograms were performed by gating on FITC positive cells. In each experiment at least 10,000 cells were analysed. Nonspecific fluorescence was determined by isotype matched mouse $IgG_1\kappa$-PE as control antibody. Dead cells showing very low forward scatter were discarded. Data acquisition and analysis were performed using the Cell Quest and Paint-a-Gate PRO software programs (Becton Dickinson Biosciences), respectively. The results were expressed as percentage values with respect to the mean fluorescence intensity observed in sham-operated rats analyzed in parallel.

Soluble ICAM-1 Determination. Measurements of soluble ICAM-1 (sICAM) concentration were carried out in plasma and in the supernatants of acinar cells after culture. For this, acinar cells were resuspended in Medium 199 supplemented with 10% of heat-inactivated calf fetal serum, streptomycin (0.1 mg/ml), and penicillin (100 U/ml); plated at a density of 4×10^6/ml on 24-well primary tissue culture plates and incubated for 4 hrs in a 5% CO_2 humidified sterile atmosphere at 37°C. sICAM concentration was measured using an enzyme-linked immunosorbent assay (ELISA) strictly following the manufacturer's recommendations.

Flow Cytometry Leukocyte CD11b/CD18 Expression. Analyses were performed using a direct immunofluorescence technique based on a double staining with MoAb directly conjugated with FITC and PE/Cy5:CD11b/CD18-FITC/CD45-PE/Cy5 as previously described (9). Briefly, peripheral blood containing around 10^6 nucleated cells was incubated with each MoAb for 15 mins in the dark at room temperature. After lysing erythrocytes by incubating with FACS lysis solution for 10 mins, stained nucleated cells were washed by centrifuging and immediately analyzed in a FACSCalibur flow cytometer (Becton Dickinson Biosciences) as previously described. Neutrophil and monocyte CD11b/CD18 expression were expressed as percentage values with respect to the mean fluorescence intensity observed in sham-operated rats.

Myeloperoxidase (MPO) Determination. Neutrophil sequestration in the pancreas was estimated by measuring tissue MPO activity (23). Briefly, tissue samples were resuspended in 50 m*M* phosphate buffer (pH 6.0) containing 0.5% HDTAB and homogenized. After four cycles of freezing and thawing, the homogenate was further disrupted by sonication. After centrifuging (10,000 *g*, 5 mins, 4°C) the supernatant was incubated with TMB reagent for 110 secs at 37°C for the MPO assay. The reaction was stopped with 0.18 *M* H_2SO_4, and the absorbance measured at 450 nm. MPO activity is expressed per unit of dry weight (fold increase over sham-operated rats).

Histological Examination. A portion of pancreas was fixed in 8% buffered formalin and embedded in paraffin. Tissue slices were stained with hematoxylin and eosin for light microscopy examination. Histological alterations were blindly evaluated in each microscopic field and graded according to a scoring system. Interstitial edema was scored as follows: 0, absent; 1, expanded interlobular septa; 2, expanded intralobular septa; and 3, separated individual acini. Infiltration of inflammatory cells, vacuolization, and necrosis were scored as the percentage of involvement of the examined area, as follows: 0, absent; 1, <10%; 2, 10%–50%; 3, >50%.

Statistical Analysis. Results are expressed as means ± SEM. Statistical analysis was carried out using ANOVA followed by the Scheffé test. *P* values of <0.05 were considered statistically significant.

Results

ICAM-1 Expression in Acinar Cells. The expression of ICAM-1 mRNA expression on acinar cells is shown in Figure 1. RT-PCR analysis revealed a baseline level in sham-operated rats that significantly ($P < 0.001$) increased 3 hrs after BPDO in both NAC-treated and non-treated rats.

At protein level, measurements were carried out on the surface of acinar cells by flow cytometry (Fig. 2) and in the culture medium by ELISA (Fig. 3). As shown in a representative example, low intensity of fluorescence due to ICAM-1 labeling was found in acinar cells from sham-operated rats (Fig. 2A). The expression of ICAM-1 on acinar cell membrane (Fig. 2B) significantly ($P < 0.001$) increased 3 hrs after BPDO, but no significant change compared with sham animals was found at longer BPDO

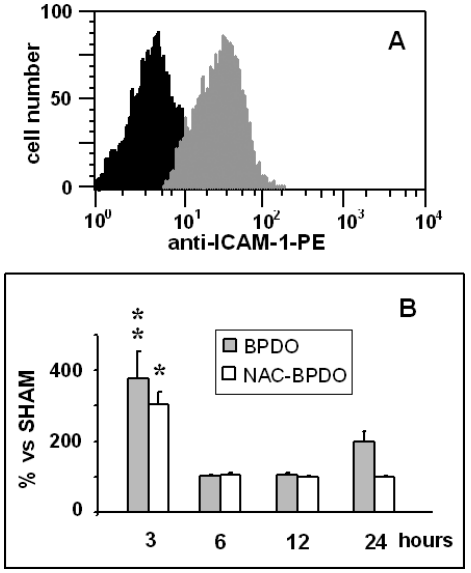

Figure 2. Flow cytometric analysis of ICAM-1 expression in pancreatic acinar cells. (A) A representative example of acinar cell distribution from sham-operated (black) and 3 hrs BPDO (grey) rats according to the intensity of fluorescence due to anti-ICAM-1-phycoerythrin (PE) labeling. (B) Percentage of variation in mean fluorescence intensity with respect to sham-operated animals. Values are expressed as means ± SEM. Number of animals per group and experimental period: 5. ANOVA followed by Scheffé test showed significant differences vs. sham-operated animals. (* $P < 0.05$; ** $P < 0.01$).

periods. Membrane-bound ICAM-1 acinar cell expression in BPDO rats did not vary by NAC treatment.

On the other hand, no variation in soluble ICAM-1 (sICAM-1) concentration was found in the culture medium of acinar cells from NAC-treated and non-treated BPDO rats with respect to sham-operated rats (Fig. 3).

sICAM Plasma Levels. sICAM plasma levels were significantly ($P < 0.001$) increased in rats subjected to BPDO from 3 hrs onwards. NAC treatment did not vary plasma sICAM concentration during BPDO-induced AP (Fig. 4).

Expression of CD11b/CD18 on Leukocytes. Figure 5A shows neutrophil and monocyte subsets (CD45 positive) well differentiated on the basis of side scatter (SSC) properties. They show a constitutive expression of CD11b/

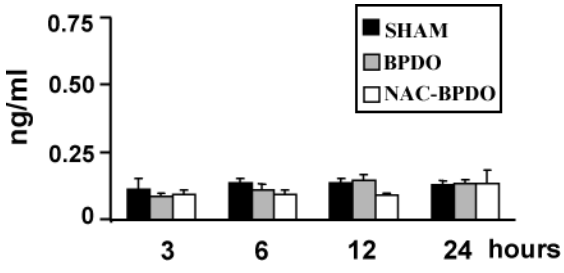

Figure 3. Soluble ICAM-1 (sICAM-1) concentration in the culture medium of acinar cells. Values are expressed as means ± SEM. Number of animals per group and experimental period: 5. ANOVA showed no statistically significant difference among the groups.

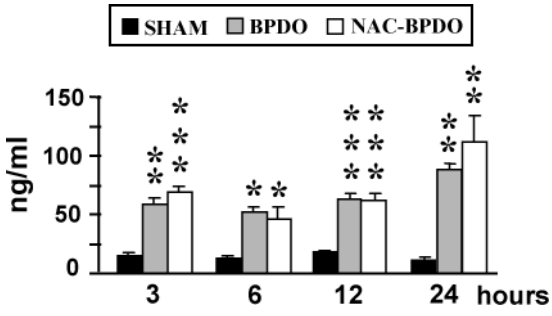

Figure 4. Plasma soluble ICAM-1 (sICAM-1) levels. Values are expressed as means ± SEM. Number of animals per group and experimental period: 6. ANOVA followed by Scheffé test showed significant differences vs. sham-operated animals. (* $P < 0.05$; ** $P < 0.01$; ***$P < 0.001$).

CD18 (Fig. 5B), which significantly ($P < 0.05$) increased from 6 hrs after BPDO onwards (Fig. 5C). CD11b/CD18 upregulation was inhibited in neutrophils and delayed and significantly reduced in monocytes by NAC treatment.

Myeloperoxidase (MPO) Activity. Neutrophil infiltration in the pancreas measured as MPO activity (Fig. 6) significantly increased from 6 hrs after BPDO onwards. NAC treatment delayed and significantly ($P < 0.05$) reduced the accumulation of neutrophils in pancreas until 24 hrs after BPDO.

Histological Assessment. Histological findings (Fig. 7) confirmed the development of acute edematous pancreatitis with a significant ($P < 0.001$) vacuolization from 3 hrs after BPDO. Leukocyte infiltration significantly

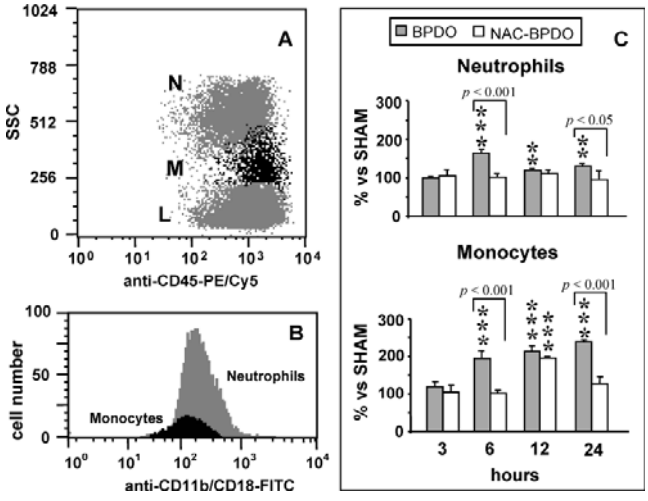

Figure 5. (A) Flow cytometric distribution of neutrophils (N), monocytes (M), and lymphocytes (L) according to side scatter (SSC) and CD45 labeling as detected by phycoerythrin/cyanin 5 (PE/Cy5). (B) Representative example of neutrophil (grey) and monocyte (black) distribution according to the intensity of fluorescence due to anti-CD11b/CD18-fluorescein isothiocyanate (FITC) labeling. (C) Percentage variation in mean fluorescence intensity with respect to sham-operated animals. Values are expressed as means ± SEM. Number of animals per group and experimental period: 5. ANOVA test followed by Scheffé test showed significant differences vs. sham-operated animals (** $P < 0.01$; ***$P < 0.001$) and vs. BPDO ($P < 0.05$; $P < 0.001$).

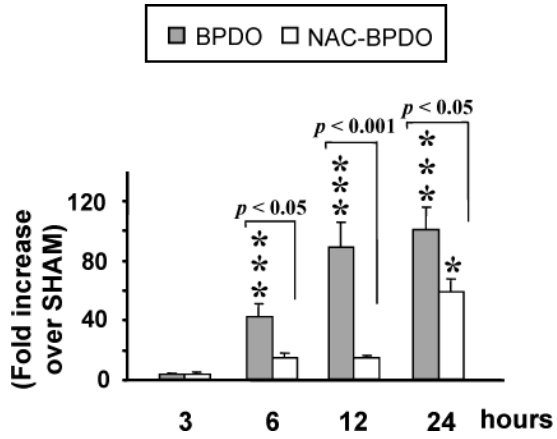

Figure 6. Myeloperoxidase (MPO) activity in pancreatic tissue. Values are expressed as changes with respect to sham animals (means ± SEM). Number of animals per group and experimental period: 6. ANOVA followed by Scheffé test showed significant differences vs. sham-operated animals (* $P < 0.05$; *** $P < 0.001$) and vs. BPDO ($P < 0.05$; $P < 0.001$).

($P < 0.001$) increased from 6 hrs after BPDO onwards and focal necrosis was found 24 hrs after BPDO. NAC treatment significantly reduced all histological alterations induced by BPDO.

Discussion

Many studies have shown that infiltrating leukocytes play a key role in the pathogenesis of AP leading to pancreatic and extrapancreatic manifestations (24). A complex cascade of events is involved in the initial recruitment of leukocytes to the damaged pancreas. It requires the activation of endothelial cells by gland-derived signals that induce the expression of adhesion molecules, which in turns act as ligands of leukocyte membrane receptors to trigger leukocyte-endothelial cell interactions. Analysis carried out in tissue samples have reported ICAM-1 increases in pancreas and lung during the progression of AP (12, 16, 17, 25). Our results showed an increase in ICAM-1 mRNA expression in acinar cells at early stages of AP, which led to the upregulation of ICAM-1 expression in acinar cell membrane only 3 hrs after inducing AP by BPDO. ICAM-1 is an inducible protein expressed on the surface of endothelial cells, which in the absence of inflammatory stimuli can be detected at very low levels on a few types of cells (11). In line with our results, Zaninovic *et al*. (17) recently reported that ICAM-1 is upregulated in acinar cells of rats with AP induced by caerulein, but in contrast with our experimental model ICAM-1 remained overexpressed during the next 6 hrs after inducing AP.

Since shedding of ICAM-1 may occur after expression by TNF-α regulated mechanisms (26), we wondered if ICAM-1 would also be continuously upregulated in acinar cells during BPDO-induced AP but would be undetectable because of their release into the extracellular milieu. Analysis carried out in the acinar cell culture medium

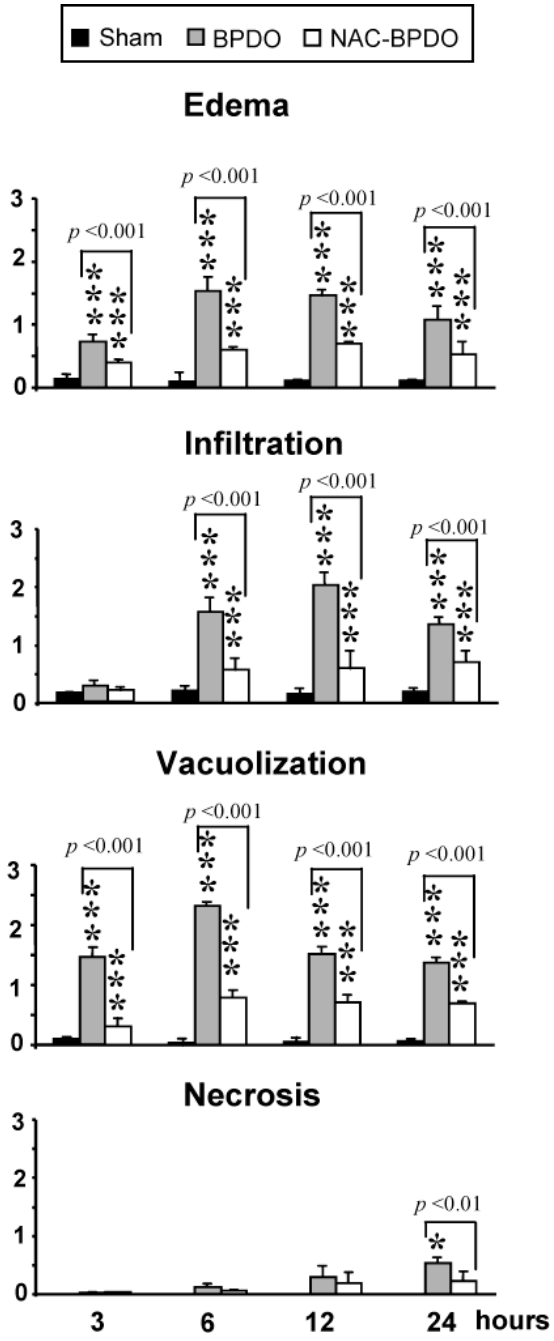

Figure 7. Histological alterations in pancreas (score ranging from 0 to 3 is described in Materials and Methods). Values are expressed as means ± SEM of the results on five animals per group and experimental period. ANOVA test followed by Scheffé test showed significant differences vs. sham-operated animals (* $P < 0.05$; *** $P < 0.001$) and vs. BPDO ($P < 0.01$; $P < 0.001$).

revealed no release of ICAM-1 throughout 24 hrs BPDO, suggesting that although acinar cells are able to produce ICAM-1 at early AP, they make a minimum contribution to the increased ICAM-1 levels found in plasma in the course of pancreatitis. Therefore, other endothelial cells, probably of vascular origin, should be considered as the main source of ICAM-1 during BPDO-induced AP.

The fact that ICAM-1 upregulation, whatever the source, preceded pancreatic neutrophil infiltration could suggest that ICAM-1 plays a key role in the recruitment of inflammatory cells to the damaged tissue during AP. In this regard, previous studies have reported a reduction of local and systemic injury associated with AP in ICAM-deficient mice (12) or after the blockade of ICAM-1 expression with monoclonal antibodies (27–29). However, the results obtained in the present study from an overall comprehensive analysis of ICAM-1 acinar expression, ICAM-1 plasma levels, and CD11b/CD18 leukocyte expression suggest that although ICAM-1 may be involved, other components are contributing to the sequestration of leukocytes within the pancreas.

This notion is supported by the fact that in contrast to ICAM-1, CD11b/CD18 is constitutively expressed in neutrophil and monocyte membrane (18) at high enough levels to interact with its counter-receptor if expressed. However, our results indicated that although ICAM-1 was upregulated from early stages of AP, it was not able to act as an effective ligand for the leukocyte receptor to promote pancreatic infiltration. These observations would explain why complete amelioration of the disease has not been achieved in studies of ICAM-1 expression blockade.

In line with our results, Folch *et al.* (30) reported the total prevention of leukocyte infiltration in the pancreas of rats with taurocholate-induced AP by blockade of P-selectin but not with anti-ICAM-1 treatment. Further studies focused on the role of selectins would be of great interest in BPDO-induced AP, since multiple pathways for leukocyte homing may be involved and their relative contribution may vary in different tissues and models of pancreatitis.

Oxygen radical production increases considerably during AP (31), mainly from acinar cells from early stages of the disease (21). The role of oxidative stress in leukocyte infiltration process during AP still remains controversial. *In vitro* studies have shown that ROS function as important messengers for ICAM-1 expression in endothelial cells, at least in part through the activation of NF-κB (19, 20). Telek *et al.* (16) reported a chronological and topographical overproduction of ROS and ICAM-1 upregulation during AP.

On this basis, several antioxidants have been used in *in vivo* and *in vitro* studies to interfere with the expression of ICAM-1, but controversial results have been reported. DMSO has been shown to be able to reduce ICAM-1 expression in pancreas of rats with necrotizing AP (32) and it also inhibited ICAM-1 gene expression in septic rats (33). Pyrrolidine dithiocarbamate (PDTC), vitamin E, and allopurinol were also able to reduce ICAM-1 expression in IFN-γ-stimulated ECV304 and SKHEP-1 cells. However, no inhibitory effect has been shown by NAC (34), a compound widely considered to be an active antioxidant by acting as a precursor of GSH and by directly scavenging ROS (35).

In accordance with the results obtained in cell lines (34) and in experimental models of ischemia/reperfusion injury (36), unexpectedly, no reduction of ICAM-1 was found in the present study, either in acinar cells or plasma, in rats with BPDO-induced AP treated with a single dose of NAC. However, NAC proved to be capable of abolishing the overproduction of ROS in acinar cells of rats subjected to the same AP model (21). Taken together, these findings suggest that ROS are not important factors mediating ICAM-1 expression.

In contrast, NAC reduced the overexpression of CD11b/CD18 in neutrophils and monocytes and pancreatic infiltration. This finding reinforces the notion that ICAM-1 is not the main molecule involved in the adhesion of leukocytes during AP induced by BPDO, since NAC treatment significantly protected the pancreas from inflammation although ICAM-1 expression was not abrogated. In addition, we can deduce that CD11b/CD18 overexpression during BPDO-induced AP appears to be triggered by oxidative-dependent mechanisms and contributes to pancreatic injury by promoting leukocyte infiltration.

In summary, the data show that ICAM-1 is upregulated at different locations, including pancreatic acinar cells, from early stages of BPDO-induced AP by oxidant-nondependent mechanisms. However, other molecules seem to be required in the recruitment of leukocytes. In contrast, there is evidence of a correlation between the infiltration of neutrophils within the pancreas and the overexpression of the β2-integrin, CD11b/CD18, in leukocytes, an event which resulted sensitive to antioxidant treatment. Multiple pathways may contribute to the sequestration of leukocytes within damaged tissue during AP. For this reason, if the different molecules mediating this phenomenon could be identified it would help to trigger therapeutic approaches used to interfere in the interaction of leukocytes and adhesion molecules, hindering local and systemic inflammation during AP.

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