Membrane-bound ICAM-1 is upregulated by trypsin and contributes to leukocyte migration in acute pancreatitis

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Membrane-bound ICAM-1 is upregulated by trypsin and contributes to leukocyte migration in acute pancreatitis. Am J Physiol Gastrointest Liver Physiol 287: G1194–G1199, 2004. First published August 12, 2004; doi:10.1152/ajpgi.00221.2004.—In acute pancreatitis, ICAM-1 is upregulated in various organs and contributes to the development of organ injury. To investigate the effects of pancreatic proteases on ICAM-1 expression and their role in the early process of leukocyte migration, human umbilical vein endothelial cells (HUVECs) were incubated with serum subjected to limited trypsin digestion and Wistar rats were injected with trypsin. Significant upregulation of membrane-bound ICAM-1 was seen on HUVECs incubated with trypsinated serum. Likewise, soluble ICAM-1 increased in the supernatant of HUVECs. Changes of membrane-bound ICAM-1 and soluble ICAM-1 were maximal with high concentrations of trypsin. HUVECs incubated with TNF-α (positive control) showed similar changes. In the pancreas and lungs of animals infused with trypsin, ICAM-1 and leukocyte sequestration were increased compared with controls. Reflecting the relevance of protease-induced ICAM-1 expression in leukocyte migration, leukocyte-endothelium interaction, as assessed by intravital microscopy, was markedly increased by trypsin. Inhibition of ICAM-1 ameliorated these changes significantly. In conclusion, trypsinated serum induces the upregulation of both membrane-bound ICAM-1 on endothelial cells and soluble ICAM-1. These changes contribute to the early steps of leukocyte migration in acute pancreatitis. The role of soluble ICAM-1 remains to be investigated.

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serum alone served as controls. Incubation was allowed to proceed for 3, 6, 9, 12, and 24 h at 37°C. After incubation, the supernatant was frozen and stored at −80°C until 70–90% confluence and then subcultured onto flat-bottom microwell plates. The plates were further incubated in the humidified incubator for 1–3 days to allow cell adherence and growth to desired density. Cells between passages 5 and 12 were used for all experiments.

**Limited trypsin digestion of serum.** Non-heat-inactivated bovine serum was subjected to limited trypsin digestion by incubation with bovine trypsin (final concentration 10⁻⁶–10⁻⁴ M, n = 5 samples per group) and simultaneous addition of soybean trypsin inhibitor twice the concentration of trypsin (both from Sigma). Incubations were performed for 20 min at room temperature. Unlike incubation of serum with trypsin alone, limited trypsin digestion has previously been shown to effectively activate complement components in human serum and to induce neutrophil activation (33). Significant residual trypsin activity is not present in these serum preparations as measured spectrophotometrically using p-toluene sulfonfyl-l-arginine methyl ester (Sigma, St. Louis, MO) as substrate (12). After completion of incubation, trypsinated serum was frozen and stored at −80°C until being used for HUVEC incubations.

**Cellular ELISA for ICAM-1.** The expression of ICAM-1 on HUVECs incubated with serum subjected to limited trypsin digestion was quantified by using a modified ELISA assay as described previously (24). Briefly, after completion of the incubation protocol and subtraction of the supernatant, cells were washed with PBS/Tween buffer (PBST), fixed with 1% paraformaldehyde (Sigma) for 30 min, and washed again with PBST. Nonfat dry milk (5%) was added to reduce nonspecific binding. Cells were incubated with 100 µl of monoclonal anti-human ICAM-1 (CD54; Pharmingen, San Diego, CA), diluted 1:1000 in culture medium for 45 min, washed 5 times with PBST, and incubated in the dark with 100 µl of biotinylated anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA) for 30 min. Subsequently, cells were washed as above and incubated in the dark with 100 µl streptavidin horseradish peroxidase (DAKO, Carpinteria, CA) for 30 min. After another washing with PBST, 100 µl of TMB peroxidase substrate (tetramethyl benzidine dihydrochloride K-Blued MAX; Neogen Lexington, KY) was added for 15–30 min. The reaction was halted by the addition of 1 M sulfuric acid (50 µl/well). All incubations were carried out at room temperature. The expression of ICAM-1 was determined by the difference in absorbance at wavelength 450 vs. 630 nm measured by an automated microplate reader.

An ELISA-based cell count was performed to correct for possible differences in the amount of HUVECs in the wells after incubation with trypsinated serum or TNF-α. After the cellular ELISA for ICAM-1, cells were washed in tap water. Crystal violet (0.08%, Sigma) was added for 5 min, followed by another thorough wash in tap water. Acetic acid (33%) was used to solubilize the nuclear stain. Optical density was read at 570 nm, representing the actual cell count per well.

Likewise, soluble ICAM-1 was determined in the supernatant of incubated HUVECs. After completion of incubations, the supernatant was transferred to separate 96-well Nunclon plates. Plates were kept overnight at 4°C to allow binding of protein to the wells. ELISA was then performed as described in *Cellular ELISA for ICAM-1*, starting with the addition of nonfat dry milk to reduce nonspecific antibody binding. Results of soluble ICAM-1 are expressed as the percentage of ICAM-1 in the supernatant at baseline.

**In Vivo Experiments**

The early effects of trypsin released into the systemic circulation in acute pancreatitis were imitated in the present study by intravenous infusion of trypsin into healthy rats. Previously, it has been shown by our laboratory (12, 15) that infusion of trypsin, as well as infusion of trypsinated serum into rats, results in pancreatic and pulmonary injury similar to that found in acute pancreatitis. Unlike in vitro incubations, which necessitate serum subjected to limited trypsin digestion (see *Limited trypsin digestion of serum*), the presence of serum containing plenty of antiproteases in the rat allows the use of trypsin infusion for in vivo experiments.

**Experimental protocol.** Bovine trypsin (Sigma) was infused intravenously into rats (n = 6) for 6 h at 60 nmol·kg⁻¹·h⁻¹. At this concentration, trypsin causes significant pancreatic and pulmonary injury, as assessed by the wet-to-dry ratio, myeloperoxidase activity, and histology (15). Infusions were divided into 3 aliquots/h, and each aliquot was freshly reconstituted in saline before injection to minimize autodigestion of trypsin. Saline was infused into control animals. At 6 h after the start of infusions, leukocyte-endothelium interaction and capillary blood flow were assessed in the head of the pancreas by intravital microscopy. In additional animals infused with trypsin, ICAM-1 expression in the pancreas and lungs was measured by Western blot analysis. Pancreatic and pulmonary leukocyte sequestration was assessed by activity of myeloperoxidase, an enzyme stored in the azurophilic granules of neutrophils. To evaluate whether changes in leukocyte-endothelium interaction induced by trypsin are related to ICAM-1 upregulation, one group of animals (n = 6) was pretreated with purified anti-rat ICAM-1 antibody (1.0 mg/kg; Pharmingen) before trypsin infusions were started.

**Animals.** Inbred male Wistar rats weighing 250–300 g were used for experiments. Care was provided in accordance with the German law for care and use of laboratory animals. The study was approved by the Regierungspräsidium Karlsruhe, Germany, committee on animal care. Animals were fasted overnight before the experiments but allowed free access to water.

**Anesthesia and catheter placement.** Surgical anesthesia was induced by intraperitoneal injection of pentobarbital sodium (10 mg/kg, Nembutal, Sanofi-Ceva, Genova, Germany) and intramuscular injection of ketamine (40 mg/kg Ketanest S; Parke-Davis, Berlin, Germany). Two polyethylene catheters (ID, 0.5 mm) were inserted into the left carotid artery and the right internal jugular vein, respectively, tunneled subcutaneously to the suprascapular area, and exited through a steel tether that allowed the animals free movement. The venous catheter was used for infusion regimens, and the arterial line was used for blood sampling and hemodynamic monitoring.

**Intravital microscopy.** Microcirculatory changes of the pancreas caused by trypsin infusion were assessed by intravital microscopy as described in detail elsewhere (16). Briefly, after a midline laparotomy was performed, the pancreas with the duodenal loop was gently exteriorized and placed in an immersion chamber containing Ringers lactate maintained at 37°C. The pancreatic microcirculation was then evaluated in epi-illumination using a fluorescence microscope (Fa. Leitz, Wetzlar, Germany). Therefore, animals were injected with FITC-labeled erythrocytes (0.5 ml/kg, hematocrit 50%) for capillary blood flow measurements and Rhodamin 6G (bolus of 0.1 ml) for in vivo staining of leukocytes and quantitation of leukocyte-endothelium interaction. Mean capillary red blood cell velocity was analyzed in four different regions of the pancreas in each rat. The mean red blood cell velocity in each area was calculated by averaging the velocity of erythrocytes in 15 ± 2 capillaries. According to their interaction with the endothelial lining, adherent and rolling leukocytes were assessed in postcapillary venules with a diameter of 25–40 µm. Adherent leukocytes (stickers) were defined as cells that did not move or detach from the endothelium within the observation period of 30 s (10). Rolling leukocytes (rollers) were defined as those white cells moving at a velocity less than two-thirds of that of erythrocytes in the
centerline of the venule (42). Both stickers and rollers were expressed as the number of cells per square millimeter of vessel surface, calculated from the diameter and length (100 μm) of the vessel segment studied. Off-line analysis of video recordings was performed in a blinded fashion using a computer-assisted microcirculation analysis system (Cap Image; Dr. H. Zeintl, Heidelberg, Germany).

**Gel electrophoresis and Western blot analysis.** Pancreas and lung tissue were homogenized in sodium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (Sigma) and 5% soybean trypsin inhibitor at a tissue-to-buffer ratio of 1:1.5. Protein concentration was determined by protein assay (Pierce, Perbio Science Deutschland, Bonn, Germany) and adjusted to a final protein concentration of 1.8 mg/ml. Electrophoresis was performed by SDS-PAGE. A total of 30 μl of tissue sample per well was loaded on the gel in nonreducing sample buffer. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, München, Germany). The blotted membrane was blocked with nonfat dry milk (5%) in TBS/Tween-buffer (TBST) and agitated for 2 h with a mouse anti-rat ICAM-1 antibody (1:100 dilution in TBST; Serotec, Düsseldorf, Germany). After samples were washed with TBST, the secondary reagent, goat anti-mouse IgG linked to horseradish peroxidase (1:10,000 dilution, Linearis, Wertheim-Bettingen, Germany) was added. After repeated washing with TBST, the membrane was rinsed with water. The method of enhanced chemiluminescence (ECL Plus; Amersham Biosciences, Freiburg, Germany) was chosen for detection, according to the manufacturer’s instruction. All incubations were carried out at room temperature.

**Myeloperoxidase activity.** Excised pancreatic and pulmonary tissues were rinsed with saline, blotted dry, snap frozen in liquid nitrogen, and stored at −80°C until thawing for determination of myeloperoxidase activity as previously described (15).

**Statistical Analysis**

Data are presented as means ± SE. One-way ANOVA was used for evaluation of differences between groups (when >2), followed by post hoc comparison of the means of the groups. Student’s t-test was used for pairwise comparison of normal, distributed parameters. Values of P < 0.05 were considered significant.

**RESULTS**

**In Vitro Experiments**

**ICAM-1 expression on HUVECs.** The expression of ICAM-1 on HUVECs incubated with trypsinated serum was significantly increased compared with controls incubated with untreated serum. Levels of ICAM-1 expression increased with higher concentrations of trypsin used for serum trypsination (Fig. 1). Highest levels of ICAM-1 expression were found at 9 h after the start of incubations. Larger amounts of trypsinated serum used for serum incubations (50 instead of 20 μl) did not further increase ICAM-1 expression. However, peak levels of ICAM-1 occurred earlier (data not shown).

HUVECs incubated with TNF-α showed similar changes and served as positive controls (Fig. 1). No significant changes of ICAM-1 expression were found when HUVECs were incubated with medium or untreated serum (Fig. 1). The ELISA-based cell count, which was performed to correct for differences in the amount of cells per well, did not show any significant differences among incubations with medium, control serum, trypsinated serum, or TNF-α.

Similar to membrane-bound ICAM-1, significant amounts of soluble ICAM-1 were found in the supernatant of HUVECs stimulated with trypsinated serum. Again, levels of soluble ICAM-1 increased with higher amounts of trypsin used for serum trypsination (Fig. 2). When trypsin was used at a concentration of 10⁻⁴ M, levels of soluble ICAM-1 peaked at 9 h of HUVEC incubation but decreased significantly thereafter. HUVECs incubated with TNF-α showed highest levels of soluble ICAM-1 at 12 h. Unlike incubations with trypsinated serum, soluble ICAM-1 levels remained elevated for the entire observation period of 24 h (Fig. 2).

**In Vivo Experiments**

In confirmation of our in vitro findings, Western blot analysis demonstrated higher levels of ICAM-1 expression in the lungs of animals injected with trypsin compared with control animals injected with saline only (Fig. 3). ICAM-1 expression was more pronounced in the lungs than in the pancreas. Likewise, animals showed significant organ injury with significant leukocyte sequestration in the pancreas and lungs as determined by myeloperoxidase activity (pancreas: 13.3 ± 1.9 vs. 6.1 ± 0.9 U/mg; lungs: 112.2 ± 9.7 vs. 47.8 ± 7.2 U/mg; both trypsin vs. control). This is in agreement with previous studies (15).

Both rollers and stickers were significantly increased in the pancreatic microvasculature of animals infused with trypsin.
compared with controls that were injected with saline only (both $P < 0.001$ vs. control, Fig. 4). At the same time, pancreatic microcirculatory perfusion decreased significantly in these animals ($P < 0.01$, Fig. 5). Importantly, macrohemodynamic parameters did not show any significant changes among the groups (data not shown).

When animals infused with trypsin were pretreated with a monoclonal antibody against ICAM-1, the number of stickers was significantly decreased compared with animals without pretreatment ($P = 0.04$, Fig. 4B). Likewise, pancreatic perfusion was less profoundly reduced with anti-ICAM-1 pretreatment ($P = 0.03$, Fig. 5). However, no significant differences were found in rolling leukocytes (Fig. 4A).

**DISCUSSION**

Systemic complications are frequently associated with SIRS in the early phase of acute pancreatitis, and neutrophils play a critical role in the pathogenesis of these complications. ICAM-1, an adhesion molecule of the immunoglobulin superfamily, contributes to the firm adhesion of leukocytes to the endothelium and their subsequent transendothelial migration (39). It is constitutively expressed on endothelial and epithelial cells and to a lesser extent on leukocytes. However, ICAM-1 expression is induced in a cell-specific manner by several proinflammatory mediators (7, 17, 30). In the present study, we have demonstrated that ICAM-1 is upregulated on endothelial cells that were stimulated by trypsinated serum. ICAM-1 expression was maximal when high trypsin concentrations were used for serum incubations and peaked at 6–9 h of HUVEC stimulation. This time interval is possibly explained by the transcriptional regulation of ICAM-1. Interestingly, ICAM-1 expression was increased in the trypsin-infused group compared to the control group (Fig. 2).

**Fig. 2.** Soluble ICAM-1 was determined in the supernatant of incubated HUVECs. Soluble ICAM-1 is expressed as the percentage of ICAM-1 in incubations with control serum.

**Fig. 3.** Immunoblots of ICAM-1 in pancreas and lung tissue. The expression of ICAM-1 in the pancreas and lungs of trypsin-infused animals and control animals was assessed by Western blot analysis. The monoclonal antibody used for blotting showed cross-reactivity with recombinant human ICAM-1, which served as a control marker.

**Fig. 4.** Rolling (A) and adherent (B) leukocytes were assessed in postcapillary venules of the pancreas with a diameter of 25–40 μm. Adherent leukocytes (stickers) were defined as cells that did not move or detach from the endothelium within the observation period of 30 s. Rolling leukocytes (rollers) were defined as those white cells moving at a velocity $\frac{2}{3}$ of that of erythrocytes in the centerline of the venule. Both stickers and rollers were expressed as the number of cells per square millimeter of vessel surface. $^*P < 0.01$ vs. control, $^\dagger P = 0.04$ vs. trypsin only.
In acute pancreatitis, ICAM-1 is upregulated in both the pancreas and the lungs, and the extent of ICAM-1 expression correlates with the severity of organ injury (26, 41). In previous studies (11, 15, 40), we have shown that the occurrence and severity of pancreatitis-associated lung injury corresponds to the levels of circulating trypsinogen and its activation to trypsin and that neutrophils are involved in the development of this injury. Furthermore, it has been demonstrated that the expression of Mac-1 (CD11b/CD18), the ligand to endothelial ICAM-1, is increased on the surface of neutrophils incubated with trypsinated serum (12). Mac-1, a membrane-bound adhesion molecule of the integrin group, is primarily expressed on the surface of neutrophils on stimulation. There is general agreement that the firm adhesion of neutrophils to the endothelial lining is, at least in part, mediated by the interaction of ICAM-1 and Mac-1 (4). With the indirect trypsin-induced upregulation of both Mac-1 and ICAM-1, we have identified trypsin as a mediator capable of inducing neutrophil and endothelium activation, followed by extravasation of cells and manifestation of organ injury in acute pancreatitis.

Serine protease trypsin has been assumed for a long time to cause local and distant organ injury in acute pancreatitis. Whereas intra-acinar and interstitial trypsinogen activation are widely accepted to be central events in the early pathogenesis of acute pancreatitis (8, 13, 37), the role of trypsin in the pathogenesis of distant organ injury is controversial. Although selective trypsin infusion caused lung injury in several animal models (5, 15, 38), systemic trypsin has been questioned as playing a significant role in acute pancreatitis due to its quick binding to protease inhibitors. In the present study, we demonstrate that trypsin-induced changes in serum can induce adhesion molecule expression and thereby initiate leukocyte migration into tissue. The limited trypsin digestion of serum, as applied in our study, included the simultaneous addition of soybean trypsin inhibitor twice the concentration of trypsin. Unlike trypsin alone, using this procedure has also been demonstrated as inducing neutrophil activation with subsequent induction of the respiratory burst (33). It may closely reflect in vivo conditions in which protease inhibitors are present in excess.

Recently, several studies (1, 27) have focused on the regulation of adhesion molecules in acute pancreatitis. Confirming our results, increased expression of Mac-1 or ICAM-1 was found on neutrophils and endothelial cells incubated with serum or ascites from animals with acute pancreatitis. The proinflammatory substances that are possibly inducing this cell activation include proteases other than trypsin, cytokines, or complement proteins. Interestingly, complement activation has been demonstrated by trypsin (2, 28, 33) and also in acute pancreatitis (18, 34). Therefore, complement proteins may mediate the trypsin-induced leukocyte activation and thereby contribute to the development of systemic organ injury in acute pancreatitis (12).

Most of the membrane-bound adhesion molecules are also known to exist in a soluble form. Soluble ICAM-1 was first described in 1991 (32, 35) in the blood of healthy human subjects. Subsequently, a number of studies (6, 20, 29) reported increased levels of soluble ICAM-1 in inflammatory disease, but little is known about soluble ICAM-1 in acute pancreatitis. Our in vitro experiments showed elevated levels of soluble ICAM-1 in the supernatant of HUVECs activated by trypsinated serum, and soluble ICAM-1 increased with higher amounts of trypsin used for serum trypsination. This finding is in agreement with the proposal that soluble adhesion molecules may reflect endothelial cell activation and the magnitude of expression of their membrane-bound counterparts. Whether the circulating levels of soluble ICAM-1 are useful markers or predictors for the severity of acute pancreatitis, as suggested by recent studies (23, 31), remains to be investigated.

In summary, we have demonstrated that trypsin induces changes in serum that result in upregulation of ICAM-1 and in leukocyte migration. Inhibition of ICAM-1 expression may be a potential therapy of systemic complications in acute pancreatitis. A diagnostic tool may be available by measurement of soluble ICAM-1 in serum of patients.

**GRANTS**

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DISCLOSURES

Data from this study were presented at the American Pancreatic Association (Chicago, IL, November 1–3, 2001) and at the combined meeting of the International Association of Pancreatology and the European Pancreas Club (Heidelberg, Germany, June 19–22, 2002).

REFERENCES