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

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Hartwig, Werner, Jens Werner, Andrew L. Warshaw, Bozena Antoniu, Carlos Fernandez-del Castillo, Martha-Maria Gebhard, Waldemar Uhl, and Markus W. Büchler. 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.

adhesion molecules; endothelial cells; proteases; systemic inflammatory response syndrome

SYSTEMIC INFLAMMATORY response syndrome (SIRS) with concomitant manifestation of multiple organ failure is frequently found in severe acute pancreatitis. A substantial number of patients develop SIRS within the first week after onset of symptoms and need intensive care treatment due to the development of cardiovascular, pulmonary, or renal insufficiency (3, 14, 21, 22). A critical role in the pathogenesis of local and distant organ injury in this setting is attributed to neutrophils. Neutrophils are activated by proinflammatory mediators and infiltrate the pancreas as well as distant organs (11, 19). The trafficking of neutrophils into tissue involves sequential leukocyte-endothelial cell interactions that are described as leukocyte rolling (low-affinity adhesion), leukocyte sticking (firm adhesion), and leukocyte transmigration. Leukocyte trafficking is mediated by complex interactions between adhesion molecules expressed on leukocytes and their corresponding ligands on endothelial cells (4, 36).

ICAM-1 (CD54), a member of the immunoglobulin superfamily, is expressed constitutively at a low extent on the surface of endothelial cells. On cell activation, ICAM-1 is upregulated and participates in the firm adhesion of leukocytes to endothelium and in transendothelial cell migration (39). In acute pancreatitis, ICAM-1 is known to be upregulated in the pancreas and lungs, and both onset and extent of ICAM-1 expression correlate with the severity of organ injury (9, 26, 41). Furthermore, genetically induced ICAM-1 deficiency or administration of monoclonal antibodies against ICAM-1 has been proven to be effective in the prevention and treatment of these complications (9, 25, 41).

Although various mediators have been implicated in the upregulation of ICAM-1 in inflammatory disease (7, 17, 30), it is yet unknown which factors are involved in this process in acute pancreatitis. Transcriptional activation of the adhesion molecules ICAM-1 and VCAM-1 expression was found when endothelial cells were incubated with ascites collected from animals with necrotizing pancreatitis (27). Recently, our laboratory (12) has demonstrated that Mac-1 (CD11b/CD18), the ligand to ICAM-1, is upregulated on neutrophils incubated with trypsinated serum, giving evidence that the serine protease trypsin induces changes in serum, which cause leukocyte activation in acute pancreatitis, possibly by complement activation. In the present study, we provide direct evidence for the central role of trypsin in the initiation of leukocyte migration and organ injury. Trypsin induces changes in serum that result in ICAM-1 expression on endothelial cells in vitro, upregulates ICAM-1 expression in the pancreas and lungs in vivo, and increases ICAM-1-dependent interaction between leukocytes and the endothelial lining.

MATERIALS AND METHODS

In Vitro Experiments

Human umbilical vein endothelial cell incubations. Human umbilical vein endothelial cells (HUVECs) were plated at a concentration of 3 × 10^5 cells/well in 96-well, flat-bottom, microwell plates (Nunclon; Fisher Scientific, Pittsburgh, PA) and grown to confluence at 37°C in a humidified incubator with 5% CO₂-95% air. For incubations, 20 μl (or 50 μl) of serum subjected to limited trypsin digestion (see Limited trypsin digestion of serum) were added to HUVECs. HUVECs incubated with TNF-α (400 and 1000 U/ml; Sigma, St. Louis, MO) or

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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 withdrawn from endothelium layers and transferred to separate Nunclon microwell plates for measurement of soluble ICAM-1. Membrane-bound as well as soluble ICAM-1 were measured quantitatively by ELISA.

Cells and cell culture. HUVECs, EBM-2 endothelial growth cell medium, and EGM-2 supplement were purchased from Clonetics (San Diego, CA). HUVECs were grown on 100 × 20-mm tissue culture-treated dishes (Corning, Corning, NY) 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 sulfonyl fluoride methyl ester (Sigma, St. Louis, MO) as substrate (12). After completion of incubation, trypsinized 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 trypsinized 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 trypsinized 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, is 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 50; 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 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
ICAM-1 regulation in acute pancreatitis

In vitro experiments

Materials and Methods

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. Interest-
upregulation of both Mac-1 and ICAM-1, we have identified ICAM-1 and Mac-1 (4). With the indirect trypsin-induced epithelial lining is, at least in part, mediated by the interaction of agreement that the firm adhesion of neutrophils to the endothelial surface of neutrophils on stimulation. There is general sion molecule of the integrin group, is primarily expressed on expression of Mac-1 (CD11b/CD18), the ligand to endothelial this injury. Furthermore, it has been demonstrated that the 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. In our in vivo 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


