Cerulein upregulates ICAM-1 in pancreatic acinar cells, which mediates neutrophil adhesion to these cells

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Cerulein upregulates ICAM-1 in pancreatic acinar cells, which mediates neutrophil adhesion to these cells. Am J Physiol Gastrointest Liver Physiol 279: G666–G676, 2000.—Neutrophil infiltration into the pancreas is a key event in pancreatitis. Here we show that intercellular adhesion molecule-1 (ICAM-1), which regulates neutrophil adhesion, is present on rat pancreatic acinar cells, is upregulated by a hormone (cerulein) and mediates direct binding of neutrophils to acinar cells. ICAM-1 was upregulated in pancreas of rats with experimental pancreatitis induced by supramaximal doses of cerulein. Furthermore, cerulein time and dose dependent stimulation of expression of ICAM-1 mRNA and protein in isolated pancreatic acinar cells. Inhibitory analysis showed that activation of transcription factor nuclear factor-κB (NF-κB) was involved in ICAM-1 upregulation by cerulein, but NF-κB did not mediate basal expression of ICAM-1 mRNA in acinar cells. With an adhesion assay, we found that neutrophils bind to isolated pancreatic acinar cells and that cerulein upregulates the extent of adhesion. Neutralizing ICAM-1 antibody blocked neutrophil binding to both control and cerulein-stimulated acinar cells, suggesting ICAM-1 involvement in this adhesion. Thus the acinar cell is capable of targeting neutrophils to its surface, a process that may be important for inflammatory and cell death responses in pancreatitis and other pancreatic disorders.

INFLAMMATORY INFILTRATION is one of the main characteristics of human and experimental pancreatitis (42, 45). Using an experimental model of pancreatitis induced in rats by infusion of supramaximal doses of the CCK analog cerulein (12), we (38) and others (10, 19) showed that neutrophil infiltration plays a key role in the development of both inflammatory and cell death responses in the disease. In particular, neutrophil depletion ameliorated the parameters of pancreatitis: pancreatic edema, vacuole formation in acinar cells, increases in blood levels of amylase and lipase, and pancreatic necrosis (38). However, the mechanisms that mediate neutrophil recruitment into the pancreas remain largely unknown.

ICAM-1 plays a central role in mediating leukocyte adhesion to endothelial and some epithelial cells (14, 41). ICAM-1 (CD54) is a cell surface glycoprotein that belongs to the immunoglobulin superfamily of adhesion molecules and binds to β2-integrin counterreceptors LFA-1 and MAC-1 (41, 44). This interaction is critical for leukocyte adhesion to endothelial cells, a first step in leukocyte transmigration into the areas of inflammation (14, 41). ICAM-1 has a restricted tissue distribution and is constitutively expressed at low levels on endothelial and some epithelial cells (1, 2, 14, 21, 44). Its expression is markedly upregulated at sites of inflammation (1, 2, 41, 44).

The ICAM-1 gene promoter/enhancer has binding sites for a number of transcription factors (8, 9, 20, 36), in particular, nuclear factor-κB (NF-κB), which is involved in cytokine-induced upregulation of ICAM-1 expression in some cell types (8, 20, 24, 27). NF-κB mediates rapid induction of cytokines and adhesion molecules implicated in the immune and inflammatory responses (4, 46). In most resting cells, NF-κB is kept silent in the cytoplasm by association with inhibitory proteins of the IκB family. On cell activation, IκB is hyperphosphorylated and rapidly degraded via a proteasome-involving pathway. The free NF-κB is then rapidly translocated into the nucleus and activates the expression of genes that have κB binding sites.

Recently, we showed (16) that pancreatic acinar cells express and produce tumor necrosis factor-α (TNF-α), a powerful cytokine. Furthermore, we showed that cerulein and CCK caused NF-κB activation, both in vitro, in isolated acinar cells, and in vivo, in pancreas from rats with experimental pancreatitis (17).

The pivotal role of ICAM-1 in regulating leukocyte recruitment has been demonstrated in a number of models of gastrointestinal and liver inflammation (2, 3, 14, 29). Patients with severe pancreatitis have elevated levels of circulating ICAM-1 (26). It has recently been reported that the severity of cerulein pancreatitis was reduced in ICAM-1 knockout mice (10). However,

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very little is known about ICAM-1 expression in pancreas as well as the mechanism(s) of its regulation. The interaction of pancreatic acinar cells with neutrophils and the possible involvement of ICAM-1 in this process have not yet been addressed.

In the present study we examined the presence of ICAM-1 in pancreatic acinar cells, its regulation by cerulein in vivo and in vitro, and whether ICAM-1 can mediate a direct interaction of pancreatic acinar cells with neutrophils. The results show that 1) ICAM-1 is present in rat pancreas and in isolated acinar cells; 2) cerulein upregulates the expression of both ICAM-1 mRNA and protein in pancreas from rats with cerulein-induced pancreatitis and in isolated acinar cells; and 3) ICAM-1 mediates neutrophil adhesion to pancreatic acinar cells in vitro, which is stimulated by cerulein.

METHODS

Experimental model of pancreatitis. Experimental pancreatitis was induced by infusion of cerulein as described previously (16, 17, 38). We randomized male Sprague-Dawley rats (Harlan, Madison, WI) weighing 270-320 g into two categories, A and B. The rats in category A received a continuous intravenous infusion of 5 μg·kg⁻¹·h⁻¹ cerulein (in physiological saline solution) for a period of up to 6 h at a rate of 0.6 ml/h. Category B consisted of control rats that, instead of cerulein, received an infusion of physiological saline solution at the same rate.

After treatment, rats were killed and blood was collected for serum amylase and lipase determinations. The pancreas was removed, and a 50- to 100-mg piece of the gland close to the spleen was cut out, rinsed in ice-cold PBS, and snap-frozen in liquid nitrogen for subsequent RNA isolation. Other portions of the pancreas were used for protein extraction and for morphological examination. Serum amylase and lipase levels were determined with a Hitachi 707 analyzer (Anotech Diagnostics, Irvine, CA).

Isolation of dispersed pancreatic acini. Dispersed rat pancreatic acini were prepared using a previously published collagenase digestion method (33). To culture acinar cells, portions of the pancreas were used for protein extraction and frozen in liquid nitrogen for subsequent RNA isolation. Other portions of the pancreas were used for immunoprecipitation and for morphological examination. Serum amylase and lipase levels were determined with a Hitachi 707 analyzer (Anotech Diagnostics, Irvine, CA).

Detection of ICAM-1 mRNA by RT-PCR. Total RNA was extracted from pancreatic tissue or isolated acinar cells using TRIzol reagent (GIBCO BRL) or by the method of Han et al. (18). RNA quality was verified by ethidium bromide staining of rRNA bands on a denaturing agarose gel. Five micrograms of total RNA were reverse-transcribed according to the manufacturer’s protocol (SuperScript II Preamplification System, GIBCO BRL, Grand Island, NY), using oligo(dT) as a primer. For ICAM-1 message, the cDNA prepared from 0.5 μg of total RNA was subjected to PCR at the annealing temperature of 56°C using rat gene-specific primers. The forward 5'-GGG-TTGAGACTAACTGGATGA and reverse 5'-GGATCGAGCTCCACTCGGTCC primers span several introns in the ICAM-1 gene and are located at nucleotide positions 208–229 and 389–370, respectively, in ICAM-1 cDNA (GenBank D00913).

The amplified RT-PCR products were separated on an agarose gel stained with ethidium bromide and densitometrically quantified with an AMBIS image analysis system (Scnalytics, San Diego, CA). For comparison between different conditions, ICAM-1 mRNA expression was normalized to the expression of the housekeeping genes for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or acidic ribosomal phosphoprotein P0 (ARP) in the same sample, as described previously (17). The RT-PCR products for GAPDH or ARP were run together with those for ICAM-1 on the same gel to normalize densitometric ICAM-1 data. The sequences of ARP and GAPDH primers used have been published (16, 17). All RT-PCR products were of expected sizes and were directly sequenced to verify their identity. Negative controls were performed by omitting the RT step or the cDNA template from PCR amplification.

Northern blot analysis. Northern blot analysis was performed using a Northern Max kit from Ambion (Austin, TX). Briefly, 10–20 μg of total RNA were fractionated on a 1% formaldehyde-agarose gel and transferred to BrightStar-Plus nylon membrane (Ambion). After ultraviolet cross-linking, the blots were prehybridized for 1 h and then hybridized for 16 h at 42°C in ULTRAHyb solution (Ambion) with rat ICAM-1 cDNA probe. Blots were washed twice with a solution containing 2× standard saline citrate (SSC) and 0.1% SDS, and then once at 42°C and once at 52°C with a wash solution containing 0.1× SSC and 0.1% SDS, each time for 15 min. After densitometry, the blots were stripped and reprobed in a similar way with a radiolabeled rat ARP cDNA probe. For the probes we used RT-PCR products for ICAM-1 and ARP, which were gel-purified with GeneClean II (BIO 101, La Jolla, CA) and labeled with [α-32P]dCTP by using Prime-It II labeling kit (Stratagene, La Jolla, CA). The densitometric analysis of band intensities was done in the PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Background correction was performed using an area adjacent to the ICAM-1 or ARP bands on the blot. The ICAM-1 band intensity was then normalized to that of ARP in the same sample.

Immunoprecipitation. Immunoprecipitation was performed essentially as described previously (16). To extract proteins from the acinar cells, freshly prepared pancreatic acini were washed twice with PBS and lysed by incubating for 20 min at 4°C in lysis buffer containing 0.15 M NaCl, 50 mM Tris (pH 7.2), 1% deoxycholic acid (wt/vol), 1% Triton X-100 (wt/vol), 0.1% SDS (wt/vol), and 1 mM phenylmethylsulfonyl fluoride (PMSF), as well as 5 μg/ml each of the protease inhibitors pepstatin, leupeptin, chymostatin, antipain, and aprotinin. The cell lysates were then centrifuged for 20 min at 15,000 g at 4°C, and the supernatants were used for immunoprecipitation. To extract proteins from tissue, the pancreas was washed with ice-cold PBS and homogenized on ice in the lysis buffer using a Dounce homogenizer. The tissue homogenates were incubated in the cold room as above for 20 min and centrifuged, and the supernatants were used for immunoprecipitation. For immunoprecipitation, the supernatants from cell or tissue lysates were incubated at 4°C overnight with primary ICAM-1 antibody (1:100 dilution) and then for 1 h with protein A-Sepharose. To immunoprecipitate ICAM-1, we used three types of monoclonal mouse anti-rat ICAM-1 antibodies from Genzyme (Cambridge, MA), R&D Systems (Minneapolis, MN), or Seikagaku (Tokyo, Japan). The protein A-Sepharose antigen precipitates were separated by centrifugation, washed three times with the lysis buffer, and resuspended in a sample buffer containing 10% glycerol (vol/vol), 2% SDS (wt/vol), and 0.0025% bromphenol blue (wt/vol) in 63 mM Tris (pH 6.8). The antigen was eluted from protein A-Sepharose by heating for 5 min at 100°C. Samples were

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centrifuged, and the supernatants containing the antigen were collected.

**Western blot analysis.** Proteins in the whole cell lysate or immunoprecipitated proteins were analyzed by immunoblotting. Proteins were separated by 8% SDS-PAGE for 2 h at 120 V using precast Tris-glycine gels and a Mini-Cell gel apparatus (Novex, San Diego, CA). Separated proteins were electrophoretically transferred to a nitrocellulose membrane for 2 h at 30 V using a Novex Blot Module. Non-specific binding was blocked by 1-h incubation of the membranes in 5% (wt/vol) nonfat dry milk in Tris-buffered saline (TBS; pH 7.5). Equal loading was checked by Ponceau staining of the blots. Blots were then incubated for 2 h with primary antibody (1:100) in an antibody buffer containing 1% (wt/vol) nonfat dry milk in TTBS (0.05% vol/vol Tween-20 in TBS), washed three times with TTBS, and finally incubated for 1 h with a peroxidase-labeled secondary antibody in the antibody buffer. For immunoblotting, we used the same three types of monoclonal mouse anti-rat ICAM.

Blots were developed for visualization using an enhanced chemiluminescence (ECL) detection kit (Amersham, Arlington Heights, IL). The intensities of the bands were quantified by densitometry using AMBIS software.

**Immunocytochemistry.** For immunostaining, isolated pancreatic acini were suspended in PBS and plated on polylysine-coated glass coverslips. They were allowed to attach for 10 min at room temperature and then were washed with 0.5 ml of PBS to remove unattached cells. The remaining acini were fixed on the coverslips by a 10-min incubation with 0.5 ml of 2% paraformaldehyde.

Slides were incubated in the blocking medium containing 1% (vol/vol) goat serum, 1% (wt/vol) bovine serum albumin, and 1% (vol/vol) gelatin for 30 min at room temperature before application of primary antibody. The preparations were then incubated for 12 h at 4°C with the primary ICAM-1 antibody (1:100 dilution) in the incubation medium containing 0.1% (vol/vol) goat serum, 0.1% (wt/vol) bovine serum albumin, 0.1% (vol/vol) gelatin, and 0.1% saponin in PBS. Slides were then washed three times with the incubation medium, covered with 50 μl of the same medium containing FITC-tagged secondary antibody (1:200), and finally incubated for 1 h at room temperature. The slides were washed three times with PBS, air dried, and mounted in Supermount.

Immunostaining for ICAM-1 was performed using monoclonal mouse anti-rat ICAM-1 antibody (Genzyme). We examined the slides with fluorescence microscopy using a Nikon Diaphot microscope. In all experiments, negative controls were performed using the secondary antibody only. The exposure and print times were the same for the experimental and control conditions.

**Preparation of nuclear extracts and electrophoretic mobility shift assay.** Nuclear protein extracts were prepared as described previously (16, 17). Briefly, pancreatic acinar cells were lysed on ice in hypotonic buffer A (17) supplemented with 1 mM PMSF and 1 mM dithiothreitol (DTT) and with a protease inhibitor cocktail containing 5 μg/ml each of pepstatin, leupeptin, chymostatin, antipain, and aprotinin. The lysate was left to swell on ice for 20–25 min, and then Nonidet P-40 was added to a final concentration of 0.3% (vol/vol). The nuclear pellet was collected by microcentrifugation for 30 s. The supernatant was removed, and the nuclear pellet was resuspended in high-salt buffer C (17) containing 20 mM HEPES (pH 7.6), 25% (vol/vol) glycerol, 0.42 M NaCl, 1.5 MgCl2, 0.2 mM EDTA, 20 mM β-glycerophosphate, 10 mM Na2MoO4, 50 μM Na2VO4, 1 mM DTT, 1 mM PMSF, and the protease inhibitor cocktail. The nuclear membranes were pelleted by microcentrifugation for 10 min, and the clear supernatant (nuclear extract) was aliquoted and stored at −80°C. Protein concentration in the nuclear extract was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

For electrophoretic mobility shift assay (EMSA), aliquots of nuclear extracts with equal amounts of protein (2–10 μg) were mixed in 20-μl reactions in a buffer containing 10 mM HEPES (pH 7.6), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% (vol/vol) glycerol, and 3 μg of poly(dI-dC). Binding reactions were started by addition of 20,000–40,000 cpm of 32P-labeled DNA probe and incubated at room temperature for 30 min. The oligo probe G668 ICAM-1 was annealed to the complementary oligonucleotide bearing a 5× G overhang, and end-labeled using Klenow DNA polymerase I. Samples were electrophoresed at room temperature in 0.5× TBE buffer (1× TBE: 89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) with loading dye on non-denaturing 5% polyacrylamide gel at 200 V. Gels were dried and directly analyzed in the phosphorimager or exposed at −80°C to Fuji RX film with intensifying screens. In this case, the intensity of bands on gel fluorograms was quantified using the AMBIS image analysis system.

**Neutrophil isolation.** Neutrophils were isolated from 20 ml of rat blood as described previously (15). Briefly, blood was drawn by venipuncture and collected in tubes containing heparin. After dextran sedimentation of red cells, the neutrophils were purified by centrifugation through Ficoll-Hypaque. Contaminating red cells were removed by NH4Cl lysis, and the neutrophils were maintained in RPMI 1640.

**In vitro assay of neutrophil adhesion to pancreatic acinar cells.** To measure neutrophil adherence to pancreatic acinar cells, we applied a procedure described previously (23) with some modifications. Acinar cells isolated from one half of a pancreas were suspended in 2 ml of medium 199 and incubated with or without ICAM-1 neutralizing antibody (1:100, Seikagaku) and with or without 10 nM cerulein for 3 h at 37°C. Unbound antibody and cerulein were removed by centrifugation, and then 200 μl of acinar cells resuspended in PBS were plated on polylysine-coated slides. Freshly isolated rat neutrophils were separately incubated for 1 h at 37°C with fluorescein-labeled anti-neutrophil antibody (1:100), and then unbound antibody was removed by centrifugation. In some experiments, 0.1 μM N-formylmethionyl-leucyl-phenylalanine (fMLP) was added to the neutrophils for the last 30 min of incubation. Acini on the slide were overlaid with fluorescein-labeled neutrophils (200 μl of neutrophil suspension containing 1×106 neutrophils/ml). The same volume of neutrophil suspension was also plated on an empty part of the slide with the same surface area to serve as the background control. The slides were incubated for 30 min at 37°C on a rotational shaker. Unattached acinar cells and neutrophils were then washed away with PBS, and the remaining cells were fixed by 10.220.33.3 on June 25, 2017 http://ajpgi.physiology.org/ Downloaded from
hematoxylin and eosin. Finally, the number of neutrophils adhered to acinar cells was expressed per 100 acinar cells.

In some experiments, the preparation was stained with rhodamine-phalloidin to visualize actin filaments in both neutrophils and acinar cells. This technique demonstrates actin filaments localized to the apical aspect of the acinar cell (32). Statistical analysis. Statistical analysis of data was done using unpaired two-tailed Student’s t-test.

Reagents. Monoclonal mouse anti-rat ICAM-1 antibodies were from Genzyme, Seikagaku, or R&D Systems. Horseradish peroxidase-conjugated goat anti-mouse IgG was from BioRad, and fluorescein-conjugated goat anti-mouse secondary antibody was from American Qualex (San Clemente, CA). FITC-conjugated rabbit anti-neutrophil antibody was from Accurate (Westbury, NY). Cerulein was from Peninsula Laboratories (Belmont, CA). Protein A-Sepharose was from Pierce (Rockford, IL). Poly(dI-dC) was from Boehringer Mannheim (Indianapolis, IN). Precast Tris-glycine gels were from Novex. RPMI 1640 and medium 199 were from Gibco BRL. \( ^{32}P \)-labeled dCTP (3,000 mCi/mmol) and the ECL detection kit were from Amersham. The proteasomal inhibitor Z-LLLH (MG-132) was from Peptide International (Louisville, KY). All other chemicals were from Sigma (St. Louis, MO).

**RESULTS**

**ICAM-1 is present in rat pancreas and is upregulated in cerulein-induced pancreatitis.** To determine whether the development of pancreatitis is associated with changes in ICAM-1 mRNA and protein, we used the well-established cerulein model of acute pancreatitis (12, 17, 38). Rat cerulein pancreatitis is characterized by edema, acinar cell vacuolization, inflammatory infiltration, and high blood levels of amylase and lipase. To verify the full development of cerulein pancreatitis, we measured blood levels of amylase and lipase, acinar cell vacuolization, and pancreatic inflammatory infiltration in saline (control) and cerulein (5 \( \mu \)g·kg\(^{-1} \)·h\(^{-1} \); 6 h)-infused rats. Serum amylase and lipase levels in cerulein-treated rats were 30,200 ± 5,000 and 6,100 ± 980 U/l, respectively, compared with 3,000 ± 470 and <750 U/l in saline-infused rats (n = 7 in each group); 27 ± 3% acinar cells contained vacuoles in cerulein-treated rats compared with <0.3% in saline-infused rats. Infiltration of inflammatory cells (neutrophils, macrophages, lymphocytes) into pancreas in cerulein-treated rats reached 5.8 ± 1.0% (i.e., 5.8 inflammatory cells per 100 acinar cells) compared with <0.1% in control animals.

The results (Fig. 1A, top) demonstrate that mRNA for ICAM-1 is expressed in both saline-infused rats and rats with cerulein pancreatitis. Semiquantitative RT-PCR (Fig. 1A, bottom) indicated that, relative to the control group, cerulein hyperstimulation moderately
(~2.5-fold) increased the level of ICAM-1 mRNA expression in pancreas.

To determine whether ICAM-1 protein was present in pancreas and whether it was upregulated in cerulein pancreatitis, we applied Western blot analysis. Protein extracts were obtained from pancreatic tissue of rats infused with saline or cerulein for different times. Proteins were separated by SDS-PAGE, transferred onto membranes, and probed with one of three types of ICAM-1 monoclonal antibody (MAb). In samples from both saline- and cerulein-treated rats, the antibody recognized a 95-kDa band (Fig. 1B, top) characteristic for ICAM-1 (44). Densitometric analysis (Fig. 1B, bottom) showed that cerulein hyperstimulation caused a time-dependent increase in the 95-kDa ICAM-1 protein in rat pancreas. The maximum increase was observed at 3–6 h, but it was already statistically significant after 1 h of cerulein infusion. In control rats, pancreatic expression of ICAM-1 protein did not change with time.

ICAM-1 is present on pancreatic acinar cells, and both ICAM-1 mRNA and protein are upregulated by cerulein in vitro. To determine whether ICAM-1 mRNA is expressed in pancreatic acinar cells and whether its expression is regulated by the hormone, we extracted total RNA from isolated acinar cells incubated for different times with and without cerulein. RT-PCR was performed as described in Fig. 1A. An RT-PCR product of expected size (182 bp) was amplified with specific ICAM-1 primers spanning several introns in the ICAM-1 gene (Fig. 2A). Its identity was verified by direct sequencing.

Figure 2A demonstrates upregulation of ICAM-1 mRNA expression by cerulein, which was most pro-

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A: ICAM-1 mRNA expression in isolated rat pancreatic acini incubated without or with 0.7 μM cerulein for different times. Shown are representative RT-PCR for the expression of ICAM-1 and the housekeeping gene ARP. cDNA was reverse-transcribed from 0.5 μg of total RNA and amplified as described in METHODS. The RT-PCR products were resolved on an agarose gel, and their densities were normalized to that of ARP in the same RNA sample. ICAM-1 mRNA expression levels are given relative to cells incubated without cerulein (lane 1). The experiment was repeated twice on different preparations of acini, with similar results. B: proteins were extracted from isolated pancreatic acini, extracts were adjusted to equal protein concentration, and ICAM-1 was immunoprecipitated and immunoblotted as in Fig. 1B; numbers are protein standard sizes in kDa. The experiment was repeated 4 times with similar results. C: for immunofluorescence, isolated pancreatic acini were plated on a glass coverslip, permeabilized, and stained for ICAM-1 with monoclonal mouse anti-rat ICAM-1 antibody (Seikagaku). Immunostaining was performed using both primary ICAM-1 antibody and FITC-tagged secondary antibody (a) or the secondary antibody alone (b; negative control). D: Western blot showing the effect of cerulein on ICAM-1 protein. Isolated pancreatic acini were incubated for 3 h without or with cerulein at indicated concentrations. Proteins were extracted, and ICAM-1 was immunoprecipitated and immunoblotted as in Fig. 1B. E: dose dependence of ICAM-1 protein levels on cerulein at 3-h incubation. The intensity of the 95-kDa ICAM-1 band on immunoblots was quantified by densitometry and expressed relative to cells incubated without cerulein (1.0). Results are means ± SE from ≥4 determinations for each concentration point; *P < 0.05 vs. cells incubated without cerulein. F: time course of the effect of 1 nM cerulein on ICAM-1 protein levels. The intensity of the 95-kDa ICAM-1 band on immunoblots was quantified by densitometry and expressed relative to cells incubated without cerulein (1.0). Results are means ± SE from ≥4 determinations for each time point; *P < 0.05 compared with cells at time 0.
nounced at 1 h of incubation of the cells with the hormone and decreased by 3 h. To obtain an estimation of these changes, the intensity of the ICAM-1 RT-PCR band was quantified by densitometry and normalized to that of the housekeeping gene ARP in the same sample. Semiquantitative RT-PCR indicated that relative to control cells, 0.7 μM cerulein increased the level of ICAM-1 mRNA expression two- to threefold.

To detect ICAM-1 protein in isolated pancreatic acinar cells, we used immunoprecipitation followed by Western blot analysis. Western blotting of ICAM-1 immunoprecipitate demonstrated a prominent band at 95 kDa (Fig. 2B) characteristic for ICAM-1 (44). To prove the specificity of this band, we performed immunoprecipitation followed by Western blot analysis using three types of monoclonal mouse anti-rat ICAM antibody from different companies (see METHODS). All three recognized this same 95-kDa band (not shown). In some samples a faint 130-kDa band was also recognized by ICAM-1 MAb (Fig. 2B); its relation to ICAM-1 remains unclear.

To confirm that ICAM-1 is localized to acinar cells, dispersed pancreatic acini were stained for ICAM-1 with the same antibodies we used in the Western blot analysis. Immunofluorescence (Fig. 2C) demonstrated predominant ICAM-1 localization to acinar cell plasma membrane. No staining was observed when secondary antibody only was used (Fig. 2C). Microscopic observations of our hematoxylin and eosin-stained preparations of isolated pancreatic acini (16) showed that acinar cells comprise >97% of the cell population, with no inflammatory cells present. In control experiments we found no staining for the endothelial cell marker platelet endothelial cell adhesion molecule-1 (data not shown).

As demonstrated in Fig. 2, D–F, cerulein upregulated ICAM-1 protein in isolated acini in a dose- and time-dependent manner. The ICAM-1 band intensity increased with cerulein, with the maximal stimulation (2.5- to 3.0-fold) observed at cerulein concentrations >1 nM (Fig. 2, D and E). With 1 nM cerulein the increase in ICAM-1 level was already statistically significant after 1 h of incubation, and it stayed elevated during the 6-h observation period (Fig. 2F).

**Blocking of NF-κB inhibits ICAM-1 expression in cerulein-treated pancreatic acinar cells.** The transcription factor NF-κB was shown to mediate ICAM-1 induction by cytokines such as TNF-α in a number of cells (20, 24, 27). Recently, we found (17) that cerulein activates NF-κB in isolated pancreatic acinar cells. To determine whether NF-κB is involved in upregulation of ICAM-1 by cerulein, we measured the effect of NF-κB inhibition on ICAM-1 mRNA expression in pancreatic acinar cells. For NF-κB inhibition we used a potent proteasomal inhibitor, peptide aldehyde MG-132, which is commonly used to inhibit NF-κB activation (4, 24, 46). Changes in NF-κB DNA binding activity were measured by EMSA.

As shown in Fig. 3A, MG-132 (10 μM) effectively inhibited both basal and cerulein-induced NF-κB binding activity in isolated acinar cells. The basal NF-κB

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Fig. 3. Nuclear factor-κB (NF-κB) is involved in cerulein-induced ICAM-1 mRNA expression in pancreatic acinar cells. Isolated rat pancreatic acini were preincubated for 1 h without or with 10 μM MG-132, a potent NF-κB inhibitor. After that, the incubation continued for indicated times without or with 0.7 μM cerulein. Nuclear protein and total RNA were isolated and analyzed for NF-κB DNA binding activity and ICAM-1 mRNA expression. The experiment was repeated twice on different preparations of acini, with similar results. A: representative electrophoretic mobility shift assay (EMSA) showing the effect of MG-132 on NF-κB activation in control and cerulein-treated acinar cells. Positions of specific NF-κB-DNA complexes and the free probe are indicated by single and double arrowhead, respectively. The values represent densitometric intensities of NF-κB-DNA complexes and the free probe are indicated by single and double arrowhead, respectively. The values are given relative to cells incubated without both MG-132 and cerulein (lanes 1 and 5). NS, nontpecific. B: representative RT-PCR showing the effect of MG-132 on mRNA expression of ICAM-1, the housekeeping gene ARP, and inducible NO synthase (iNOS) in control and cerulein-treated acinar cells. Densitometric intensity of ICAM-1 RT-PCR band was normalized to that of ARP in the same RNA sample. These values are given relative to cells incubated without both MG-132 and cerulein (lanes 1 and 5).
activity was inhibited by 70–80%, and the cerulein-induced activation of NF-κB was completely prevented.

Treatment with MG-132 inhibited cerulein-induced stimulation of ICAM-1 mRNA expression, which was particularly pronounced at 1 h of incubation with the hormone (Fig. 3B). The inhibition was less pronounced at 3 h, when the transitory effect of cerulein itself is already fading away. However, blocking of NF-κB with MG-132 did not inhibit the basal mRNA expression of ICAM-1 in isolated acinar cells at all (Fig. 3A). These results suggest that NF-κB is involved in stimulation of ICAM-1 expression by cerulein, but it may not play a major role in the regulation of basal transcription from ICAM-1 gene in pancreatic acinar cells. An alternative explanation is that the residual level of NF-κB activity in the presence of MG-132 (Fig. 3A) is enough to support the basal expression of ICAM-1.

Interestingly, NF-κB appears to regulate both the basal and cerulein-induced mRNA expression of some other inflammatory molecules that we found expressed in the pancreatic acinar cell (5, 6). For comparison with ICAM-1 expression, in Fig. 3B we show that in these same samples MG-132 significantly inhibited not only cerulein-induced but also basal mRNA expression of the inflammatory enzyme iNOS.

ICAM-1 message stability in pancreatic acinar cells is not affected by MG-132. To investigate whether the inhibitory effect of MG-132 could be caused by a decrease in the stability of ICAM-1 message, we studied ICAM-1 mRNA degradation in isolated acinar cells. The cells were incubated with 0.7 μM cerulein for 1 h in the absence and presence of 10 μM MG-132, after which actinomycin D (10 μg/ml) was added to stop mRNA synthesis, and incubation continued for indicated times. A similar experiment was performed for control cells. Total RNA was isolated and subjected to Northern blot analysis with radiolabeled probes for ICAM-1 and the housekeeping ARP (Fig. 4). The latter was used to normalize levels of ICAM-1 mRNA.

In pancreatic acinar cells, we detected ICAM-1 messages of ~3.3 and ~2.4 kb. In both control and cerulein-treated cells, there was no degradation of ICAM-1 mRNA during 3 h of observation. MG-132 had no significant effect on ICAM-1 mRNA degradation.
Neutrophils bind to pancreatic acinar cells in vitro, and this adhesion is mediated by ICAM-1 and stimulated by cerulein. To assess the functional role of ICAM-1 expression on pancreatic acinar cells, we asked whether neutrophils can attach to acinar cells and whether ICAM-1 mediates this adhesion. For this purpose, we used a previously published in vitro adhesion assay (23) with modifications. Isolated pancreatic acini were incubated with or without cerulein and with or without neutralizing ICAM-1 antibody and then plated on a slide and overlaid with FITC-labeled neutrophils as described in METHODS. The slides were incubated for 30 min at 37°C to allow neutrophil attachment to acinar cells. Unattached cells were then washed away, and the remaining cells were fixed and analyzed under a fluorescence microscope.

To characterize neutrophil binding to pancreatic acinar cells, we visualized actin in both neutrophils and acinar cells by staining the preparation with rhodamine-phalloidin. In neutrophils, actin is evenly distributed all over the cell; in acinar cells, it is localized along the apical membrane (Fig. 5), in agreement with previously published data (25, 32, 40). Thus the data in Fig. 5 demonstrate close attachment of neutrophils to basal membranes of acinar cells. On the other hand, the absence of filamentous actin staining around acinar cells again indicates (cf. Fig. 2C) that our preparation of pancreatic acini is not contaminated with endothelial cells, a possible source of ICAM-1.

Quantitative analysis of neutrophil attachment to control and cerulein-treated acinar cells (Fig. 6) showed that the hormone increased neutrophil binding about threefold. To block binding via ICAM-1, we used the same neutralizing ICAM-1 MAb that we applied in the Western blot analysis. In both control and cerulein-stimulated acini, ICAM-1 antibody inhibited neutrophil binding to acinar cell (Fig. 6). The antibody did not affect the background neutrophil attachment to the empty slide. In the control experiment, incubation of acini with an unrelated mouse anti-rat IgG (diluted to the same concentration as the ICAM-1 MAb) did not influence neutrophil binding to acinar cells (data not shown). The results indicate that neutrophils bind to acinar cells in vitro, that the binding is mediated by ICAM-1, and that cerulein stimulates this adhesion via ICAM-1.

It was shown previously (2, 7) that neutrophil adhesion to endothelial and some epithelial cells increases when neutrophils are activated with fMLP. For the neutrophil adhesion to pancreatic acinar cells we also observed such stimulation: preincubation of neutrophils with fMLP increased their binding to cerulein-treated acinar cells (Fig. 6).

Figure 6 also shows the effect of NF-κB inhibition with the proteasomal inhibitor MG-132 on neutrophil adhesion to acinar cells. MG-132 significantly (by ~50%) inhibited cerulein-stimulated neutrophil attachment, but it did not affect neutrophil binding to control acinar cells. These results correlate well with the data on the effect of MG-132 on ICAM-1 expression (Fig. 3).

DISCUSSION

In this study we showed that ICAM-1, a molecule that mediates neutrophil adhesion to endothelial and some epithelial cells (14, 34, 44), is present on rat pancreatic acinar cells and is upregulated by the hormone cerulein. The results indicate that ICAM-1 ex-
expression is functional and mediates neutrophil binding to acinar cells in vitro, which is stimulated by cerulein. Pancreatic expression of both ICAM-1 mRNA and protein is upregulated in cerulein-induced pancreatitis.

Although a significant role of neutrophils in the development of cerulein pancreatitis was demonstrated by us (38) and others (11, 19), the mechanisms of neutrophil activation and transmigration into the pancreas, as well as the role of acinar cells in this process, have not been addressed. Our finding that ICAM-1 is upregulated in pancreas by cerulein hyperstimulation suggests that it is involved in the recruitment of neutrophils into pancreas. Indeed, it was recently reported (10) that cerulein pancreatitis in ICAM-1 knockout mice displayed decreased neutrophil infiltration and improvement in parameters of the disease.

Despite the presence of ICAM-1, there are essentially no neutrophils in normal pancreas (38), indicating that by itself, constitutive ICAM-1 expression does not induce neutrophil infiltration. The level of basal ICAM-1 expression in pancreas may be not sufficiently high, or, more probably, other adhesion and inflammatory molecules, in addition to ICAM-1, are important for neutrophil recruitment into pancreas. In particular, neutrophil chemoattractants may play a critical role in this process. We recently found (17) that pancreatic expression of chemokine KC, a murine analog of interleukin (IL)-8/growth-related oncogene (GRO)-α and a potent neutrophil chemoattractant, greatly increased in rat cerulein pancreatitis. We (16) and others (see Ref. 30) also showed that another chemoattractant, TNF-α, is upregulated and plays a role in cerulein-induced and other experimental models of pancreatitis. Thus cerulein hyperstimulation results in combined upregulation of both inflammatory cytokines/chemokines and adhesion molecules, which can coordinate activation of neutrophils and their recruitment to the sites of inflammation and acinar cell injury in pancreatitis.

Our data demonstrate that the pancreatic acinar cell itself is a source for ICAM-1 in the pancreas. The expression of both ICAM-1 mRNA and ICAM-1 protein in isolated acinar cells is upregulated by cerulein. Recently we (5, 6, 16, 38) and others (13) showed that acinar cells express and produce the proinflammatory cytokines/chemokines platelet-activating factor, TNF-α, KC, Mob-1, and MCP-1. Thus pancreatic acinar cells express both classes of molecules regulating neutrophil activation and recruitment, adhesion molecules and chemotactic cytokines. This suggests that acinar cells play an active part in regulating neutrophil transmigration into the pancreas.

Recently, we found (17) that cerulein greatly activates NF-κB both in experimental pancreatitis and in isolated pancreatic acini. "Supershift" EMSA experiments showed that p65/p50 and p50/p50 dimers, but not c-Rel or p52 complexes, were manifest in cerulein pancreatitis and in isolated acinar cells (17). NF-κB was shown to mediate ICAM-1 gene expression in a number of cells (7, 8, 24, 46). Therefore, we asked whether NF-κB is involved in regulation of ICAM-1 expression in pancreatic acinar cells. We found that blocking of NF-κB nuclear translocation with proteasomal inhibitor MG-132 did inhibit cerulein-induced expression of ICAM-1 mRNA. However, it did not influence the basal expression of ICAM-1 mRNA in acinar cells. This may indicate that in these cells cerulein-induced and basal transcription from ICAM-1 gene are regulated by different sets of transcription factors and that NF-κB mediates the former but not the latter process. Alternatively, the residual level of NF-κB activity remaining in the presence of MG-132 may be enough to support the basal expression of ICAM-1.

Fig. 6. Neutrophil attachment to pancreatic acinar cells is mediated by ICAM-1 and is stimulated by cerulein. Isolated rat pancreatic acini were preincubated for 1 h without or with ICAM-1 neutralizing antibody (1:100) and in the absence or presence of the proteasomal inhibitor MG-132 (0.7 μM). After that, incubation continued for 3 h without or with 0.7 μM cerulein. Acinar cells were then plated on polylysine-coated slides. Freshly isolated rat neutrophils were labeled for 1 h with fluorescein-conjugated anti-neutrophil antibody, without or with (for the last 30 min) 0.1 μM N-formylmethionyl-leucyl-phenylalanine (fMLP). Neutrophils were then plated over the layer of acinar cells or directly on the polylysine-coated slides (background control) as described in METHODS. The slides were incubated for 30 min at 37°C, unattached cells were washed away, and the remaining cells were fixed and analyzed under fluorescence microscope as described in METHODS. Values are means ± SE from ≥4 experiments for each condition. *P < 0.05 vs. untreated cells; #P < 0.05 vs. cells incubated with cerulein only.
In addition to NF-κB, transcription factors known to regulate ICAM-1 expression by various inducers include AP-1, C/EBP, and Ets proteins (8, 9, 20, 27, 36). In one and the same cell, different transcription factors mediate ICAM-1 expression in response to different stimuli (27, 36). There are examples of NF-κB being not involved in regulation of ICAM-1 gene expression even when activated by a stimulus (28, 43). Different regulatory elements in the ICAM-1 gene are implicated in its basal vs. cytokine-stimulated expression (20, 22). It remains to be elucidated which transcription factors, other than NF-κB, mediate the basal and hormone-induced ICAM-1 mRNA expression in pancreatic acinar cells.

With Northern blot analysis, we observed ICAM-1 mRNAs of ~3.3 and ~2.4 kb in pancreatic acinar cells. Multiple-size ICAM-1 messages have been reported in human cell lines (22). In both control and cerulein-stimulated acinar cells, there was no significant mRNA degradation over the observation period, indicating a half-life of >3 h for ICAM-1 mRNA in these cells. MG-132 had no significant effect on ICAM-1 mRNA degradation. This indicates that its effect on ICAM-1 mRNA expression is not caused by a decreased stability of the message.

The 3′-untranslated region of ICAM-1 mRNA contains elements (in particular, AU-rich elements; Ref. 37) regulating its stability (31). Depending on the stimulus, these elements may or may not be involved in ICAM-1 mRNA stabilization (31). The absence of the effect of MG-132 may indicate that NF-κB does not regulate the stability of ICAM-1 message in acinar cells.

Over the past several years, evidence has been accumulating that not only endothelial but also epithelial cells can play an active role in leukocyte (in particular, neutrophil) transmigration (1, 7, 21, 34). We found that neutrophils adhere to pancreatic acinar cells in vitro and that this binding is stimulated by cerulein. The adhesion was greatly inhibited by a neutralizing anti-ICAM-1 antibody. Furthermore, blocking NF-κB activation with MG-132 significantly inhibited cerulein-stimulated neutrophil adhesion to acinar cells. Our results show a strong correlation between the effects of MG-132 on ICAM-1 expression and neutrophil adhesion: MG-132 inhibited both ICAM-1 expression and neutrophil adhesion in cerulein-stimulated but not in control cells. These results suggest that cerulein stimulates neutrophil adhesion to pancreatic acinar cells through NF-κB activation and subsequent ICAM-1 up-regulation.

ICAM-1 expression in various epithelia has been reported mainly in response to cytokines (21, 24, 27, 35). To our knowledge, this is the first example of ICAM-1 stimulation by a hormone in epithelial cells. CCK is a pleiotropic hormone that activates multiple signal transduction pathways and triggers various biological responses. Our data demonstrate a novel role for CCK—it’s ability to regulate expression of adhesion molecules.

In summary, the results obtained show that ICAM-1 is present on pancreatic acinar cells. Cerulein upregulates ICAM-1 both in vivo, in pancreas of rats with experimental pancreatitis, and in vitro, in isolated acinar cells. The transcription factor NF-κB is involved in cerulein-induced ICAM-1 upregulation. Neutrophils adhere to pancreatic acinar cells in vitro. ICAM-1 mediates this adhesion, which is stimulated by the hormone. Upregulation of ICAM-1 expression in pancreatitis may mediate parenchymal cell damage by facilitating attachment of neutrophils. Moreover, acinar cells themselves may contribute to pancreatic inflammation through recruitment of neutrophils into pancreas.

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