

## **Pancreatic acinar cells produce, release, and respond to tumor necrosis factor-alpha. Role in regulating cell death and pancreatitis.**

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### Research Article

The aim of this study was to determine whether tumor necrosis factor-alpha (TNFalpha) and receptors for TNFalpha are expressed in the exocrine pancreas, and whether pancreatic acinar cells release and respond to TNFalpha. Reverse transcription PCR, immunoprecipitation, and Western blot analysis demonstrated the presence of TNFalpha and 55- and 75-kD TNFalpha receptors in pancreas from control rats, rats with experimental pancreatitis induced by supramaximal doses of cerulein, and in isolated pancreatic acini. Immunohistochemistry showed TNFalpha presence in pancreatic acinar cells. ELISA and bioassay measurements of TNFalpha indicated its release from pancreatic acinar cells during incubation in primary culture. Acinar cells responded to TNFalpha. TNFalpha potentiated NF-kappaB translocation into the nucleus and stimulated apoptosis in isolated acini while not affecting LDH release. In vivo studies demonstrated that neutralization of TNFalpha with an antibody produced a mild improvement in the parameters of cerulein-induced pancreatitis. However, TNFalpha neutralization greatly inhibited apoptosis in a modification of the cerulein model of pancreatitis which is associated with a high percentage of apoptotic cell death. The results indicate that pancreatic acinar cells produce, release, and respond to TNFalpha. This cytokine regulates apoptosis in both isolated pancreatic acini and experimental pancreatitis.

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# Pancreatic Acinar Cells Produce, Release, and Respond to Tumor Necrosis Factor- $\alpha$

## Role in Regulating Cell Death and Pancreatitis

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### Abstract

The aim of this study was to determine whether tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and receptors for TNF $\alpha$  are expressed in the exocrine pancreas, and whether pancreatic acinar cells release and respond to TNF $\alpha$ . Reverse transcription PCR, immunoprecipitation, and Western blot analysis demonstrated the presence of TNF $\alpha$  and 55- and 75-kD TNF $\alpha$  receptors in pancreas from control rats, rats with experimental pancreatitis induced by supramaximal doses of cerulein, and in isolated pancreatic acini. Immunohistochemistry showed TNF $\alpha$  presence in pancreatic acinar cells. ELISA and bioassay measurements of TNF $\alpha$  indicated its release from pancreatic acinar cells during incubation in primary culture. Acinar cells responded to TNF $\alpha$ . TNF $\alpha$  potentiated NF- $\kappa$ B translocation into the nucleus and stimulated apoptosis in isolated acini while not affecting LDH release. In vivo studies demonstrated that neutralization of TNF $\alpha$  with an antibody produced a mild improvement in the parameters of cerulein-induced pancreatitis. However, TNF $\alpha$  neutralization greatly inhibited apoptosis in a modification of the cerulein model of pancreatitis which is associated with a high percentage of apoptotic cell death. The results indicate that pancreatic acinar cells produce, release, and respond to TNF $\alpha$ . This cytokine regulates apoptosis in both isolated pancreatic acini and experimental pancreatitis. (*J. Clin. Invest.* 1997. 100:1853–1862.) Key words: TNF $\alpha$  • apoptosis • NF- $\kappa$ B • TNF $\alpha$  receptor • pancreatitis

### Introduction

Over the past few years, significant evidence has accumulated that cytokines can mediate many pathophysiological responses of an organism to injury or disease (1, 2). In particular, TNF $\alpha$  is a major mediator of the acute inflammatory response that is generated during many disease states, including infection and inflammation (3–5). In vitro TNF $\alpha$  has also been shown to

stimulate death through necrosis and/or apoptosis in many cell types (6–13).

Intracellular TNF $\alpha$  signaling is initiated by interaction of TNF $\alpha$  with two distinct receptors on the cell surface, a 55-kD receptor (TNFR1)<sup>1</sup> and a 75-kD receptor (TNFR2), which have recently been cloned (6, 13–18). Activation of the transcription factor NF- $\kappa$ B is one of the first steps involved in TNF $\alpha$  signaling (9, 19–21). NF- $\kappa$ B activation is believed to mediate the effects of TNF $\alpha$ , in particular, TNF $\alpha$ -induced cell injury. Macrophages/monocytes are considered a major source of TNF $\alpha$  production (4). Recently, however, TNF $\alpha$  has also been found to be expressed in adipose tissue (22), human muscle (23), endotoxin-stimulated myocardium (24), IL-1-stimulated  $\beta$  cell lines (25), and astrocytes (26).

It has not been determined whether TNF $\alpha$  can be produced by the pancreas, whether pancreatic acinar cells can synthesize and release TNF $\alpha$ , whether acinar cells respond to this cytokine, or whether TNF $\alpha$  can regulate acinar cell function.

Previous studies have demonstrated that TNF $\alpha$  levels are increased in the serum of patients with pancreatitis as well as in animal models of pancreatitis (27–31). These results suggest a role for this cytokine in the disease. Because pancreatitis is associated with inflammatory infiltration (32, 33), inflammatory as well as epithelial cells could be a source of cytokine production in the disease. The role of cytokines and, in particular, TNF $\alpha$  in the development of pancreatitis has not been determined.

Recently, we and others (33–36) have found that both types of cell death, necrosis and apoptosis, occur in experimental pancreatitis. The role of TNF $\alpha$  in mediating either type of cell death in pancreatitis has not been determined.

The purpose of this study was to determine: (a) whether TNF $\alpha$  and receptors for TNF $\alpha$  are expressed in the rat pancreas; (b) whether acinar cells can release and respond to TNF $\alpha$ ; and (c) whether TNF $\alpha$  has a role in regulating acinar cell death in vitro and in an experimental model of pancreatitis. Experimental pancreatitis was induced by infusion of supramaximal doses of cerulein, a stable cholecystokinin analogue. This model has been widely used and well characterized (37).

The results showed that the rat pancreas, in particular, pancreatic acinar cells, can produce, release, and respond to TNF $\alpha$ . This cytokine regulates apoptosis in both isolated pancreatic acini and in experimental pancreatitis.

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1. Abbreviations used in this paper: ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; H&E, hematoxylin and eosin; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT, reverse transcription; TBS, Tris-buffered saline; TNFR1, 55-kD TNF $\alpha$  receptor; TNFR2, 75-kD TNF $\alpha$  receptor.

## Methods

**Isolation of dispersed pancreatic acini.** Dispersed pancreatic acini from rats were prepared using a previously published collagenase digestion method (38). To culture acinar cells, dispersed pancreatic acini were washed and finally resuspended in 199 medium supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), and 0.5% BSA. Cells were plated at a concentration of  $5 \times 10^5$ /ml in 25-ml tissue-culture flasks and incubated for the indicated time in a 5% CO<sub>2</sub> humidified atmosphere at 37°C.

**Immunoprecipitation.** 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-HCl (pH 7.2), 1% deoxycholic acid (wt/vol), 1% Triton X-100 (wt/vol), 0.1% SDS (wt/vol), and 1 mM PMSF, as well as 5 µg/ml each of 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 supernatants were used for immunoprecipitation.

For immunoprecipitation, the supernatants from cell or tissue lysates were incubated at 4°C overnight with primary antibodies, then for 1 h with protein A-Sepharose. 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% bromophenol blue (wt/vol) in 63 mM Tris-HCl (pH 6.8). Sample buffer for TNFα immunoprecipitate was supplemented with 5% β-mercaptoethanol. The antigen was eluted from protein A-Sepharose by heating for 5 min at 100°C. Samples were centrifuged, and supernatants containing the antigen were collected.

TNFα was immunoprecipitated with polyclonal rabbit anti-mouse TNFα antibody (IP-410; Genzyme Corp., Cambridge, MA) in 1:100 dilution. TNFα receptors were immunoprecipitated with hamster anti-mouse TNFα receptor mAbs (Genzyme Corp.) in 1:50 dilution. To test whether the antibody amounts in our immunoprecipitations were limiting, TNFα was immunoprecipitated from tissue lysates using 1:100 and 1:50 dilutions of the TNFα antibody in parallel experiments. Both dilutions gave the same intensities of bands on immunoblots. A similar experiment was performed for TNFα receptors. The increase in the amount of antibody did not change the intensity of the bands on immunoblots.

**Western blot analysis.** Immunoprecipitated proteins, proteins in the whole cell lysate, and recombinant TNFα were analyzed by immunoblotting. Proteins were separated by SDS-PAGE for 2 h at 120 V using precast Tris-glycine gels and a Mini-Cell gel apparatus (Novex, San Diego, CA). Samples for gel electrophoresis were usually prepared by mixing protein sample with equal volume of sample buffer which contained 2% SDS. However, in the experiments designed to reveal TNFα oligomeric structure (see Fig. 1 C), we used sample buffer prepared without SDS, since TNFα oligomers are known to dissociate in the presence of 1% SDS (39, 40). Separated proteins were transferred electrophoretically to nitrocellulose membrane for 2 h at 30 V using a Blot Module (Novex). Nonspecific binding was blocked by 1-h incubation of nitrocellulose membranes in 5% (wt/vol) nonfat dry milk in Tris-buffered saline (pH 7.5) (TBS). Blots were then incubated for 2 h with primary antibodies in 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.

Immunoblotting of TNFα was performed using rabbit anti-mouse TNFα antibody in 1:250 dilution. Immunoblotting of 55- and 75-kD TNFα receptors was performed using three types of antibodies against each receptor: hamster anti-mouse mAbs in 1:250 dilution,

goat anti-human antibodies in 1:250 dilution, and mouse anti-human antibody in 1:100 dilution.

Blots were developed for visualization using an ECL Detection Kit (Amersham Corp., Arlington Heights, IL). The intensities of the bands were quantified by densitometry using AMBIS software (Scanalytics, San Diego, CA).

**DNA extraction and gel electrophoresis.** Isolated pancreatic acinar cells were collected by centrifugation, lysed by resuspension in a buffer containing 10 mM Tris-HCl (pH 8.0), 10 mM NaCl, 10 mM EDTA, 300 µg/ml proteinase K, and 1% (wt/vol) SDS, and incubated at 48°C until the mixture became clear. DNA was purified by phenol/chloroform extraction (1:1, vol/vol), precipitated overnight at -20°C with 0.3 M sodium acetate, and collected by centrifugation at 15,000 *g* for 15 min at 4°C. The pellet containing RNA and DNA was resuspended in TE buffer [10 mM Tris-HCl (pH 8.0), 1.0 mM EDTA] and treated subsequently with RNase (200 µg/ml) for 2 h at room temperature, followed by an incubation overnight with proteinase K (200 µg/ml) at 48°C. Finally, the mixture was reextracted with phenol/chloroform and chloroform, precipitated with ethanol, and resuspended in TE buffer. DNA fragments were separated electrophoretically on 1.8% agarose gel containing 0.5 µg/ml ethidium bromide in 0.5× TBE buffer (TBE: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA).

**Detection of mRNA for TNFα and TNFα receptors by reverse transcription and PCR (RT-PCR).** Total RNA was extracted from pancreatic tissue and from isolated acinar cells using the method of Han et al. (41) or TRIzol reagent (GIBCO BRL, Gaithersburg, MD). RNA quality was verified by ethidium bromide staining of rRNA bands on denaturing agarose gel. 5 µg of total RNA was reverse-transcribed according to the manufacturer's protocol (SuperScript II Pre-amplification System; GIBCO BRL), using oligo(dT) as a primer. For TNFα message, the cDNA prepared from 1 µg total RNA was subjected to PCR for 32 cycles at 56°C using rat-specific primers. The forward 5'-GAGATGTGGAAGTGGCAGAGG and reverse 5'-GGTACAGCCCATCTGCTGGTA primers spanned several introns in the TNF gene, and were located at nucleotides 23–43 and 386–406, respectively, of rat TNFα cDNA (GenBank accession number X66539).

For mRNA expression of TNFR1 and TNFR2, the same amount of cDNA was subjected to hot-start PCR for 35 cycles at 56°C using murine-specific intron-spanning primers. The forward 5'-TGGT-GCTCCTGGCTCTGCT and reverse 5'-ACCTGGAACATTTCTTT-CCGAC primers for TNFR1 were located at nucleotides 275–293 and 551–572, respectively, of rat TNFR1 cDNA (accession number M63122/M75862). The forward 5'-ATGAGAAATCCCAGGATG-CAG and reverse 5'-CTACAGACGTTCCAGATGCAG primers for TNFR2 were located at nucleotides 957–977 and 1192–1212, respectively, of mouse TNFR2 cDNA (accession number M60469), and are 100% identical to the rat sequence (U55849). The amplified PCR products were separated on agarose gel stained with ethidium bromide, purified with GeneClean II kit (Bio 101, La Jolla, CA), subcloned into pGEM-T vector (Promega Corp., Madison, WI), and sequenced using the Sequenase Version 2.0 sequencing kit (USB Biologicals, Cleveland, OH).

**Preparation of nuclear extracts and electrophoretic mobility shift assay (EMSA).** Nuclear protein extracts were prepared essentially as described previously (42). A sample of isolated pancreatic acinar cells was washed with ice-cold PBS and lysed on ice in hypotonic buffer A (42) by five strokes in a glass Dounce homogenizer. Just before use, buffer A was supplemented with PMSF and DTT to a final concentration of 1 mM each and with the protease inhibitor cocktail described above. The homogenate was left on ice for 15 min, after which NP-40 was added to the final concentration of 0.3–0.4% (vol/vol). The sample was briefly vortexed, incubated on ice for 1–2 min, and crude nuclear pellet was collected by centrifugation for 30 s in a microfuge. The supernatant was removed, and the nuclear pellet was resuspended in high-salt buffer C (42) containing 20 mM Hepes (pH 7.6), 25% (vol/vol) glycerol, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM

EDTA, 20 mM  $\beta$ -glycerophosphate, 10 mM  $\text{Na}_2\text{MoO}_4$ , 50 mM  $\text{Na}_3\text{VO}_4$ , 1 mM DDT, 1 mM PMSF, and the protease inhibitors. After rotating at 4°C for up to 1 h, 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 protein assay (Bio-Rad Laboratories, Hercules, CA) based on the method of Bradford.

In the EMSA, aliquots of nuclear extracts with equal amount of protein (2–10  $\mu\text{g}$ ) were mixed in 20- $\mu\text{l}$  reactions with 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  $\mu\text{g}$  poly [d(I-C)]. After incubating on ice for 5 min, binding reactions were initiated by the addition of 20,000–40,000 cpm (10 fmol) of  $^{32}\text{P}$ -labeled DNA probe and allowed to proceed for 25–30 min at room temperature. The oligo probe 5'-GCAGAGGGGACTTCCGAGA containing a  $\kappa\text{B}$ -binding motif (bold) was annealed with the complementary oligonucleotide bearing 5' G overhang, and labeled using Klenow DNA polymerase I. For cold competition, a 60–300-fold molar excess of nonlabeled  $\kappa\text{B}$  oligonucleotide was added to the reaction together with the probe. Samples were electrophoresed in 0.5 $\times$  TBE buffer with loading dye on nondenaturing 4.5% polyacrylamide gel at 200 V. Gels were dried and exposed at -80°C to RX film (Fuji Photo Film Co., Tokyo, Japan) with intensifying screens. The intensity of bands on gel fluorograms was quantitated with the use of image analysis system AMBIS.

**Quantitation of DNA fragmentation.** Isolated pancreatic acinar cells were collected by centrifugation and then suspended in TE lysis buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.2% (wt/vol) Triton X-100. High and low molecular weight DNA were separated by centrifuging the samples for 15 min at 13,000 g. Supernatants containing fragmented DNA and pellets containing high molecular weight DNA were each incubated overnight at 4°C in 1.0 ml TE lysis buffer and 0.25 ml 50% (vol/vol) TCA. DNA in precipitates from both supernatants and pellets was hydrolyzed by heating at 70°C for 20–25 min in 1.0 ml 0.5 M  $\text{HClO}_4$  and quantitated by the diphenylamine method of Burton (43).

**Cell viability.** Cell viability was determined by release of lactate dehydrogenase (LDH) into incubation medium. LDH activity was measured spectrofluorometrically as the production of NAD from pyruvic acid and NADH. Values for LDH release are presented as the percentage of total cellular LDH determined by permeabilizing cells with Triton X-100.

**TNF $\alpha$  measurements.** TNF $\alpha$  was measured using both bioassay and ELISA techniques. The bioassay measured the lytic effect of TNF $\alpha$  on WEHI 164 mouse fibrosarcoma cell line (44). WEHI 164 cells were resuspended at  $5 \times 10^5$  cells/ml in RPMI 1640 medium containing 10% bovine serum, 1 mM L-glutamine, and 0.5  $\mu\text{g}/\text{ml}$  actinomycin D. 100  $\mu\text{l}$  WEHI cell suspension was added to wells of a 48-well microtiter plate. 100  $\mu\text{l}$  recombinant TNF $\alpha$  or samples for analysis were added to the wells containing WEHI cells. Cell suspensions were then incubated for 20 h at 37°C in a humidified 5%  $\text{CO}_2$  atmosphere. To measure WEHI cell viability, 20  $\mu\text{l}$  3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well at a concentration of 5 mg/ml in PBS. MTT penetrates the cells. Viable cells with active mitochondria cleave the tetrazolium ring into a dark blue formazan reaction product, which can then be measured spectrophotometrically. The plates were incubated with MTT for 4 h, the medium was removed from each well by aspiration, and 200  $\mu\text{l}$  0.04 N HCl in isopropanol was added to each well. The plates were then stored overnight at room temperature. Absorbance was read at 550 nm. TNF $\alpha$  values for experimental samples were determined from a standard curve generated using mouse recombinant TNF $\alpha$ .

In another assay, immunoreactive TNF $\alpha$  was quantified by ELISA according to the manufacturer's protocol. In brief, a 48-well microtiter plate with wells precoated with anti-rat TNF $\alpha$  mAbs was used to capture rat TNF $\alpha$  from standards and test samples. After washing the plate to remove unbound material, a peroxidase-conjugated polyclonal TNF $\alpha$  antibody which binds to captured rat TNF $\alpha$  was added. To remove unbound material, each well was washed

again, and a substrate solution was added to initiate a peroxidase-catalyzed color reaction which was stopped by acidification. Absorbance was measured at 450 nm for both standards and experimental samples. Concentrations of rat TNF $\alpha$  were determined using the standard curve.

**Immunohistochemistry.** 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 were then washed with 0.5 ml PBS to remove unattached cells. The remaining acini were fixed on the cover slips and permeabilized by a 10-min incubation with 0.5 ml methanol at -20°C. For immunostaining of the pancreatic tissue, the pancreas was cut into 2–3-mm pieces and fixed in 4% paraformaldehyde on ice for 2 h. The tissue was then frozen in liquid nitrogen. Serial cryostat sections were cut at 8  $\mu\text{m}$  and mounted on glass slides.

Slides were incubated in the blocking medium containing 0.25 ml PBS, 5% (vol/vol) goat serum, 1% (wt/vol) BSA, and 0.1% (vol/vol) gelatin for 1 h at room temperature before application of primary antibody. The tissue sections or cell smears were then incubated for an additional 2 h at room temperature with primary antibodies against TNF $\alpha$  in the blocking solution. Slides were then washed three times with blocking medium, covered with 50  $\mu\text{l}$  blocking medium containing FITC-tagged secondary antibody, and finally incubated for 1 h at room temperature. The slides were washed three times with PBS, air dried, and mounted in Supermount (BioGenex, San Ramon, CA).

Immunostaining for TNF $\alpha$  was performed using rabbit anti-mouse TNF $\alpha$  antibody in 1:100 dilution (IP-400; Genzyme Corp.). Fluorescein-labeled goat anti-rabbit secondary antibody was used in 1:100 dilution; by itself, it did not stain inflammatory or any other cells on pancreatic tissue sections. The slides were washed three times with PBS, air dried, and mounted in Supermount.

To test the ability of exogenous TNF $\alpha$  to block the antibody labeling, 20 ng of TNF $\alpha$  in 1 ml blocking medium was mixed with 20  $\mu\text{l}$  TNF $\alpha$  antibody, rotated at 4°C for 4 h, and applied for staining.

To identify macrophages in the suspension of isolated pancreatic acinar cells, cell smears were stained using rabbit anti-rat macrophage antibody (Accurate Chemical and Science Corp., Westbury, NY) and HistoMark BLACK Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD). To identify neutrophils on pancreatic tissue sections, we stained the sections with FITC-tagged rabbit anti-rat polymorphonuclear leukocyte antibody (Accurate Chemical and Science Corp.).

We examined the slides with light or fluorescence microscopy using an FE1 microscope (Nikon, Inc. Instr. Group, Melville, NY). In all experiments negative controls were performed using secondary antibody only. The exposure and print times were the same for the experimental and control conditions.

**Experimental model of pancreatitis.** We divided rats randomly into four groups, each of which fell into two main categories, A and B. Category A consisted of rats that received continuous intravenous infusion of cerulein (in physiologic saline solution plus 30 U/liter heparin) at a dose of 5  $\mu\text{g}/\text{kg}/\text{h}$  for a period of 6 h at a rate of 0.6 ml/h. Category B consisted of rats that received an infusion of physiologic saline solution plus 30 U/liter heparin at the same rate. There were four groups of rats in each category. In A, group 1 contained rats that received cerulein only. Group 2 contained rats that, in addition to cerulein, were treated with an intravenous bolus of polyclonal rabbit anti-mouse neutralizing TNF $\alpha$  antibody. TNF $\alpha$  antibody (10  $\mu\text{l}$  diluted in 990  $\mu\text{l}$  PBS) was injected immediately before cerulein infusion. Group 3 contained rats that were neutrophil-depleted before the administration of cerulein. Neutrophil depletion was achieved, as we described previously (33), by an intraperitoneal injection of rabbit anti-rat neutrophil serum at a dose of 0.5 ml/100 g of body weight 24 h before the initiation of cerulein infusion. Only rats with neutrophil counts of < 200 were used. Group 4 contained rats that received combined treatment of anti-neutrophil serum 24 h before the cerulein infusion and an intravenous bolus of polyclonal rabbit anti-mouse TNF $\alpha$  neutralizing antibody immediately before cerulein infusion.

In category B, group 5 contained rats that received saline solution alone; group 6 contained rats treated with TNF $\alpha$  antibody immediately before saline infusion. Group 7 contained rats that were neutrophil depleted for 24 h prior to the saline infusion. Group 8 contained rats that were neutrophil-depleted as described above and that also received an intravenous bolus of polyclonal rabbit anti-mouse TNF $\alpha$  neutralizing antibody before the saline infusion. Each group consisted of at least three rats.

**Serum amylase and lipase.** Serum amylase and lipase were assessed by the Diagnostic Laboratory at the San Diego and West Los Angeles VA Medical Centers, using a Vet Test 8008 chemistry analyzer model.

**Vacuolization.** Pancreatic tissue was fixed immediately in 10% buffered formaldehyde. The tissue embedded in paraffin was sectioned and stained with hematoxylin and eosin (H&E). Values were obtained by counting the percentage of acinar cells containing vacuoles.

**Neutrophil infiltration.** The number of neutrophils on the pancreatic tissue sections stained was determined after staining for neutrophils (see *Immunohistochemistry*). Values were obtained by counting neutrophils in an average of 50 fields at a magnification of 40, accounting for at least 1,000 total cells.

**Quantitation of apoptosis.** Quantitation of apoptosis was performed as described previously (35) on sections of pancreatic tissue or isolated acini stained with 8  $\mu$ g/ml Hoechst 33258 in H<sub>2</sub>O. The stained sections were examined by fluorescent microscopy at excitation 380 nm and emission 510 nm. Scoring and classification of nuclei were performed as a function of the condensation state of chromatin. Pancreatic nuclei containing condensed and/or fragmented chromatin were considered to be apoptotic.

**Statistical analysis of data.** Statistical analysis of data was done using unpaired Student's *t* test.

**Reagents.** Polyclonal rabbit anti-mouse TNF $\alpha$  antibodies (Western blot, IP-410; neutralizing, IP-400), hamster anti-mouse mAbs against TNF $\alpha$  receptors TNFR1 and TNFR2, recombinant mouse TNF $\alpha$ , and TNF $\alpha$  ELISA kit were from Genzyme Corp.; goat anti-human antibodies against TNF $\alpha$  receptors were from R&D Systems (Minneapolis, MN); mouse anti-human mAbs against TNF $\alpha$  receptors were generously provided by Dr. M. Brockhaus (Hoffmann-La Roche Inc., Basel, Switzerland). Anti-neutrophil serum and FITC-tagged rabbit anti-rat polymorphonuclear leukocyte antibody were from Accurate Chemical and Science Corp. Fluorescein-conjugated goat anti-rabbit and horseradish peroxidase-conjugated goat anti-hamster secondary antibodies were from American Qualex Antibodies (San Clemente, CA); goat anti-mouse, goat anti-rabbit, and rabbit anti-goat IgG horseradish peroxidase conjugates were from Bio-Rad Laboratories. HistoMark BLACK test system for immunohistochemical staining was from Kirkegaard & Perry Laboratories. Cerulein was from Peninsula Laboratories (Belmont, CA). NADH disodium salt, Hoechst 33258, diphenylamine, antipain, aprotinin, chymostatin, leupeptin, pepstatin, PMSF, and LPS from *Escherichia coli* serotype were from Sigma Chemical Corp. (St. Louis, MO). Protein A-Sepharose was from Pierce Chemical Co. (Rockford, IL). Proteinase K and poly[d(I-C)] were from Boehringer Mannheim (Indianapolis, IN). Pre-cast Tris-glycine gels were from Novex. Medium 199 and RPMI 1640 were from GIBCO BRL. ECL detection kit and [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol) were from Amersham Corp.

## Results

**TNF $\alpha$  mRNA and protein expression in pancreas.** To determine whether TNF $\alpha$  mRNA is expressed in rat pancreas, the total RNA was extracted, and RT-PCR was performed as described in Methods. As shown in Fig. 1 A, a PCR product of expected size (384 bp) was amplified with specific TNF $\alpha$  primers spanning several introns in the TNF $\alpha$  gene. The amplified product was subcloned, sequenced, and its identity was confirmed by

comparing with published rat TNF $\alpha$  cDNA sequence (GenBank accession number X66539). PCR controls performed without template or with the RT reaction omitted were both negative, indicating absence of contamination (not shown).

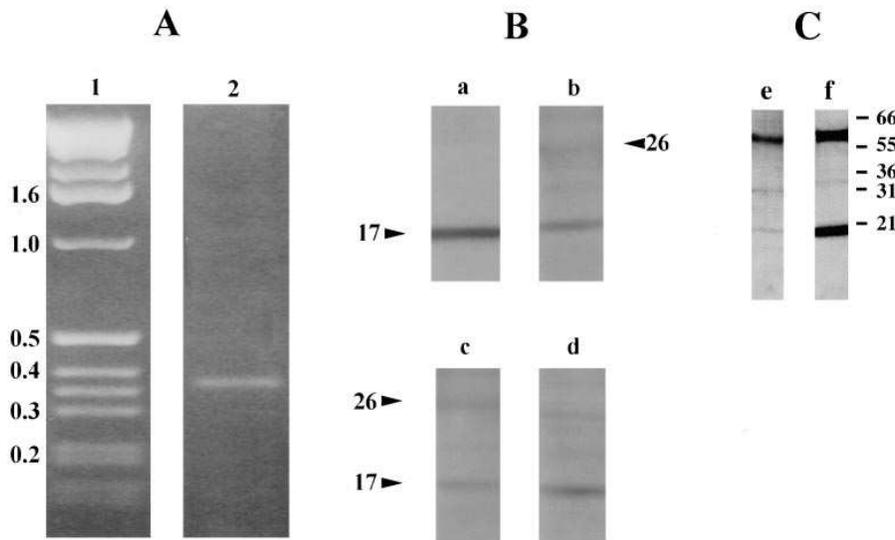
To demonstrate that the TNF $\alpha$  protein is produced by the pancreas, we enriched TNF $\alpha$  in tissue or cell lysates by immunoprecipitation and analyzed it using Western blot. Fig. 1 B shows that antibody against TNF $\alpha$  recognized a 17-kD protein in immunoprecipitates from acinar cell lysates, from normal pancreatic tissue, and from tissue from rats treated with cerulein. The protein had the same molecular size as the recombinant TNF $\alpha$  (lane *a*). The 26-kD band recognized by the antibody against TNF $\alpha$  may represent an unprocessed membrane-bound form of TNF $\alpha$  which was reported previously (19, 45).

The data presented in Fig. 1 demonstrate that both TNF $\alpha$  mRNA and protein are present in normal rat pancreas.

We compared the intensities of the 17-kD band in the immunoprecipitates obtained from equal amounts of total protein from pancreata of cerulein-treated versus control rats (Fig. 1 B, lanes *d* and *c*, respectively). The ratio of the intensities of these bands was  $2.2 \pm 0.4$  ( $n = 5$ ,  $P < 0.03$ ), indicating upregulation of TNF $\alpha$  in cerulein pancreatitis. To check if this ratio could be affected by possible differences in TNF $\alpha$  recovery from tissue lysates under different treatment conditions, pancreatic tissue lysates from both saline- and cerulein-treated animals were spiked with the same amount of recombinant TNF $\alpha$ , followed by immunoprecipitation and Western blotting. Immunoblots showed that the same amount of TNF $\alpha$  was recovered in both pancreatic and control conditions (not illustrated).

The biologically active native form of TNF $\alpha$  is a noncovalently linked trimer which dissociates into monomers under denaturing conditions, in particular in the presence of SDS (39, 40, 45). We wanted to test whether the TNF $\alpha$  antibody we applied for immunolocalization of TNF $\alpha$  in pancreas (see below) recognizes the native form of TNF $\alpha$ . For this, we used conditions (see Methods) under which the TNF $\alpha$  monomer-oligomer equilibrium in tissue lysate was shifted towards trimer formation. In these conditions (0.05% SDS, instead of regular 1%), Western blot analysis of both recombinant TNF $\alpha$  and total pancreatic protein revealed a prominent band at 55–60 kD (the size of TNF $\alpha$  trimer) and also two weak bands which correspond to dimeric (36 kD) and monomeric (17 kD) forms of the protein (Fig. 1 C). The immunoblot did not show any other prominent band in the whole range up to 200 kD. The ability of this antibody to recognize both trimeric and monomeric forms of TNF $\alpha$  justified its use for immunohistochemical localization of TNF $\alpha$ .

**Cellular source for TNF $\alpha$  in pancreas.** To determine if TNF $\alpha$  was present in pancreatic acinar cells, we isolated acini from control rats and incubated them for up to 6 h with or without LPS, a potent inducer of TNF $\alpha$  synthesis in macrophages (3, 4). Staining performed using the primary antibody characterized above (Fig. 1 C) and FITC-tagged secondary antibody showed TNF $\alpha$  localization in acinar cells (Fig. 2, *a* and *b*). If TNF $\alpha$  were produced mainly by macrophages or any other minor cell population present in acinar cell preparations, there should have been a few cells with high intensity of staining. However, this was not the case: both in the absence (Fig. 2 *a*) or presence (not shown) of LPS, we observed an even staining on cell smears. Microscopic observations of H&E-stained preparations of isolated dispersed pancreatic acini showed that



**Figure 1.** Both TNF $\alpha$  mRNA and protein are expressed in rat pancreas. (A) Total RNA was extracted from normal rat pancreas, and RT-PCR was performed as described in Methods. One half of the 50  $\mu$ l PCR reaction was resolved on an agarose gel stained with ethidium bromide. Lane 1, DNA standards in kb pairs; lane 2, PCR product amplified with specific rat TNF $\alpha$  primers. The data are representative of four similar RT-PCR experiments performed on different RNA preparations. (B) Proteins were extracted from isolated pancreatic acinar cells, the pancreas of control rats, and the pancreas of rats with cerulein pancreatitis, as described in Methods. The extracts were adjusted to equal protein concentration, and TNF $\alpha$  was immunoprecipitated as described in Methods. Proteins from the immunoprecipitate were fractionated on 4–20% SDS-polyacrylamide gels, and immunoblotted with polyclonal rabbit anti-mouse TNF $\alpha$  antibody (IP-410; Genzyme Corp.). Immunoreactive bands were visualized by ECL. Lanes are as follows: (a) recombinant TNF $\alpha$ ; (b) isolated pancreatic acinar cells; (c) pancreas from control (saline-infused) rats; and (d) pancreas from rats with cerulein pancreatitis. Arrowheads, Protein sizes in kD. The data are representative of five similar experiments. (C) Pancreatic tissue lysate from normal rat (e) and recombinant TNF $\alpha$  (lane f) were subjected to Western blot analysis under conditions favoring TNF $\alpha$  oligomerization (see Methods). In both samples, the final concentration of SDS was 0.05% instead of regular 1%. The proteins were separated by 4–20% PAGE and immunoblotted with polyclonal rabbit anti-mouse TNF $\alpha$  antibody (IP-400; Genzyme Corp.). Immunoreactive bands were visualized by ECL. The numbers shown are protein standard sizes in kD. The experiment was repeated twice with similar results.

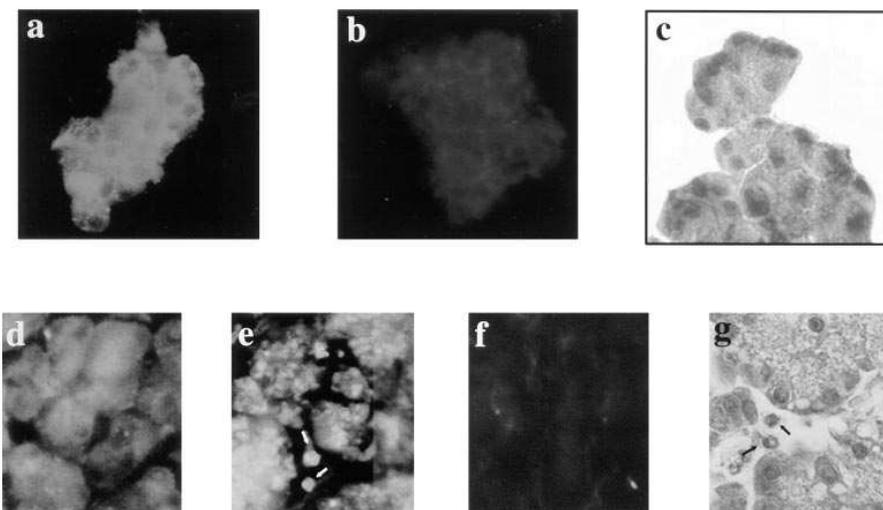
munoblotted with polyclonal rabbit anti-mouse TNF $\alpha$  antibody (IP-410; Genzyme Corp.). Immunoreactive bands were visualized by ECL. Lanes are as follows: (a) recombinant TNF $\alpha$ ; (b) isolated pancreatic acinar cells; (c) pancreas from control (saline-infused) rats; and (d) pancreas from rats with cerulein pancreatitis. Arrowheads, Protein sizes in kD. The data are representative of five similar experiments. (C) Pancreatic tissue lysate from normal rat (e) and recombinant TNF $\alpha$  (lane f) were subjected to Western blot analysis under conditions favoring TNF $\alpha$  oligomerization (see Methods). In both samples, the final concentration of SDS was 0.05% instead of regular 1%. The proteins were separated by 4–20% PAGE and immunoblotted with polyclonal rabbit anti-mouse TNF $\alpha$  antibody (IP-400; Genzyme Corp.). Immunoreactive bands were visualized by ECL. The numbers shown are protein standard sizes in kD. The experiment was repeated twice with similar results.

acinar cells comprise > 97% of the cell population. Staining of acinar cell preparations for macrophages (see Methods) did not reveal the presence of inflammatory cells in dispersed acini from control rats (not illustrated).

Fig. 2 c shows the morphology of acinar cells in primary culture on H&E preparations. It has been shown previously that acinar cells aggregate within minutes after cell isolation and during incubation in culture (46). In accordance with this, we observed cell clusters in acinar cell preparations after 3 h incubation in 199 medium.

Staining of acini for TNF $\alpha$  was observed in pancreatic tissue from both control and cerulein-treated rats; in rats with pancreatitis, staining was more intense and had more of a

punctate character (Fig. 2, d and e). Staining was abolished when the TNF $\alpha$  antibody was preabsorbed with exogenous TNF $\alpha$  (Fig. 2 f), or when secondary antibody alone was applied (not shown). As can be seen in Fig. 2 g, pancreas from rats with cerulein pancreatitis displayed infiltration of inflammatory cells. This is a characteristic feature of pancreatitis: in rat cerulein pancreatitis, inflammatory cells comprise ~ 8% of total cells in the pancreas, including 5% neutrophils (33). Pancreatic tissue sections from cerulein-treated rats displayed comparable intensity of staining for TNF $\alpha$  in both acinar and inflammatory cells (Fig. 2 e). Thus, both acinar and inflammatory cells are sources for TNF $\alpha$  in pancreas. The relative contribution of these two sources to TNF $\alpha$  bioactivity in pancreas,



**Figure 2.** TNF $\alpha$  is localized in rat pancreatic acini. (a–c) Dispersed pancreatic acini isolated from normal rats were incubated for 3 h at 37°C in 199 medium, plated on glass coverslips, permeabilized, and stained for TNF $\alpha$  (a and b) or with H&E (c). Immunostaining was performed using both the primary TNF $\alpha$  antibody (IP-400; Genzyme Corp.) and FITC-tagged secondary antibody (a) or the secondary antibody alone (b; negative control). The slides were examined with fluorescence (a and b) or light (c) microscopy;  $\times 40$ . (d–g) Sections of pancreatic tissue from saline- (d) or cerulein-treated (e–g) rats were stained for TNF $\alpha$  (d–f) or with H&E (g). Immunostaining was performed using the same primary and secondary antibodies as in a and b. In f, the TNF $\alpha$  antibody was preabsorbed with an excess of recombinant

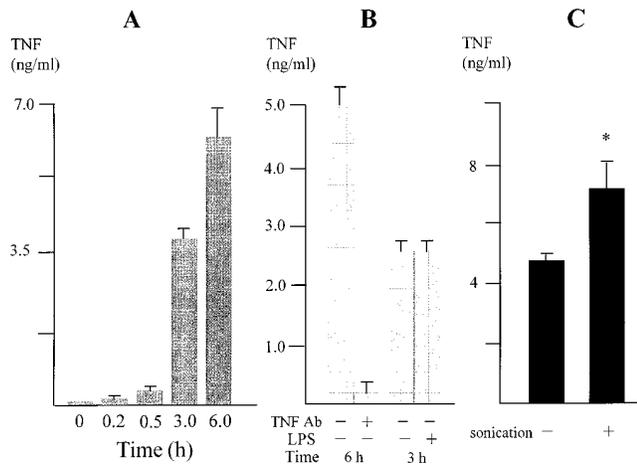
TNF $\alpha$  (see Methods). Arrowheads (e and g), Inflammatory cells in the pancreas of rats with cerulein pancreatitis. The slides were examined with fluorescence (d–f) or light (g) microscopy;  $\times 40$ .

which is dependent on the rates of TNF $\alpha$  release by these cell types, remains to be determined.

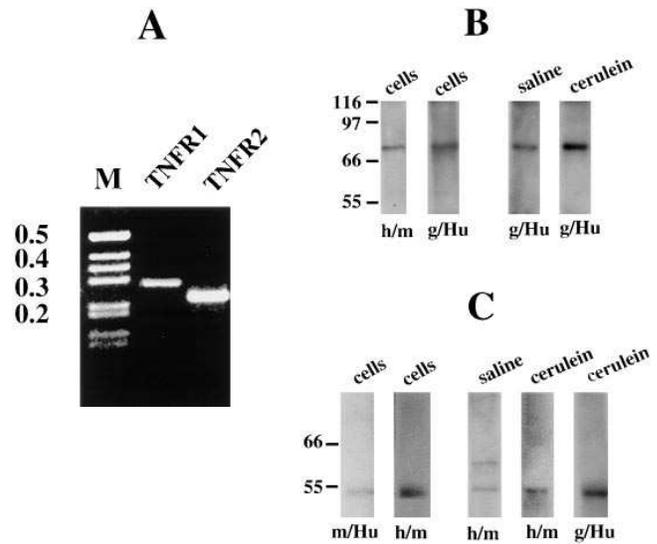
**TNF $\alpha$  release from pancreatic acinar cells.** To test whether the pancreas could release TNF $\alpha$ , we incubated freshly isolated pancreatic acini for different time periods in 199 medium and measured TNF $\alpha$  in the extracellular medium. Both ELISA and bioassay detected TNF $\alpha$  released from acinar cells (Fig. 3).

In these and other experiments, we used dispersed pancreatic acini cultured in 199 medium as described in Methods. The morphology of acinar cells in this primary culture was shown above in Fig. 2 c. The cell viability during 6 h incubation was measured by LDH release and was > 92% (see Fig. 5). To test further whether acinar cells after 6 h incubation retained their functions, we measured Ca<sup>2+</sup> response to the neurotransmitter carbachol. In cells incubated for 1, 4, and 6 h, 0.1 mM carbachol induced the same threefold increase in cytosolic Ca<sup>2+</sup> (not shown).

TNF $\alpha$  accumulation in the extracellular medium increased with incubation time, from 0.1 ng/ml after 10 min up to 6.3 ng/ml after 6 h incubation (Fig. 3 A). Specificity of the bioassay TNF $\alpha$  detection in our conditions was confirmed by adding an anti-TNF $\alpha$  neutralizing antibody to the incubation medium. In the presence of the antibody, TNF $\alpha$  activity determined by the bioassay decreased more than 100-fold compared with the



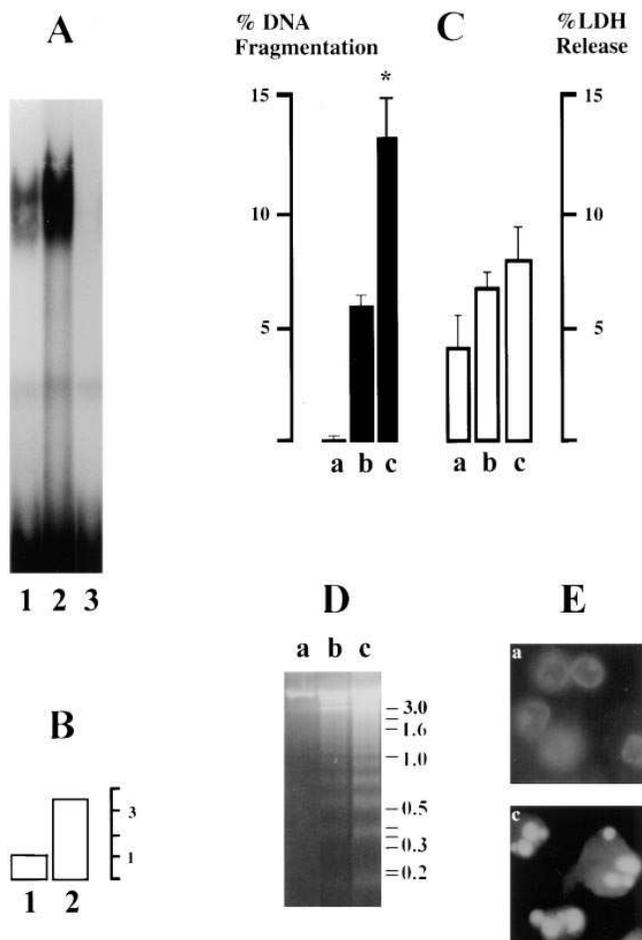
**Figure 3.** Pancreatic acinar cells produce and release TNF $\alpha$ . (A) Dispersed acini from normal rat were incubated in 199 medium at 37°C at a concentration of  $5 \times 10^5$  cells/ml, and TNF $\alpha$  levels in the extracellular medium were determined by ELISA at indicated time points. The results represent means  $\pm$  SE for triplicate determinations. A similar time curve was obtained in a second independent experiment. (B) Dispersed acini from normal rat were incubated for 3 or 6 h in 199 medium at 37°C in the presence or absence of 5  $\mu$ l neutralizing TNF $\alpha$  antibody or 10  $\mu$ g/ml LPS. TNF $\alpha$  levels in the extracellular medium were determined by bioassay (see Methods). The results represent means  $\pm$  SE for three determinations. (C) Dispersed acini from normal rat were incubated for 6 h in 199 medium at 37°C, then the cell suspension was divided equally into two portions, and TNF $\alpha$  levels were measured by ELISA. In one portion, the cells were permeabilized by sonication, and the total amount of TNF $\alpha$  in the medium was measured. In the second, the amount of TNF $\alpha$  released from intact cells (without sonication) was measured in the extracellular medium. The data represent means  $\pm$  SE for triplicate determinations. Similar results were obtained in a second independent experiment on a different preparation of acinar cells. \* $P < 0.03$ .



**Figure 4.** Both 55- and 75-kD TNF $\alpha$  receptors are expressed in rat pancreas on mRNA and protein level. (A) Total RNA was extracted from normal rat pancreas, and RT-PCR was performed as described in Methods. PCR products for TNFR1 (expected size 298 bp) and TNFR2 (expected size 257 bp) were amplified with rat-specific intron-spanning primers. One half of the 50- $\mu$ l PCR reaction was resolved on an agarose gel stained with ethidium bromide. Lane M, DNA standards in kb pairs. The data are representative of three similar RT-PCR experiments performed on different RNA preparations. (B–C) Proteins were extracted from isolated pancreatic acinar cells (cells), from the pancreas of control rats (saline) or from rats with cerulein pancreatitis (cerulein). The extracts were adjusted to equal protein concentration, and TNF $\alpha$  receptors were immunoprecipitated with hamster anti-mouse antibodies against 75- (B) or 55-kD TNF $\alpha$  receptor (C). Precipitates were solubilized, fractionated on 8% SDS-polyacrylamide gels, and immunoblotted with hamster anti-mouse (h/m), goat anti-human (g/Hu), or mouse anti-human (m/Hu) antibodies against TNFR1 and TNFR2. Immunoreactive bands were visualized by ECL. The numbers shown are protein standard sizes in kD. The data are representative of three to five similar experiments.

same sample incubated without the TNF $\alpha$  antibody (Fig. 3 B). Similar results (not shown) were obtained when the TNF $\alpha$  neutralizing antibody was added into the samples after 6 h incubation of acinar cells in 199 medium, just before testing for TNF $\alpha$  bioactivity with WEHI cells. Addition of the same amount of unrelated antibody, a rabbit anti-rat IgG, did not affect TNF $\alpha$  activity determined by the bioassay. Of note, LPS did not stimulate TNF $\alpha$  secretion from acinar cells (Fig. 3 B), suggesting that the release of pancreatic TNF $\alpha$  was regulated differently from macrophage TNF $\alpha$ .

To determine the total amount of TNF $\alpha$  in dispersed pancreatic acini, the cells were incubated for 6 h, permeabilized by sonication, and then TNF $\alpha$  concentration was measured in the medium (Fig. 3 C). In a parallel experiment, TNF $\alpha$  released during 6 h incubation from intact cells (without sonication) was measured in the extracellular medium. It appeared that after 6 h incubation, the total amount of TNF $\alpha$  in the cell suspension (TNF $\alpha$  within the cells plus TNF $\alpha$  in the extracellular medium) was only 1.5 times that of the released TNF $\alpha$  (Fig. 3 C); that is, acinar cells released about the same amount of TNF $\alpha$  as they contained. On the other hand, these data make it unlikely that the released TNF $\alpha$  leaked out of damaged cells.



**Figure 5.** Effects of TNF $\alpha$  on parameters of injury and viability of isolated pancreatic acinar cells. Pancreatic acini isolated from normal rats were incubated at 37°C for 6 h in 199 medium at the concentration of  $5 \times 10^5$  cells/ml in the absence or presence of 100 ng/ml TNF $\alpha$ . The cells were then collected, and both pellets and supernatants were used separately to measure NF- $\kappa$ B activation (A and B), the percentage (C) and pattern (D) of DNA fragmentation, and LDH release (C). The morphology of apoptosis in the acinar cell suspension was evaluated by staining with Hoechst 33258 (E). (A) NF- $\kappa$ B electrophoretic mobility shift assay was performed on nuclear extracts containing 7  $\mu$ g of protein from cells incubated without (lane 1) or with TNF $\alpha$  (lane 2). The binding reaction shown in lane 2 was repeated in the presence of 100-fold molar excess of cold  $\kappa$ B oligonucleotide used as a competitor (lane 3). The results are representative of two similar experiments performed on different acinar cell preparations. (B) Total counts for the NF- $\kappa$ B band presented in lane 2 (cells incubated with TNF $\alpha$ ) were normalized to the intensity of NF- $\kappa$ B band in lane 1 (cells incubated without TNF $\alpha$ ). Densitometric quantitation of gel fluorograms was done using the image analysis system AMBIS. (C) DNA fragmentation (filled bars) and LDH release (open bars) were measured in freshly isolated acinar cells (a) and cells incubated for 6 h in the absence (b) or presence (c) of 100 ng/ml TNF $\alpha$ . For quantitation of DNA fragmentation, low and high molecular weight DNA were separated by 15 min centrifugation at 13,000 g. The amount of DNA in supernatants and pellets was determined with the diphenylamine method as described in Methods. The data represent the percentage of fragmented DNA per total DNA. LDH released from the cells was measured spectrophotometrically in supernatants. Total cellular LDH was determined in cell suspension treated with 0.5% Triton X-100. The values represent the percentage of total cellular LDH release in the incubation medium. The results represent means  $\pm$  SE of at least three different determinations. \* $P < 0.05$  compared with

(Note again that LDH release after 6 h incubation did not exceed 8%).

**TNF $\alpha$  receptors on pancreatic acinar cells.** mRNA expression of both TNFR1 and TNFR2 in the normal rat pancreas was detected by RT-PCR (Fig. 4 A) as described in Methods. PCR products of expected size (298 bp for TNFR1 and 257 bp for TNFR2) were amplified with rat-specific intron-spanning primers, subcloned, and sequenced by the dideoxy chain termination method (47). Their sequences were identical to published rat cDNAs for TNFR1 (GenBank accession number M63122/M75862) and TNFR2 (U55849). PCR controls performed with the omission of the RT step were negative. Interestingly, the amount of PCR product for the 75-kD TNF $\alpha$  receptor was higher than for the 55-kD receptor (Fig. 4 A); both were amplified in identical conditions, from the same amount of cDNA obtained in one RT reaction. RT-PCR products of the sizes expected for TNFR1 and TNFR2 were also amplified from RNA isolated from dispersed pancreatic acini (not shown).

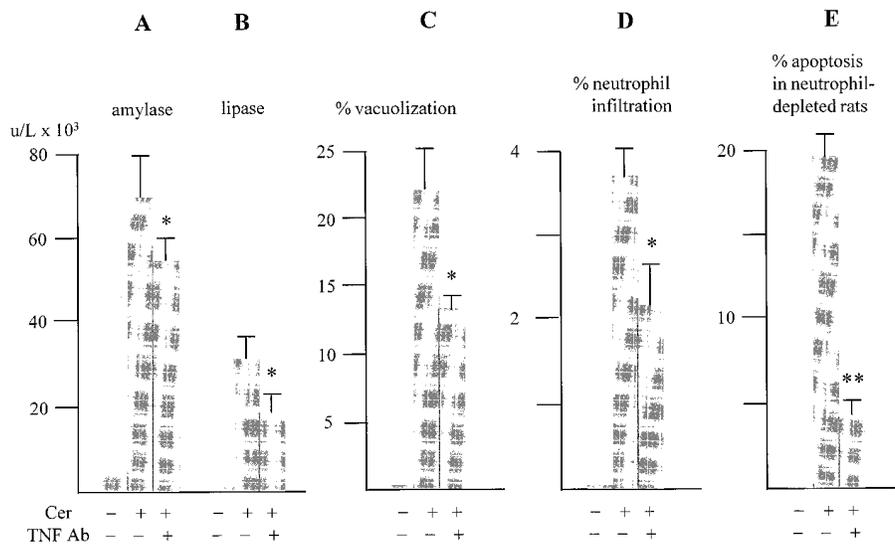
To demonstrate that the TNF $\alpha$  receptors were present on pancreatic acinar cells, we used immunoprecipitation (to enrich TNF $\alpha$  receptor concentrations in cell or tissue lysates) followed by Western blot analysis. To immunoprecipitate TNF $\alpha$  receptors, we used hamster anti-mouse mAbs against 55-kD and 75-kD TNF $\alpha$  receptors. Immunoprecipitates were probed with three different types of antibodies against TNF $\alpha$  receptors: hamster anti-mouse, goat anti-human, and mouse anti-human. All of them recognized TNFR1 and TNFR2 both in pancreatic tissue and acinar cells (Fig. 4, B and C).

The intensities of TNFR1 and TNFR2 bands were compared for saline- versus cerulein-treated rats using immunoprecipitates obtained from equal amounts of pancreatic protein. Densitometry showed that in pancreas from cerulein pancreatitis, the amount of 75-kD TNF $\alpha$  receptor (Fig. 4 B) increased  $1.80 \pm 0.28$  times ( $n = 4$ ,  $P < 0.05$ ) relative to control. The same ratio for the 55-kD TNF $\alpha$  receptor (Fig. 4 C) varied from 1.3- to 3.0-fold ( $n = 4$ ).

**Effects of TNF $\alpha$  on pancreatic acinar cells.** To determine whether TNF $\alpha$  affects pancreatic acinar cell functioning, we measured the effects of exogenous TNF $\alpha$  on NF- $\kappa$ B activation, apoptosis, and viability of acinar cells (Fig. 5). NF- $\kappa$ B translocation into the nucleus (a measure of activation) was determined using EMSA. Apoptosis was quantitated by measuring percentage of fragmented DNA. Oligonucleosomal DNA fragmentation is considered a hallmark of apoptosis (48); therefore, the pattern of DNA fragmentation was determined by gel electrophoresis. Effect of TNF $\alpha$  on cell viability was estimated by measuring LDH release.

Isolated acinar cells were incubated in serum-free medium for 6 h in the presence or absence of 100 ng/ml TNF $\alpha$ , and indicated parameters were measured. The results (Fig. 5, A and B)

DNA fragmentation in the absence of TNF $\alpha$ . (D) DNA was isolated from freshly isolated acinar cells (a) and cells incubated for 6 h in the absence (b) or in the presence (c) of 100 ng/ml TNF $\alpha$ . DNA fragments were separated on 1.8% agarose gel. DNA molecular size standards in kb pairs are shown to the right. The data are representative of three similar experiments on different acinar cell preparations. (E) Pancreatic acini were plated on glass coverslips, fixed with methanol, and stained with Hoechst 33258 as described in Methods. (a) freshly isolated cells; (c) cells incubated for 6 h in the presence of 100 ng/ml TNF $\alpha$ .



**Figure 6.** Effects of TNF $\alpha$  blockade on the characteristics of pancreatitis: plasma amylase and lipase, vacuolization, neutrophil infiltration, and apoptosis. (A–D) Animals received saline infusion, cerulein infusion, or were treated with an intravenous bolus of polyclonal rabbit anti-mouse neutralizing TNF $\alpha$  antibody immediately before cerulein infusion (see Methods). (E) Rats were first depleted of neutrophils by infusion of rat anti-neutrophil serum and then received saline, cerulein, or TNF $\alpha$  antibody plus cerulein. (A and B) Amylase and lipase levels in U/liter; (C) percentage of acinar cells with vacuoles; (D) percentage of neutrophils per total cells in pancreatic tissue sections; and (E) percentage of apoptotic cells per total cells in pancreatic tissue sections. The results represent means  $\pm$  SE of the values reported from at least three animals. \* $P < 0.05$ , \*\* $P < 0.01$  compared to the same parameters in the absence of TNF $\alpha$  antibody.

show a slight activation of NF- $\kappa$ B in control cells incubated for 6 h in serum-free medium, and a pronounced NF- $\kappa$ B translocation caused by TNF $\alpha$  (a 3.7-fold increase). Thus, pancreatic acinar cells respond to TNF $\alpha$  by NF- $\kappa$ B activation.

Incubation of control acinar cells in serum-free medium for 6 h resulted in  $\sim 6\%$  DNA fragmentation which was undetectable in freshly isolated cells (Fig. 5 C). The observed DNA fragmentation suggested that dispersed pancreatic acini undergo apoptosis in primary culture. This finding was confirmed by oligonucleosomal degradation of DNA extracted from acinar cells incubated in serum-free medium for 6 h (Fig. 5 D, lane b).

As shown by LDH release, after 6 h incubation, viability of control cells was almost the same ( $> 92\%$ ) compared with freshly isolated acinar cells (Fig. 5 C). Longer ( $> 12$  h) incubation of primary cells in the conditions used resulted in the death of  $> 80\%$  of acinar cell population. For this reason, the effects of TNF $\alpha$  were measured at 6 h.

Incubation of dispersed acini with TNF $\alpha$  under conditions stated increased the DNA fragmentation twofold (Fig. 5 C). TNF $\alpha$  also potentiated DNA laddering (Fig. 5 D, lane c). In accordance with the data on DNA laddering, cells with morphology of apoptosis were found in preparations of dispersed pancreatic acini incubated with TNF $\alpha$  but not in freshly isolated cells (Fig. 5 E). Fewer apoptotic cells were detected in preparations of acinar cells incubated for 6 h without TNF $\alpha$ . Thus, TNF $\alpha$  stimulates apoptosis in pancreatic acinar cells.

It has been shown that for some cell types, inhibition of protein synthesis increases cell sensitivity for TNF $\alpha$  (49). In our experiments, actinomycin D (0.5  $\mu$ g/ml) did not affect DNA fragmentation either in control acinar cells or in the presence of TNF $\alpha$  (not shown). Under the conditions used, TNF $\alpha$  increased slightly the percentage of LDH released from dispersed pancreatic acini (Fig. 5 C), which could be associated with both apoptosis and necrosis.

*Effects of TNF $\alpha$  neutralization on the parameters of pancreatitis.* To test if TNF $\alpha$  was involved in the mechanism of pancre-

creatitis, we infused rats with neutralizing TNF $\alpha$  antibody just before cerulein infusion. The data presented in Fig. 6 demonstrate that TNF $\alpha$  neutralization results in a mild improvement in characteristics of cerulein pancreatitis. The increase in serum levels of amylase and lipase was inhibited by 25 and 41%, respectively. Vacuolization and neutrophil infiltration decreased twofold. These findings suggest that TNF $\alpha$  is involved in the development of pancreatitis, although it apparently does not play a key role in the process. The same treatment with the TNF $\alpha$  antibody of saline-infused rats (group 6) did not change the indicated parameters compared with the saline-infused rats (group 5, not shown).

The cerulein model of pancreatitis is characterized by low levels of both apoptosis and necrosis (33, 36). Previously, we showed that the percentage of apoptosis drastically increased in rats depleted of neutrophils (33). We used this model to determine if TNF $\alpha$  would mediate apoptosis in the pancreas. Fig. 6 E demonstrates that TNF $\alpha$  antibody greatly inhibited apoptosis in cerulein-treated rats depleted of neutrophils, indicating the role of TNF $\alpha$  in mediation of apoptosis in this model of pancreatitis. The same treatment with TNF $\alpha$  antibodies of neutrophil-depleted rats which received saline infusion (group 8) did not show any changes in the parameters compared with the control rats (group 7, not shown).

## Discussion

Using RT-PCR, Western blot analysis, and immunohistochemistry, we found that TNF $\alpha$  mRNA is expressed and that TNF $\alpha$  protein is produced in both normal rat pancreas and dispersed pancreatic acini. RT-PCR and Western blotting showed that both 55- and 75-kD TNF $\alpha$  receptors were present in pancreatic acinar cells. Dispersed acini responded to TNF $\alpha$ , again demonstrating that TNF $\alpha$  receptors are present and functionally active in acinar cells. Activation of the transcription factor NF- $\kappa$ B is one of the first steps in TNF $\alpha$  signaling (9, 20, 21), which is believed to mediate, at least partially, the effects of

TNF $\alpha$ . Our data demonstrated that TNF $\alpha$  stimulated NF- $\kappa$ B translocation into the nuclei and potentiated apoptotic cell death in acinar cells incubated for 6 h in serum-free medium.

TNF $\alpha$  is not only produced but is also secreted by isolated acinar cells. In contrast to what was found for macrophages (3–5, 19), TNF $\alpha$  secretion was not increased by LPS. TNF $\alpha$  was released from isolated acinar cells during incubation of control cells in 199 medium without addition of any specific exogenous stimulus.

To our knowledge, these results are the first identification of TNF $\alpha$  production and release by pancreatic acinar cells. TNF $\alpha$  mRNA was not detected in normal mouse pancreas (29), and there is only one abstract communication (50) reporting the presence of this cytokine in acinar cells in rat tissue with experimental pancreatitis using *in situ* hybridization technique.

Our data suggest a physiological role for TNF $\alpha$  in pancreatic acinar cell functioning, namely, mediating apoptosis in these cells. In many cell types, TNF $\alpha$  was shown to stimulate death through necrosis and/or apoptosis (6–13). Signals which trigger apoptosis in acinar cells and TNF $\alpha$  release are yet to be determined; however, it is known that pancreatic acinar cells are very difficult to keep responsive and viable in long-term culture (46, 51). These cells usually undergo dedifferentiation and die during the first days after isolation. We found that apoptosis is one of the pathways of acinar cell death in culture. The mechanisms of pancreatic acinar cell death are currently under investigation in our laboratory. One can speculate that the loss of organ structure, cell–cell and cell–extracellular matrix contacts during cell isolation trigger TNF $\alpha$  release from acinar cells, and that TNF $\alpha$  mediates the process of death or dedifferentiation in primary culture. Supporting this speculation is the finding that addition of exogenous TNF $\alpha$  to acinar cell suspension resulted in activation of NF- $\kappa$ B translocation and potentiated apoptosis.

The data show an increase in the amount of TNF $\alpha$  in pancreas from rats with experimental pancreatitis compared with control. In tissue from rats with pancreatitis, both acinar and inflammatory cells stained for TNF $\alpha$  with similar intensity. The relative contribution of these cell types in TNF $\alpha$  bioactivity in the pancreas during pancreatitis depends, among other factors, on the rates of TNF $\alpha$  release by inflammatory and acinar cells, which remain to be determined.

From Western blot analysis, we found an upregulation of TNF $\alpha$  receptors in cerulein pancreatitis. The upregulation of TNF $\alpha$  and TNF $\alpha$  receptors in the pancreas can mediate a number of events in pancreatitis, in particular, recruitment of inflammatory cells in the area, regulation of cytokine production, and acinar cell death.

To evaluate a possible role for TNF $\alpha$  in pancreatitis, we measured the effects of TNF $\alpha$  neutralization on the parameters of cerulein pancreatitis. The results showed a mild improvement of pancreatitis. The rise in serum amylase and lipase was inhibited by 25–41%, and vacuolization and neutrophil infiltration decreased approximately twofold. This suggests that TNF $\alpha$  contributes to the development of pancreatitis, although it does not play a critical role in the induction of the injury. The role of TNF $\alpha$  in different experimental models of pancreatitis has not been studied in detail. The data obtained thus far suggest that in addition to cerulein pancreatitis, TNF $\alpha$  partially mediates experimental pancreatitis induced by bile infusion (31, 52, 53) and necrotizing pancreatitis in mice

(54). Our results show that TNF $\alpha$  released from pancreatic acinar cells can contribute to the development of pancreatitis by mediating the early stages which precede inflammatory infiltration. It is worth noting that TNF $\alpha$  was found to induce the mRNA expression of a pancreatitis-associated protein PAP-I in acinar cell line AR-42J (55).

Recently, we have shown that in rats depleted of neutrophils, cerulein-induced pancreatitis is associated with a high percentage of acinar cell apoptosis (33). Using this model of pancreatitis, we showed in this study that TNF $\alpha$  neutralization greatly inhibits apoptotic cell death in the pancreas. These results and the ability of TNF $\alpha$  to potentiate apoptosis in acinar cells in suspension suggest that TNF $\alpha$  mediates apoptosis in pancreas *in vivo*.

In conclusion, this study shows that pancreatic acinar cells produce, release, and respond to TNF $\alpha$ . TNF $\alpha$  is involved in the development of pancreatitis, and it mediates apoptosis in acinar cell suspension *in vitro* and also *in vivo* in the cerulein model of pancreatitis. Recently, we and others have found (33, 56) that another cytokine, platelet-activating factor, also mediates the development of experimental pancreatitis. Platelet-activating factor was shown to be produced by pancreatic acini (33, 56). One can hypothesize that at early stages of cell injury, pancreatic acinar cells produce and release cytokines/chemokines that initiate the chain of events resulting in the development of pancreatitis. These events include activation and recruitment of inflammatory cells in the area and induction of apoptotic and necrotic cell death.

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