Stimulation of stellate cells by injured acinar cells: a model of acute pancreatitis induced by alcohol and fat (VLDL)

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Siech M, Zhou Z, Zhou S, Bair B, Alt A, Hamm S, Gross H, Mayer J, Beger HG, Tian X, Kornmann M, Bachem MG. Stimulation of stellate cells by injured acinar cells: a model of acute pancreatitis induced by alcohol and fat (VLDL). Am J Physiol Gastrointest Liver Physiol 297: G1163–G1171, 2009. First published September 24, 2009; doi:10.1152/ajpgi.90468.2008.—Mechanisms leading to acute pancreatitis after a fat-enriched meal combined with excess alcohol are incompletely understood. We have studied the effects of alcohol and fat (VLDL) on pancreatic acinar cell (PAC) function, oxidative stress, and repair mechanisms by pancreatic stellate cells (PSC) leading to fibrogenesis. To do so, PAC (rat) were isolated and cultured up to 24 h. Ethanol and/or VLDL were added to PAC. We measured PAC function (amylase, lipase, injury (lactic dehydrogenase), apoptosis (TUNEL, Apo2.7, annexin V binding), oxidative stress, and lipid peroxidation (conjugated dienes, malondialdehyde, chemoluminescence); we also measured PSC proliferation (bromodeoxyuridine incorporation), matrix synthesis (immunofluorescence of collagens and fibronectin, fibronectin immunoaassay), and fatty acids in PAC supernatants (gas chromatography). Within 6 h, cultured PAC degraded and hydrolyzed VLDL completely. VLDL alone (50 μg/ml) and in combination with alcohol (0.2, 0.5, and 1% vol/vol) induced PAC injury (LDL, amylase, and lipase release) within 2 h through generation of oxidative stress. Depending on the dose of VLDL and alcohol, apoptosis and/or necrosis were induced. Antioxidants (Trolox, Probucol) reduced the cytotoxic effect of alcohol and VLDL. Supernatants of alcohol/VLDL-treated PAC stimulated stellate cell proliferation and extracellular matrix synthesis. We concluded that, in the presence of lipoproteins, alcohol induces acinar cell injury. Our results provide a biochemical pathway for the clinical observation that a fat-enriched meal combined with excess alcohol consumption can induce acinar cell injury (acute pancreatitis) followed by repair mechanisms (proliferation and increased matrix synthesis in PSC).

THE MECHANISM OF ALCOHOLIC PANCREATITIS is incompletely understood. Despite the fact that about 40% of acute pancreatitis and 70% of chronic pancreatitis in humans are induced by alcohol, most alcoholics will never be affected by any of these diseases. As calculated by Ammann et al. (1), only 5% of all alcohol abusers will develop alcoholic pancreatitis. Interestingly enough, this can also be observed in setups with animals where acute pancreatitis cannot be induced by alcohol itself (8, 15, 25). Therefore, additional factors accompanying alcohol intake must be coresponsible for the induction of the disease. Some of these cofactors such as stimulation of pancreatic secretion and/or obstruction of pancreatic outflow have been experimentally observed by us and others (8, 12, 25, 26).

Clinical experience has taught us that a large number of patients presenting an acute event of alcoholic pancreatitis have drunk lots of alcohol together with an opulent (fat enriched) meal the day before onset of the disease. In the present study, we focused on the effects of lipids in combination with alcohol on acinar cell injury and fibrogenesis.

In experimental alcoholic liver disease, pronounced free radical formation precedes pathological liver injury and seems to be responsible for most of the damage (11, 21). Pancreatic acinar cells (PAC) also metabolize ethanol by oxidative (10, 18) and nonoxidative pathway (9). We offered the hypothesis that exposure of PAC to ethanol induces the production of free radicals, which could further react with double bonds of unsaturated fatty acids, generating lipid peroxides. Both free oxygen radicals and lipid peroxides have a high potential to induce cell damage. We therefore added alcohol alone and in combination with fat (VLDL) to cultured rat PAC and measured the oxidative stress, amylase and lipase release, acinar cell injury [lactic dehydrogenase (LDH) release], and apoptosis.

In the second part of our experimental setup, we investigated the impact of injured acinar cells on fibrogenesis. Pancreatic stellate cells (PSC) are responsible for the production of the majority of extracellular matrix in acute and chronic pancreatitis (4, 16). We were interested to know whether acinar cells incubated with alcohol and VLDL produce fibrogenic mediators, which stimulate proliferation and matrix synthesis of stellate cells. On the basis of our data, we present a mechanism how ethanol might interact with lipids, leading to acinar cell damage and finally fibrosis.

MATERIALS AND METHODS

Reagents

Materials were purchased from the following sources: collagenase I and monoclonal anti-c fibronectin from Sigma Chemical (München, Germany); OptiPrep from Nycomed Pharma (Oslo, Norway); Ficoll from Pharmacia LKB (Freiburg, Germany); horseradish peroxidase (HRP)-anti-mouse, HRP-anti-rabbit, fluorescein-conjugated streptavi-
Preparation and Modification of VLDL

Total VLDL were isolated by ultracentrifugation (24 h, 4°C, 48,000 revolution/min; Beckman Ultracentrifuge L-60, Rotor Ti502) at a density of >1.019 g/mL (18.37 mg KBr is added to 1 mL plasma) from human EDTA plasma of healthy volunteers. To avoid the presence of chylophilic, at least 6 h after the last meal 100 mL EDTA-blood was taken and centrifuged at 1,800 × g to obtain EDTA-plasma. To eliminate KBr, isolated VLDL fraction was dialyzed twice against PBS (volume 400-fold) in the presence of 0.2 mM EDTA. To obtain enzymatically degraded VLDL (edVLDL), VLDL samples were incubated for 2 h at 37°C with trypsin (final concentration 7.7 mg/mL). Samples were incubated with neuraminidase (162 mU/mL; Dade Behring, Marburg, Germany) for 14 h at 37°C, and, finally, samples were diluted with DMEM to 10-fold of the initial volume. The increase of free fatty acids in edVLDL was quantified by gas chromatography. VLDL protein concentration was determined with a biuret reaction.

Animals

Wistar rats were obtained from the breeding colony of Ulm University Animal Facilities and were kept at 24°C under a 12-h:12-h light/dark cycle. The animals received a standard diet, and tap water was offered ad libitum. All experiments were approved by the local Animal Use and Care Committee (Ethics Commission Committee of the University of Ulm, no. 211/2002).

Cell Isolation

PAC and PSC were prepared from the same pancreas. Rats were euthanized, and collagenase-containing Eagle medium (1 mg/mL) was added to the pancreas. The distended pancreas was removed and shaken in an Erlenmeyer flask (37°C for 15 min). After this first digestion, the pancreas was minced followed by a second digestion with collagenase (1.75 mg/mL for 45 min). Dissection was accomplished by up and down suction through tubes with decreasing diameter. After dissociation, the acini and cells were filtered through a 250-μm nylon cloth and centrifuged after layering the filtrate on top of a dextran-Eagle-HEPES density gradient. After centrifugation, PAC were obtained from the bottom of the gradient, resuspended in DMEM, and cultured in fibronectin-coated six-well plates (or in eight-well culture slides for stainings), and cultured at 37°C in an oxygenized humidified surrounding.

After centrifugation, PSC were collected from the top of the gradient, washed twice, resuspended in Tris-buffered saline, and transferred on top of an Iodixanol density gradient. After another centrifugation, PSC were collected from the top of the gradient, washed, suspended in DMEM/Ham’s F12 (1:1, vol/vol) with 10% fetal calf serum (FCS), 2% l-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1% amphotericin, and seeded with a density of 4 × 10^5 cells/cm².

PSC Culture

PSC were cultured at 37°C in a 5% CO₂ humidified incubator. The medium consisted of DMEM/Ham’s F12 (1:1, vol/vol) with 10% FCS, 2% l-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 1% amphotericin. Medium was changed the day after seeding and each second day thereafter. After reaching confluence, cells were subcultured after trypsinization using a 0.025% trypsin solution containing 0.01% EDTA in PBS. To study cell proliferation and matrix synthesis, cells were seeded in 24-well plates (2 cm²/well; 1 mL medium; 30–50 × 10⁴ cells/cm²). To perform immunofluorescence microscopy, cells were seeded on 1-cm² glass coverslips in six-well (10 cm²/well; 2 mL medium) plates (two to three glass coverslips per well). The purity of PSC preparations was assessed on the basis of translucent fat droplets in the cytoplasm, their typical stellate-like morphology, and after passage by stainings of vimentin (100% positive), desmin (20–40% positive), and α-smooth muscle actin (>90% positive). (5)

Demonstration of PAC Apoptosis

Within 1 h after cell isolation, alcohol and fat (VLDL) were added either separately or mixed to cultured PAC. Six hours after the addition of alcohol and/or VLDL, PAC were washed carefully with PBS and fixed for 1 h in PBS-buffered 4% formaldehyde. After washing, nonspecific binding was blocked with 10 mM Tris, 150 mM NaCl, 0.5% bovine albumin, pH 7.4 (TNB buffer) for 45 min at room temperature (RT).

Annexin V binding. Fixed PAC were incubated with biotinylated annexin V (diluted 1/30 in TNB with 2 mM CaCl₂) for 1 h at RT. After washing with TNB buffer for 45 min at room temperature (RT), PAC were incubated with streptavidin-phycoerythrin (1/100 in TNB with 2 mM CaCl₂) for 1 h at RT. After washing with TNT buffer in the presence of CaCl₂, nuclei were counterstained with 5 μM Hoechst 33258 for 30 min at RT.

Ap2.7 expression. Cell membranes of formaldehyde-fixed PAC were permeabilized using 70% ethanol (1 h, at RT). Thereafter, unspecific bindings were blocked with TNT buffer. Ap2.7 was stained using anti-Ap2.7 diluted 1/10, anti-mouse HRP (1/50), followed by TSA. After washing, PAC were stained with streptavidin-phycocerythin (1/100 in TNB with 2 mM CaCl₂) for 1 h at RT. After washing with TNT buffer in the presence of CaCl₂, nuclei were counterstained with 5 μM propidium iodide/RNAse solution for 30 min at RT in the dark. AP2.7-positive and -negative cells were visualized and photographed using an epifluorescence microscope (C. Zeiss, Oberkochen, Germany).

TUNEL reaction. To stain the PAC for TUNEL-reaction, we used the APO-DIRECT kit. Formalin-fixed PAC were washed (3 times), and cell membranes were permeabilized by incubation with 70% (vol/vol) ethanol in PBS for 1 h. Staining was performed by incubating the cells overnight in the dark at 22°C with a solution consisting of 10 μL of terminal deoxynucleotidyl transferase (TdT)-reagent, 0.75 μL TdT-enzyme, 8 μL FITC deoxy-uridine-triphosphate, and 32 μL distilled water. The following day cells were incubated twice for 30 min with 50 μL of rinse buffer and then incubated with 50 μL propidium iodide/RNAse solution for 30 min at RT in the dark. Finally, the cells were washed (3 times) with PBS and analyzed by fluorescence microscopy.
Time-lapse microscopy of annexin V binding. Using time-lapse microscopy, annexin V binding was observed and digitally recorded during the next 6 h after addition of VLDL and alcohol. Each 30 min a picture of the same area was taken using a motorized inverted research microscope (Olympus IX81) equipped with a humidified CO₂ incubator and digital camera.

Furthermore, Trypan blue exclusion and LDH activity in the supernatant were used to ensure >95% viability of the PAC throughout the experiment.

Determination of Fibronectin Synthesis

Fibronectin concentration in PSC supernatants was measured by time-resolved fluorescence immunoassay as described (16, 23, 24). Fibronectin concentration was put in reference to the DNA content in the corresponding culture well. All measurements (standards, controls, and samples) were repeated. Deviation resulting by the double measurement was between 0.5% and 5% and did not exceed 8%.

Immunofluorescence Microscopy of Collagen Types I and III and Fibronectin

To demonstrate the effect of acinar cell supernatants on cell-associated collagens and fibronectin, cultured PSC, grown in the presence of 0.1% FCS on glass coverslips, were incubated with 120 µl/ml acinar cell-conditioned media. After 48 h PSC cultures were acetone fixed and immunostained for collagen types I and III and fibronectin as described earlier (24).

TSA reagent was used for staining the collagens. For collagen type I, the staining sequence was rabbit-anti-rat-collagen I (1:20), anti-rabbit HRP (1:50), biotin-TSA reagent (1:50), and streptavidin-FITC (1:50). The staining sequence for collagen type III was biotin-anti-rat collagen III (1:20), SA-HRP (1:50), biotin-TSA reagent (1:50), and streptavidin-FITC (1:50). Fibronectin was stained using rabbit-anti-fibronectin (1:50), biotin-anti-rabbit (1:50), and streptavidin-FITC (1:50). Nuclear counterstaining was performed using Hoechst33258. Staining was observed using epifluorescence microscopy (C. Zeiss). Photographs were taken using Ektachrome 400 film. To compare different staining intensities, exposure time was always the same. Nonspecific staining was controlled by including rabbit-nonimmune serum instead of specific first antibody.

The average staining intensity per picture was measured using Cell R Imaging Software for Life Science Microscopy, Version 1.2 (Olympus Biosystems, Planegg, Germany). The result is presented as the fold of control cells.

Quantitative Determination of Extracellular Matrix Synthesis

C-fibronectin was measured by time-resolved fluorescence immunoassay. Briefly, 96-well microtiter plates (Nunc-Maxi Sorp, Roskilde, Denmark) were coated overnight at 4°C with gelatin (10 µg/ml) in coating buffer (0.05 M NaHCO₃, pH 9.1) and thereafter blocked using assay buffer (0.005 M Tris, 0.15 M NaCl, and 0.5% radioimmunoassay-grade albumin, pH 7.5). One hundred microliters of standard (19–5,000 ng/ml) and culture supernatants diluted in assay buffer (1/20 to 1/200) were added and incubated for 3 h at RT. Thereafter, the plates were washed four times with 0.05% Tween 20 and NaCl solution (0.9%) followed by incubation for another 3 h with a monoclonal mouse antifibronectin (diluted 1/1,000 in assay buffer). After four washings, the plates were incubated for 2 h with the second antibody (100 µl/well; biotin-labeled anti-mouse IgG diluted 1/1,000 in assay buffer). Thereafter, a Europium-labeled streptavidin (diluted 1/1,000 in assay buffer) was added and incubated for 1 h. After five additional washing steps, 200 µl of enhancement solution was added for 30 min at RT, and, thereafter, time-resolved fluorescence of the Europium chelate was measured using a Delfia Flurometer model 1232 (Wallac Oy).

Quantitative Determination of Ethanol, Acetaldehyde, Malondialdehyde, TORC, Free Oxygen Radicals, Amylase, Lipase, LDH

Ethanol and acetaldehyde were measured in the supernatant by gas chromatography according to generally known proceedings. Malondialdehyde (MDA) levels were determined using the method of Yagi (32). Therefore, concentration was related fluorometrically to the protein content of PACs.
Total reductive capacity (TORC) was also measured fluorometrically by generating a fluorescence adduct of 96H9262M arachidonyl-thio phosphatidylcholine and 185H9262M Monobromobiman, put in assay buffer, consisting of HEPES 16.8 mM, pH 7.4, NaCl 33 mM, CaCl2 89.6 mM, Triton X-100 0.8 mM, and the same volume Propantriol 6% (17).

Additionally free oxygen radicals were measured in the supernatant of ethanol-treated PAC using chemoluminescence. After cell preparation, ethanol was added in different concentrations. Then lucigenin 0.01 M was added, and the peak radical emission was measured using a Lumistar fluorometer (BMG Labtechnologies, Offenburg, Germany).

According to the manufacturer’s instructions, amylase, lipase, and LDH were measured using the Clinical Chemistry Analyzer Dimension RxL (Siemens Medical Solutions Diagnostics, Eschborn, Germany).

Statistical Analysis

All quantitative measurements (c-fibronectin, collagen type I, and DNA) were performed in duplicate. The results are presented as means ± SD of at least three independent experiments. Each condition in the experiments was tested using three or four cultures (three or four wells). A variance test was applied for comparison of different groups.

RESULTS

PAC

More than 95% of PAC survived in culture media for at least 4 h. To see whether ethanol or VLDL or a combination of both induce acinar cell injury, we added ethanol (0.1, 0.5, 1.0% vol/vol) and human VLDL (20, 50, 100 μg/ml) alone or together to freshly isolated PAC in culture and measured the enzyme activities of LDH, amylase, and lipase at different time points in cell supernatants. The culture media were aspirated 5 min, 2 h, 4 h, and 6 h after addition of ethanol and VLDL. As shown in Fig. 1 50 μg/ml VLDL (with and without 0.5% ethanol) increased LDH (Fig. 1A), amylase (Fig. 1B), and lipase activity (Fig. 1C) in PAC supernatants within 2 h.

Fig. 2. Ethanol administration to freshly isolated PAC. A: ethanol decay in a control solution (upper curve) and PAC (lower curve) (values are given as means ± SE, n = 12). B: acetaldehyde increase as the first metabolite of ethanol in the same control solution (lower curve) and in acinar cells (upper curve). C: total reductive capacity (TORC) of PAC decreases within 4 h of ethanol or combined ethanol + VLDL exposure (**P < 0.05 to control). D: concentrations of ethanol in rat acinar cell culture medium 3 h after addition of ethanol and ethanol plus VLDL. The concentrations were 5 mg/ml ethanol (0.5%) and 50 μg/ml VLDL. The data of 2 wells are presented.

Fig. 3. Effect of either ethanol and/or VLDL on the production of malondialdehyde (MDA) (A) and the induction of oxidative stress (B) in cultured PAC. A: MDA increased sharply within 15 min after ethanol and/or VLDL exposure (**P ≤ 0.01 to control) and stayed up to 4 h of experiment (values are given as means ± SE, n = 12). B: direct assessment of free oxygen radicals in PAC by chemoluminescence using lucigenin. There is a moderate increase by ethanol alone (bottom curve), a marked increase by VLDL (middle curve) and a high and earlier increase in ethanol plus VLDL (upper curve). nVLDL, native VLDL (50 μg/ml); edVLDL, enzymatically degraded VLDL degraded by lipase, cholesterol-esterase, and trypsin (equivalent to 50 μg/ml nVLDL).
Enzyme activity further increased after 4 and 6 h (Fig. 1). Ethanol alone did not show any effect on the activity of these enzymes. Furthermore, addition of a supramaximal concentration (0.1 μM) of the secretagog cerulein to cultured PAC 3 or 5 h after VLDL and ethanol did not induce a further release of amylase and lipase (data not shown). In the presence of antioxidants (or serum), the enzyme release induced by VLDL in combination with ethanol was less pronounced (data not shown). To demonstrate that VLDL is not contaminated by LPS/endotoxin, the LAL gel-clot test was performed. All of our VLDL preparations were negative for LPS. In addition, to see whether LPS could induce similar effects as VLDL or VLDL plus ethanol on isolated rat PAC, 10 ng/ml LPS was added to the cultured PAC, and the activity of LDH, amylase, and lipase was measured after 6 h. Compared with control medium, neither LPS nor LPS plus ethanol induced an increase of LDH, amylase, and lipase. In strong contrast, VLDL or VLDL plus ethanol induced a dramatic increase in the three enzymes in the cultured PAC medium (Fig. 1D).

As shown in Fig. 2A, PAC metabolized ethanol. Ethanol concentration in cell supernatant decreased to 50% of the initial concentration within 3 h. The presence of VLDL did not influence the magnitude of ethanol metabolism (Fig. 2D). Acetaldehyde, which is produced by PAC through ethanol oxidation, is released in part into cell supernatant. We measured acetaldehyde in cell supernatant (Fig. 2B). The fact that (1) acetaldehyde vaporizes already above 20°C and (2) part of the acetaldehyde is metabolized intracellularly to acetate and acetyl-CoA might explain the discrepancy between ethanol decrease and acetaldehyde increase. In addition, nonoxidative ethanol metabolism hereby generating fatty acid ethyl esters might also play a role in pancreas (see Discussion).

The radical scavenging system of PAC was altered by the combination of ethanol and VLDL (Fig. 2C). TORC of PAC decreased significantly within 4 h after VLDL and ethanol exposure (Fig. 2C). The reduction of TORC was not observed after addition of both compounds alone (data not shown).

Because VLDL are degraded to fatty acids, cholesterol, glycerol, and peptides by enzymes produced by acinar cells, in the next set of experiments we added complete VLDL or edVLDL to cultured PAC. MDA, which is produced by reactive oxygen species through degradation of polyunsaturated lipids, increased significantly within the first hour of alcohol exposure when either VLDL or edVLDL were present (Fig. 3A, P ≤ 0.01). In the absence of ethanol, VLDL did not influence MDA production (data not shown). The increase in MDA was completely inhibited by the antioxidant Trolox (a water-soluble vitamin E analog) (Fig. 3A, P ≤ 0.01).

The direct assessment of radical production was performed using a chemoluminescence method. This set of experiments...
demonstrated a stimulated radical production as early as 10–15 min after addition of edVLDL (Fig. 3B). Ethanol accelerated and enhanced radical production in all 12 experiments performed (Fig. 3B). In all experiments the peak showed up earlier and higher compared with edVLDL alone. Radical production was markedly reduced by Trolox (data not shown).

Because ethanol in combination with VLDL induced oxidative stress and cell injury (change in cell morphology, shrinking, lysis, and LDH release), the next experiments should answer whether apoptosis and/or necrosis are induced in cultured PAC by ethanol in combination with VLDL. As shown in Fig. 4, after 6 h in culture there was no sign of apoptosis (Apo2.7 expression, annexin V binding, positive TUNEL reaction) in control PAC (Fig. 4, A, B, and C), and only very few cells showed apoptosis 6 h after addition of 0.5% ethanol (Fig. 4, D, E, and F) or VLDL (Fig. 4, G, H, and I). However, the combination of alcohol and VLDL strongly induced all the measured apoptosis markers (Fig. 4, J, K, and L). As expected the antioxidant Trolox (Fig. 4, M, N, and O) and the iron-chelating compound Desferal strongly inhibited apoptosis induction. Higher amounts of VLDL (>100 μg/ml) rather led to necrosis than to apoptosis (not shown). Time-lapse microscopy demonstrated apoptosis of PAC as early as 2 h after addition of VLDL/ethanol (Fig. 5D). At that time point apoptosis was absent in control PAC (Fig. 5C). Apoptosis increased during the following 4 h in VLDL/ethanol-treated PAC (Fig. 5, F and H). At 6 h almost all PAC were apoptotic or necrotic (positively stained for annexin V, Fig. 5H).

**PSC**

In the next set of experiments, we tested whether the supernatants of cultured PAC treated with ethanol and/or VLDL stimulate PSC proliferation and matrix synthesis. Acinar cell supernatant with and without ethanol and/or VLDL was therefore added to cultured subconfluent PSC. PSC proliferation was measured by bromodeoxyuridine (BrdU) incorporation, Fig. 5. Time-lapse microscopy of annexin V binding in control acinar cells (A, C, E, and G) and acinar cells treated with ethanol (0.5%) and VLDL (50 μg/ml) (B, D, F, and H). FITC-labeled annexin V was added together with ethanol/VLDL. Each 30 min, a picture of the same area was taken using a motorized inverted research microscope (Olympus IX81) equipped with a humidified CO2 incubator and digital camera.
and matrix synthesis was demonstrated by immunofluorescence microscopy and fibronectin immunoassay.

As shown in Fig. 6, supernatants of injured PAC stimulated the proliferation of cultured PSC. Moderate stimulation of BrdU incorporation in PSC was seen after either ethanol, or VLDL, supplementation of PAC. Highest stimulation of proliferation was observed by the combination of ethanol and VLDL. Interestingly, stimulated proliferation of PSC induced by acinar cell supernatants was highest if radical scavengers (Probucol and Trolox) were added together with VLDL/ethanol to cultured PAC (Fig. 6).

By immunofluorescence microscopy we demonstrated that these stimulated stellate cells abundantly synthesized cell-associated extracellular matrix such as fibronectin and collagen type I and type III (Fig. 7). Depending on the components added to cultured acinar cells (ethanol < VLDL < ethanol + VLDL), the magnitude of stimulated cell-associated matrix is defined (Fig. 7). The most intense stimulation of PSC-associated extracellular matrix was observed after adding supernatants of acinar cells treated with ethanol and VLDL (Fig. 7, M, N, O, and P).

The quantitative measurement of the soluble c-fibronectin fraction in PAC supernatant confirmed the synthesis of extracellular matrix after stimulation with supernatants of PAC treated with VLDL or the combination of VLDL with ethanol (Fig. 8).

DISCUSSION

To our knowledge this is the first study demonstrating that pathophysiological concentrations of ethanol lead to functional and structural damage of cultured acinar cells if native VLDL or degraded VLDL are also present. We further show for the first time that supernatant of ethanol- and VLDL-treated PAC alters the function of PSC.

Even though alcohol is causing the disease in almost half of the patients with acute pancreatitis and more than 70% of chronic pancreatitis, it was not possible under experimental conditions to induce any of these diseases only by alcohol. The conclusion of all these experiments was that alcohol is acting as a cofactor or at least needs a cofactor to induce the disease, meaning that there are some coconditions necessary to induce pancreatitis (20). The clinical experience that alcohol is mostly consumed together with fat-enriched meals led to the experimental design of this study. Alcohol caused already a certain amount of free radical production in vivo (31). Alcohol has

**Fig. 6.** Quantitative assessment of bromodeoxyuridine (BrdU) incorporation as a measure of proliferation in pancreatic stellate cells (PSC). PAC were treated with VLDL and/or ethanol for 6 h. Supernatants of these PAC were added to the cultures of PSC. Stellate cells proliferated especially in the presence of supernatants of PAC treated with both ethanol and VLDL (values are given as means ± SE, n = 4). *P ≤ 0.05; §P ≤ 0.05. Prob, Probucol; Tro, Trolox; Alc, alcohol; SN, supernatants.

**Fig. 7.** Effect of acinar cell supernatant (AC-SN) on cell-associated fibronectin, collagen type I, and collagen type III. PAC supernatants (D, E, and F), alcohol-treated PAC supernatants (G, H, and I), VLDL-treated PAC supernatants (J, K, and L), and ethanol/VLDL-treated PAC supernatants were added to cultured PSC. 48 h later cultures were stopped and stained for fibronectin (A, D, G, J, and M), collagen type I (B, E, H, K, and N), and collagen type III (C, F, I, L, and O). Control PSC are shown in A, B, and C. P: average staining intensity per picture (measured by Cell R Imaging Software for Life Science Microscopy; Olympus Biosystems, Planegg, Germany). The result is presented as the fold of control cells.
recently been found to cause cellular damage by free oxygen radicals in the gastrointestinal mucosa (6), in the liver (22, 28), and in the pancreas (19). As our findings and those by others show, radical production induced by ethanol is obviously not high enough to cause the structural damage of the PAC. However, ethanol together with VLDL, in particular VLDL with a high content of polyunsaturated fatty acids (more double bonds), induces structural damage to cells if oxidative stress is present. Accordingly, Tsukamoto et al. (27) observed hypergranulation and apoptosis of PAC after chronic alcohol and fat diet feeding in rats (27). This is somewhat opposite to the general belief that polyunsaturated fatty acids are always beneficial for human health (20).

We show that addition of VLDL alone or in combination with ethanol to cultured PAC gradually increased amylase, lipase, and LDH activity in cell supernatants. Ethanol alone did not show any effects on the activity of these enzymes. However, because also LDH activity increased in parallel to the enzymes amylase and lipase, we suggest that the enzyme release is not a consequence of enzyme secretion but a result of cell injury. This interpretation is supported by two other sets of experiments.

First, addition of a supramaximal concentration (0.1 μM) of the secretagog cerulein to cultured PAC 3 or 5 h after VLDL and ethanol did not induce a further release of amylase and lipase. Second, in the presence of antioxidants (or serum), the enzyme release induced by VLDL in combination with ethanol was less pronounced.

Hamamoto et al. (10) have shown that fatty acid ethyl ester synthase activity is much higher in pancreas compared with liver (1,348 vs. 23 nmol/h per mg protein). According to Haber et al. (9), the rate of oxidative metabolism of ethanol in cultured rat PAC was 21-fold higher than that of nonoxidative metabolism. In agreement with this, Laposata et al. (14) and Werner et al. (30) have presented data showing that nonoxidative metabolism of ethanol-generating fatty acid esters might also play a role in cultured PAC. We conclude from these and our data that part of ethanol is metabolized by nonoxidative pathways by PAC in particular in the presence of fatty acids to form fatty acid ethyl esters (see Fig. 9).

It was interesting to learn that supernatant of ethanol/VLDL-treated acinar cells activates PSC to proliferate (Fig. 6) and produce extracellular matrix (Figs. 7 and 8). A similar result was obtained experimentally in rat liver by a high-fat diet (rich in polyunsaturated fatty acids) combined with alcohol (29). PSC might be activated directly by ethanol or its metabolite acetaldehyde (2, 3). Abundant extracellular matrix is the most characteristic finding in pancreas fibrosis, and PSC represent the major matrix-producing cells in acute and chronic pancreatitis and also in pancreas carcinoma (4, 16). Some consistent data supporting the hypothesis that free oxygen radicals play a role in human chronic pancreatitis were recently published by Casini et al. (7). They evaluated 4-hydroxynonenal (HNE, a product of free radical action) in resected specimen of patients with chronic alcoholic pancreatitis. They found HNE in acinar cells adjacent to pancreatic fibrosis, and, furthermore, they observed that activated PSC underlay HNE-stained acinar cells (7).

The compounds able to stimulate PSC need to be evaluated further. Although we found an increased radical production in cultured PAC in the presence of ethanol and fat (VLDL), which could be reduced by antioxidants, the synthesis of extracellular matrix by PSC treated with acinar cell supernatants could not be inhibited if antioxidants were added together with VLDL and ethanol to PAC. This indicates that, beside oxidative stress-induced factors, also other mediators (probably growth factors) produced by PAC might be relevant to stimulate proliferation and matrix synthesis in PSC. However, by gas chromatography a modified fatty acid was detected in the supernatants of PAC after addition of VLDL and ethanol. This modified fatty acid was absent after addition of antioxidants. Presently the exact molecular structure of this fatty acid is unclear and needs to be further characterized by chemical and biochemical methods.

Our presented in vitro data are supported by in vivo experiments by Kono et al. (13). They fed rats with ethanol, unsaturated fatty acids, or both for 8 wk. Radical adducts were found in the pancreas, and after 8 wk fibrosis and collagen I expression was significantly enhanced in the ethanol plus unsaturated fatty acids group (13).

Our conclusion is summarized in Fig. 9. Alcohol is degraded by acinar cells via oxidative and nonoxidative pathways. Hereby acetaldehyde, fatty acid ethyl esters, and MDA are...
generated. Fatty acid ethyl esters and MDA are produced in particular if fatty acids are present. Fatty acids might be generated through VLDL degradation by enzymes released from acinar cells. Oxidative stress, MDA, oxidized fatty acids, and fatty acid ethyl esters might be responsible for inducing acinar cell injury. Depending on the dose of ethanol and fat (VLDL), apoptosis and necrosis might be induced. Acinar cell secretions e.g., of growth factors and/or modified fatty acids, stimulate PSC to proliferate and induce repair mechanisms (production of extracellular matrix).

Our results provide a biochemical pathway for the two clinical observations that 1) a fat-rich meal combined with excess alcohol is able to alter function and integrity of PAC

2) ethanol induces acute pancreatitis in patients suffering from hyperlipidemias types I, IV, or V.

DISCLOSURES
No conflicts of interest are declared by the author(s).

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