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

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Abstract

Aims: 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 (very low density lipoproteins = VLDL) on pancreatic acinar cell (PAC) function, oxidative stress, and repair mechanisms by pancreatic stellate cells (PSC) leading to fibrogenesis.

Methods: 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 (LDH), apoptosis (TUNEL, Apo2.7, Annexin V binding), oxidative stress and lipid peroxidation (conjugated dienes, malondialdehyde, chemoluminescence); PSC proliferation (BrdU-incorporation) and matrix synthesis (immunofluorescence of collagens and fibronectin, fibronectin immunoassay); fatty acids in PAC-supernatants (gas chromatography).

Results: Within 6 h cultured PAC degraded and hydrolyzed VLDL completely. VLDL alone (50µg/ml) and in combination with alcohol (0.2, 0.5, 1% v/v) 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.

Conclusion: 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).
Introduction

The mechanism of alcoholic pancreatitis is incompletely understood. In spite of 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 (1), only 5% of all alcohol abusers will develop alcoholic pancreatitis. Interestingly enough, this can also be observed in set-ups with animals where acute pancreatitis cannot be induced by alcohol itself (8, 15, 25). Therefore, additional factors accompanying alcohol intake must be co-responsible 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 also metabolize ethanol by oxidative (10, 18) and nonoxidative pathway (9). We put up the hypothesis that exposure of pancreatic acinar cells (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 = very low density lipoproteins) to cultured rat PAC and measured the oxidative stress, amylase and lipase release, acinar cell injury (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 stimulating proliferation and matrix synthesis of PSC.
Our data indicate that alcohol induces oxidative stress and acinar cell injury particularly in the presence of fat (VLDL). Furthermore, supernatants of acinar cells treated with ethanol and fat contain fibrogenic mediators which in turn stimulate proliferation and extracellular matrix synthesis of stellate cells. Based on 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 Co. (Munich, Germany); OptiPrep from Nycomed Pharma (Oslo, Norway); Ficoll from Pharmacia LKB (Freiburg, Germany); horseradish peroxidase (HRP)-anti-mouse, HRP-anti-rabbit, fluorescein-conjugated streptavidin, biotinylated rabbit anti-mouse immunoglobulin G (IgG), HRP-conjugated streptavidin, HRP-conjugated rabbit antifluorescein, rabbit polyclonal antidesmin; rabbit anti-rat collagen type III, rabbit anti-rat collagen type I, and biotin-labeled rabbit anti-rat collagen type III from Chemicon International (Temecula, CA); polyclonal antifibronectin from Dade-Behring (Marburg, Germany); Tyramide Signal Amplification (TSA) from New England Nuclear Life Science Products (Boston, MA); Delfia Eu-labeled streptavidin, Delfia Eu-labeled anti-mouse IgG, Delfia Eu-labeled anti-rabbit IgG, and enhancement solution from Wallac Oy (Turku, Finland). The APO-DIRECT™ kit was from Pharmingen (San Diego, CA), the anti-Apo2.7 monoclonal antibody was obtained from Immunotech (Marseille, France) and biotinylated Annexin-V was from Hoelzel Diagnostica (Cologne, Germany). LPS (E. coli O26:B6) was from Sigma-Aldrich, St. Louis, MO.

Preparation and modification of VLDL
Total VLDL were isolated by ultracentrifugation (24 h, 4°C, 48,000 rpm, Beckman UZ model 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 chylomicrons at least 6 h after the last meal 100 ml EDTA-blood was taken and centrifuged at 1800g to obtain EDTA-plasma. To eliminate KBr isolated VLDL fraction was dialyzed 2x against PBS (volume 400fold) 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 (Sigma, Deisenhofen, Germany, final concentration 7.7 µg/ml) and cholesterol esterase (Roche Diagnostics, Tutzing, Germany, 57.8 µg/ml). Thereafter, the pH was adjusted to 5.5 by addition of morpholinoethanesulfonic acid buffer (50 mM). Samples were incubated with neuraminidase (Dade Behring, Schwalbach, Germany, 162 mU/ml) for 14 h at 37°C, and finally, samples were diluted with DMEM to the 10-fold of the initial volume. The increase of free fatty acids
in enzymatically degraded VLDL was quantified by gas chromatography. VLDL protein concentration was determined with a modified Lowry protein assay (BioRad, Freiburg, Germany). VLDL concentrations were referred to their protein content; concentrations of edVLDL were referred to their initial protein concentration (prior to enzymatic degradation). To examine whether the VLDL preparations were contaminated with LPS/endotoxin, the Limulus Amebocyte Lysate (LAL) gel-clot test with 0.125 ng/ml sensitivity (Associates of CAPE CO., East Falmouth, MA) was performed according to the manufacturer’s instruction.

Animals
Wistar rats were obtained from the breeding colony of Ulm University Animal Facilities and were kept at 24°C under a 12-h light/dark cycle. The animals received a standard diet, tap water was offered ad libitum. All experiments were performed according to the guidelines of the local Animal Use and Care Committee.

Cell isolation
Pancreatic acinar cells and pancreatic stellate cells were prepared from the same pancreas: Rats were sacrificed, and collagenase-containing Eagle medium (1 mg/5 mL) was instilled intraductally into 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/5 mL for 45 min). Dispersion 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, distributed in fibronectin-coated 6-well plates (or in 8-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, and 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 x 10^4 cells/cm^2.

**PSC culture**

PSC were cultured at 37°C in a 5% CO_2 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 confluency, cells were subcultured after trypsinization using a 0.025% trypsin solution containing 0.01% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS). To study cell proliferation and matrix synthesis, cells were seeded in 24-well plates (2 cm^2/well; 1 mL medium; 30-50 x 10^4 cells/cm^2). To perform immunofluorescence microscopy, cells were seeded on 1-cm^2 glass coverslips in 6-well (10 cm^2/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. 6 h 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 non-specific binding was blocked with TNB-buffer (10 mM Tris, 150 mM NaCl, 0.5 % bovine albumin, pH 7.4) 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 CaCl_2) for 1 h at RT, washed (3x) with TBT-buffer (TBS-buffer containing 0.05% Tween 20 and 2 mM CaCl_2), and then incubated with streptavidin-phycoerythrin (1/100 in TNB with 2 mM CaCl_2) for 1 h at RT. After washing with TNT-buffer in the presence of CaCl_2, nuclei were counterstained with 5 µM Hoechst 33258 for 30 min at RT. Apo2.7 expression: cell membranes of formaldehyde-fixed PAC were permeabilized using 70% ethanol (1 h, at RT). Thereafter unspecific bindings were blocked with
TNB-buffer. Apo2.7 was stained using anti-Apo2.7 diluted 1/10, anti-mouse HRP (1/50), followed by TSA. After 5 washing steps with TNT-buffer streptavidin-FITC (1/50) was added and incubated for 1 h. After another 5 washing steps nuclei were counterstained with 50 µl propidium iodide/RNAse solution for 30 min at RT in the dark. APO 2.7-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 (3x) and cell membranes were permeabilized by incubation with 70% (v/v) 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 terminal deoxynucleotidyl transferase (TdT)-reagent, 0.75 µl TdT-enzyme, 8 µl FITC deoxy-uridin-triphosphate (dUTP) 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 (3x) 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, III and fibronectin**

In order 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, 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 (1:100). 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, Oberkochen, Germany). Photographs were taken using Ektachrome 400 film. To compare different staining intensities exposure time was always the same. Non-specific staining was controlled by including rabbit-non-immune 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 GmbH, 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 - 5000 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 anti-cellular fibronectin (anti-cFN) (diluted 1/1000 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 1/1000 in assay buffer). Thereafter, a Europium-labeled streptavidin (diluted 1/1000 in assay buffer) was added and incubated for 1 h. After five additional washing steps, 200 µL enhancement solution was added for 30 min at RT, and thereafter, time-resolved
fluorescence of the Europium chelate was measured using a Delfia Fluorometer model 1232 (Wallac Oy, Turku, Finland).

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 levels were determined using the method of Yagi (32). Therefore, concentration was related fluorimetrically to the protein content of PAC’s.

TORC, the total reductive capacity, was also measured fluorimetrically by generating a fluorescence adduct of 96 µM arachidonyl-thio-phosphatidylcholine and 185 µM Monobrombiman, put in assay-buffer, consisting of HEPES 16.8 mM, pH 7.4 and NaCl 33 mM, CaCl₂ 89.6 mM, Triton X-100 0.8 mM and 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 fluorimeter (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

A. Pancreatic acinar cells (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% v/v) 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. 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 secretagogue 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 Limulus Ameocyte Lysate (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, the activity of LDH, amylase, and lipase was measured after 6 h. Comparing 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 all 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 metabolization (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 (i) acetaldehyde vaporizes already above 20°C and (ii) part of the acetaldehyde is metabolized intracellularly to acetate and acetyl-CoA might explain the discrepancy between ethanol decrease and acetaldehyde increase. In addition,
non oxidative ethanol metabolization hereby generating fatty acid ethyl esters might also play a role in pancreas (see Discussion).

The radical scavenging system of pancreatic acinar cells was altered by the combination of ethanol and VLDL (Fig. 2C). TORC of pancreatic acinar cells 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. Malondialdehyde (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 \leq 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 analogue) (Fig. 3A, p \leq 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 enzymatically degraded VLDL (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 to 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, LDH-release) the next experiments should answer whether apoptosis and/or necrosis is 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. 4A, B, C) and only very few cells showed apoptosis 6 h after addition of 0.5% ethanol (Fig. 4D, E, F) or VLDL (Fig. 4G, H, I). However, the combination of alcohol and VLDL strongly induced all the measured apoptosis markers (Fig. 4J, K, L). As expected the antioxidant Trolox (Fig. 4M, N, 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. 5F, H). At 6 h almost all PAC were apoptotic or necrotic (positively stained for Annexin-V, Fig. 5H).
B. Pancreatic stellate cells (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 BrdU-incorporation and matrix synthesis was demonstrated by immunofluorescence microscopy and fibronectin immunoassay.

As shown in Fig. 6, supernatants of injured pancreatic acinar cells stimulated the proliferation of cultured pancreatic stellate cells. 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. 7M, N, O, P).

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

To our knowledge this is the first study demonstrating that pathophysiologic concentrations of ethanol leads to functional and structural damage of cultured acinar cells if native very low density lipoproteins (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 co-factor or at least needs a co-factor to induce the disease, meaning that there are some co-conditions 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 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 pancreatic acinar cells. However, ethanol together with VLDL, in particular VLDL with a high content of polyunsaturated fatty acids (more double bonds), induce structural damage to cells if oxidative stress is present. Accordingly, Tsukamoto et al. observed hypogranulation 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 2 other sets of experiments. First, addition of a supramaximal concentration (0.1 µM) of the secretagogue 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 to liver (1348 vs. 23 nmol/h/mg protein). According to Haber et al. (9) the rate of oxidative metabolism of ethanol in cultured rat pancreatic acinar cells was 21-fold higher than that of non-oxidative metabolism. In agreement with this Laposata et al. (14) and Werner et al. (30) have presented data showing that non oxidative metabolism of ethanol generating fatty acid ethyl 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 activate pancreatic stellate cells to proliferate (Fig. 6) and produce extracellular matrix (Fig. 7, Fig. 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 pancreatic stellate cells 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 eight weeks. Radical adducts were found in the pancreas and after 8 weeks fibrosis and collagen I expression was significantly enhanced in the ethanol plus unsaturated fatty acids group (13).

Conclusion:

Our conclusion is summarized in figure 9. Alcohol is degraded by acinar cells via oxidative and non-oxidative pathways. Hereby acetaldehyde, fatty acid ethyl esters and malondialdehyde are generated. Fatty acid ethyl esters and malondialdehyde 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, malondialdehyde, 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 pancreatic stellate cells to proliferate and induce repair mechanisms (production of extracellular matrix).

Our results provide a biochemical pathway for the two clinical observations that (i) a fat rich meal combined with excess alcohol is able to alter function and integrity of pancreatic acinar cells and (ii) induction of acute pancreatitis by ethanol in patients suffering from hyperlipidemias types I or IV or V.
References


Fig. 1 Effect of ethanol and/or VLDL on the activity of LDH (A), amylase (B), and lipase (C) in the culture medium of isolated rat pancreatic acinar cells.

0.5% ethanol, 50 µg/mL VLDL, or VLDL plus ethanol were added to the PAC. The culture media were aspirated after 5 min, 2 h, 4 h and 6 h and enzymes were measured by standard methods on RxL-Dimension. Values are expressed as mean ± SD; 4 experiments have been performed in triplicate.

Effect of LPS (10 µg/mL) and/or ethanol in comparison with ethanol and/or VLDL on the activity of LDH, amylase, and lipase after 6 h is presented in D.

Fig. 2 Ethanol administration to freshly isolated pancreatic acinar cells.

A Ethanol decay in a control solution (upper curve) and pancreatic acinar cells (lower curve) (values are given as mean ± SEM, 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 pancreatic acinar cells decrease within 4 h of ethanol or combined ethanol + VLDL exposure (*p < 0.05 to control, **p < 0.01 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 ethanol 5 mg/ml (0.5%) and VLDL 50 µg/ml. The data of 2 wells are presented.

Fig. 3 Effect of either ethanol and/or VLDL on the production of malondialdehyde (A) and the induction of oxidative stress (B) in cultured pancreatic acinar cells.
A Malondialdehyde 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 mean ± SEM, n = 12)

B Direct assessment of free oxygen radicals in pancreatic acinar cells 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 very low density lipoprotein (50 µg/ml).
edVLDL, VLDL degraded by lipase, cholesterol-esterase and trypsin (equivalent to 50 µg/ml nVLDL).

Fig. 4 Fluorescence staining after induction of acinar cells apoptosis (Apo2.7 expression = green, Annexin-V binding = red, TUNEL reaction = yellow) by VLDL and ethanol.

Cells were fixed 6 h after addition of ethanol/VLDL. There is some apoptosis induced by VLDL that becomes advanced by additional administration of ethanol. Ethanol/VLDL induced apoptosis was almost completely inhibited by the antioxidant Trolox.

Fig. 5 Time-lapse-microscopy of Annexin-V-binding in control acinar cells and acinar cells treated with ethanol (0.5%) and VLDL (50 µg/ml).

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.

Fig. 6 Quantitative assessment of bromodeoxyuridine incorporation as a measure of proliferation in pancreatic stellate cells.

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 mean ± SEM, n = 4)
Fig. 7  Effect of acinar cell supernatant on cell associated fibronectin, collagen type I and III.

PAC supernatants (D, E, F), alcohol-treated PAC supernatants (G, H, I) VLDL-treated PAC supernatants (J, K, 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, M), collagen type I (B, E, H, K, N) and collagen type III (C, F, I, L, O). Control PSC (A, B, C).

P: Average staining intensity per picture (measured by Cell R Imaging Software for Life Science Microscopy, Olympus Biosystems GmbH, Planegg, Germany). The result is presented as the fold of control cells.

Fig. 8  Quantitative assessment of soluble c-fibronectin in PSC supernatant 24 h after addition of acinar cell supernatants (20 or 120 µl/ml).

Significant stimulation of fibronectin synthesis was observed after addition of VLDL treated PAC supernatant and ethanol/VLDL treated PAC supernatant (*p < 0.01 compared to control). 10% fetal calf serum (FCS) was used as positive control.

Fig. 9  Induction of pancreatic acinar cell injury by ethanol and fat (VLDL) and stimulation of stellate cell activation (proliferation and matrix synthesis) by fatty acid ethyl ester, oxidized fatty acids and probably other fibrogenic mediators (growth factors) released by acinar cells.

FA, fatty acid; PSC, pancreatic stellate cell.
Fig. 1
Fig. 2

A. Graph showing the decrease in ethanol concentration over time for control and cells.

B. Graph showing an increase in acetald sehed concentration over time for control and cells.

C. Bar graph showing the decrease in TORC (in μg/g Protein) at different time points (0 h, 1 h, 2 h, 3 h, 4 h) for control and experimental samples.

D. Bar graph showing the concentration of ethanol (g/L) for control, eth, and eth+VLDL samples.
Fig. 3
Fig. 4
Fig. 5
Fig. 6
Fig. 8
Fig. 9

- Fat (VLDL) + Ethanol → FA-ethyl esters
- Trypsin lipase
- Oxidative metabolism
- Acetaldehyde
- Ox. stress
- Trolox
- Desferal
- Growth factors?
- Acinar cell injury
- Trolox
- Desferal
- Activation
- Proliferation
- Matrix synthesis