Liver steatosis is a characteristic feature of important liver diseases such as chronic hepatitis C, alcoholic liver disease, and non-alcoholic steatohepatitis (NASH). There is a line of evidence that hepatic steatosis contributes to progression of chronic hepatitis C,1–4 and NASH has been proposed as a possible cause of liver cirrhosis and hepatocellular carcinoma.5

The pathogenesis of hepatic steatosis is multifactorial and involves insulin resistance, accumulation of intracellular fatty acids, adenosine triphosphate depletion, mitochondrial dysfunction, and oxidative stress.6–8 The cytokine tumour necrosis factor α (TNF-α) is an important mediator of insulin resistance and mediates not only the early stages of fatty liver disease but also the transition to more advanced stages of liver injury.9 However, administration of TNF-α to healthy animals promotes proliferation of hepatocytes rather than hepatic steatosis.10 Even though TNF-α appears to be a key factor for the development of fatty liver disease in alcohol related liver injury, NASH and chronic hepatitis C, obviously a network of cytokines rather than TNF-α alone is necessary to induce hepatic steatosis.

In addition to TNF-α, other members of the TNF family are involved in the pathogenesis of liver diseases. The receptor/ligand pair Fas (CD95)/FasL (CD95L) has been described as playing an important role in the apoptosis of hepatocytes in viral hepatitis and in alcoholic liver injury. Recently Feldstein et al showed that Fas is strongly overexpressed in hepatocytes in liver specimens from NASH patients, indicating that high Fas activity is involved in apoptosis of hepatocytes in NASH.11

While hepatocytes are constitutively susceptible for FasL mediated apoptosis, expression of tumour necrosis factor related apoptosis inducing ligand (TRAIL) does not result in apoptosis of hepatocytes in healthy livers.12–14 We recently demonstrated that TRAIL mediated apoptosis of hepatocytes needs to be triggered through viral infection and that overexpression of TRAIL enables the organism to selectively eliminate virally infected hepatocytes.15 Recently, Higuchi et al reported that bile acids upregulate TRAIL receptor DR5 expression16–17 and Zheng et al demonstrated that TRAIL mediated apoptosis of hepatocytes in Con-A and Listeria induced liver injury,18 indicating that TRAIL is also an important mediator of apoptosis in non-viral liver diseases.

TNF receptor 1 (TNFR1), Fas, and the TRAIL receptors 1 (DR4) and 2 (DR5) recruit the key adapter protein FADD to the cell membrane, thereby inducing the caspase cascade. But in contrast with Fas, TNFR1 and TRAIL receptors also bind the adapter molecule TRADD,19 20 which explains the potent activation of nuclear factor κB by TNF-α and TRAIL in contrast with FasL.21 The molecular mechanisms in signal transduction by TNF-α and TRAIL are very similar, so it is tempting to speculate that TRAIL may also be involved in the pathogenesis of fatty liver disease.

Consequently, it was the aim of our study to investigate the role of TRAIL in hepatic steatosis. TRAIL expression neither induces apoptosis nor steatosis in healthy livers. However, in virally infected livers and in animals after alcohol intake, TRAIL, but not FasL, expression results in hepatic steatosis. In viral hepatitis, TRAIL mediated steatosis is associated with

Abbreviations: TRAIL, tumour necrosis factor related apoptosis inducing ligand; HCV, hepatitis C virus; TNF-α, tumour necrosis factor α; TNFR1, TNF receptor 1; NASH, non-alcoholic steatohepatitis.
apoptosis of hepatocytes while expression of TRAIL after alcohol intake induces hepatic steatosis without triggering apoptosis, suggesting two distinct functional pathways for hepatic steatosis and apoptosis in TRAIL signalling.

MATERIAL AND METHODS

Cell lines and adenovirus preparation

The human hepatocellular carcinoma cell line HuH7 and the embryonal kidney cell line 293 were obtained from the American Type Culture Collection. Cells were maintained in growth medium (Dulbecco's modified Eagle's medium/glutamax (GibcoBRL, Gaithersburg, Maryland, USA), supplemented with 10% heat inactivated fetal bovine serum (GibcoBRL), 100 units/ml of penicillin, and 100 μg of streptomycin) at 37°C in 5% CO2.

Construction and generation of adenoviruses Ad-FasL, Ad-TRAIL, and Ad-GFP have been described previously.14 The adenoviral vector Ad5-CMV-LacZ was kindly provided by Dr D Brenner (Chapel Hill, USA). Viral infectivity was determined by plaque assays. Endotoxin contaminations were excluded following the protocol provided by the manufacturer. Virus preparations were stored at −20°C in 25% glycerol, 10 mM Tris/HCl, pH 7.4, and 1 mM MgCl2.

Human liver tissues

Specimens of normal human liver were obtained from two patients who underwent partial hepatectomy because of colorectal liver metastases. Specimens of livers from patients with liver cirrhosis due to hepatitis C infection were obtained during liver transplantation at the Medizinische Hochschule Hannover. Anti-hepatitis C virus (HCV) was detected by second or third generation enzyme linked immunosorbent assay (Abbott, Chicago, Illinois, USA), and HCV-RNA was assessed by reverse transcriptase-polymerase chain reaction. Liver specimens were immediately snap frozen in liquid nitrogen after hepatectomy and stored at −80°C until analysis.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western blot analysis

Protein concentrations of liver extracts were measured by Bio Rad Microassay (Bio Rad, München, Germany). Protein extracts (10 μg) were separated on a 10% sodium dodecyl sulphate-polyacrylamide gel and blotted onto Hybond N membrane (Millipore, Franklin, Germany). The following primary antibodies were used: AF837 against DR5 human and mouse (R&D, Wiesbaden, Germany), AF630 against DcR1 mouse (R&D), anti-DcR2 against DcR2 mouse (ProSci, Poway, California, USA), C-11 against β-actin (Santa Cruz, Santa Cruz, California, USA), rabbit polyclonal FasL antibody Ab-1 (Oncogene, Calbiochem, California, USA), and H-257 against TRAIL human and mouse (Santa Cruz). The antigen-antibody complexes were visualised using the ECL detection system, as recommended by the manufacturer (Amersham, Braunschweig, Germany).

Animal experiments

Pathogen free male Balb/c mice (aged 6–8 weeks) were obtained from the Animal Research Institute of the Medizinische Hochschule Hannover. All experiments were performed in agreement with German legal requirements. Mice were fed a normal standard diet (Altromin, Lage, Germany) given ad libitum. Exposure to ethanol for four days was performed by adding ethanol to the drinking water (20% vol/vol). Metformin was applied orally (350 μg/kg/day) (Merck, Darmstadt, Germany).

For infection experiments, viruses Ad-LacZ, Ad-GFP, Ad-FasL, or Ad-TRAIL were prepared, purified, and titred as described previously.14 Prior to infection the virus was dialysed twice against a solution containing 10 mM Tris-HCl, pH 8.0, 1 mM MgCl2, 140 mM NaCl at 4°C. Infection of mice was carried out by administration of 0.3 ml of virus solution into the tail vein with 1×106 to 1×1010 pfu/g at the time points indicated, mice were sacrificed and livers were harvested for preparation of whole cell extracts and cryosections, respectively. To evaluate the efficacy of Ad vectors infecting hepatocytes in vivo, liver specimens of Ad-lacZ infected mice were frozen on dry ice and subsequently frozen sections were stained with β-gal substrate X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (Sigma, Taufkirchen, Germany).

Monoclonal anti-TRAIL antibody N2B2 (eBioscience, San Diego, California, USA) or an isotype matched control rat IgG antibody was injected into the tail vein (125 μg or 250 μg/mouse) one hour prior to application of Ad-TRAIL.

Fat red staining and TUNEL assay

Liver tissue of infected mice was embedded in OCT compound (Sakura, Netherlands) and shock frozen in liquid nitrogen. Samples were stored at −80°C. Sections (7 μm) were prepared and fixed in phosphate buffered saline buffered paraformaldehyde solution (4%) for 20 minutes at room temperature. For fat red staining, sections were exposed for one hour to fat red (Sigma, Taufkirchen, Germany) counterstained with hemalaun (Merck) for 10 minutes.

Figure 1 Overexpression of tumour necrosis factor related apoptosis inducing ligand (TRAIL) and steatosis in the livers of patients with chronic hepatitis C. (A) Whole cell extracts of normal livers (C1–2) and livers from patients with chronic hepatitis C infection (P1–6) were investigated for protein expression by western blot experiments. TRAIL was overexpressed in the livers of patients with chronic hepatitis C infection. (B) Assessment of hepatic steatosis in the corresponding tissues by fat red staining (magnification 400×).
For TUNEL assays we used the In Situ Cell Death Detection Kit, Fluorescein (Boehringer, Mannheim, Germany). After incubation with proteinase K and Triton X-100, sections were stained as described in the protocol provided by the manufacturer. The number of TUNEL positive cells was assessed for each liver by counting TUNEL positive cells in three sections. For each section 10 fields (magnification ×400) were examined. The results were processed using the Student's t test, with p<0.05 accepted as denoting statistical significance.

Assessment of hepatic steatosis

Hepatic steatosis was evaluated using a scale of 0–4. A score of 0 indicated no vesicular fat was present; scores of 1, 2, 3, and 4 corresponded to the following number of cells (expressed as a percentage of the total number of cells) that contained vesicular fat: ≤25, 25–50, 50–75, and 75–100. For each liver, three sections and for each section, 10 fields (magnification ×200) were examined. The results were processed using the Student's t test, with p<0.05 accepted as denoting statistical significance.

Assessment of caspase 8 activity in liver tissue

For determination of caspase 8 activity in the mouse liver, freshly harvested organs were subjected to a buffer containing 25 mM HEPES, 5 mM MgCl₂, 5 mM EDTA, 2 mM dithiothreitol, 0.1% CHAPS, 0.5 mM Pefabloc, 0.1 mg/ml leupeptin, and 0.1 mg/ml pepstatin in a w/v ratio of 1:3. For preparation of whole cell extracts, tissue was homogenised on ice and subsequently centrifuged at 12 000 g for 10 minutes (4°C). Total protein (100 µg) from the supernatant was used to measure caspase 8 activity by the ApoAlert Caspase Fluorescent Assay kit (BD Bioscience, Heidelberg, Germany). The specificity of the results was controlled by adding a caspase 8 inhibitor (C 8 inhi.), as suggested by the manufacturer.

Three livers from three independent experiments were used in each group. The results were processed using the Student's t test to compare differences between groups. A p value of <0.05 was considered statistically significant.

RESULTS

Expression of TRAIL in virally infected livers induces hepatic steatosis and apoptosis

Liver steatosis is characteristic of HCV infection. To investigate whether TRAIL is upregulated in liver with hepatitis C associated hepatic steatosis, we performed western blot experiments in liver tissues from patients with chronic hepatitis C. Compared with normal liver, TRAIL was overexpressed in the liver of patients with hepatitis C. However, the quantity of fat accumulation in hepatocytes did not tightly correlate with hepatic TRAIL expression (fig 1).

To determine whether expression of TRAIL resulted in hepatic steatosis, we used adenoviral vectors expressing FasL or TRAIL (fig 2A). There was no evidence of fatty liver disease or apoptosis of hepatocytes after infection of healthy livers with low amounts of Ad-TRAIL, confirming our previous observation that TRAIL, in contrast with FasL, was not harmful to healthy livers (fig 2B).
It has been hypothesised that the interplay of different cytokines rather than one particular ligand may be necessary for the development of fatty liver disease. Consequently, we explored whether viral infection may be a predisposing condition for TRAIL mediated hepatic steatosis. Prior infection of mice with high doses of adenoviruses (Ad-lacZ) caused downregulation of the TRAIL decoy receptor DcR2 and upregulation of TRAIL receptor DR5 expression, and sensitised hepatocytes to TRAIL mediated steatosis and apoptosis, as shown in fig 3A and 3B. In contrast with adenoviral hepatitis, administration of Ad-TRAIL did not enhance caspase 8 activity in the liver, suggesting distinct functional pathways for TRAIL regarding regulation of apoptosis and steatosis (fig 4B). To confirm that TRAIL mediated steatosis is not dependent on apoptosis under these circumstances, we assessed caspase 8 activity in the livers of the animals. In contrast with adenoviral hepatitis, administration of Ad-TRAIL did not enhance caspase 8 activity in the liver.

Expression of TRAIL after alcohol intake results in hepatic steatosis without apoptosis

TNF-α plays an important role in alcoholic fatty liver disease. Consequently, we investigated whether expression of TRAIL after alcohol intake would result in hepatic steatosis. Feeding of mice with 20% alcohol for four days did not lead to significant regulation of TRAIL or TRAIL receptors in the liver (fig 4A). Nevertheless, alcohol intake also sensitised the liver to TRAIL mediated steatosis, as shown in fig 4B. However, in contrast with viral hepatitis, expression of TRAIL in animals after alcohol intake resulted only in accumulation of fat within the hepatocytes, but not in apoptosis of hepatocytes, suggesting distinct functional pathways for TRAIL regarding regulation of apoptosis and steatosis (fig 4B). To confirm that TRAIL mediated steatosis is not dependent on apoptosis under these circumstances, we assessed caspase 8 activity in the livers of the animals. In contrast with adenoviral hepatitis, administration of Ad-TRAIL did not enhance caspase 8 activity in the liver.

Figure 3 Viral hepatitis sensitised for tumour necrosis factor related apoptosis inducing ligand (TRAIL) mediated apoptosis and steatosis. (A) Viral hepatitis was induced by intravenous application of $1 \times 10^7$ pfu/g of Ad-lacZ into Balb/c mice. After 40 hours whole cell extracts of the livers were isolated and investigated for protein expression of TRAIL, DR5, DcR1, DcR2, and β-actin by western blot experiments. (B) Adenoviral vectors AdGFP, AdFasL, and Ad-TRAIL ($1 \times 10^7$ pfu/g) were administered into the tail vein of Balb/c mice 24 hours after pretreatment with $5 \times 10^7$ pfu/g of Ad-lacZ. Twenty four hours after the last injection, apoptosis of hepatocytes was analysed by TUNEL assay (magnification 200×) and steatosis by fat red staining (magnification 200×). Figure shows representative data of four independent experiments. (C) Adenoviral vectors AdGFP, AdFasL, and Ad-TRAIL ($1 \times 10^7$ pfu/g) were administered into the tail vein of Balb/c mice 24 hours after pretreatment with $5 \times 10^7$ pfu/g of Ad-lacZ. Twenty four hours after the last injection, livers were harvested for caspase 8 assays. FasL and TRAIL significantly (*) induced caspase 8 activity. C 8 inh., caspase 8 inhibitor. (D) In animals with adenoviral hepatitis, one hour prior to Ad-TRAIL application, anti-TRAIL antibody N2B2 (250 μg/mouse) or isotype control antibody (250 μg/mouse) was injected into the tail vein. Hepatic steatosis was assessed by fat red staining. Administration of TRAIL neutralising antibody significantly (*) inhibited TRAIL mediated hepatic steatosis. Figure shows representative data of four independent experiments (magnification 600×).
indicating that TRAIL mediated steatosis is independent of caspase 8 activity (fig 4C).

TNF-α induces hepatic steatosis by mediating insulin resistance through its effects on several important sites of insulin action. As it has been shown that metformin improves insulin resistance and fatty liver disease in ob/ob mice, we investigated whether metformin is capable of preventing hepatic steatosis in animals after alcohol intake and after administration of Ad-TRAIL. As shown in fig 4D, metformin did not protect the liver against TRAIL mediated steatosis. According to the experiments in viral hepatitis, application of anti-TRAIL antibodies significantly prevented TRAIL mediated steatosis in animals after alcohol intake, as shown in fig 4E.

**DISCUSSION**

TRAIL has received particular attention because early reports suggest that it induces apoptosis in a variety of cancer cells with little or no effect on normal cells. As a consequence, recombinant soluble TRAIL was believed to be safe for use as a cancer therapy agent without damaging intact tissues. Preclinical studies in mice have been very promising but some investigators described TRAIL mediated toxicity in normal human cells, such as keratinocytes, hepatocytes, and brain cells, raising the concern that TRAIL might cause unexpected toxicity in humans. Actually, it became apparent that different recombinant TRAIL preparations exhibit significant biological differences and recent results indicate that administration of TRAIL lacking exogenous sequence tags or leucine zipper domains is effective in tumour therapy but is unlikely to cause toxicity to healthy human tissues.

The physiological role of TRAIL expression is largely unknown. The main function of TRAIL appears to be the negative regulation of the immune system to prevent autoimmunity but some investigators propose that TRAIL may also serve as an important effector molecule on activated T and B lymphocytes, natural killer cells, monocytes, and dendritic cells. We recently showed that TRAIL and TRAIL receptor DR5 are involved in apoptosis of hepatocytes in viral
hepatitis. In contrast with FasL, TRAIL mediated apoptosis of hepatocytes in vivo needs triggering through viral infection, confirming the hypothesis that TRAIL is an essential tool of an innate defence mechanism against viruses.

In this report we demonstrated that hepatic expression of TRAIL, in contrast with FasL, induced fatty liver disease in viral hepatitis and after alcohol intake. Expression of TRAIL did not induce steatosis in untreated animals, indicating a precondition of an environment in which other cytokines sensitises hepatocytes for TRAIL mediated steatosis. The molecular basis for this sensitisation remains uncertain but it may resemble, at least in part, the mechanisms involved in the susceptibility of the liver to TNF-α mediated steatosis.

Under normal conditions, hepatocytes are resistant to TNF-α induced steatosis but several cytokines released by macrophages such as interferon γ and interleukin 12 are known to promote TNF-α mediated steatohepatitis. As type I or II interferons strongly induce TRAIL expression on several effector cells of the immune system, TRAIL may also enhance TNF-α induced steatosis during viral hepatitis.

In our study, TRAIL mediated apoptosis was associated with hepatic steatosis only in viral hepatitis while expression of TRAIL induced neither apoptosis nor caspase 8 activity in animals after alcohol intake. These results suggest that TRAIL mediated apoptosis and steatosis may be independently modulated by additional cytokines released during viral infection and after alcohol intake. It has been shown that CD40 engagement triggers apoptosis of human hepatocytes and may amplify Fas dependent hepatocyte apoptosis in inflammatory liver diseases. In addition, CD40 ligation can induce functional expression of Fas ligand, TRAIL, and TNF-α in cancer cells, indicating an important role for CD40 as a modifying signal in receptor mediated apoptosis. It appears likely that CD40 engagement is also involved in our animal models and the contribution of CD40 to TRAIL mediated apoptosis and steatosis should be investigated in further studies.

Our results demonstrate that TRAIL is a new mediator of hepatic steatosis in viral hepatitis and after alcohol intake. As a consequence of this study, hepatotoxicity has to be considered in clinical trials with TRAIL in patients with viral hepatitis, alcoholic liver disease, or other liver diseases that may activate TRAIL sensitivity in hepatocytes. In this regard it is important to note that even a short duration of alcohol consumption was sufficient to increase the susceptibility of hepatocytes to TRAIL mediated steatosis in our experiments. However, in both of our animal models of TRAIL mediated steatosis, application of neutralising anti-TRAIL antibodies was capable of inhibiting liver steatosis. Thus our results also suggest that, in addition to TNF-α, TRAIL may be a new attractive therapeutic target for inhibition of hepatic steatosis.

ACKNOWLEDGEMENTS

Research was supported by grants of the Deutsche Forschungsgemeinschaft (KU 1213/ 3-1) and the Deutsche Krebshilfe (10-2078-Ku2).

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Conflict of interest: None declared.

REFERENCES


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EDITOR’S QUIZ: GI SNAPSHOT

A calcified and cystic peripancreatic mass

Clinical presentation
A 33 year old woman with no previous medical history presented with acute epigastric pain and nausea. Her physical examination was essentially unremarkable. Chest x-ray and urgent blood investigations were normal, including serum amylase, lipase, and hepatic enzymes. Abdominal ultrasound was performed and calcified masses was found anterior to the pancreas. An abdominal computed tomography scan was requested (fig 1). After surgical resection, histopathological examination was performed (fig 2).

Question
What is the diagnosis?
See page 1629 for answer

This case is submitted by:

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doi: 10.1136/gut.2005.068502

Figure 1  Computed tomography scan of the abdomen showing a cystic calcified mass.

Figure 2  Histopathological specimen showing the muscular layer in the right upper side and bone tissue in the left lower side (orange).