Involvement of the CD95 (APO-1/Fas) Receptor and Ligand in Liver Damage

By Peter R. Galle,* Walter J. Hofmann,‡ Henning Walczak,§
Heinz Schaller,‖ Gerd Otto,¶ Wolfgang Stremmel,*
Peter H. Krammer,§ and Laura Runkel‖

From the *University Hospital, Department of Gastroenterology, 69115 Heidelberg; ‡Institute of
Pathology, 69120 Heidelberg; §Tumorimmunology Program, German Cancer Research Center,
69120 Heidelberg; ‖Center of Molecular Biology, 69120 Heidelberg; and ¶University Hospital,
Department of Surgery, 69120 Heidelberg, Germany

Summary

Apoptosis occurs in the normal liver and in various forms of liver disease. The CD95 (APO-1/
Fas) (CD95) receptor mediates apoptosis, and liver cells in animal models are acutely sensitive
to apoptosis initiated by this receptor. We have used primary human hepatocytes as a model
system to investigate CD95-mediated apoptotic liver damage. Treatment of fresh human hepa-
tocytes with low concentrations of agonistic antibodies against CD95 resulted in apoptosis of
>95% of the cultured liver cells within 4 and 7.5 h. Immunohistology of a panel of explanted
liver tissues revealed that hepatocytes in normal livers ($n = 5$) and in alcoholic cirrhosis ($n =
13$) expressed low constitutive levels of CD95. CD95 receptor expression was highly elevated
in hepatocytes in hepatitis B virus–related cirrhosis ($n = 9$) and in acute liver failure ($n = 8$). By
in situ hybridization CD95 ligand messenger RNA expression was absent in normal liver but
detected at high levels in livers with ongoing liver damage. In cases of hepatitis B virus–related
cirrhosis and acute hepatic failure, ligand expression was found primarily in areas with lympho-
cytic infiltration. In contrast, in patients with alcoholic liver damage, high CD95 ligand mes-
senger RNA expression was found in hepatocytes. These findings suggest that liver destruction
in hepatitis B may primarily involve killing of hepatocytes by T lymphocytes using the CD95
receptor–ligand system. In alcoholic liver damage, death of hepatocytes might occur by fratri-
cide and paracrine or autocrine mechanisms mediated by the hepatocytes themselves.

Proliferation and apoptosis of cells play an essential role
in development and homeostasis of multicellular or-
ganisms (for review see references 1 and 2). Thus, apoptosis
is found in the metamorphosis of the tadpole and in the de-
velopment of the nervous and of the immune system, for
example (3–5). Apoptosis may also serve as a defense
against viral invasion. Certain viral infections lead to the
initiation of an intrinsic death program (6–8), which spares
other cells of the same organism from subsequent infection.
In addition, cytotoxic T lymphocytes and NK cells elimi-
nate their targets by apoptosis (9–13).

Apoptosis is mediated through activation of the CD95
receptor system. CD95, a member of the TNF receptor/
nerve growth factor receptor family is a type I transmem-
brane protein (14), and it acts as an inducer of apoptosis in
CD95-expressing cells in response to agonistic antibodies
or ligand binding (15–19). The ligand for CD95 has been
recently cloned and found to be a type II transmembrane
protein that belongs to the TNF family. The ligand is ex-
pressed in various cells, including activated thymocytes and
splenocytes, as well as in the testis, lung, and kidney (20,
21). The CD95 receptor–ligand system plays an important
role in B and T lymphocyte development and maturation
(5). In addition, the CD95 and the perforin system mediate
T cell cytotoxicity (10–12, 22–27). CD95 is constitutively
expressed in a wide range of tissues (28). Therefore, it may
have a general biological role beyond its function in the
immune system.

Many nonlymphoid tissues, such as liver, are CD95 re-
ceptor positive. The liver is highly sensitive to induction of
apoptosis by the agonistic anti-Fas antibody. Mice injected
intraperitoneally with 10–100 µg anti-Fas died rapidly,
within several hours (29). Biochemical, histological, and
electron microscopic analysis revealed that severe liver
damage by apoptosis was the most likely cause of death.
These findings suggest that CD95 is involved in apoptosis
in the liver, and they pose the question of whether CD95-
mediated cell death plays a role in liver failure in humans.

Apoptosis has been demonstrated in the acinar zone 3 of
the liver as a mechanism to eliminate senescent hepatocytes

(30). In addition, it is found during tissue remodeling in the course of liver regeneration or during involution of the liver after cessation of treatment with lipophilic compounds such as phenobarbital, which initially caused hypertrophy and hyperplasia of this organ (1). Furthermore, apoptosis in the liver has been observed during viral infection (1, 8). Councilman bodies, a characteristic pathological feature in viral hepatitis, closely resemble apoptotic cells or fragments. During viral hepatitis, activated T cells attack hepatocytes (31). The CD95 ligand (CD95L)[1] is expressed in T cells upon activation, and the CD95 receptor is constitutively expressed in liver cells (28). Therefore, activated T cells might kill hepatitis B virus (HBV) antigen–expressing hepatocytes by CD95 ligand–receptor interaction and, thus, clear HBV from the liver. Since HBV-infected individuals can either become chronic virus carriers or can clear the virus, individual differences may exist in the induction of liver cell apoptosis by cytotoxic T cells (and NK cells). Furthermore, various states of hepatitis exist in which liver cell apoptosis might be disregulated, possibly involving the CD95 receptor, ligand, or signaling pathway.

In the present study, we investigated induction of apoptosis in primary human hepatocytes upon CD95 activation. Receptor activation by addition of low amounts of agonistic antibodies to hepatocyte cultures rapidly and efficiently killed these cells. Immunohistology of liver tissue from patients with chronic HBV infection or acute hepatic failure showed highly elevated CD95 receptor expression in hepatocytes relative to normal livers. In situ hybridization detected CD95L messenger RNA (mRNA) in lymphocytes in HBV-related and acute liver damage. It is interesting to note that, in alcoholic cirrhosis, high ligand mRNA levels were found in hepatocytes directly.

Materials and Methods

Primary Human Hepatocyte Cultures. Primary human liver cells were prepared from healthy liver tissue obtained during hepatic surgery. The study was approved by the Ethics Committee of the Medical Faculty of the University of Heidelberg (Heidelberg, Germany). Cells were isolated as described (32, 33). Briefly, the resected piece of liver was perfused via a central vessel, first with S-MEM (GIBCO BRL, Paisley, Scotland) for 10–15 min, and then with Williams' medium E (WME; GIBCO BRL) containing 0.05% collagenase (Sigma Chemical Co., Munich, Germany) and 5 mmol/liter calcium for another 10–15 min. Subsequently, nonperfused parts were discarded, and the cell suspension was filtered through gauze, washed four times with ice-cold WME containing 5% FCS (Sigma Chemical Co.) by differential centrifugation, and seeded in maintenance medium at a density of 1 × 10^5 viable cells/cm² on collagen-coated tissue culture dishes (Costar Corp., Cambridge, MA). Viability was determined by trypan blue dye exclusion (34). The medium was changed 0.5 h after plating and every 1–2 d thereafter. For seeding and maintenance of cells, WME was used, supplemented with 0.066 μmol/

liter insulin (Serva Biochemicals, Heidelberg, Germany), 0.1 μmol/liter glucagon (Eli Lilly, Giessen, Germany), 0.1 μmol/liter triiodothyronine (Serva Biochemicals), 5 mmol/liter glutamine (Flow Laboratories GmbH, Meckenheim, Germany), 37 μmol/liter inosine (Serva Biochemicals), 10 μg/ml gentamycin (Flow Laboratories GmbH), 100 μg/ml streptomycin (Flow Laboratories GmbH), 100 μg/ml penicillin (Flow Laboratories GmbH) and 20 mmol/liter Hepes (Flow Laboratories GmbH). Cultures were incubated at 37°C in 5% CO_2 in air.

To investigate the capability of human hepatocytes to undergo apoptosis upon CD95 stimulation, cells were treated 12 or 48 h after plating with the mAb anti–APO-1 (IgG3-containing hybridoma supernatant) at concentrations of 1, 10, 20, or 100 ng/ml. Unless otherwise indicated, 20 ng/ml was used. This antibody has been shown to induce apoptosis in CD95 antigen–expressing cells by receptor cross-linking (15, 35). Control cultures were treated with an mAb at the same concentrations recognizing an influenza viral epitope.

Liver Tissues. All tissues examined in this study were obtained from hepatectomy specimens during liver transplantation, immediately snap frozen in liquid nitrogen and stored at −80°C until analyzed. The diagnosis alcoholic cirrhosis was based on histologically proven cirrhosis and a daily intake of >60 g alcohol in women and >80 g in men for >10 yr in the absence of serological markers for HBV or hepatitis C virus (HCV). The diagnosis of chronic HBV infection was based on a long-standing history of chronic hepatitis, the presence of serological markers for HBV, and immunohistological detection of hepatitis B surface Ag and/or hepatitis B core Ag in the liver tissue.

The criterion used for the diagnosis of acute hepatic failure was the development of hepatic encephalopathy within 12 wk from the onset of jaundice in the absence of preexisting liver disease (36). Hyperacute liver failure (LF) was assumed in cases in which encephalopathy developed within 7 d, acute LF in cases with an interval of between 8 and 28 d, and subacute LF in cases with an interval of between 5 and 12 wk. The patients with acute hepatic failure are listed in Table 1. This group consisted of three patients with HCV infection, one with HBV infection, one with hepatitis A virus (HAV) infection, and three cases with unknown etiology.

DAPI Staining and Analysis of DNA Fragmentation. For DAPI (4',6-diamidino-2-phenylindole; Sigma Chemical Co.) staining of DNA, cells were plated on plastic coverslips. After removal of the culture medium, cells were briefly dipped twice into PBS

Table 1. *Cases with Acute Liver Failure*

Case	Etiology*	Course
1	HCV	Subacute LF
2	?	Subacute LF
3	HCV	Subacute LF
4	HAV	Hyperacute LF
5	HCV	Subacute LF
6	?	Hyperacute LF
7	HBV	Acute LF
8	?	Acute LF

---

[1] *Abbreviations used in this paper:* CD95L, CD95 ligand; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; LF, liver failure; mRNA, messenger RNA; WME, Williams' medium E.

*As detected by HCV, HCV RNA in liver tissue; HAV, anti-HAV IgM in the absence of other viral markers; HBV, hepatitis B surface Ag and hepatitis B core Ag in liver tissue; ?, unknown etiology.

containing 1 mM MgCl at 37°C. The coverslips were then fixed in methanol for 10 min at −20°C followed by acetone for 5 min at −20°C and air dried. Staining with DAPI was performed according to the protocol of the manufacturer.

For DNA electrophoresis, ∼7 × 10⁶ detached cells were harvested after 7.5 h of treatment with 20 ng/ml anti–APO-1 and washed twice in PBS, and DNA was extracted as described (37). Electrophoresis was carried out in a 2% agarose gel, and the gel was subsequently stained with ethidium bromide.

Immunohistochemistry. CD95 antigen detection was performed using a monoclonal IgG₁ antibody directed against CD95 (35). The avidin–biotin peroxidase method was applied. Cryosections (4–5 μm thick) were fixed immediately after sectioning for 5 min in cold acetone, air dried overnight, and either used directly for immunolabeling or stored at −80°C until used. Fixed and, in case of storage, thawed sections were washed in PBS. Sections were then preincubated with streptavidin and biotin for 15 min to block endogenous liver avidin (Avidin Biotin Blocking Kit; Genex, Gaithersburg, MD). After another wash, sections were incubated for 60 min at room temperature with the primary mAb at a concentration of 10 μg/ml. Bound mAbs were visualized by the avidin–biotin peroxidase method using VECTASTAIN Elite Kit (Vector Laboratories, Inc., Burlingame, CA) and amino-ethylcarbazol as chromogen. Counterstaining was performed with hemalaun.

The liver tissues were graded as follows: 0, absence of CD95 expression; 1, low constitutive CD95 expression (28); 2, intermediate CD95 expression; 3, high CD95 expression.

RNA In Situ Hybridization. Cryosections (5 μm thick) were prepared from human liver tissues that had been stored at −80°C since explantation. Sections were transferred onto siliconized glass slides and fixed in 4% (wt/vol) paraformaldehyde/PBS (20 min, room temperature), and then briefly washed in PBS (room temperature) and dehydrated by sequential transfer through 70 and 95% ethanol baths. The in situ hybridization procedure was performed essentially as described by Bosch et al. (38) using an in vitro–synthesized, ³⁵S-labeled antisense RNA corresponding to the sequence coding for amino acids 100–246 of the human CD95L (39). Controls for the specificity of the ligand signal were performed by parallel treatment of cryosections with a 20-fold molar excess of unlabeled, competitor antisense RNA. This resulted in a marked reduction in the strength of ligand signals in expressing cells. In situ hybridizations were dipped into liquid film emulsion (K5; Ilford Ltd., Basildon, UK) and exposed for 3–4 wk. Development of the slides was performed according to the instructions of the manufacturer. The developed in situ hybridizations were counterstained with hematoxylin and eosin and were prepared for microscopy.

Results

Induction of Apoptosis by Anti–Apo-1. Initial experiments were aimed at determining the sensitivity of human hepatocytes to the induction of apoptosis by the agonistic antibody anti–APO-1. Freshly plated cells were treated with 1, 10, 20, or 100 ng/ml anti–APO-1. Control cultures were treated with an irrelevant antibody directed against an influenza viral epitope. Treatment for ∼7.5 h with concentrations of 10 ng/ml or above resulted in extensive cell death (>95%) as exemplified in Fig. 1 *b* for 20 ng/ml. Control cultures remained unaffected (Fig. 1 *a*). The first morphological changes were noted after 4–4.5 h, when cells became round in shape, started to lose contact with
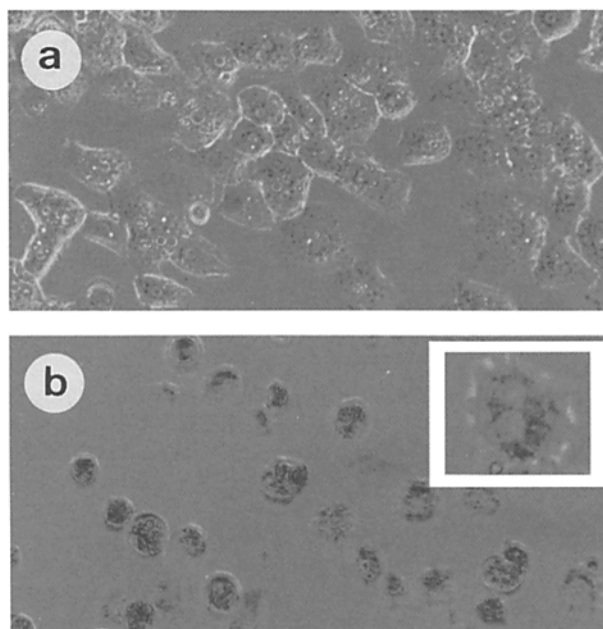

Figure 1. Primary human hepatocytes treated with a control antibody (*a*) or with 20 ng/ml anti–APO-1 (*b*) for 7.5 h. Phase contrast, ×160. The inset in *b* shows a rounded-up hepatocyte with cytoplasmic blebs after 5 h of treatment at 360-fold magnification.

neighboring cells, and formed cytoplasmic "blebs" (Fig. 1 *b, inset*). These changes were pronounced after 5.5 h, and after 7.5 h essentially all cells were dead and mostly detached (Fig. 1 *b*).

A quantification of the anti–APO-1 effect using liver cells 12 h after plating is shown in Fig. 2 *a*. Nearly all hepatocytes died within a short period of 4–7.5 h after the beginning of the antibody treatment. In contrast, control cultures maintained 90% viability throughout the entire culture period. Treatment with anti–APO-1 48 h after plating resulted in similar kinetics of death but affected only ∼60% of the cells (Fig. 2 *b*). This demonstrates a loss in responsiveness to CD95 stimulation with time in culture, which might reflect the loss of differentiated characteristics typically encountered in aging liver cells in primary culture (40).

To investigate nuclear morphology, DAPI staining of cellular DNA was performed. Control cultures maintained their nuclear morphology throughout the culture period (Fig. 3 *a*). In contrast, anti–APO-1 treatment resulted in progressive condensation and fragmentation of the nuclei, changes characteristic of apoptosis (Fig. 3 *b*, after 5 h of treatment). Biochemical analysis of chromosomal DNA in dead cells that had detached from the culture plates after 7.5 h of treatment demonstrated fragmentation of DNA, yielding a typical "ladder" pattern in agarose gel electrophoresis (Fig. 4).

CD95 Expression in Patients with Active Liver Disease. We investigated CD95 receptor expression in liver tissues from patients who underwent liver transplantation. A panel of tissues derived from patients with liver failure due to chronic HBV infection–related cirrhosis (*n* = 9), alcoholic
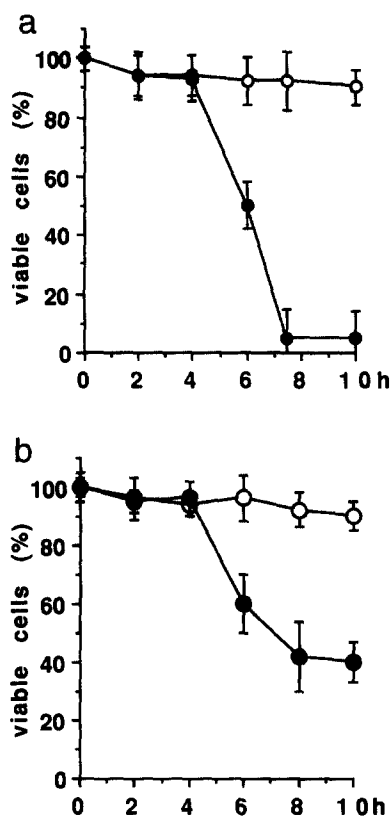
Figure 2. Viability of hepatocytes in the presence (●) or absence (○) of 20 ng/ml anti–APO-1. Treatment was started 12 h (a) or 48 h (b) after plating. Viability was assessed morphologically after trypan blue staining. The data represent mean ± SD of three independent wells.

Figure 3. DNA staining with DAPI in controls (a) and anti-APO-1–treated (b) liver cells after 5 h of treatment. ×400.

cirrhosis (n = 13), or acute hepatic failure (n = 8) was analyzed by immunohistology. In addition, normal liver tissues (n = 5) were examined. Normal liver and the majority (12 out of 13) of the cases with alcoholic liver disease showed a low constitutive level of CD95 expression on the hepatocytic cell membrane, homogeneously distributed throughout the liver sections, as exemplified in Fig. 5 a for alcoholic cirrhosis. In contrast, in patients with HBV-related cirrhosis (8/9), highly up-regulated CD95 expression was detected on the cell membrane of hepatocytes (Fig. 5 b). In the eight patients with acute hepatic failure, only small islands of dying hepatocytes surrounded by monocytic cells were seen. These islands displayed CD95 expression at high levels in all cases (Fig. 5 c). The data on CD95 expression levels in the panel of human liver tissues are summarized in Table 2.

CD95L mRNA Expression in Patients with Chronic and Acute Liver Failure. To determine whether the CD95L is expressed in cases of ongoing liver damage, our panel of liver tissues was also examined for expression of CD95L mRNA. CD95L mRNA expression was not detectable in normal liver (zero out of three cases examined). However, easily detectable or high levels of CD95L mRNA were found in livers with ongoing cell damage. It is interesting
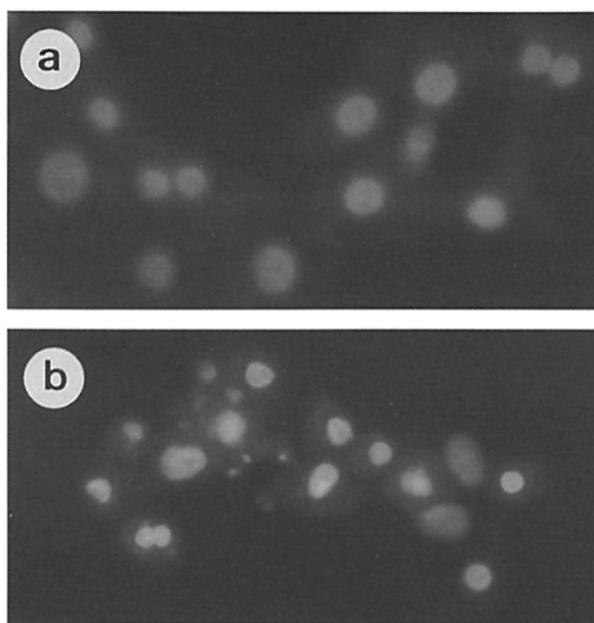
to note that two different patterns of expression were discerned. In cases with HBV-related cirrhosis (three out of three), CD95L mRNA expression was found in the periphery of the pseudolobules at the border to the periportal tracts at the side of piecemeal cell death (Fig. 6, a and a'). Thus, CD95L mRNA expression localizes to the area of lymphocytic infiltration. Similarly, in one out of three cases (Table 1, case No. 4) with virus-induced, acute hepatic failure, ligand mRNA expression was detected in lymphocytes surrounding islands of dying hepatocytes (not shown). In the other two cases with acute LF examined for CD95L expression, the tissue destruction was too advanced for morphological analysis. In contrast to this picture, in the cases of alcoholic cirrhosis (three out of three), very high CD95L mRNA expression was detected in the center of the cirrhotic pseudolobule (Fig. 6, b and b'). Thus, CD95L mRNA was mainly found in areas where no lymphocytes but only hepatocytes and mesenchymal liver cells (i.e.,
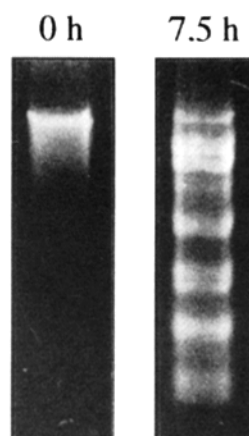

Figure 4. Agarose gel electrophoresis of cellular DNA from hepatocytes after 7.5 h of treatment with anti–APO-1 (*right*) or a control antibody (*left*).
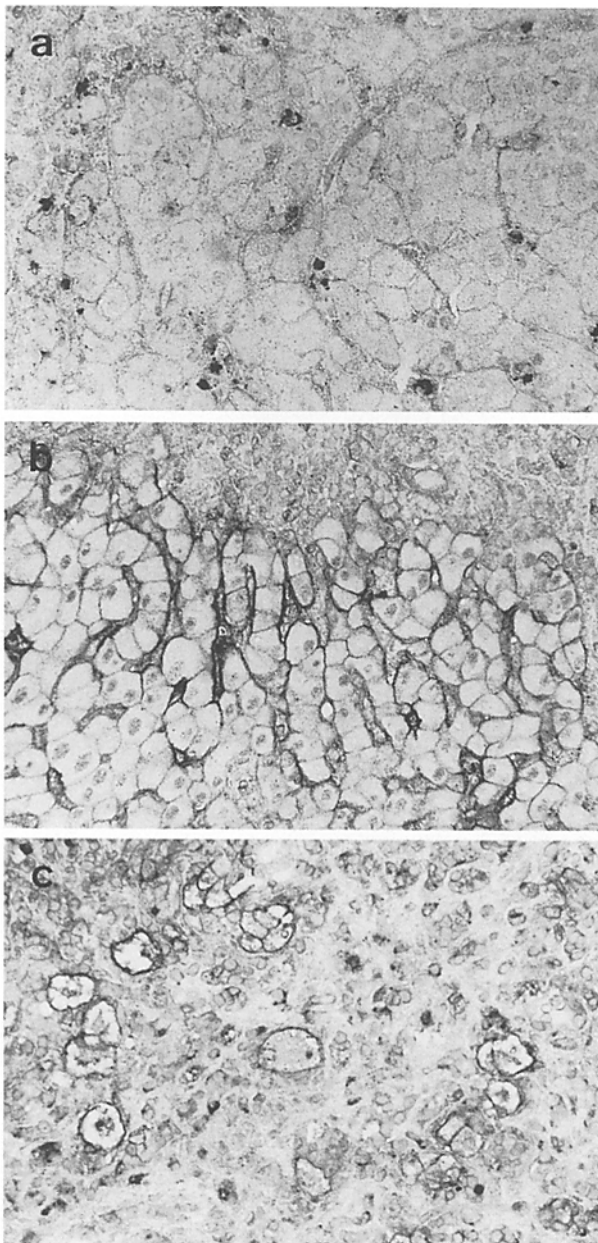
Figure 5. CD95 receptor expression in livers with chronic liver disease and liver failure. (*a*) Alcoholic cirrhosis with faint expression of CD95 on the membranes of hepatocytes. The black dots indicate unspecific staining of bile in this cholestatic cirrhosis. (*b*) Cirrhosis with chronic HBV-induced hepatitis. Shown is the strong expression of CD95 antigen on the membrane of hepatocytes, accentuated at sites of inflammation and contact with infiltrating lymphocytes. (*c*) Subacute, cryptogenic liver failure (case No. 2 in Table 1). Only few intact hepatocytes are visible with marked expression of CD95 antigen on their cell membranes. Some infiltrating lymphocytes also show CD95 antigen expression. ×180.

Table 2. *CD95 Receptor Expression in Human Liver*

Liver tissue of	n	CD95 receptor expression*
Normal liver	5	0.8
Alcoholic cirrhosis	13	1.15
HBV-related cirrhosis	9	2.45
Acute liver failure	8	2.5

This table gives the mean on the basis of the immunohistological grading of the human liver tissues as outlined in Materials and Methods. The SE was always <0.4.
*Score for immunohistology: 0, no expression; 1, weak expression; 2, intermediate expression; 3, high expression.

their large round nuclei localized centrally within a broad cytoplasm.

Discussion

This paper demonstrates that triggering of the CD95 receptor by agonistic anti–APO-1 antibodies rapidly induces massive apoptotic cell death in primary human hepatocytes. This finding in human hepatocytes reflects the acute sensitivity of the liver to CD95 stimulation previously observed in the mouse in vivo (29) and in vitro (41), and it shows that constitutive receptor expression levels are functionally sufficient for CD95-mediated apoptosis.

These observations raise the question of whether liver damage in humans in vivo is mediated by the CD95 system. In this context, we examined explanted liver tissues from patients with normal livers, HBV-related and alcoholic cirrhosis, and acute liver failure. Our results confirm the observation of constitutive low expression of CD95 in normal human liver (28). In contrast, in livers from patients with chronic HBV infection or acute hepatic failure but not with alcoholic cirrhosis, CD95 receptor expression was found to be up-regulated. Increased CD95 expression as observed in livers with ongoing cell damage in this study may have several implications. Enhanced receptor expression might initiate the disease. Alternatively, CD95 expression might be increased in response to a primary stimulus and render the hepatocyte more susceptible to CD95L stimulation. This assumption is supported by the observation that induction of CD95 expression occurs as a result of chronic, lymphohistiocytic inflammation in different epithelial cells (28).

To assess CD95L synthesis and to define the ligand-producing effector cell, mRNA in situ hybridization was performed. In patients with chronic HBV infection, CD95L mRNA was found to be expressed in areas with lymphocytic infiltration, in contrast to normal liver, where no signal was detectable. In vitro evidence exists that activated T cells express the CD95L. In addition, the CD95 pathway constitutes a major mechanism of T cell–mediated cytotoxicity (10, 20, 21, 27, 42–45). Moreover, expression of
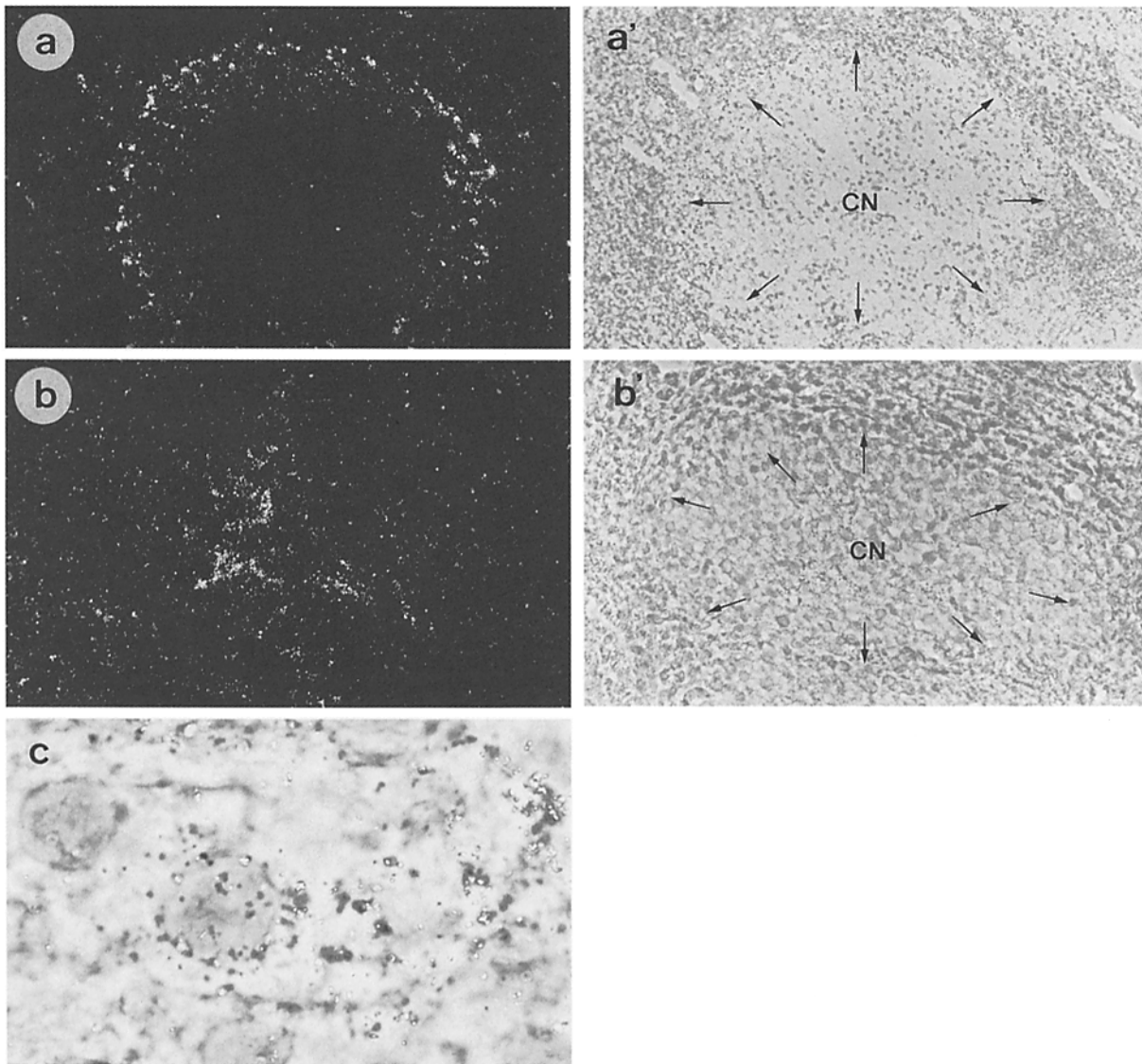
Kupffer cells and endothelial cells) are present. This was confirmed by investigation of hematoxylin and eosin–stained parallel sections (not shown). This finding strongly suggests that the CD95L can be expressed in hepatocytes. Indeed, higher magnification revealed the presence of silver grains in hepatocytes (Fig. 6 *c*), as morphologically identified by

Figure 6. CD95L mRNA expression in chronic liver disease. (*a*) Dark field; (*a'*) bright field view of an HBV-related cirrhosis with chronic hepatitis. A cirrhotic nodule (*CN*) is shown surrounded by a dense inflammatory infiltrate. A prominent ligand signal is detectable at the border (*arrows*) of the same cirrhotic nodule in dark field view. (*b*) Dark field; (*b'*) bright field view of an alcoholic cirrhosis. A cirrhotic nodule (*CN*) is surrounded by fibrous bands and fibrotic portal tracts (*arrows*). The silver grain signal in the dark field view is found in the center of the same cirrhotic nodule. (*c*) Higher magnification of *b'* with evidence of CD95L mRNA within a hepatocyte. (*a*, *a'*, *b*, and *b'*) ×30; (*c*) ×980.

the CD95L has been shown recently for liver-infiltrating mononuclear cells in patients with hepatitis C (46). Therefore, activated T-killer cells may kill HBV-infected hepatocytes using the CD95 system. A different degree of CD95-mediated cytotoxicity may account for various disease stages. Such different levels of cytotoxicity may explain ineffective viral clearance in chronic hepatitis, regular clearance in acute, self-limiting hepatitis, or over-effective clearance in fulminant hepatitis. Indeed, in chronic HBV-induced liver failure, a poor response to HBV antigens has been described for MHC class I– and II–restricted T cells (47).

Unexpectedly, in alcoholic hepatitis, the CD95L was expressed at high levels in hepatocytes directly. The presence of ligand mRNA and receptor in the same cell points to a

new mechanism of liver cell damage. Initial investigation of ligand expression in the rat revealed that the CD95L is not expressed in the liver (20). However, our data demonstrate that, in alcoholic liver damage, human hepatocytes themselves can up-regulate the CD95 system. The CD95L might be expressed as a membrane-bound form and might mediate apoptosis as "fratricide" interacting with the CD95 receptor on neighboring cells. Alternatively, CD95-mediated apoptosis may occur in an autocrine or paracrine fashion via a soluble form of the ligand. Indeed, soluble ligand activity capable of inducing apoptosis has been detected in supernatants of anti-CD3–stimulated T cells (43). Our data show that the above forms of CD95-mediated killing are not restricted to lymphocytes but may also occur in non-

lymphoid cells such as hepatocytes. Therefore, it is conceivable that the CD95 system is highly relevant for apoptosis of various tissues throughout the body.

The involvement of the CD95 pathway in hepatocellular damage offers a new fundamental understanding of destructive liver disease, particularly in alcoholic cirrhosis, and it may provide new therapeutic approaches based on interference with CD95 receptor–ligand interaction or with the signaling pathway.

References

1. Bursch, W., F. Oberhammer, and R. Schulte-Hermann. 1992. Cell death by apoptosis and its protective role against disease. *Trends Pharmacol. Sci.* 13:245–251.
2. Evans, V.G. 1993. Multiple pathways to apoptosis. *Cell Biol. Int.* 17:461–475.
3. Vaux, D.L., G. Haecker, and A. Strasser. 1994. An evolutionary perspective on apoptosis. *Cell.* 76:777–779.
4. Schwartz, L.W., and B.A. Osborne. 1993. Programmed cell death, apoptosis and killer genes. *Immunol. Today.* 12:582–590.
5. Krammer, P.H., I. Behrmann, P. Daniel, J. Dhein, and K.M. Debatin. 1994. Regulation of apoptosis in the immune system. *Curr. Opin. Immunol.* 6:279–289.
6. Pickup, D.J., B.S. Ink, W. Hu, C.A. Ray, and W. Kinoklik. 1986. Hemorrhage in lesions caused by cowpox virus is induced by a viral protein that is related to plasma protein inhibitors of serine proteases. *Proc. Natl. Acad. Sci. USA.* 83:7698–7702.
7. White, E., P. Sabbatini, M. Debbas, W.S.M. Wold, D.I. Kusher, and L.R. Gooding. 1992. The 19kD adenovirus E1B transforming protein inhibits programmed cell death and prevents cytolysis by TNF. *Mol. Cell Biol.* 12:2570–2580.
8. Roberts, J.M., J.W. Searle, and W.G. Cooksley. 1993. Histological patterns of prolonged hepatitis C infection. *Gastroenterology.* 26:37–41.
9. Apasov, S., F. Redegeld, and M. Sitkovsky. 1993. Cell-mediated cytotoxicity; contact and secreted factors. *Curr. Opin. Immunol.* 5:404–410.
10. Lowin, B., M. Hahne, C. Mattmann, and J. Tschopp. 1994. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. *Nature (Lond.).* 370:650–652.
11. Kägi, D., B. Ledermann, K. Bürki, P. Seiler, B. Odermatt, K.J. Olsen, E.R. Podack, R.M. Zinkernagel, and H. Hengartner. 1994. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. *Nature (Lond.).* 369:31–37.
12. Kägi, D., F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner, and P. Golstein. 1994. Fas and perforin pathways as major mechanisms of T-cell-mediated cytotoxicity. *Science (Wash. DC).* 265:528–530.
13. Heusel, J.W., R.L. Wesselschmidt, S. Stresta, J.H. Russel, and T.J. Ley. 1994. Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogenic target cells. *Cell.* 76:977–987.
14. Smith, C.A., T. Farrah, and R.G. Goodwin. 1994. The TNF receptor superfamily of cellular and viral proteins: activation, costimulation and death. *Cell.* 76:959–962.
15. Trauth, B.C., C. Klas, A.M.J. Peters, S. Matzku, P. Möller, W. Falk, K.M. Debatin, and P.H. Krammer. 1989. Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science (Wash. DC).* 245:301–305.
16. Yonehara, S., A. Ishii, and M. Yonehara. 1989. A cell-killing monoclonal antibody (anti-Fas) to cell surface antigen co-downregulated with the receptor of tumor necrosis factor. *J. Exp. Med.* 169:1747–1756.
17. Itoh, N., S. Yonehara, A. Ishii, M. Yonehara, S.I. Mizuchima, M. Sameshima, A. Hase, Y. Seto, and S. Nagata. 1991. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell.* 66:2333–2343.
18. Oehm, A., I. Behrmann, W. Falk, M. Pawlita, G. Maier, C. Klas, M. Li-Weber, S. Richards, J. Dhein, B.C. Trauth, et al. 1992. Purification and molecular cloning of the APO-1 surface antigen, a member of the TNF/NGF receptor superfamily. *J. Biol. Chem.* 267:10709–10715.
19. Suda, T., and S. Nagata. 1994. Purification and characterization of the Fas-ligand that induces apoptosis. *J. Exp. Med.* 179:873–879.
20. Suda, T., T. Takahashi, P. Golstein, and S. Nagata. 1993. Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell.* 75:1169–1178.
21. Lynch, D.H., M.L. Watson, M.R. Alderson, P.R. Baum, R.E. Miller, T. Tough, M. Gibson, T. Davis Smith, C.A. Smith, K. Hunter, et al. 1994. The mouse Fas-ligand gene is mutated in gld mice and is part of a TNF family gene cluster. *Im-*

munity. 1:131–136.

22. Rouvier, E., M.-F. Luciani, and P. Golstein. 1993. Fas involvement in Ca^{2+}-independent T cell–mediated cytotoxicity. *J. Exp. Med.* 177:195–200.

23. Gilette-Ferguson, I., and C.L. Sidman. 1994. A specific intercellular pathway of apoptotic cell death is defective in the mature peripheral T cells of autoimmune lpr and gld mice. *Eur. J. Immunol.* 24:1181–1185.

24. Hanabuchi, S., M. Koyanagi, A. Kawasaki, N. Shinohara, A. Matsuzawa, Y. Nishimura, Y. Kobayashi, S. Yonehara, H. Yagita, and K. Okumura. 1994. Fas and its ligand in a general mechanism of T-cell-mediated cytotoxicity. *Proc. Natl. Acad. Sci. USA.* 91:4930–4934.

25. Ju, S.T., H. Cui, D.J. Pauka, R. Ettinger, and A. Marshak-Rothstein. 1994. Participation of target Fas protein in apoptosis pathway induced by $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells. *Proc. Natl. Acad. Sci. USA.* 91:4185–4189.

26. Stalder, T., S. Hahn, and P. Erb. 1994. Fas antigen is the major target molecule for $CD4^+$ T-cell mediated cytotoxicity. *Immunology.* 152:1127–1133.

27. Vignaux, F., and P. Golstein. 1994. Fas-based lymphocyte-mediated cytotoxicity against syngeneic activated lymphocytes: a regulatory pathway? *Eur. J. Immunol.* 24:923–927.

28. Leithäuser, F., J. Dhein, G. Mechtersheimer, K. Koretz, S. Brüderlein, C. Henne, A. Schmidt, K.M. Debatin, P.H. Krammer, and P. Möller. 1993. Constitutive and induced expression of APO-1, a new member of the NGF/TNF receptor superfamily, in normal and neoplastic cells. *Lab. Invest.* 69:415–429.

29. Ogasawara, J., R. Watanabe-Fukunaga, M. Adachi, A. Matsuzawa, T. Kasugai, Y. Kitamura, N. Itoh, T. Suda, and S. Nagata. 1993. Lethal effects of the anti-Fas antibody in mice. *Nature (Lond.).* 364:806–809.

30. Benedetti, A.A., M. Jezaquel, and F. Orlandi. 1988. Preferential distribution of apoptotic bodies in acinar zone 3 of normal human and rat liver. *J. Hepatol.* 7:319–324.

31. Chisari, F.V. 1992. Hepatitis B virus biology and pathogenesis. *Mol. Genet. Med.* 2:67–104.

32. Galle, P.R., L. Theilmann, R. Raedsch, G. Otto, and A. Stiehl. 1990. Ursodeoxycholate reduces hepatotoxicity of bile salts in primary human hepatocytes. *Hepatology.* 12:486–491.

33. Galle, P.R., J. Hagelstein, B. Kommerell, M. Volkmann, P. Schranz, and H. Zentgraf. 1994. In vitro experimental infection of primary human hepatocytes with hepatitis B virus. *Gastroenterology.* 106:664–673.

34. Freshnez, R.I. 1987. Culture of Animal Cells: A Manual of Basic Technique. Alan R. Liss, Inc., New York. 245–246.

35. Dhein, J., P.T. Daniel, B.C. Trauth, A. Oehm, P. Möller, and P.H. Krammer. 1992. Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on cross-linking of APO-1 cell surface antigens. *J. Immunol.*

149:3166–3173.

36. O'Grady, J.G., S.W. Schalm, and R. Williams. 1993. Acute liver failure: redefining the syndromes. *Lancet.* 342:273–275.

37. Sanchez, V., M. Lucas, A. Sanz, and R. Goberna. 1992. Decreased protein kinase C activity is associated with programmed cell death (apoptosis) in freshly isolated rat hepatocytes. *Biosci. Rep.* 12:199–206.

38. Bosch, F.X., N. Udvarhelyi, E. Venter, C. Herold-Mende, A. Schumann, H. Maier, H. Weidauer, and A.I. Born. 1993. Expression of the histone H3 gene in benign, semi-malignant and malignant lesions of the head and neck: a reliable proliferation marker. *Eur. J. Cancer.* 29A:1454–1461.

39. Takahashi, T., M. Tanaka, J. Inazawa, T. Abe, T. Suda, and S. Nagata. 1994. Human Fas ligand: gene structure, chromosomal location and species specificity. *Int. Immunol.* 6:1567–1574

40. Galle, P.R., H.-J. Schlicht, C. Kuhn, and H. Schaller. 1989. Replication of duck hepatitis B virus in primary duck hepatocytes and its dependence on the state of differentiation of the host cell. *Hepatology.* 10:459–465.

41. Ni, R., Y. Tonita, K. Matsuda, A. Ichihara, K. Ishimura, J. Ogasawara, and S. Nagata. 1994. Fas mediated apoptosis in primary cultured mouse hepatocytes. *Exp. Cell Res.* 215:332–337.

42. Anel, A., M. Buferne, C. Boyer, A.-M. Schmitt-Verhulst, and P. Golstein. 1994. T cell receptor induced Fas ligand expression in cytotoxic T-lymphocyte clones is blocked by protein tyrosin kinase inhibitors and cyclosporin A. *Eur. J. Immunol.* 24:2469–2476.

43. Dhein, J., H. Walczak, C. Bäumler, K.M. Debatin, and P.H. Krammer. 1995. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature (Lond.).* 373:438–441.

44. Ramsdell, F., M.S. Seaman, R.E. Miller, T.W. Tough, M.R. Alderson, and D.H. Lynch. 1994. gld/gld mice are unable to express a functional ligand for Fas. *Eur. J. Immunol.* 24:928–933.

45. Takahashi, T., M. Tanaka, C.I. Brannan, N.G. Copeland, N.A. Jenkins, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell.* 76:969–976.

46. Mita, E., N. Hayashi, S. Iio, T. Takehara, T. Hijioka, A. Kasahara, H. Fusamoto, and T. Kamada. 1994. Role of Fas ligand in apoptosis induced by hepatitis C virus infection. *Biochem. Biophys. Res. Commun.* 204:468–474.

47. Nayersina, R., P. Fowler, S. Guilhot, G. Missale, A. Cernay, H.J. Schlicht, A. Vitiello, R. Chesnut, J.L. Person, A.G. Redeker, et al. 1993. HLA A2 restricted cytotoxic T lymphocyte responses to multiple hepatitis B surface antigen epitopes during hepatitis B virus infection. *J. Immunol.* 150:4659–4671.