Regulation of CD95 (APO-1/Fas) Receptor and Ligand Expression by Lipopolysaccharide and Dexamethasone in Parenchymal and Nonparenchymal Rat Liver Cells

MARKUS MÜSCHEN,[1] ULRICH WARSKULAT,[1] PATRICE DOUILLARD,[2] EMMANUELLE GILBERT,[2] AND DIETER HÄUSSINGER[1]

The effect of lipopolysaccharide (LPS) on the expression of CD95 (APO-1/Fas) receptor and ligand (CD95L) was studied in primary cultures of rat liver Kupffer cells (KCs), sinusoidal endothelial cells (SECs), and parenchymal cells (PCs) at the messenger RNA (mRNA) level and by means of immunocytochemistry. LPS treatment of KCs and SECs led to a three- to five-fold increase in CD95L mRNA levels within 6 hours, which declined thereafter. Within 24 hours, the number of KCs and SECs staining positive for CD95L strongly increased. After a lag phase of 12 hours after LPS addition, in both cell types the mRNA levels for the soluble CD95 isoform increased approximately 10-fold; however, the number of KCs and SECs staining positive for transmembrane CD95 remained low and did not significantly increase. Compared with nonparenchymal cells, CD95L mRNA levels in primary hepatocyte cultures were low in the absence and presence of LPS. On the other hand, functionally active CD95 expression markedly increased in response to LPS in these cells. Dexamethasone diminished the LPS-induced stimulation of CD95L expression in nonparenchymal cells but markedly stimulated CD95L expression in PCs. Apoptosis of PCs and thymic lymphocytes was stimulated by the addition of supernatants derived from LPS-treated KC or SEC cultures and was apparently mediated by CD95L as assessed by its sensitivity to inhibitors of the CD95-dependent apoptotic pathway in PCs. The data suggest a complex and timely coordinated interplay between the various liver cell populations with respect to LPS-induced activation of the apoptotic machinery with potential relevance for immunoregulation. (HEPATOLOGY 1998;27:200-208.)

Abbreviations: LPS, lipopolysaccharide; KC, Kupffer cell; SEC, sinusoidal endothelial cell; PC, parenchymal cell; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rHPRT, rat hypoxanthine-guanine phosphoribosyltransferase; PCR, polymerase chain reaction; PBS, phosphate buffered saline; sCD95, soluble CD95 isoform; TUNEL, TdT-mediated deoxyuridine triphosphate nick end labeling; Ac-YVAD-cmk, acetyl-Tyr-Val-Ala-Asp-chloromethylketone.

From the [1]Department of Gastroenterology and Infectiology, Heinrich-Heine Universität Düsseldorf, Düsseldorf, Germany; and [2]INSERM Unité 437, Immunointervention dans les Allo- et Xénotransplantations, Nantes, France.

Received March 18, 1997; accepted September 16, 1997.

Supported by the Deutsche Forschungsgemeinschaft, the Gottfried-Wilhelm-Leibniz-Prize, and the Fonds der Chemischen Industrie.

Address reprint requests to: Prof. Dr. D. Häussinger, Medizinische Universitätsklinik, Heinrich Heine Universität, Moorenstrasse 5, D-40225 Düsseldorf, Germany. Fax: (49) 211-8118838.

0270-9139/98/2701-0031$3.00/0

Apoptosis (for review, see references 1-8) can be mediated through activation of the CD95 (Fas/Apo-1) receptor/ligand system. CD95 belongs to the tumor necrosis factor (TNF) receptor/nerve growth factor receptor family and acts as an inducer of apoptosis in CD95-expressing cells when activated by CD95 antibodies or the CD95 ligand (CD95L).[9-14] CD95 is also involved in apoptosis in liver. Here, apoptosis may serve the physiological removal of senescent hepatocytes[15] and the remodeling of the organ during development or after liver regeneration.[1] In line with this, targeted mutation of the CD95 gene causes hyperplasia of the liver.[16] However, apoptosis may also play an important role in liver pathology, such as viral hepatitis,[17-19] acute liver failure,[8] or alcohol-induced liver injury.[8] Further, hepatocytes are sensitive to CD95 antibodies,[20] and in acute liver failure and chronic hepatitis B, but not in alcoholic cirrhosis, CD95 is up-regulated.[17] Apoptosis of liver cells can be induced by T-lymphocytes, which express CD95L on activation. Such killing of hepatocytes by lymphocytes via the CD95 receptor and CD95L system in viral hepatitis may help to clear infected hepatocytes. Glucocorticoids such as dexamethasone induce apoptosis in T-lymphocytes but paradoxically inhibit activation-induced apoptosis by preventing up-regulation of CD95L.[21,22]

Recent studies have pointed to a role of sinusoidal endothelial and Kupffer cells in the phagocytosis of circulating neutrophils undergoing apoptosis.[23] When Kupffer cells become activated in response to lipopolysaccharide (LPS), they produce a variety of mediators. Among them, TNF-α mediates apoptosis in nonparenchymal liver cells,[24] but the role of the CD95/CD95L system is not definitely established. The expression patterns for TNF-α and the CD95 receptor/CD95L system can overlap in many cells, but the pattern of induction may indicate distinct roles during cellular immune and activation responses.[12] However, little is known about the role of resident nonparenchymal cells in liver with respect to CD95 receptor and CD95L expression, which was investigated in the present study. The data show a sequential expression of CD95L and CD95 receptor in nonparenchymal cells in response to LPS, but only of the CD95 receptor in parenchymal cells. Furthermore, dexamethasone leads to an up-regulation of CD95L in parenchymal cells, but inhibits the expression of CD95L in nonparenchymal cells. The findings may point to an important role of Kupffer and sinusoidal endothelial cells in the regulation of apoptosis of circulating white blood cells and hepatocytes.

MATERIALS AND METHODS

Reagents and Antibodies. Polybead microspheres (2.5% solid latex, 1 μm diameter) were obtained from Polysciences Limited (St. Goar, Germany). RPMI 1640 medium (without phenol red) and fetal bovine serum were from Biochrom (Berlin, Germany). Phytohemagglutinin was from Seromed (Berlin, Germany). The salts required for preparation of the Krebs-Henseleit buffer were from Merck (Darmstadt, Germany). Williams' E medium, anti-rabbit peroxidase immunoglobulin G, and all other chemicals were from Sigma (Deisenhofen, Germany). Oligonucleotides were synthesized by Birsner and Grob (Freiburg, Germany). Rabbit polyclonal antibodies used for immunocytochemical procedures raised against a peptide corresponding to amino acids 308 to 327 mapping at the carboxy terminus of membrane-bound CD95 (Fas/APO-1) receptor of mouse origin and against a peptide corresponding to amino acids 2 to 19 mapping at the amino terminus of CD95 receptor ligand (FasL) of rat origin were from Santa Cruz Biotechnology (Santa Cruz, CA). BB-3103, an inhibitor of a variety of matrix metalloproteinases, was a gift from British Biotech (Oxford, England).

Isolation and Culture of Rat Liver Parenchymal Cells. Male Wistar rats (body wt, 300-400 g) were raised in the local institute for laboratory animals and held according to the rules of the local ethical guidelines. Liver parenchymal cells were prepared from livers of male Wistar rats, fed *ad libitum* on stock diet, by collagenase perfusion as described previously[25] and plated on collagen-coated cluster 6 dishes (Costar, Cambridge, MA) at a density of approximately 1×10^6 cells/mL. Cell viability was more than 95% as assessed by trypan blue exclusion. The cells were then cultured in Williams' E medium, 37°C, 5% CO_2, pH 7.4, plus glucose (5 mmol/L), supplemented with 5% fetal bovine serum for 24 hours. Thereafter, cells were maintained under various test conditions for 6, 24, or 48 hours.

Isolation and Culture of Kupffer and Sinusoidal Endothelial Cells. Cells were prepared by collagenase-pronase perfusion and separated by a single Nycodenz gradient and centrifugal elutriation as described previously.[26,27] The viability of the cells was more than 95% as assessed by trypan blue exclusion. The purity of the respective Kupffer cell (KC) and sinusoidal endothelial cell (SEC) cultures was more than 98% as assessed by morphological examination and by the ability to ingest latex particles.[28] Whereas in KC cultures more than 98% of the cells avidly phagocytosed latex particles, phagocytosis occurred in less than 4% of the cells contained in the SEC cultures, indicating negligible contamination by KCs. Contamination of the KC and SEC cultures with parenchymal cells was less than 1% as assessed by light microscopy.

KCs were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS on cluster 6 dishes (Costar, Cambridge, MA) for 48 hours. SECs were cultured in the same medium on collagen-coated cluster 6 dishes for 24 hours. The experiments were performed during the following 48 hours using Krebs-Henseleit hydrogen carbonate buffer (pH 7.4) containing 1% heat-inactivated FBS and 10 mmol/L glucose. In one set of experiments, LPS-treated KCs were simultaneously incubated with 1 μmol/L of the matrix metalloproteinase inhibitor BB-3103 (British Biotech, Oxford, England) to prevent the release of CD95L into the medium.

Isolation and Culture of Thymic Lymphocytes. Freshly removed rat thymus was cut into several pieces in RPMI medium and then pressed with the plunger of a 10-mL syringe against the bottom of a petri dish until mostly fibrous tissue remained. Clumps were dispersed by drawing up and expelling the suspension several times through a 70-μm cell strainer. After repeated washing with medium, the suspension was centrifuged for 10 minutes at 200*g*. The pellet was resuspended in RPMI medium and centrifuged again. Thereafter, the resuspended cells were cultured for 24 hours in RPMI 1640 medium supplemented with 10% heat-inactivated FBS and 2.4 μg/mL phytohemagglutinin (PHA-L) in 250-mL cell culture flasks (Greiner, Solingen, Germany). Thereafter, cells were maintained under various test conditions for 24 hours. The viability of the cells was more than 98% as assessed by trypan blue exclusion at the end of cell isolation.

Quantitative Titration of Complementary DNA by Amplification Reactions Run to Saturation. The messenger RNA (mRNA) expression of membrane-bound CD95 receptor (Fas/APO-1), soluble CD95 receptor (sCD95), CD95L, and rHPRT in parenchymal and nonparenchymal rat liver cells was detected using quantitative titration of resulting cDNA by amplification reactions run to saturation.[29,30] The complementary DNA (cDNA) to be assayed was co-amplified with known amounts of an internal standard DNA, which was apart from a deletion of four bases identical to the assayed DNA. The read-out of the amplification involved one additional oligonucleotide, which allows to discriminate between these two DNA species. The level of gene expression was measured using a respective fluorescent oligonucleotide as primer in run-off reactions together with an automated sequencer. In detail, the quantitative titration method was carried out as follows:

Quantitative Reverse-Transcription Polymerase Chain Reaction. Total RNA from near-confluent plates of cells was isolated by using a total RNA extraction kit (Qiagen, Hilden, Germany). One microgram of RNA was subjected to reverse-transcription (RT) using a first-strand cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany), and the resulting cDNA was subjected to polymerase chain reaction (PCR) amplification (40 cycles of 94°C, 20 seconds; 60°C, 45 seconds; 72°C, 45 seconds, with 5 minutes extension time at 72°C on cycle 40). Table 1 shows the oligonucleotide primers used for amplification. Amplification reactions were performed in a 20-μL mixture containing 20 units/mL of *Taq* polymerase (Eurogentec, Seraing, Belgium), 200 μmol/L of dNTP, 0.5 μmol/L of each of the two primers, and 1.5 μmol/L Mg^{2+} in Eurogentec reaction buffer and were carried out in a Perkin Elmer 9600 Thermocycler.

Construction of Standard and Quantification of Transcripts. DNA standards were constructed essentially as described in Pannetier[29] and Delassus.[30] Briefly, for DNA standard for CD95 isoforms, CD95L and rHPRT, respective oligonucleotides (3′ and 5′ primers; see Table 1) were used to amplify a specific 201–, 320–, or 425–base pair fragment in a rat spleen derived total cDNA (Table 1). A 1,000-fold dilution of this product was reamplified using the respective 3′ primer and an additional construct primer which was made of 44 nucleotides and contained a four-base deletion compared with the wild-type sequence. Each of the deleted specific wildtype products

TABLE 1. Sequence of the Oligonucleotides Used for Quantitative RT-PCR

Gene	Sequence	Length (Nucleotides) of Longer Wild-Type PCR Products	Length (Nucleotides) of Run-off Products: Wild-Type Standard
CD95 ligand mRNA expression			
CD95L-5′	ATGGAACTGCTTTGATCTCTGG		
CD95L-3′	AGATTCCTCAAAATTGATCAGAG	320	
CD95L-RO*	TGGCTTAGGGGCTGGCTGTT		130/126
CD95-isoform mRNA expression			
CD95/sCD95-5′	GATATGCTGTGGATCATGGC		
CD95-3′	AACTTTTCGTTCACCAG	201	
sCD95-3′	TTCATGTGAACCTACCAGGC	201	
CD95/sCD95-RO*	GGACTGATAGCATCTTTGAGG		131/126
Hypoxanthine–guanine phosphoribosyltransferase mRNA expression			
rHPRT-5′	TGCTGGATTACATTAAAGCGC		
rHPRT-3′	CTTGGCTTTTCCACTTTCGC	425	
rHPRT-RO*	GTTGACTGGTCATTACAGTAGC		100/96

*Fluorescent dye–labeled (FAM).

was electrophoresed, electroeluted, and precipitated. It was dissolved in water and its concentration was derived from its absorbance value at 260 nm. The number of copies per milliliter was then calculated. Subsequent dilutions of this standard DNA were carried out in a solution of 10 μg/mL herring sperm DNA.

For quantification of each transcript, a constant amount of cDNA corresponding to the reverse transcription of 50 ng of total RNA was mixed with 10^1, 10^2,. . ., 10^9, or 0 copies of the standard and then amplified to saturation using the respective 3′ and 5′ primer. It should be noted that the respective 20 bases long 5′ primer was a part of the standard construct primer and that the amplified fragments differed in the sequence only by a four-base deletion close to its end.

Run-off Reaction and Analysis. The amplified solution (2 μL) was mixed in final reaction volume of 10 μL containing 0.1 μmol/L fluorescent dye-labeled (FAM) oligonucleotide (CD95L-RO, CD95-RO, or rHPRT-RO; see Table 1), 20 U/mL *Taq* polymerase, 200 μmol/L dNTP, and 3 μmol/L Mg^{2+} in Eurogentec reaction buffer. Run-off reactions were carried out in a Perkin Elmer 9600 Thermocycler (5 cycles of 94°C, 20 seconds; 60°C, 45 seconds; 72°C, 45 seconds, with 3 minutes extension time at 72°C on cycle 5). The run-off reaction products were mixed with an equal volume of 20 mmol/L ethylenediaminnetetraacetate formamide solution, heat-denatured at 80°C for 10 minutes, and 4 μL of the resulting mixture was loaded on a 6% acrylamide, 8 mol/L urea gel and electrophoresed for 6 hours using an Applied Biosystems 373A DNA sequencer. The fluorescent profiles of the run were recorded, and the profile areas were analyzed using Immunoscope software, kindly provided by Dr. C. Pannetier (Unité de Biologie Moléculaire du Gène, INSERM Unité 277, Institut Pasteur, Paris, France).[29,30]

Immunocytochemical Procedures. The cells grown on a Lab-Tek Tissue Culture Chamber Slide (Nunc, Naperville, OH) were washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde for 30 minutes at room temperature, and washed in PBS for 5 minutes. The sections were then treated with 0.3% H_2O_2 in methanol for 30 minutes. Thereafter, the cells were washed with PBS, incubated for 30 minutes in 1% bovine serum albumin in PBS and then exposed to 2 μg/mL rabbit polyclonal antibody against CD95 (Fas/APO-1) or CD95L for 30 minutes. After two rinsings in PBS, the slides were incubated 1 hour with 1:200 diluted anti-rabbit peroxidase (POD) immunoglobulin G. The cells were then washed in PBS, and the immunocomplexed POD was visualized by incubation for 15 minutes in substrate solution ABTS (1 mg/mL; Boehringer Mannheim, Mannheim, Germany). The number of cells staining positive for CD95 and CD95L was determined by light microscopy. The cell cultures were graded as follows: 0, absence of CD95 (CD95L) expression; 1, less than one third of the cells stained positive for CD95 (CD95L); 2, between one and two thirds of the cells stained positive for CD95 (CD95L); 3, more than two thirds of the cells stained positive for CD95 (CD95L).

Detection of Apoptosis in Parenchymal Liver Cells and Thymic Lymphocytes. The functional relevance of the observed sCD95/CD95 expression in rat liver parenchymal cells (PCs) was tested by using an agonistic antibody rhsAPO-1/Fas ligand (Alexis, San Diego, CA). Apoptosis of PCs was measured after incubation with 0, 10, 25, 50, or 100 ng/mL antibody for 24 hours under the various test conditions.

In another set of experiments it was examined whether supernatants derived from LPS-treated rat KCs or SECs can stimulate apoptosis in lymphocytes or PCs. Cultured KCs or SECs were incubated without LPS (control), with 1 μg/mL LPS, or with LPS plus dexamethasone for either 24 hours or 48 hours. For this last condition, 1 μmol/L dexamethasone was present in the 24- or 48-hour LPS-treated KCs or SECs only during the first 6 or 24 hours, respectively. The supernatants obtained from the 6- to 24-hour or 24- to 48-hour incubations, respectively, were then added to rat thymic lymphocytes or PCs for 24 hours. The supernatant obtained from 10^6 KCs or SECs was added to 10^6 PCs or lymphocytes resulting in an effector/target cell ratio of approximately 1. In another set of experiments, thymic lymphocytes cocultured with KCs or SECs were incubated for 24 hours under the conditions described above and apoptosis was measured during the following 24 hours. In order to test whether apoptosis in PCs is mediated by CD95, PCs were preincubated for 6 hours with 500 μmol/L tetrapeptide Ac-YVAD-cmk (Bachem, Torrance, CA), which as a noncleavable substrate inhibits interleukin 1β-converting enzyme (ICE)-related protease and accordingly CD95-induced apoptosis.[31]

Apoptosis was detected by the TdT-mediated fluorescein deoxyuridine triphosphate nick end labeling (TUNEL) method[32] using an *in situ* cell-death detection kit (Boehringer Mannheim). The incorporated fluorescein was detected by measuring the cell-associated fluorescence. The excitation wavelength was 490 nm. The cell cultures were graded as follows: 0, no apoptotic cells were detected; 1, less than one third of the parenchymal cells or lymphocytes stained positive for apoptosis; 2, between one and two thirds of the cells stained positive for apoptosis; 3, more than two thirds of the cells stained positive for apoptosis.

Statistics. Data are expressed as means ± SEM (n = number of cell preparations from different animals). Statistical analysis was performed using Student's *t* test. $P < .05$ was considered to be statistically significant.

RESULTS

Effect of LPS and Dexamethasone on the Expression of CD95 (APO-1/Fas) Receptor and CD95L in Rat Liver Macrophages (Kupffer Cells) and Sinusoidal Endothelial Cells. The mRNA levels for CD95 receptors (CD95, sCD95) and CD95L were measured using the quantitative RT-PCR technique as described in Materials and Methods. The hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene was used for standardization, and its expression was similarly measured. Northern blot analysis revealed that HPRT mRNA levels were comparable between the three liver cell types and were not significantly affected by addition of 1 μg/mL LPS in KCs, SECs and PCs, respectively (data not shown). This indicates that rough comparison can be made between the different cell types with regard to the amount of CD95 receptor and CD95L mRNA levels.

As shown in Fig 1A, addition of LPS (1 μg/mL) to KC cultures led within 6 hours to a four- to five-fold increase of the CD95L mRNA levels, when compared to untreated controls. CD95L mRNA abundances decreased rapidly within the next 6 hours and returned to control values after 48 hours of LPS treatment. Whereas under control conditions only a few of the KCs stained positive for CD95L, most KCs stained positive for CD95L after 24 hours of LPS treatment (Fig. 2A, Table 2). The positive staining for CD95L in KCs after LPS treatment was more intensive in presence of the matrix metalloproteinase inhibitor BB-3103, indicating that CD95L accumulated in these cells (Fig. 2A). The membrane-bound CD95L is known to be converted to soluble form by the action of a matrix metalloproteinase-like enzyme.[33]

The mRNA levels for the membrane-bound (CD95) and soluble (sCD95) isoforms of the CD95 receptor were very low in unstimulated KCs (Fig. 1B). Stimulation of KCs by LPS had no effect on the membrane-bound CD95, but increased the mRNA levels for sCD95 about five-fold after a lag phase of 12 hours (Fig. 1B). Thus, the induction of CD95L mRNA precedes the induction of sCD95 mRNA by LPS. On the other hand, LPS treatment had no effect on the number of KCs staining positive for CD95 (Table 2).

Similar findings were obtained in cultured SECs. Here, LPS treatment also resulted in a rapid two- to three-fold increase in CD95L mRNA levels, which slowly declined thereafter

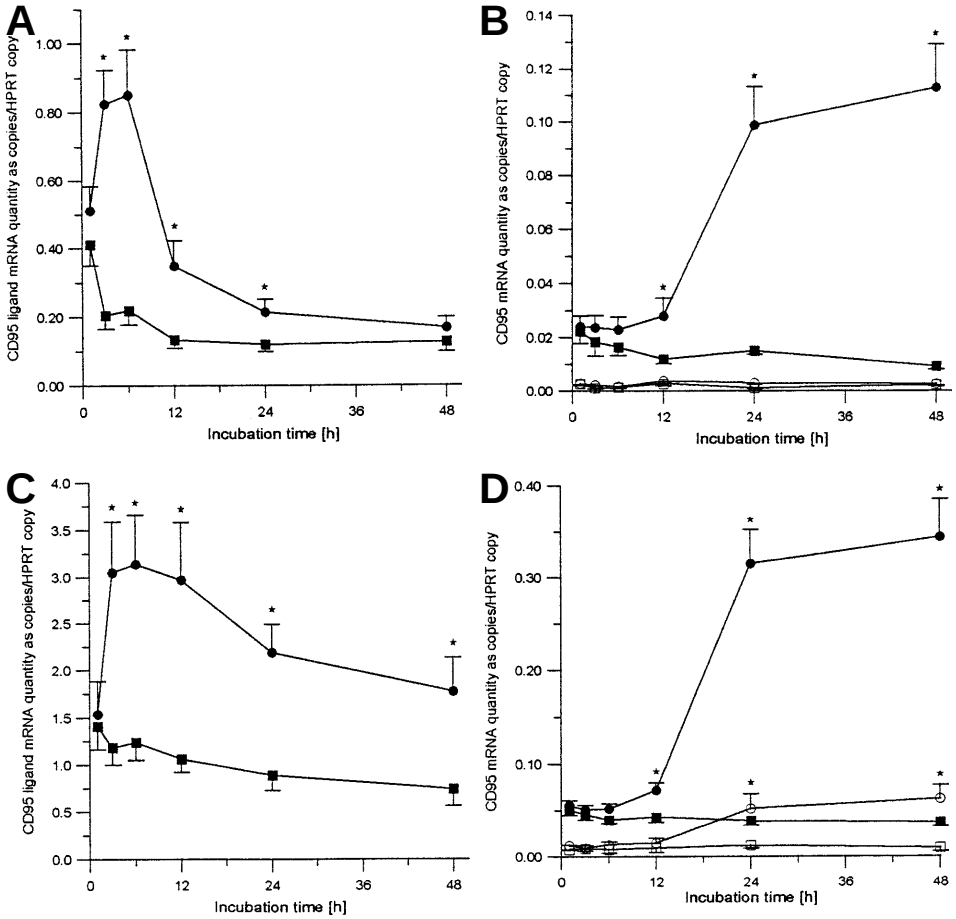

FIG. 1. Modulation of CD95L, transmembrane and soluble CD95-isoform mRNA expression in response to LPS in (A and B) Kupffer cells and (C and D) sinusoidal endothelial cells. Total RNA was extracted from LPS-treated (1 μg/mL) and untreated (control) rat KCs and SECs at the time points indicated, reverse-transcribed, and quantified by PCR as described in Materials and Methods. A and C: CD95L mRNA expression in (1 μg/mL; ●) LPS-treated and (control; ■) untreated (A) rat KCs and (C) SECs. B and D: mRNA expression of CD95 isoforms in (1 μg/mL; ● and ○) LPS-treated and (control; ■ and □) untreated (B) rat KCs and (D) SECs. Open symbols refer to the transmembrane isoform of CD95; closed symbols refer to soluble CD95. Results are expressed as the ratio of number of CD95L or CD95 isoform transcripts obtained with the indicated primers to the number of HPRT transcripts. Data are given as means ± SEM and are from three separate experiments for each condition. *Significantly different from control ($P < .05$).

(Fig. 1C), and the number of SECs staining positive for CD95L increased strongly within 24 hours of the LPS challenge (Fig. 2B, Table 2). However, the number of mRNA copies for CD95L was approximately fourfold higher in relation to HPRT mRNA in SECs compared with KCs (compare Fig. 1A). LPS treatment of SECs had little effect on the CD95 mRNA isoforms during the first 12 hours; however, thereafter a significant increase in mRNA levels for CD95 and sCD95 occurred (Fig. 1D). As in KCs, the amount of cells staining positive for CD95 was not significantly affected by LPS (Table 2).

Dexamethasone Modulated LPS (1 μg/mL)-Induced Stimulation of CD95L Expression. When 1 μmol/L dexamethasone was added 30 minutes before 6-hour LPS incubation, dexamethasone largely inhibited the LPS (1 μg/mL)-induced increase in CD95L mRNA in KCs and SECs, i.e. from 0.94 ± 0.12 to 0.32 ± 0.05 and 3.14 ± 0.52 to 1.19 ± 0.14 copies/HPRT mRNA copies (n = 3), respectively. The mRNA levels under control conditions were 0.18 ± 0.02 for KCs and 1.24 ± 0.19 copies/HPRT mRNA copies for SECs (n = 3) (Fig. 1A and C). Dexamethasone also diminished the LPS-induced increase in the number of cells staining positive for CD95L (Table 2). However, when KCs were stimulated with higher doses of LPS (10 μg/mL), addition of dexamethasone did no longer diminish LPS-induced increase in CD95L mRNA (data not shown).

Effect of LPS and Dexamethasone on the Expression of CD95 (APO-1/Fas) Receptor and CD95L in Rat Liver PCs. As shown by immunocytochemistry, approximately one third of cultured hepatocytes expressed CD95 (Fas/APO-1), but only about 1% of the cells expressed the CD95L (Fig. 3A and Table 2). On stimulation with LPS, the expression of the CD95L weakly increased (Table 2); however, most hepatocytes stained positive for CD95 (Fig. 3B and Table 2). Likewise, the mRNA levels for the CD95 isoforms were up-regulated by LPS (Table 3). The CD95 receptor was functionally active, as shown in experiments using the agonistic antibody rhsAPO-1/Fas ligand. When PCs were incubated with antibody concentrations of 10 and 25 ng/mL, the number of apoptotic cells markedly increased in presence of LPS compared with control conditions without LPS (Fig. 4).

The mRNA levels for CD95L were very low under control conditions, but slightly increased in response to LPS within 6 hours (Table 3). A marked increase in CD95L mRNA levels was observed in response to dexamethasone in the presence and absence of LPS (Table 3), and dexamethasone markedly increased the number of hepatocytes staining positive for CD95L (Table 2). Although the possibility must be considered that the LPS-induced increase in CD95L mRNA is due to contaminating nonparenchymal cells, the dexamethasone-induced increase in CD95L mRNA strongly suggests CD95L expression in parenchymal cells, because dexamethasone did inhibit the LPS-induced increase in CD95L mRNA in KCs and SECs.

Induction of Apoptosis in Parenchymal Liver Cells and Thymic Lymphocytes by Supernatants Derived From Nonparenchymal Cell Cultures. To test whether LPS-stimulated nonparenchymal rat liver cells induce apoptosis in rat liver PCs and lymphocytes, cultured KCs were incubated with 1 μg/mL LPS for 24 or 48 hours. The supernatants from these cells were then given to cultured rat PCs or thymic lymphocytes for 24 hours. As

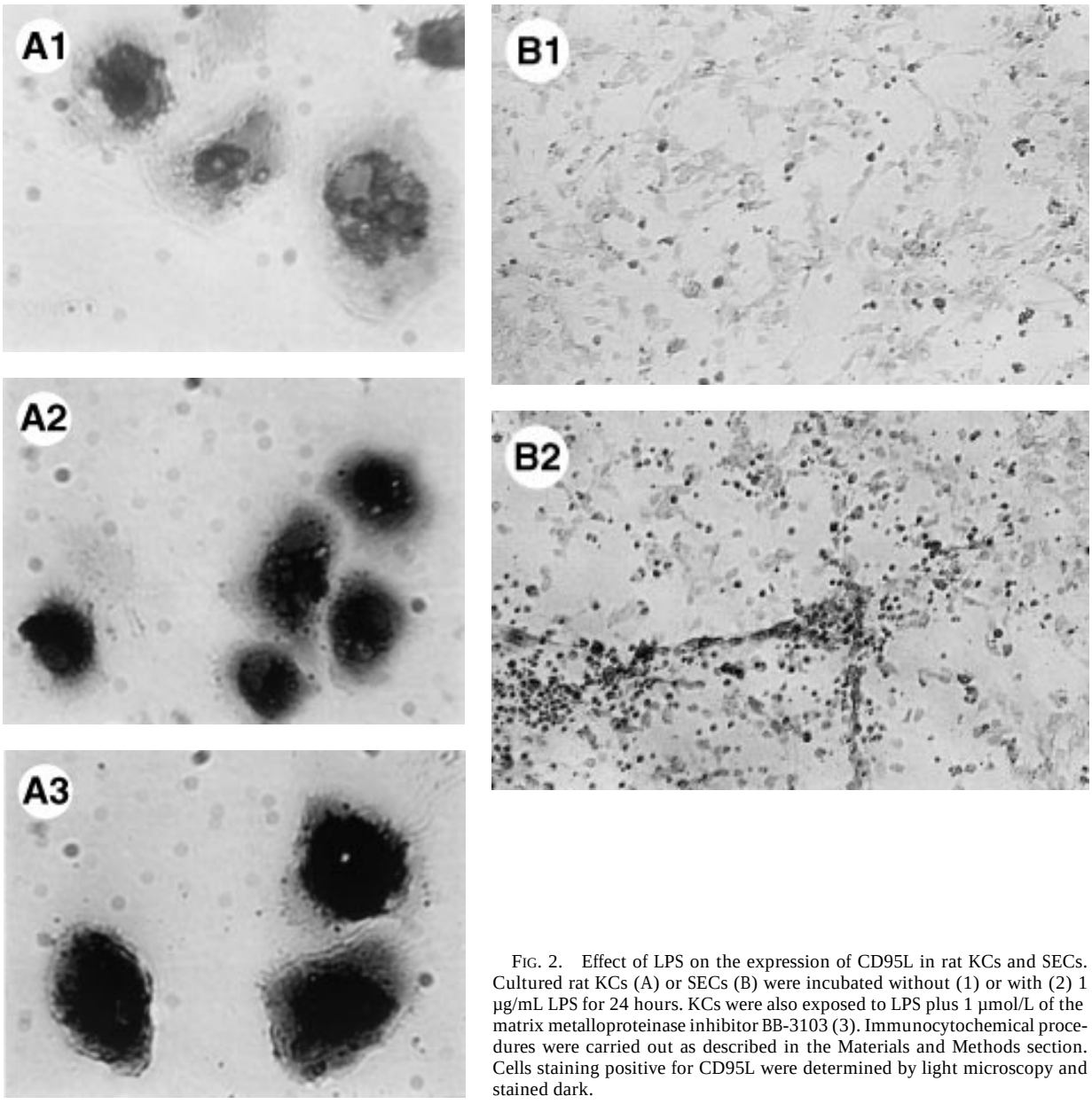

FIG. 2. Effect of LPS on the expression of CD95L in rat KCs and SECs. Cultured rat KCs (A) or SECs (B) were incubated without (1) or with (2) 1 μg/mL LPS for 24 hours. KCs were also exposed to LPS plus 1 μmol/L of the matrix metalloproteinase inhibitor BB-3103 (3). Immunocytochemical procedures were carried out as described in the Materials and Methods section. Cells staining positive for CD95L were determined by light microscopy and stained dark.

shown in Tables 4 and 5, supernatants derived from KCs were able to stimulate apoptosis in PCs and thymic lymphocytes. When these cells were cultured with supernatants derived from untreated KCs, only a few apoptotic cells were detected, whereas many cells underwent apoptosis after treatment with supernatants derived from 24 hours LPS-stimulated KCs (Tables 4 and 5). The induction of apoptosis in PCs was suppressed when these cells were preincubated for 6 hours with 500 μmol/L Ac-YVAD-cmk, an inhibitor of the ICE-related protease,[31] suggesting that LPS-dependent stimulation of apoptosis in PCs is mediated by CD95 (Table 4). In line with this, few apoptotic cells were also detected when PCs were exposed to supernatants derived from 24-hour LPS-stimulated KCs that were simultaneously treated with 1 μmol/L BB-3103 (Table 4), which inhibits the matrix metalloproteinase being responsible for CD95L shedding. On the other hand, supernatants derived from KCs or SECs, which were maintained under various test conditions for 48 hours, i.e., a period allowing for the formation of soluble CD95 (Fig. 1), induced apoptosis in only a few lymphocytes (Table 5).

Similar findings for lymphocyte apoptosis were also obtained with supernatants derived from SECs (Table 5). Apoptosis in thymic lymphocytes was also induced when the cells were cocultured with KCs or SECs and simultaneously stimulated with LPS for 24 hours (Table 5).

Furthermore, a marked decrease of the apoptotic response was observed in lymphocytes and PCs when supernatants from 24-hour LPS-treated KCs were used, which were simultaneously incubated with 1 μmol/L dexamethasone during the first 6 hours (Tables 4 and 5).

DISCUSSION

This study addressed the effect of LPS on the CD95 receptor and CD95L system in primary cultures of liver nonparenchymal (KCs and SECs) and PCs at the level of mRNA and by immunocytochemistry. As in studies with

TABLE 2. Immunocytochemical Evaluation of CD95 (Fas/APO-1) and CD95 Ligand Expression in Parenchymal and Nonparenchymal Rat Liver Cells

Condition	CD95	CD95 Ligand
Kupffer cells		
Control	0.3	0.8
LPS (1 μg/mL)	0.3	2.8*
Dexamethasone (1 μmol/L)	0.3	0.8
LPS + dexamethasone	0.5	1.5*,†
Sinusoidal endothelial cells		
Control	0.3	1.0
LPS (1 μg/mL)	0.5	2.5*
Dexamethasone (1 μmol/L)	0.5	1.0
LPS + dexamethasone	0.5	1.3*,†
Parenchymal cells		
Control	1.3	0.3
LPS (1 μg/mL)	3.0*	0.5
Dexamethasone (1 μmol/L)	1.0	2.3*
LPS + dexamethasone	1.5†	2.8*,†

NOTE. Cells were incubated for 24 hours with the addition indicated. Dexamethasone was added 30 minutes before LPS incubation. Immunocytochemical procedures were carried out as described in Materials and Methods. The cells staining positive for CD95 and CD95 ligand were detected by light microscopy. Score for immunocytology: 0, absence of CD95 (CD95L) expression; 1, less than one third of cells stained positive for CD95 (CD95L); 2, between one third and two thirds of cells stained positive for CD95 (CD95L); 3, more than two thirds of cells stained positive for CD95 (CD95L). Data are from 4 separate experiments for each condition; SEM was always <0.3.

*Significantly different from control ($P < .05$).

†Statistically significant difference ($P < .05$) versus LPS condition without dexamethasone.

other cell types,[35] HPRT mRNA levels were used for standardization. As shown by Northern blot analysis, HPRT mRNA levels were not significantly affected by LPS addition and were similar in KCs, SECs, and PCs. Thus, within the individual cell type alterations of CD95 and CD95L mRNA levels relative to those of HPRT mRNA levels reflect alterations in the cellular CD95/CD95L mRNA levels.

The present findings suggest that LPS treatment of KCs and SECs leads during the first 12 hours to a significant increase in CD95L mRNA levels and a marked increase in the amount of cells that stain positive for CD95L. Whereas CD95L mRNA levels returned to the baseline level after 12 hours, the mRNA levels for the soluble isoform of the CD95 receptor (sCD95) increased only after a lag of 12 hours. This suggests a sequential induction of CD95L and sCD95 in KCs and SECs after stimulation with LPS. Apparently, sCD95 is released rapidly into the medium, because the number of cells staining for CD95 remained unaffected. The sCD95 molecule lacks the transmembrane domain that characterizes the membrane CD95 receptor[35] and may serve as a scavenger for CD95L. Many cell types, such as lymphocytes,[35] produce the sCD95 for protection against CD95L-induced apoptosis.

Hepatic cells are sensitive to anti-CD95 when nontransformed,[20] whereas transformed liver cells frequently are resistant.[37] In human hepatocellular carcinoma and hepatoma cell lines, the sensitivity to anti-CD95 is not related to sCD95 expression but to the membrane-bound CD95 expression.[36,37] However, stable transfectants of hepatocellular carcinoma cells overexpressing sCD95 are less sensitive to CD95L than control cells.[37] In the present study, expression of CD95, mainly of the soluble isoform, was stimulated by LPS in PCs already within 6 hours and persisted for 48 hours (Table 3). On the other hand, CD95L mRNA levels increased only slightly in the PC cultures, and the number of CD95L mRNA copies per copy of HPRT mRNA was only 5% and 1% of that found in KCs or SECs, respectively. These findings suggest a very low level of CD95L expression even in the presence of LPS, as also confirmed by immunocytochemistry (Table 3). It is well conceivable that the low CD95L expression in PCs is due to contamination of PC cultures by nonparenchymal cells. Indeed, if one takes into account a 1% contamination of PC cultures with nonparenchymal cells, the low level of CD95L expression in PC cultures can fully be attributed to this contamination. Thus, we conclude that CD95L expression in PCs is negligible in response to LPS.

The present observations suggest a complex and timely coordinated interplay between liver cell populations with respect to LPS-induced activation of the CD95 receptor/CD95L system. CD95-mediated apoptosis of PCs can be induced by the CD95L contained in cell culture supernatants from LPS-treated KCs. This is similar to the induction of apoptosis in Jurkat cells by supernatants from bleomycin-treated HepG2 hepatoma cells.[38]

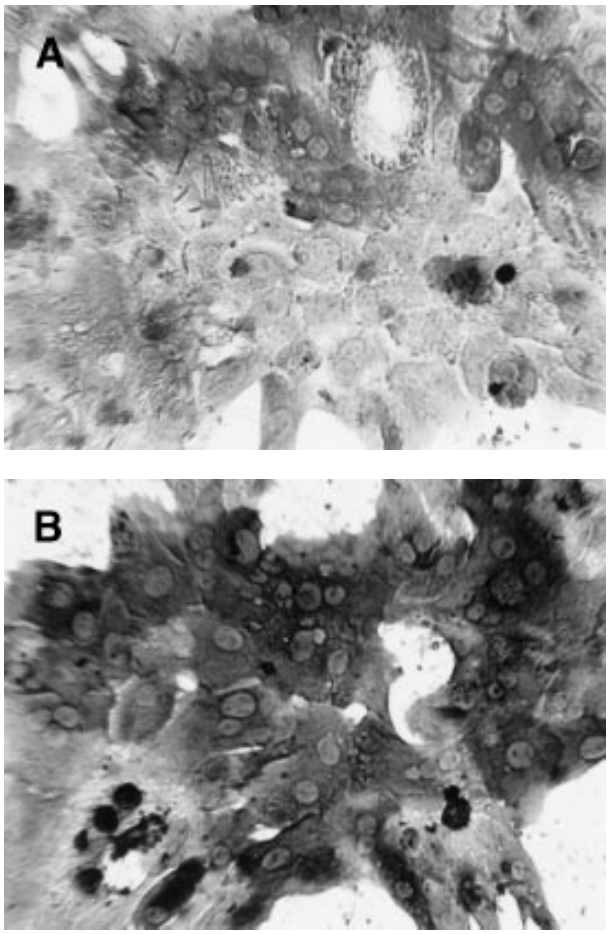

FIG. 3. Effect of lipopolysaccharide (LPS) on the expression of CD95 receptor in rat PCs. Cultured rat PCs were incubated (A) without or (B) with 1 μg/mL LPS for 24 hours. Immunocytochemical procedures were carried out as described in Materials and Methods. Cells staining positive for CD95 (Fas/APO-1) were determined by light microscopy and stained dark.

TABLE 3. Modulation of CD95 Ligand and Membrane-Bound and Soluble CD95 Isoform mRNA Expression in Response to LPS and Dexamethasone in Rat Liver Parenchymal Cells

mRNA Expression (Copies/HPRT mRNA copy)	6 h	48 h
CD95 ligand		
Control	0.01 ± 0.01	0.01 ± 0.01
Dexamethasone	0.13 ± 0.02*	0.17 ± 0.03*
LPS	0.04 ± 0.01*	0.01 ± 0.01
LPS + dexamethasone	0.12 ± 0.02*,†	0.18 ± 0.03*,†
Membrane-bound CD95 isoform		
Control	0.02 ± 0.01	0.02 ± 0.01
LPS	0.08 ± 0.01*	0.04 ± 0.01*
LPS + dexamethasone	0.02 ± 0.01†	0.02 ± 0.01†
Soblule CD95 isoform		
Control	0.03 ± 0.01	0.03 ± 0.01
LPS	0.14 ± 0.02*	0.33 ± 0.05*
LPS + dexamethasone	0.06 ± 0.01*,†	0.19 ± 0.04*,†

NOTE. Cultured liver parenchymal cells were incubated with or without 1 μg/mL LPS for 6 and 48 hours. In some experiments, 1 μmol/L dexamethasone was added 30 minutes before and throughout the 6- and 48-hour incubation periods. At the time indicated, total RNA was extracted, reverse-transcribed, and quantified by PCR as described in Materials and Methods. Results are expressed as the ratio of number of CD95 ligand or membrane-bound or soluble CD95 isoform transcripts to the number of rHPRT transcripts. Data given as means ± SEM from 3 separate experiments for each condition.

*Significantly different from the control ($P < .05$).

†Statistically significant different ($P < .05$) versus LPS condition without dexamethasone.

TABLE 4. Induction of Apoptosis in Rat Liver Parenchymal Cells by Supernatants Derived From Kupffer Cell Cultures

	Apoptosid of Liver Parenchymal Cells	
	−Ac-YVAD-cmk	+Ac-YVAD-cmk
Control	1.0	0.3
BB-3103	0.7	0.3*
LPS	2.0*	0.7
LPS + BB-3103	1.0	0.7
LPS + Dex	1.3*	0.7
LPS + BB-3103 + Dex	1.0	0.7

NOTE: Cultured rat KCs were incubated as indicated without supplement (control), with 1 μg/mL LPS, and/or with 1 μmol/L matrix metalloproteinase inhibitor BB-3103 for 24 hours, and the supernatants were then added to liver PCs for 24 hours. In some experiments, 1 μmol/L dexamethasone was added 30 minutes before LPS stimulation and during the first 6 hours (LPS + Dex). Furthermore, PCs were also preincubated for 6 hours with 500 μmol/L Ac-YVAD-cmk (+Ac-YVAD-cmk). Apoptosis was measured as described in Materials and Methods. Cells that stained positive for apoptosis were detected by fluorescence microscopy. Score for immunocytology: 0, no apoptotic cells detected; 1, less than one third of cells stained positive for apoptosis; 2, between one third and two thirds of cells stained positive for apoptosis; 3, more than two thirds of cells stained positive for apoptosis. Data are from 3 separate experiments for each condition; SEM was always <0.3.

*Statistically significant difference ($P < .05$) from control without LPS and Ac-YVAD-cmk. The number of cells stainined positive was statistically significantly different ($P < .05$) under all conditions versus the LPS condition without Ac-YVAD-cmk.

The present findings may point to an important role of KCs and SECs not only in the regulation of apoptosis of liver parenchymal cells but also of apoptosis of circulating white blood cells: supernatants derived from LPS-treated KC and SEC cultures stimulated apoptosis in rat thymic lymphocytes, although it remains unknown whether this occurs also *in vivo*. The elimination of lymphocytes may be a novel important KC and SEC function. In line with this, phagocytosis of circulating apoptotic neutrophils and peripheral blood

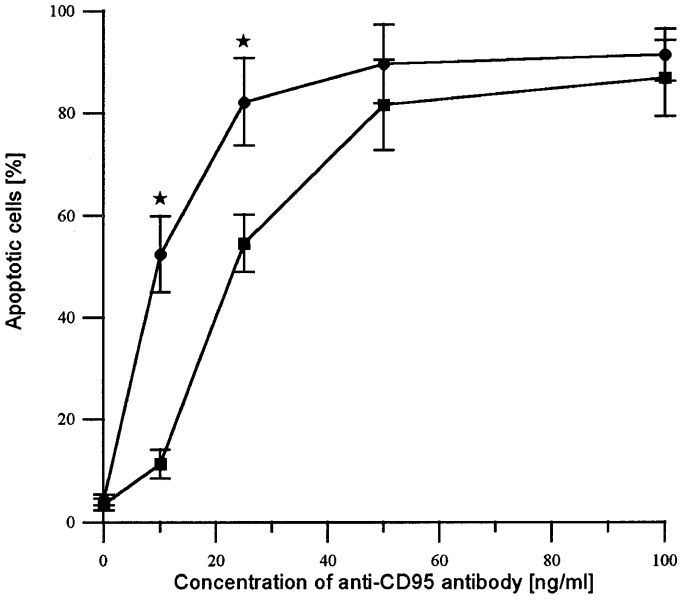

FIG. 4. Effect of lipopolysaccharide (LPS) on anti-CD95 antibody-induced apoptosis of rat PCs. Cultured rat PCs were incubated (■) without or (●) with 1 μg/mL LPS and various concentrations of anti-CD95 antibody (rhsAPO-1/Fas ligand, Alexis, San Diego, CA) for 24 hours. Apoptosis was detected by the TUNEL method as described in Materials and Methods. Data are given as means ± SEM and are from three separate experiments for each condition. *Significantly different from control ($P < .05$).

TABLE 5. Induction of Apoptosis in Thymic Lymphocytes by Superantants Derived From Nonparenchymal Cell Cultures

	Apoptosis of Lymphocytes		
	Control	LPS	LPS + Dex
1 without KC/SEC supernatant	0.3		
2 KC supernatant 6-24 h	0.5	2.8*	1.5*,†
3 KC supernatant 24-48 h	1.0	1.0	0.5
4 KC/T-cell coculture	1.0	2.5*	2.0*,†
5 SEC supernatant 6-24 h	0.8	2.8*	1.5*,†
6 SEC superantant 24-48 h	0.5	1.0	1.0
7 SEC/T-cell coculture	0.8	2.8*	2.0*,†

NOTE. Cultured rat KCs or SECs were incubated without (control), with 1 μg/mL LPS, or with LPS plus dexamethasone for either 24 or 48 hours. For the latter condition, 1 μmol/L dexamethasone was present to the 24- or 48-hour LPS-treated KCs or SECs only during the first 6 or 24 hours. The supernatants obtained from the 6- to 24- or 24- to 48-hour incubations, respectively, were then added to rat thymic lymphocytes for 24 hours. In another set of experiments, thymic lymphocytes cocultured with KCs (row 4) or SECs (row 7) were incubated for 24 hours under the conditions described above, and apoptosis was measured during the following 24 hours. Immunocytochemical procedures were carried out as described in Materials and Methods. Cells staining positive for apoptosis were detected by fluorescence microscopy. Score for immunocytology was identical to that in Table 4. Data are from 4 separate experiments for each condition; SEM was always <0.3.

*Significantly different from the control ($P < .05$).

†Statistically significant difference ($P < .05$) versus LPS condition without dexamethasone.

lymphocytes by KCs was shown.[23,39] Many lymphocytes became apoptotic after treatment with supernatants derived from 24-hour LPS-stimulated KCs or SECs. However, supernatants derived from 48-hour stimulated cells, i.e., for a period that allowed the release not only of CD95L but also of soluble CD95 receptor (Fig. 1), induced apoptosis in only a few lymphocytes, indicating that the subsequently formed soluble CD95 receptor may have inactivated the initially formed CD95L.

In T-lymphocytes, dexamethasone induced apoptosis but paradoxically inhibited activation-induced apoptosis by preventing up-regulation of CD95L.[21,22] The effects of dexamethasone are different on CD95L expression in the three liver cell types. Whereas dexamethasone diminished the LPS-induced stimulation of CD95L expression in nonparenchymal cells, it markedly stimulated CD95L expression in PCs. In line with reduced CD95L mRNA levels, lymphocyte and PC apoptosis was diminished when supernatants from KCs or SECs were used, which were incubated for 24 hours with LPS plus 1 μmol/L dexamethasone. Further studies are required to clarify the question of whether these different dexamethasone-dependent CD95L expressions in liver cells also occur *in vivo*.

The physiological significance of the timely coordinated induction of CD95 receptor and CD95L in the different liver cell populations after LPS challenge also remains to be established. However, one might speculate that it could function to terminate a cellular immune response after an LPS challenge. The early induction of CD95L in KCs and SECs may augment apoptotic lymphocyte removal and simultaneous protection of PCs may be achieved by an early induction of soluble CD95 receptor. Down-regulation of CD95L synthesis and induction of soluble CD95 receptor in KCs and SECs starting 12 hours after the LPS challenge may then terminate the apoptotic signal. Thus, given that LPS induces lymphocyte proliferation, it also appears that an early mechanism for lymphocyte elimination becomes activated in liver. Detailed analysis of the mechanisms of the regulation of CD95 receptor/CD95L–mediated biological responses in the liver will help to explain the pathogenesis of several diseases, including autoimmune diseases or viral infections.

Acknowledgment: The authors thank Dr. Christophe Pannetier for provision of the Immunoscope software and Drs. Ralf Kubitz, Torsten Peters-Regehr, and Johannes Bode for kindly helping in preparation of parenchymal and nonparenchymal rat liver cells.

REFERENCES

1. Payne CM, Bernstein C, Bernstein H. Apoptosis overview emphasizing the role of oxisative stress, DNA damage and signal transduction pathways. Leuk Lymphoma 1995;19:43-93.
2. Crispe IN. Fatal interactions: Fas-induced apoptosis of mature T cells. Immunity 1994;1:347-349.
3. Lynch DH, Ramsdell F, Alderson MR. Fas and FasL in the homeostatic regulation of immune responses. Immunol Today 1995;16:569-574.
4. Cory S. Fascinating death factor. Nature 1994;367:317-318.
5. Steller H. Mechanisms and genes of cellular suicide. Science 1995;267: 1445-1448.
6. Evan GI, Brown L, Whyte M, Harrongton E. Apoptosis and the cell cycle. Curr Opin Cell Biol 1995;7:825-834.
7. Que FG, Gores GJ. Cell death by apoptosis: basic concepts and disease relevance for the gastroenterologist. Gastroenterology 1996;110:1238-1243.
8. Bursch W, Oberhammer F, Schulte-Hermann R. Cell death by apoptosis and its protective role against disease. Trends Pharmacol Sci 1992;13:245-251.
9. Trauth BC, Klas C, Peters AMJ, Matzku S, Möller P, Falk W, Debatin KM, Krammer PH. Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 1989;245:301-305.
10. Yonehar AS, IshiI A, Yonehar AM. A cell-killing monoclonal antibody (anti-Fas) to cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp Med 1989;169:1747-1756.
11. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizuchima SI, Sameshima M, Hasa A, Seto Y, Nagata S. The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 1991;66:2333-2343.
12. Gruss HJ. Molecular, structural, and biological characteristics of the tumor necrosis factor ligand superfamily. Int J Clin Lab Res 1996;26:143-159.
13. Suda T, Nagata S. Purification and characterization of the Fas-ligand that induces apoptosis. J Exp Med 1994;179:873-879.
14. Krammer PH, Behrmann I, Daniel P, Dhein J, Debatin KM. Regulation of apoptosis in the immune system. Curr Opin Immunol 1994;6:279-289.
15. Benedetti AA, Jezaquel M, Orlandi F. Preferential distribution of apoptotic bodies in acinar zone 3 of normal human and rat liver. J Hepatol 1988;7:319-324.
16. Adachi M, Suematsu S, Kondo T, Ogasawara J, Tanaka T, Yoshida N, Nagata S. Targeted mutation in the Fas gene causes hyperplasia in peripheral lymphoid organs and liver. Nat Genet 1995;11:294-299.
17. Galle PR, Hofmann WJ, Walczak H, Schaller H, Otto G, Stremmel W, Krammer PH, Runkel L. Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. J Exp Med 1995;182:1223-1230.
18. Mochizuki K, Hayashi N, Hiramatsu N, Katayama K, Kawanishi Y, Kasahara A, Fusamoto H, Kamada T. Fas antigen expression in liver tissues of patients with chronic hepatitis B. J Hepatol 1996;24:1-7.
19. Ohishi M, Sakisaka S, Koji T, Nakana PK, Tanikawa K. The localization of HCV and the expression of Fas antigen in the liver of HCV-related chronic liver disease. Acta Histochem Cytochem 1995;28:341-348.
20. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, et al. Lethal effects of the anti-Fas antibody in mice. Nature 1993;364:806-809.
21. Yang Y, Mercep M, Ware CF, Ashwell JD. Fas and activation-induced Fas ligand mediate apoptosis of T cell hybridomas: inhibition of Fas ligand expression by retinoic acid and glucocorticoids. J Exp Med 1995;181: 1673-1682.
22. Geley S, Hartmann BL, Kapelari K, Egle A, Villunger A, Heidacher D, Greil R, et al. The interleukin 1β-converting enzyme inhibitor CrmA prevents Apo-1/Fas- but not glucocorticoid-induced poly (ADP-ribose) polymerase cleavage and apoptosis in lymphoblastic leukemia cells. FEBS Lett 1997;402:36-40.
23. Shi J, Fujieda H, Kokubo Y, Wake K. Apoptosis of neutrophils and their elimination by Kupffer cells in rat liver. HEPATOLOGY 1996;24:1256-1263.
24. Takei Y, Kawano S, Nishimura Y, Goto M, Nagai H, Chen SS, Omae A, et al. Apoptosis: a new mechanism of endothelial and Kupffer cell killing. J Gastroenterol Hepatol 1995;10:S65-S67.
25. Meijer AJ, Gimpel JA, Deleeuw GA, Tager JM, Williamson JR. Role of anion translocation across the mitochondrial membrane in the regulation of urea synthesis from ammonia by isolated rat hepatocytes. J Biol Chem 1975;250:7728-7738.
26. Zhang F, Warskulat U, Wettstein M, Schreiber R, Henninger HP, Decker K, Häussinger D. Hyperosmolarity stimulates prostaglandin synthesis and cyclooxygenase-2 expression in activated rat liver macrophages. Biochem J 1995;312:135-143.
27. Eyhorn S, Schlayer HJ, Henninger HP, Dieter P, Hermann R, Woort-Menker M, Becker H, et al. Rat hepatic sinusoidal endothelial cells in monolayer culture. J Hepatol 1988;6:23-35.
28. Dieter P, Schulze-Specking A, Decker K. Differential inhibition of prostaglandin and superoxide production by dexamethasone in primary cultures of rat Kupffer cells. Eur J Biochem 1986;159:451-457.
29. Pannetier C, Delassus S, Darche S, Saucier C, Kourilsky P. Quantitative titration of nucleic acids by enzymatic amplification reactions run to saturation. Nucleic Acids Res 1993;21:577-583.
30. Delassus S, Couthino G, Saucier C, Darche S, Kourilsky P. Differential cytokine expression in maternal blood and placenta during murine gestation. J Immunol 1994;152:2411-2420.
31. Enari M, Hug H, Nagata S. Involvement of an ICE-like protease in Fas-mediated apoptosis. Nature 1996;375:78-81.
32. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493-501.
33. Tanaka M, Suda T, Haze K, Nakamura N, Sato K, Kimura F, Motoyoshi K, et al. Fas ligand in human serum. Nat Med 1996;3:317-322.

34. Carrera L, Gazzinelli RT, Badolato R, Hieny S, Müller W, Kühn R, Sacks D. Leishmania promastigotes selectively inhibit interleukin 12 induction in bone marrow-derived macrophages from susceptible and resistant mice. J Exp Med 1996;183:515-526.
35. Cheng J, Zhou T, Liu C, Shapiro JP, Brauer MJ, Kiefer MC, Barr PJ, Mountz JD. Protection from Fas-mediated apoptosis by a soluble form of the Fas molecule. Science 1994;263:1759-1762.
36. Yano H, Fukuda K, Haramaki M, Momosaki S, Ogaswara S, Higaki K, Kojiro M. Expression of Fas and anti-Fas-mediated apoptosis in human hepatocellular carcinoma cell lines. J Hepatol 1996;25:454-464.
37. Natoli G, Ianni A, Costanzo A, De Petrillo G, Ilari I, Chirillo P, Balsano C, Levrero. Resistance to Fas-mediated apoptosis in human hepatoma cells. Oncogene 1995;11:1157-1164.
38. Strand S, Hofmann WJ, Hug H, Müller M, Otto G, Strand D, Mariani SM, et al. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells—a mechanism of immune evasion? Nat Med 1996;12:1361-1366.
39. Falasca L, Bergamini A, Serafino A, Balabaud C, Dini L. Human Kupffer cell recognition and phagocytosis of apoptotic peripheral blood lymphocytes. Exp Cell Res 1996;224:152-162.