Cardiotrophin-1 Promotes a High Survival Rate in Rabbits with Lethal Fulminant Hepatitis of Viral Origin[▽][†]

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Rabbit hemorrhagic disease virus (RHDV) causes lethal fulminant hepatitis closely resembling acute liver failure (ALF) in humans. In this study, we investigated whether cardiotrophin-1 (CT-1), a cytokine with hepatoprotective properties, could attenuate liver damage and prolong survival in virus-induced ALF. Twenty-four rabbits were infected with 2×10^4 hemagglutination units of RHDV. Twelve received five doses of CT-1 (100 μg/kg) starting at 12 h postinfection (hpi) (the first three doses every 6 h and then two additional doses at 48 and 72 hpi), while the rest received saline. The animals were analyzed for survival, serum biochemistry, and viral load. Another cohort ($n = 22$) was infected and treated similarly, but animals were sacrificed at 30 and 36 hpi to analyze liver histology, viral load, and the expression of factors implicated in liver damage and repair. All infected rabbits that received saline died by 60 hpi, while 67% of the CT-1-treated animals survived until the end of the study. Treated animals showed improved liver function and histology, while the viral loads were similar. In the livers of CT-1-treated rabbits we observed reduction of oxidative stress, diminished PARP1/2 and JNK activation, and decreased inflammatory reaction, as reflected by reduced expression of tumor necrosis factor alpha, interleukin-1β, Toll-like receptor 4, VCAM-1, and MMP-9. In addition, CT-1-treated rabbits exhibited marked upregulation of TIMP-1 and increased expression of cytoprotective and proregenerative growth factors, including platelet-derived growth factor B, epidermal growth factor, platelet-derived growth factor receptor β, and c-Met. In conclusion, in a lethal form of acute viral hepatitis, CT-1 increases animal survival by attenuating inflammation and activating cytoprotective mechanisms, thus representing a promising therapy for ALF of viral origin.

Acute liver failure (ALF) arises as a result of extensive hepatocellular damage that exceeds the liver's capacity to regenerate. Depending on the interval between the onset of jaundice and that of encephalopathy, ALF can be categorized into hyperacute (less than 1 week), acute (between 1 and 4 weeks) and subacute (more than 4 weeks). Etiologic factors include viral infections, drugs, biological toxins, metabolic disorders, and ischemia, but it is not unusual for this syndrome to arise without any known causative agent (22).

ALF is one of the most challenging human conditions requiring critical care. Therapy is merely supportive and oriented to the correction of complications. Survival is poor and liver transplantation is the only definitive treatment for patients with severe ALF. However, the indication for liver transplantation relies on prognostic assessment, and this lacks accuracy. In transplanted patients mortality is about 10%, but ca. 30% of patients with ALF die without having access to transplantation (24). Thus, novel medical treatments for this condition are

urgently needed. With the exception of *N*-acetylcysteine, which can prevent glutathione depletion in paracetamol overdose (11), pathogenic therapies able to attenuate liver cell necrosis and stimulate regeneration are lacking.

Viral infections due to hepatitis B virus, hepatitis A virus, and hepatitis E virus are common causes of ALF, mainly in particular geographical areas of the world (22). The mechanisms responsible for liver injury in acute severe viral hepatitis leading to ALF are complex and include the virus cytopathic effect, the damage induced by a vigorous inflammatory response, and the cytotoxicity of immune effectors. Most animal models of ALF, including the administration of hepatotoxins, the injection of substances that activate immune cells (e.g., concanavalin A [ConA]), and the infusion of molecules that directly promote apoptosis of hepatocytes (e.g., Fas ligand), do not reproduce the complexity of cell-damaging mechanisms activated in patients with severe acute viral hepatitis. An important difficulty for testing innovative therapeutic approaches for ALF of viral origin is the lack of appropriate animal models that develop massive hepatocellular necrosis as result of viral infection.

Recently, it has been shown that the rabbit hemorrhagic disease virus (RHDV), a member of the *Caliciviridae* family, causes a life-threatening form of viral hepatitis that recapitulates many of the features of human ALF, including a tendency to bleeding, encephalopathy, and intracranial hypertension (7, 31). More than 90% of infected adult rabbits die as result of

massive liver damage within 3 days. Since RHDV infection constitutes a highly reproducible model of virus-induced ALF, it offers a valuable scenario to test the therapeutic potential of hepatoprotective therapies in this condition.

Cardiotrophin-1 (CT-1) is a member of the interleukin-6 (IL-6) family of cytokines, which is endowed with potent cytoprotective properties. CT-1 activates different cell survival pathways, including STAT-3, AKT, and ERK1/2, and induces the expression of antiapoptotic factors (4, 15). It has been reported that CT-1 defends the liver against ConA challenge and ischemia/reperfusion damage (4, 15). However, it is not known whether this cytokine displays therapeutic effects in acute severe viral hepatitis. Therefore, the aim of the present study was to investigate whether CT-1 is able to attenuate liver damage and improve survival in a relevant model of virus-induced ALF.

MATERIALS AND METHODS

Animals. Nine-week-old New Zealand White rabbits were kept in the animal facility of the University of Leon with 12-h light cycle at 21 to 22°C and 50% relative humidity. They were given water and standard dry rabbit food *ad libitum*. Animals received care according to the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, 1985). The study protocols were reviewed and approved by the University of Leon Animal Care Committee.

Experimental Procedure. For survival studies, 24 rabbits (cohort 1) were injected intramuscularly with 2×10^4 hemagglutination units of a RHDV isolate (32). At 12, 18, 24, 48, and 72 h postinfection (hpi), 12 animals received an intravenous injection of rat CT-1 (100 μg/kg [body weight] dissolved into 3 ml of saline; Dro Biosystems, San Sebastian, Spain), and the other 12 animals received the same volume of vehicle (saline). Animals from both groups were left to die spontaneously, and all of the surviving rabbits from the CT-1-treated group were sacrificed at 7 days postinfection.

To investigate the molecular changes taking place in liver tissue in the infected untreated and treated groups, we used another cohort of animals (cohort 2, *n* = 22) which were infected and treated with saline or CT-1 as described above. Surviving animals from this cohort were sacrificed at 30 hpi (four from the saline group and six treated with CT-1) or 36 hpi (five from the saline group and six that received CT-1). A group of noninfected rabbits (*n* = 6) of the same age and gender were used as healthy controls and were sacrificed at the time of the study. In addition, a group of six noninfected normal rabbits received three injections of CT-1 (100 μg/kg [body weight]) every 6 h and were sacrificed 6 h after the last injection in order to investigate the effects of CT-1 in normal rabbit liver.

Cell culture. SIRC rabbit corneal cells (ATCC CL-60) were grown in Dulbecco minimum essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). SIRC cells were treated with 25 ng of rat CT-1/ml and were collected at different time points.

Biochemical and virological analysis. Blood samples from cohort 1 were collected from the marginal ear vein of surviving animals in heparin tubes at 12, 18, 24, 36, and 48 hpi, and at 7 days postinfection for determination of aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, and glucose. The RHDV viral load was measured in plasma and in liver extract by quantitative real-time PCR (for primers, see Table S1 in the supplemental material). The copy number was determined by extrapolation from the cycle threshold of each sample on a standard curve of known concentration. The standard was generated by insertion of the RHDV amplicon in a pCR2.1-TOPO vector (TOPO TA cloning kit; Invitrogen).

Histological analysis. Liver samples from surviving animals at 7 days postinfection (cohort 1), and animals sacrificed at 30 hpi (cohort 2) were stained with hematoxylin and eosin for histological examination. Immunohistochemical analysis of antigen Ki-67 (MM1 clone; Novocastra, Newcastle, United Kingdom) as a marker of cellular proliferation was carried out in liver specimens. Liver histology was evaluated by an experienced veterinary pathologist and semiquantitative assessment of different histological parameters was performed.

Western blot analysis. Western blot analyses in liver tissue homogenates were performed as described previously (7) using the following antibodies: anti-poly-(ADP-ribose)polymerase-1 (PARP1/2), anti-HGF, anti-PDGFRβ, and anti-Lamin-B (Santa Cruz Biotechnology, Santa Cruz, CA), horseradish peroxidase-conjugated antibody (Dako, Glostrup, Denmark), anti-β-actin (Sigma), and anti-JNK and anti-phospho-JNK$^{Thr183/Tyr185}$ (Cell Signaling, Danvers, MA). The density of the specific bands was quantified with an imaging densitometer (Scion Image, Frederick, MD).

RNA extraction and reverse transcription-PCR. Total RNA extraction from liver tissue and cDNA synthesis were performed as described previously (4). cDNA was amplified using TaqMan Universal PCR MasterMix (primers and probes in Table S2 in the supplemental material) or QuantiTect SYBR green PCR master mix (see primers in Table S1 in the supplemental material) (Applied Biosystems, Foster City, CA). Relative changes in gene expression levels were determined by using the $2^{\Delta\Delta CT}$ method as described previously (19). The results were normalized according to GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Oxidative stress parameters. Oxidized and reduced glutathione analysis was performed fluorimetrically by the method of Hissin and Hilf (14). Lipid peroxidation products were quantified in 250 mg of tissue extract by determining thiobarbituric acid reactive substances (TBARS) (34).

Statistical analysis. Results are expressed as mean values ± the standard errors of the mean. Statistical analyses were performed using nonparametric (Kruskal-Wallis and Mann-Whitney U) tests. All *P* values were two tailed and considered significant if <0.05. Kaplan-Meier plots and log-rank tests were used to analyze survival. Values were analyzed with the statistical package Statistica 7.0 (Statsoft, Inc., Tulsa, OK).

RESULTS

Treatment with CT-1 reduces liver damage and improves survival in RHDV-induced acute liver failure. Twelve hours after intramuscular administration of 2×10^4 hemagglutination units of RHDV, the infected rabbits started to show prostration, side recumbency, respiratory agitation, and tachycardia. In infected animals given saline, these symptoms were followed by neurological disturbances (convulsions, ataxia, and posterior paralysis), which rapidly progressed to coma and death. Eight rabbits from this group died before 48 hpi, and no animal survived at 60 hpi. In sharp contrast, 67% (*n* = 8) of animals that received CT-1 survived until they were sacrificed at the end of the study period (day 7 postinfection) (Fig. 1A).

Serum transaminases (which reflect the extent of hepatocellular death) experienced a striking elevation after 24 hpi, but the values were significantly less increased in RHDV-infected animals treated with CT-1 than in those receiving saline (Fig. 1B and C). Similarly, serum bilirubin levels (whose elevation results from the liver failure to transport bilirubin to bile) increased markedly at 36 and 48 hpi in the two groups, but the rise was significantly lower in CT-1-treated animals (Fig. 1D). In CT-1-treated animals that survived, the biochemical parameters approached normal values at day 7 postinfection (Fig. 1B to D).

In ALF, plasma hypoglycemia develops because of impaired gluconeogenesis and reduced insulin clearance (23, 28). In untreated infected rabbits, plasma glucose dropped profoundly as result of liver failure, while it was maintained at significantly higher values in animals which received CT-1 therapy (Fig. 1E).

Effect of CT-1 therapy on liver histopathology. At 30 hpi the livers of untreated RHDV-infected rabbits exhibited a severe acute inflammatory reaction with extensive areas of hepatocellular necrosis, intense hyperemia, edema and hemorrhage, and heterophil infiltration (rabbit heterophil leukocytes are equivalent to human neutrophils). Portal tracts showed moderate cell infiltration. Non-necrotic hepatocytes manifested cytoplasmic swelling and vacuolation occurring mainly in the centrolobular area. In infected rabbits given CT-1 therapy, the livers showed lobular inflammation with heterophil infiltration, but necrotic areas were markedly reduced compared to those
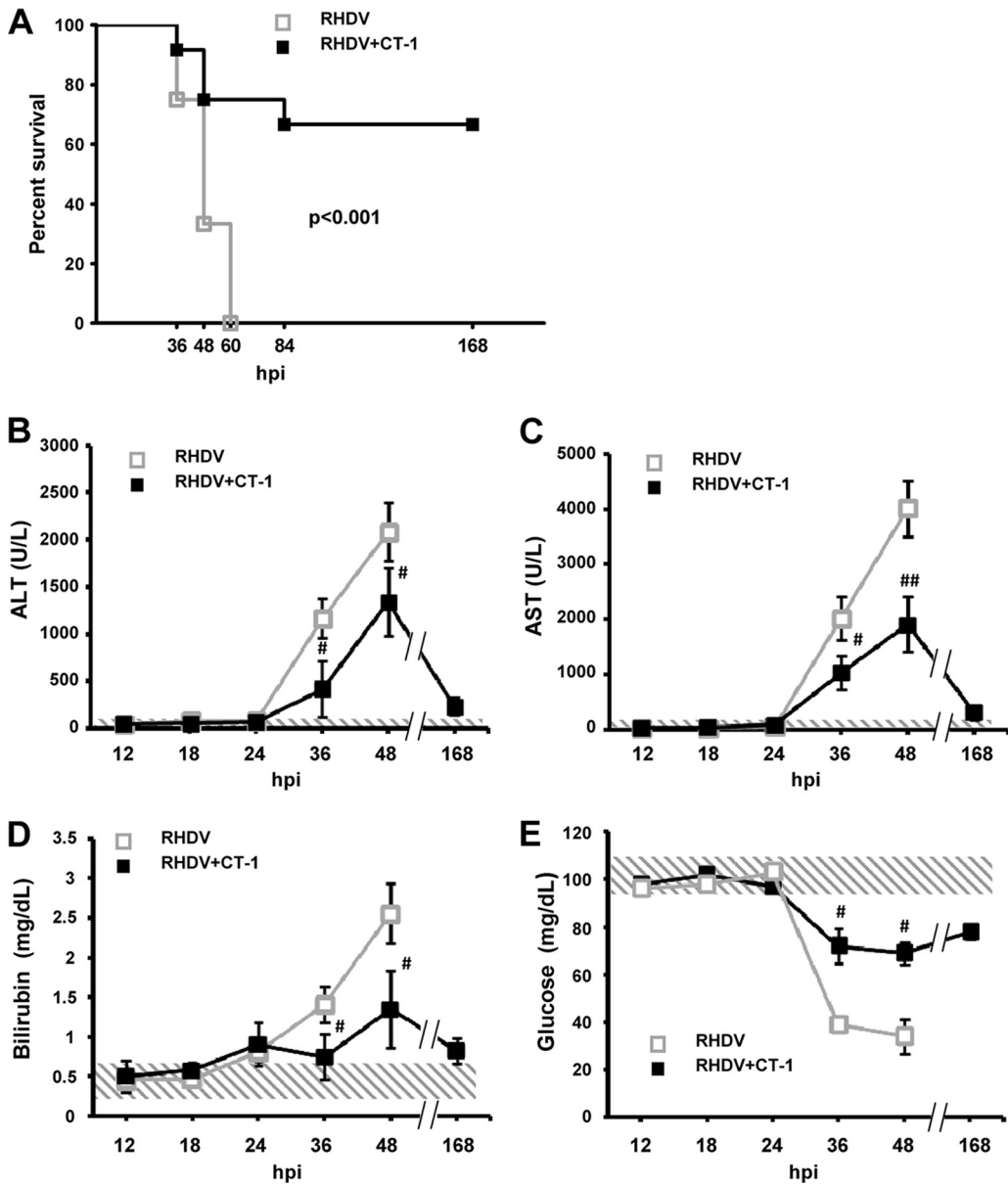
FIG. 1. Survival and liver function in RHDV-infected animals treated with saline or CT-1. (A) Percentage of surviving animals after RHDV infection that were treated with CT-1 or saline. Alanine aminotransferase (ALT) (B), aspartate aminotransferase (AST) (C), bilirubin (D), and glucose (E) plasma levels in animals after RHDV infection untreated or CT-1 treated (cohort 1) were determined. Shaded areas represent the range of normal values (means + standard deviations). #, $P < 0.05$; ##, $P < 0.01$ (CT-1-treated RHDV-infected rabbits versus untreated RHDV-infected animals).

of infected controls, and cell swelling and vacuolation were almost absent (Fig. 2). In normal noninfected rabbits receiving a similar dose of CT-1, the liver histology (and routine biochemical data) was comparable to that of normal rabbits given saline (Fig. 2A and B and data not shown). At day 7 postinfection, the livers of the surviving CT-1-treated animals showed inflammatory infiltrate in portal tracts and moderate portal and periportal fibrosis, together with the presence of some inflammatory foci in the lobule composed of mononuclear cells and heterophils (Fig. 2E). In these animals, numerous Ki-67-positive nuclei were present in hepatocytes, a finding

consistent with the activation of a vigorous postnecrotic regenerative process (Fig. 2F).

CT-1 has no influence on RHDV replication. To determine whether improved survival in CT-1 treated rabbits was related to inhibition of viral replication, RHDV viral load was quantified in plasma and liver tissue from CT-1-treated and untreated animals. We found that RHDV-RNA plasma levels during the course of the infection were similar in all RHDV-infected rabbits irrespectively of the treatment they received (saline or CT-1) (Fig. 3A). Moreover, the viral load in liver tissue at 30 and 36 hpi was comparable in the two groups (Fig.
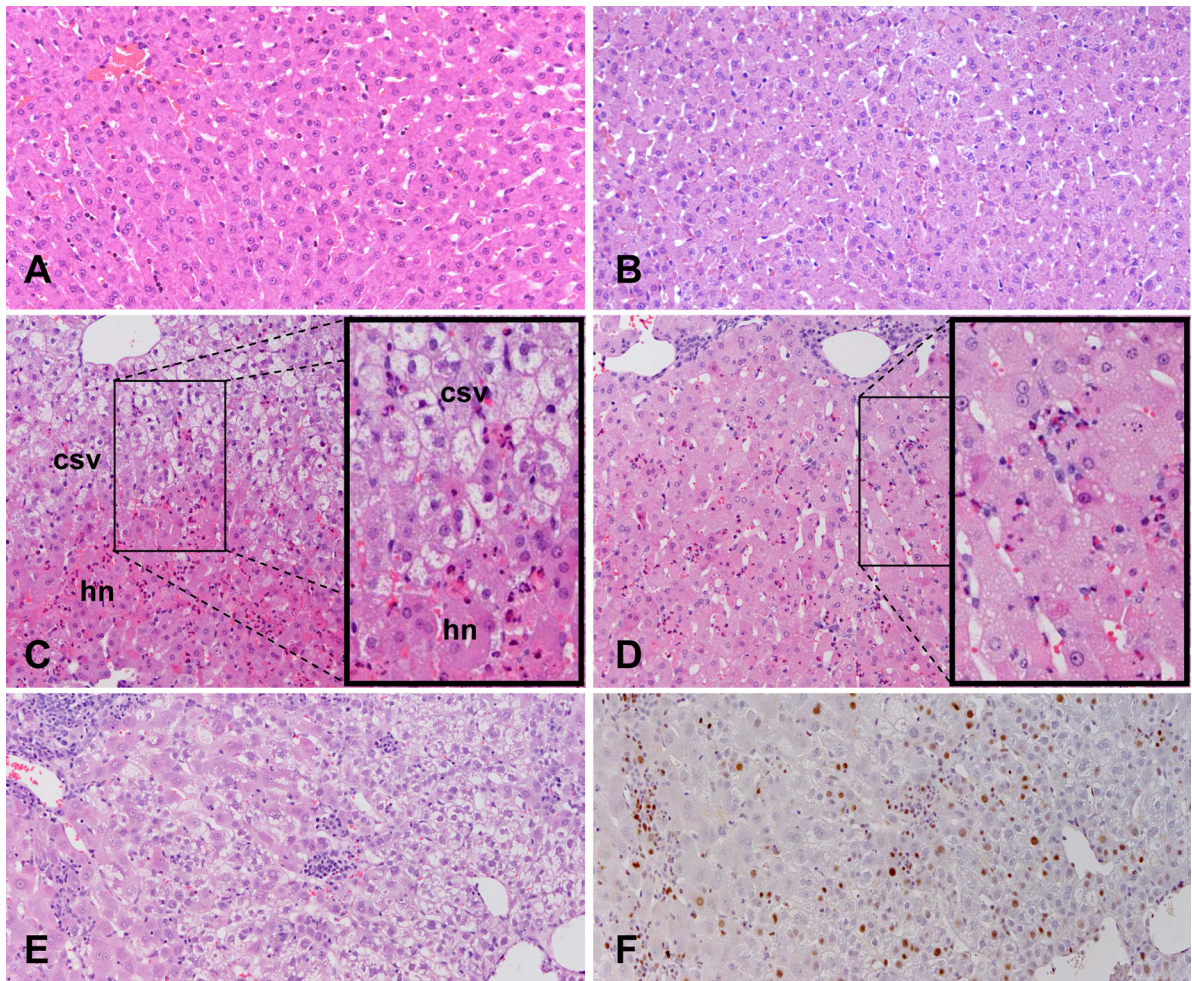
FIG. 2. Liver histology from normal rabbits treated or not treated with CT-1 and from RHDV-infected animals treated with saline or CT-1. Hematoxylin and eosin staining of liver samples from a healthy rabbit (A), a normal rabbit treated with CT-1 (see Materials and Methods) (B), an RHDV-infected rabbit treated with saline at 30 hpi (C), an RHDV-infected rabbit treated with CT-1 at 30 hpi (D), and an RHDV-infected rabbit treated with CT-1 that survived at day 7 postinfection (E) and Ki-67 staining of RHDV-infected rabbit treated with CT-1 that survived at day 7 postinfection (F) was performed. Cytoplasmic swelling and vacuolation (csv) and extensive areas of hepatocellular necrosis (hn) are seen in panel C, while these changes are markedly attenuated in panel D. Residual foci of inflammation and increased numbers of Ki67-positive nuclei (in brown) are seen in panels E and F. Images show representative microphotographs from six animals in panel A, six animals in panel B, four animals in panel C, six animals in panel D, and eight animals in panels E and F.

3B). Plasma RHDV-RNA levels reached values of 10^8 genomes/ml in both treated and untreated rabbits at 48 hpi, when mortality was very high in the untreated group. Virus titers declined subsequently in the surviving CT-1-treated animals to reach low levels (10^3 viral genomes/ml) by day 7 when the animals were sacrificed. Thus, the survival benefit afforded by CT-1 appears to be due to factors other than interference in RHDV replication.

CT-1 attenuates oxidative stress and the inflammatory reaction in the liver in rabbits with RHDV-induced acute liver failure. As noted above, RHDV-induced ALF is characterized by a severe acute inflammatory reaction in the liver. High levels of proinflammatory mediators ignite the production of reactive oxygen species (ROS) leading to persistent JNK phosphorylation, PARP activation, and necrapoptosis (17). We evaluated the extent of oxidative stress by determining the oxidized/reduced glutathione (GSSG/GSH) ratio and the lev-

els of TBARS (as a reflection of lipid peroxidation) in liver tissue from untreated and CT-1-treated infected animals sacrificed at 30 and 36 hpi. At both time points, GSSG/GSH ratio and TBARS values were significantly increased in RHDV-infected rabbits given saline compared to healthy livers. In contrast, in RHDV-infected animals treated with CT-1, both the TBARS values and the GSSG/GSH ratio were significantly lower than in animals given saline and were similar to those of healthy controls (Fig. 4A and B). Interestingly, JNK phosphorylation was prominent at 30 hpi in livers from RHDV-infected rabbits but was markedly attenuated in four of six animals treated with CT-1 (Fig. 4C). In parallel with these findings, PARP1/2 cleavage was prominent at both 30 and 36 hpi in the group of infected rabbits given saline but inconspicuous in four of six animals treated with CT-1 (Fig. 4C and data not shown). Thus, RHDV infection causes strong oxidative stress, JNK activation, PARP1/2 cleavage, and massive necrapoptosis. The
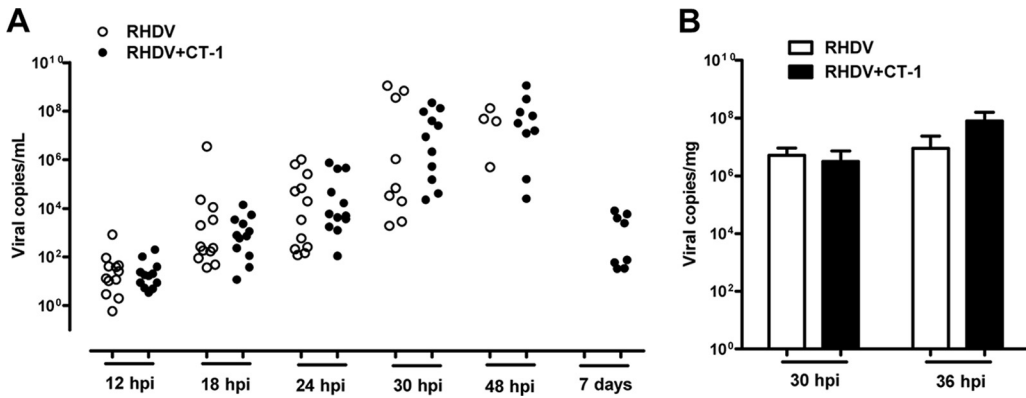
FIG. 3. RHDV viral load in RHDV-infected rabbits treated with saline or CT-1. RHDV viral load in plasma from animals of cohort 1 (A) and in liver tissue from animals of cohort 2 (B). Rabbits were RHDV infected and treated with CT-1 (RHDV+CT-1) or with saline (RHDV).

surviving benefit afforded by CT-1 therapy is associated with marked attenuation of this chain of events.

Proinflammatory cytokines have been shown to play a key role in acute liver injury of various etiologies (2, 35). We found that the mRNA levels of IL-1β, IL-6, and tumor necrosis factor alpha (TNF-α) were strongly elevated in RHDV-infected livers compared to healthy controls and that CT-1 therapy resulted in a significant reduction in the expression of all of these molecules (Fig. 5A, B, and C). Importantly, liver expression of endogenous CT-1 dropped markedly in all infected animals

(possibly as a result of the profound hepatocellular damage) without differences between those that were treated with saline or CT-1 (data not shown).

Toll-like receptor 4 (TLR4) is also an important trigger of the inflammatory reaction. TLR4 is activated by both pathogen-associated molecular patterns and endogenous ligands derived from extracellular matrix (ECM) degradation and necro/apoptotic cells (30). Marked TLR4 upregulation has been described in different viral infections (13, 39). Activation of TLR4 may ignite cascades of proinflammatory cytokines, thus
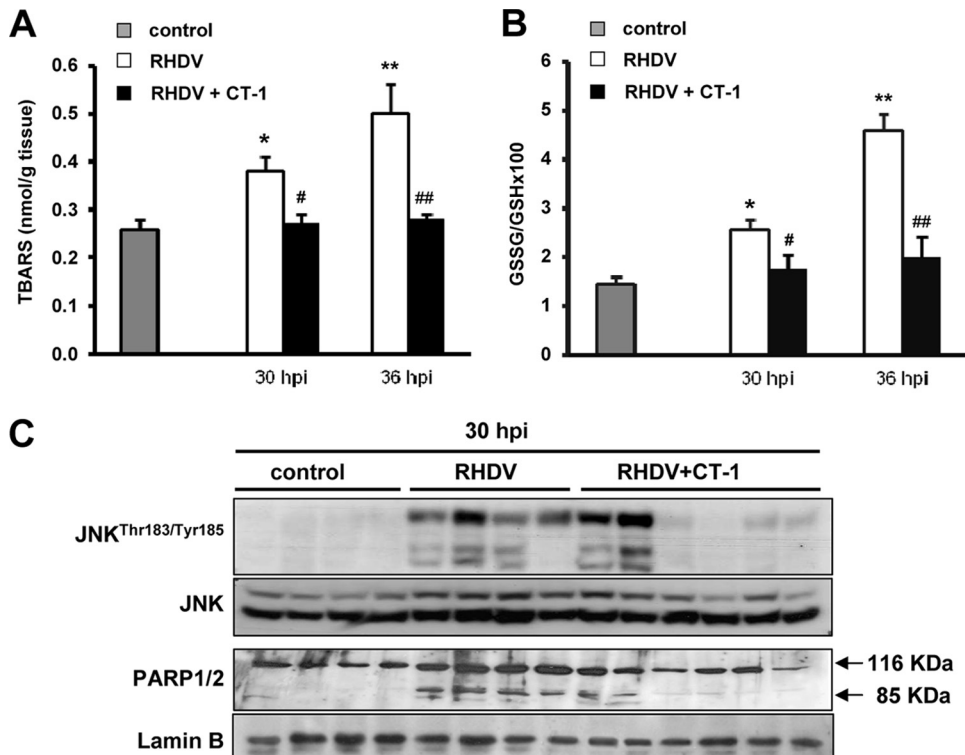

FIG. 4. Oxidative stress, JNK, and PARP1/2 in the livers of normal rabbits and RHDV-infected animals treated with saline or CT-1. TBARS (A) and GSSG/GSH ratio (B) are shown. (C) Western blot analysis of JNK activation and PARP1/2 cleavage in liver tissue from noninfected (control), RHDV-infected animals treated with CT-1 (RHDV+CT-1) or with saline (RHDV). #, $P < 0.05$; ##, $P < 0.01$ (CT-1-treated RHDV-infected rabbits versus RHDV-infected animals that received saline). *, $P < 0.05$; **, $P < 0.01$ (RHDV-infected animals versus noninfected controls).
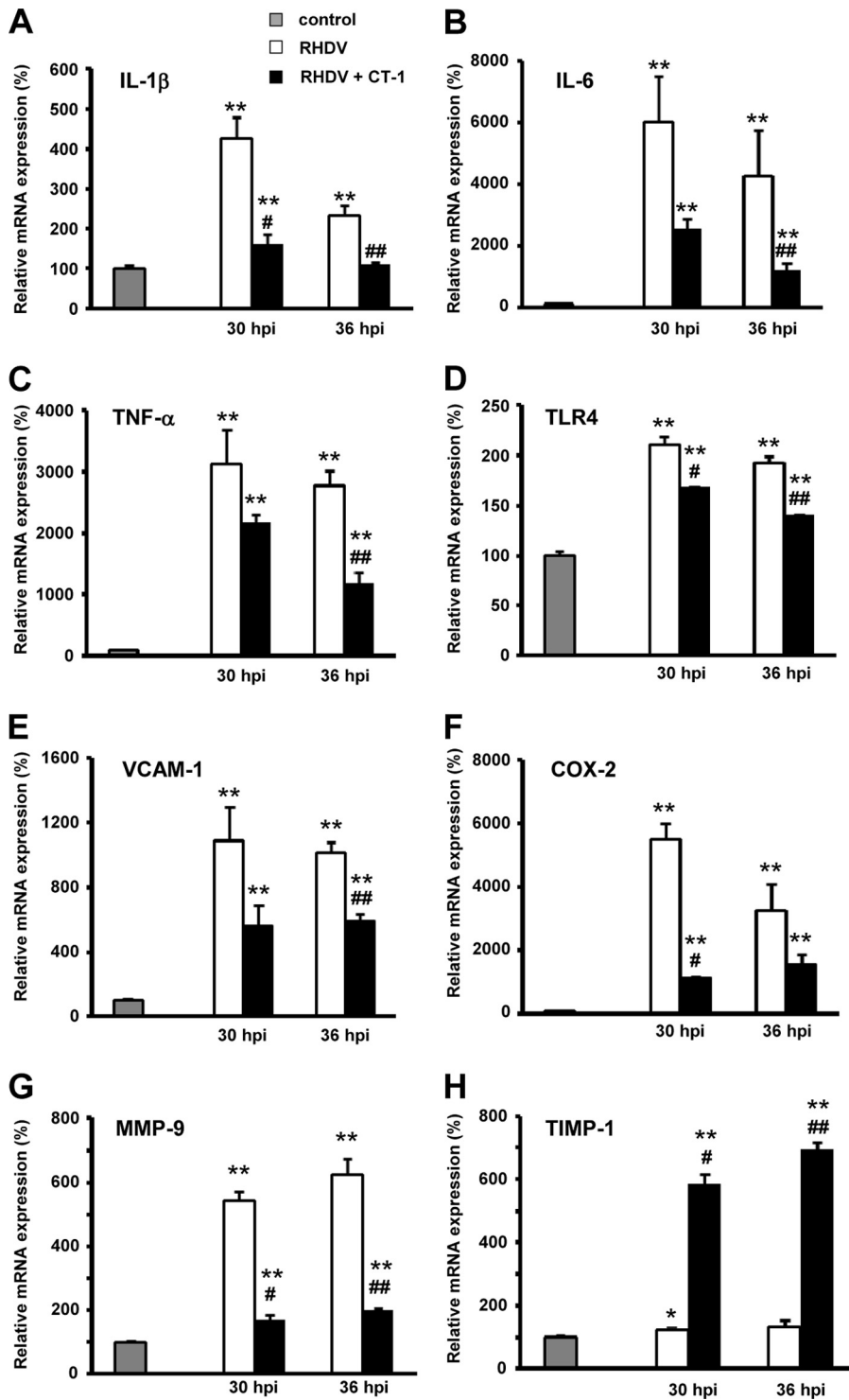
FIG. 5. Expression of inflammatory markers in livers from normal rabbits and RHDV-infected animals treated with saline or CT-1. mRNA expression of IL-1β (A), IL-6 (B), TNF-α (C), TLR4 (D), VCAM-1 (E), COX-2 (F), MMP-9 (G), and TIMP-1 (H) in liver tissue from uninfected rabbits (control) and RHDV-infected animals treated with saline (RHDV) or with CT-1 (RHDV+CT-1). #, $P < 0.05$; ##, $P < 0.01$ (CT-1-treated RHDV-infected rabbits versus RHDV-infected rabbits given saline). *, $P < 0.05$; **, $P < 0.01$ (RHDV-infected animals versus noninfected controls).

aggravating hepatocellular damage in severe forms of acute liver disease (10). Accordingly, gene deletion of TLR4 has been shown to palliate liver injury in experimental models of acute liver damage (38). We found that CT-1 therapy signifi-

cantly dampened the elevation of TLR4 expression that occurred in livers from RHDV-infected rabbits (Fig. 5D). A similar effect was observed with respect VCAM-1 expression. This membrane molecule is involved in transendothelial mi-
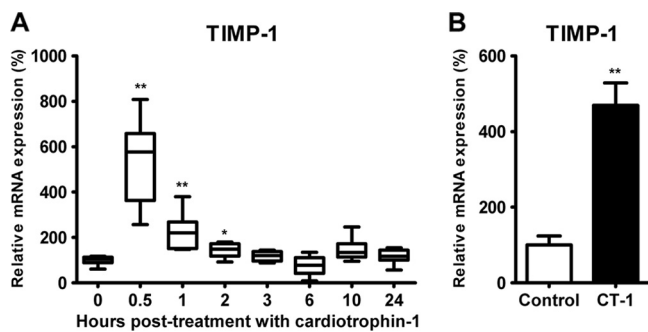
FIG. 6. Induction of TIMP-1 mRNA expression after treatment with CT-1. (A) Kinetics of TIMP-1 mRNA expression in SIRC rabbit corneal cells treated with 25 ng of CT-1/ml. (B) Expression of TIMP-1 mRNA in liver tissue from normal rabbits that received treatment with CT-1 or were left untreated (control). $*$, $P < 0.05$; $**$, $P < 0.01$ (CT-1-treated versus untreated rabbits).

gration of leukocytes and ROS production (21). We found that it is highly induced in RHDV infection and that CT-1 therapy strongly inhibited its expression (Fig. 5E). COX-2 is also a key driver of inflammation whose expression is enhanced by pro-inflammatory cytokines (18). COX-2 was markedly upregulated in the livers of rabbits with RHDV infection, and this effect was significantly reduced by CT-1 (Fig. 5F).

Activation of matrix metalloproteinases (MMPs), particularly MMP-9, is a critical event in the development of experimental ALF. By degrading ECM, MMPs facilitate leukocyte influx, collapse of sinusoids, and parenchymal hemorrhage. MMP inhibitors or genetic deletion of MMP-9 defend mice against ALF elicited by LPS/D-(+)-galactosamine or TNF-α/D-(+)-galactosamine (33, 37). TIMP-1 is an endogenous inhibitor of MMPs, but it also exerts potent cell survival and growth-promoting activities independently of its MMPs blocking effects (9, 12). In the livers of RHDV-infected animals, we observed a vigorous overexpression of MMP-9 without changes in TIMP-1 mRNA values. Strikingly, in the liver of RHDV-infected rabbits which received CT-1, MMP-9 upregulation was considerably attenuated, whereas TIMP-1 was highly hyperexpressed (Fig. 5G and H). TIMP-1 appears to be a target gene of CT-1. In fact, we observed that incubation of rabbit corneal cells with this cytokine elicited a striking upregulation of TIMP-1 gene expression (Fig. 6A). Confirming these data, we found that normal noninfected rabbits treated with CT-1 (three injections of 100 µg/kg separated by 6 h) and sacrificed 6 h after the last dose showed a robust elevation of TIMP-1 mRNA in liver tissue (Fig. 6B).

CT-1 stimulates cytoprotective and proregenerative factors in RHDV-infected livers. Tissue defense against noxious insults is orchestrated by a diversity of growth factors and cytokines, including HGF (8), EGFR ligands (16), PDGF (29), and CT-1 (4), among others. In the livers of RHDV-infected rabbits given saline, we found a marked decrease of HGF at 36 hpi and of its receptor c-Met at both 30 and 36 hpi. The infected rabbits treated with CT-1 exhibited higher hepatic expression of HGF at 36 hpi and of c-Met at 30 and 36 hpi than the infected animals which received saline (Fig. 7D and E). Also, the PDGF-B gene expression and protein levels of PDGFRβ in liver tissue dropped intensely at 30 and 36 hpi in control

RHDV-infected rabbits while those treated with CT-1 showed significantly higher values of both PDGF-B and its receptor at both time points (Fig. 7A, C, and E). Furthermore, CT-1 therapy prevented the profound decline of hepatic EGF mRNA levels taking place in RHDV infection, with values at 36 hpi that were significantly higher in rabbits treated with CT-1 than in RHDV-infected rabbits given saline (Fig. 7B).

DISCUSSION

RHDV infection is a unique model that closely reproduces the histopathological, biochemical, and clinical manifestations of human fulminant viral hepatitis (31). Therefore, we selected RHDV-induced ALF to determine whether CT-1 might represent a potential therapy for this condition and to define the responsible mechanisms.

Previously, we reported that CT-1 is a potent hepatoprotective cytokine with the ability to attenuate ConA hepatitis, Fas-induced hepatocellular damage, and ischemia/reperfusion injury (4, 15, 20). However, its role in fulminant viral hepatitis has never been tested. In the present study, RHDV-infected animals received five doses of CT-1, the first one at 12 hpi. This time point was selected because of the rapid evolution of RHDV infection. Later in the course of the disease, rabbits left untreated were so ill and prostrated that it was technically difficult to administer intravenous injections.

Notably, while 100% of rabbits with RHDV died within 60 h, CT-1 treatment allowed 67% survival at day 7 postinfection, which was the end of the study period (unpublished observations from our group indicate that, after this time point, the animals that were not sacrificed survived normally). This remarkable therapeutic efficacy could not be ascribed to any antiviral effect of the cytokine since the plasma viral load was similar in treated and untreated animals up to 48 hpi, a time point where mortality was much higher in untreated than in CT-1-treated rabbits. Also, RHDV-RNA abundance in liver tissue was comparable at 30 and 36 hpi in the two groups of animals. Our data indicate that the beneficial effect of CT-1 appears to be due to the mitigation of inflammation, the reduction of oxidative stress, and the activation of endogenous cytoprotective and growth-promoting mechanisms. Indeed, CT-1-treated animals exhibited a significant reduction in the expression of key drivers of inflammation, such as TNF-α, IL-1β, COX-2, and TLR4, which are known to be relevant mediators of tissue damage in the inflamed liver (2, 10, 18). On the other hand, in the treated rabbits the alleviation of the inflammatory reaction within the liver was accompanied by a significant decrease in oxidative stress as reflected by decreased levels of lipid peroxidation products and reduced GSSG/GSH ratio. The decrement of oxidative stress might be a consequence of the anti-inflammatory properties of CT-1, but it could also be due to the reported ability of this cytokine to stimulate antioxidant genes (15). Persisting production of ROS in the inflamed tissues causes prolonged JNK phosphorylation, leading to PARP1/2 activation and necrapoptosis (17). This chain of events appears to be inhibited by CT-1 administration. Supporting this view, we found that JNK activation and PARP1/2 cleavage were readily detectable in liver tissue at 30 hpi in untreated RHDV-infected rabbits but not in four out of six CT-1-treated infected animals.
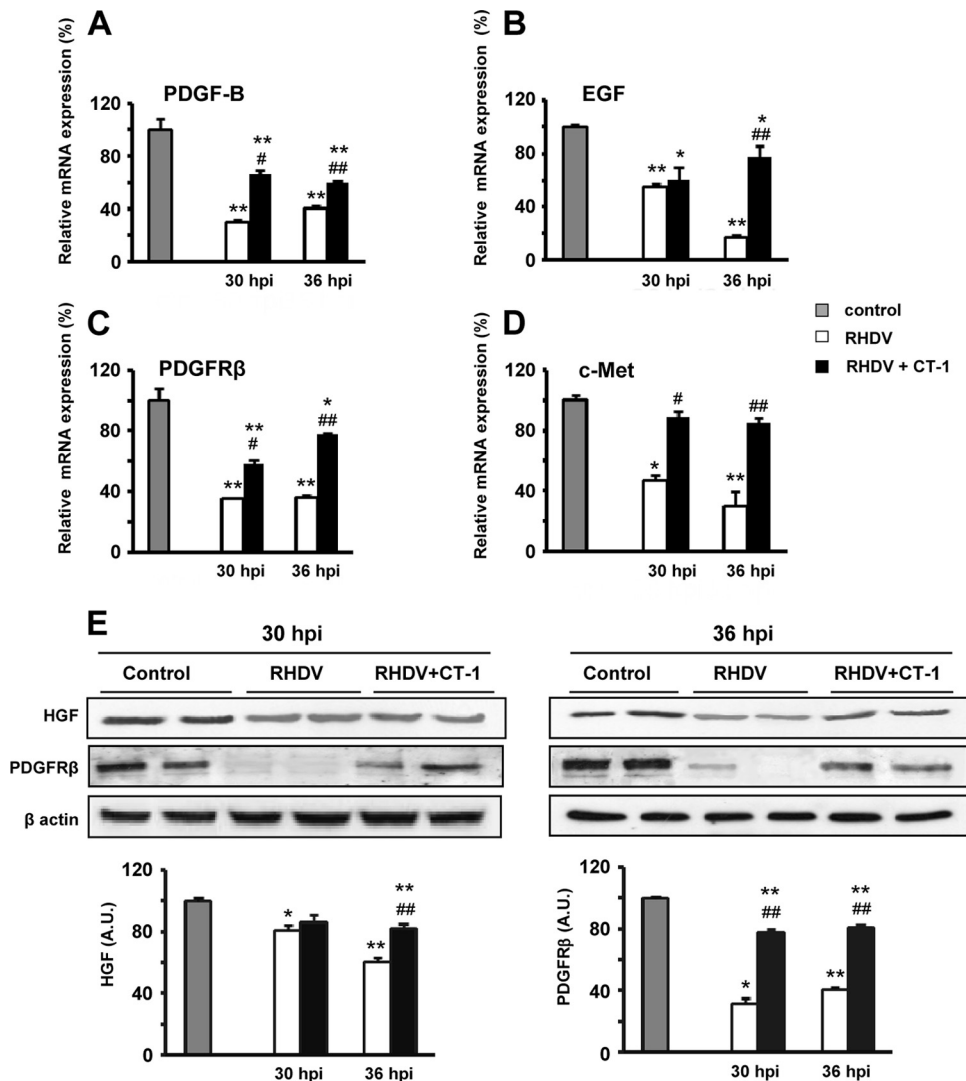
FIG. 7. Expression of growth factors and growth factor receptors in the liver from normal rabbits and from RHDV-infected animals treated with saline or CT-1. mRNA expression of PDGF-B (A), EGF (B), PDGFRβ (C), and c-Met (D) was examined. (E) Representative Western blots of HGF and PDGFRβ normalized with β-actin. The densitometric analysis corresponds to data from all cohort 2 animals. Analyses were performed in liver tissue from uninfected rabbits (control) and RHDV-infected rabbits treated with CT-1 (RHDV+CT-1) or saline (RHDV). #, $P < 0.05$; ##, $P < 0.01$ (CT-1-treated RHDV-infected rabbits versus untreated RHDV-infected animals). *, $P < 0.05$; **, $P < 0.01$ (RHDV-infected animals versus noninfected controls).

In addition to these effects, we observed that CT-1 therapy prevented the intense MMP-9 upregulation that occurred in RHDV infection. Recently, it has been found that IL-1-mediated MMP-9 induction plays an essential pathogenic role in models of ALF by promoting degradation of ECM, which leads to apoptosis of hepatocytes and parenchymal hemorrhage (37). In addition, the release of ECM-derived peptides triggers the influx and activation of polymorphs which enhance tissue damage (36). Importantly, CT-1 not only prevented MMP-9 overexpression but also provoked a dramatic increase of TIMP-1 mRNA levels. TIMP-1 appears to be a target gene of CT-1, since its expression is readily stimulated in the liver of normal rabbits treated with this cytokine, and it is also enhanced in cultured rabbit corneal cells incubated in the presence of CT-1. TIMP-1 is an inhibitor of MMPs but also acts as

a potent antiapoptotic and proregenerative molecule (9, 12). In fact, production of TIMP-1 by neoplastic cells facilitates tumor progression and resistance to chemotherapy (5). Thus, inhibition of MMP-9 expression, together with the upregulation of TIMP-1, may constitute key mechanisms by which CT-1 attenuates liver injury in RHDV infection.

In addition to the effects described above, CT-1 therapy triggered the expression in the inflamed liver of growth factors and growth factors receptors, including PDGF-B and PDGFRβ. Increased levels of these molecules have been well documented in acute liver damage (25). PDGF-B is endowed with cytoprotective activity and promotes hepatocyte proliferation (3). In patients with ALF it has been shown that PDGF-B plasma levels correlate positively with survival (29), suggesting that upregulation of this growth factor may foster recovery. We

also found that CT-1 induced the expression of EGF and c-Met. The latter is the receptor for HGF, a potent hepatoprotective factor (8) whose protein levels are increased in the liver of CT-1-treated rabbits. EGF, on the other hand, exerts antiapoptotic effects and acts as a potent hepatomitogen (16). Thus, it is likely that upregulation of all of these molecules contributes to diminish hepatocellular damage and to stimulate regeneration following severe acute liver injury.

It has been shown that, in addition to hepatocytes, RHDV can also infect endothelial cells and intravascular macrophages by promoting the activation and apoptosis of these cells, which may play a pathogenic role in RHDV-induced ALF (1, 27). It has been reported that CT-1 reduces macrophage activation (6) and exerts a protective role on endothelial cells (26). These effects on nonparenchymal cells might also contribute to the attenuation of liver damage observed in the infected animals treated with CT-1.

To summarize, we have shown that, in rabbits with lethal viral hepatitis, CT-1 therapy was able to diminish liver necrosis and to improve survival dramatically. This beneficial effect was associated with attenuation of inflammation, reduction of prooxidant injury, downregulation of MMP-9, upregulation of TIMP-1, and overexpression of several growth factors and receptors. Since CT-1 does not affect viral replication, it seems that modulation of innate immunity and inflammation may significantly reduce hepatocellular damage in acute severe viral hepatitis. In conclusion, our data point to CT-1 as a molecule of potential therapeutic value for patients with ALF of viral origin.

ACKNOWLEDGMENTS

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