A Recombinant Adenovirus Encoding Hepatitis C Virus Core and E1 Proteins Protects Mice Against Cytokine-Induced Liver Damage

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Hepatitis C virus (HCV) infection has a strong tendency to evolve to chronicity despite up-regulation of proapoptotic cytokines in the inflamed liver. The mechanisms responsible for persistent viral replication in this inflammatory environment are obscure. It is conceivable that viral replication would be facilitated if the infected hepatocytes are rendered resistant to cytokine-induced cytotoxicity. In this study, we investigated if an adenovirus encoding HCV core and E1 (RAdCE1) could reduce liver cell injury in different *in vivo* models of cytokine-mediated hepatotoxicity in mice. We show that RAdCE1 markedly attenuates hepatocellular apoptosis and the increase in serum transaminase levels after concanavalin A (con A) challenge. This protective effect is accompanied by an inhibition of nuclear translocation of nuclear factor κB (NF-κB); reduced expression of inducible nitric oxide synthase (iNOS); decreased hepatic messenger RNA levels of chemokines macrophage inflammatory protein 2 (MIP-2), monocyte chemoattractant protein 1 (MCP-1), and interferon-inducible protein 10 (IP-10); and abrogation of liver leukocyte infiltration. RAdCE1 also causes a reduction in serum transaminase levels and inhibits hepatocellular apoptosis in mice given tumor necrosis factor (TNF)-α plus D-galactosamine. In conclusion, HCV structural antigens can protect liver cells against the proapoptotic effects of proinflammatory cytokines. The antiapoptotic status of infected liver cells may represent a mechanism favoring viral persistence. Our findings also suggest that, in chronic hepatitis C, the burden of hepatocellular damage mainly affects noninfected liver cells. (HEPATOLOGY 2003;37:461-470.)

Hepatitis C virus (HCV) is a single-stranded RNA virus[1] characterized by its ability to cause persistent infection and chronic hepatitis. Chronic HCV infection constitutes a major health problem in both western and developing worlds because of its high prevalence and the propensity of chronic hepatitis C to evolve to liver cirrhosis and eventually to hepatocellular carcinoma.[2] Persisting infection is facilitated by a diversity of mechanisms that allow the virus to escape host adaptive immunity.[3-5]

In most patients with chronic HCV infection, there is an active inflammatory reaction in the liver with up-regulation of proinflammatory cytokines, notably tumor necrosis factor (TNF)-α and interferon (IFN)-γ,[6,7] which display both cytotoxic and antiviral effects. This process contributes to the hepatocellular injury seen in most HCV-infected patients but does not prevent HCV replication in liver cells. Mechanisms that make the cytokine response inefficient in controlling the infection have not been elucidated.

The 9.6-kilobase genome of HCV contains highly conserved untranslated regions at both 5′ and 3′ termini, which flank a large open reading frame encoding for 3 structural (core, E1, and E2) and at least 6 nonstructural (NS2, NS3, NS4a, NS4b, NS5a, and NS5b) proteins.[8] Although extensive study of the HCV genome has shown the contribution of translated viral proteins to the viral life

Abbreviations: HCV, hepatitis C virus; TNF, tumor necrosis factor; IFN, interferon; NF-κB, nuclear factor κB; con A, concanavalin A; IP-10, interferon-inducible protein 10; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling; iNOS, inducible nitric oxide synthase; pfu, plaque-forming units; MIP-2, macrophage inflammatory protein 2; MCP-1, monocyte chemoattractant protein 1; TNFR1, tumor necrosis factor α receptor 1.

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cycle, the effect of HCV proteins on the functions of infected cells is relatively unknown. Previous *in vitro* studies have shown that HCV proteins affect many signal pathways. Among them, HCV core and NS4B seem to be the most active at interfering cell signaling,[9] but the *in vivo* consequences of the interactions between viral and cellular proteins have not yet been analyzed. It has been reported that HCV core protein binds to the cytoplasmic domain of certain members of the TNF-α receptor superfamily, modulating the sensitivity of infected cells to the stimulus provided by their respective ligands.[10-13] However, based on *in vitro* data, there is controversy with respect to the role of HCV core protein on the TNF-α receptor superfamily signaling pathways. Thus, it has been reported that core protein activates[10,12,13] or inhibits[9,14,15] TNF-α–induced apoptosis. Similarly, there are contradictory reports regarding the role of HCV core protein on TNF-α–induced nuclear factor κB (NF-κB) activation, and it has been reported that HCV core protein inhibits,[10,12,16] has no effect,[17] or even activates[9,15,18] NF-κB. It is likely that these discrepancies may be related to differences in the cell lines used and/or differences in culture conditions.

In this study, we investigated whether transduction of the mouse liver with an adenovirus encoding HCV core and E1 (RAdCE1) proteins could protect liver cells from cytokine-induced hepatocellular damage in 2 experimental models of TNF-α–mediated hepatic injury. Our results show that RAdCE1 strongly reduces liver cell death in mice challenged with concanavalin A (con A) or with TNF-α plus D-galactosamine, suggesting that HCV-infected cells might resist the apoptotic effect of cytokines, becoming a privileged site for continuing viral replication within an inflammatory environment.

Materials and Methods

Mice. Six-week-old female BALB/c mice were purchased from IFFA Credo (Barcelona, Spain). The mice were hosted in appropriate animal care facilities and handled following institutional guidelines required for experimentation with animals.

Recombinant Adenoviruses. Recombinant adenoviruses RAdCE1, expressing HCV core and E1 proteins, and RAdLacZ, expressing β-galactosidase, have been previously described.[19] Viruses were propagated on 293 cells, purified in a CsCl isopyknic banding step, and kept in aliquots at $-80°C$.

Induction of Acute Liver Injury. Acute liver injury was induced by intravenous administration of 100 mg/kg con A (Sigma Chemical Co., St. Louis, MO) in 250 μL saline via the tail vein or by intravenous administration of 0.5 μg TNF-α/mouse (Peprotech, London, England) plus intraperitoneal administration of 25 mg D-galactosamine/mouse (Sigma Chemical Co.) (TNF-α/D-galactosamine). Six hours after administration of con A or TNF-α/D-galactosamine, mice were bled for biochemical analyses and killed for histopathologic studies. Livers sections were stored in different conditions: (1) fixed in formol for paraffin inclusion, (2) frozen in OCT for immunohistochemical analysis, and (3) frozen in liquid nitrogen for RNA or protein isolation.

Liver Function Tests and Cytokines. Liver injury was estimated by determining the serum levels of alanine aminotransferase using a colorimetric method based on the capacity of 2,4-dinitrophenylhydrazine to react with pyruvate (Sigma Chemical Co.). Alanine aminotransferase values were expressed as Sigma-Frankel units per milliliter. Determination of total bilirubin in serum was performed in the presence of dimethyl sulfoxide by diazo reaction with diazotized sulfanilic acid (ABX Diagnostics, Montpelier, France). Serum concentration of IFN-γ and TNF-α was measured using commercially available enzyme-linked immunosorbent assays (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's instructions.

Measurement of Apoptosis. Apoptosis was measured in liver sections by using the *In Situ* Cell Death Detection Kit, POD (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Briefly, tissue sections were fixed with 4% paraformaldehyde, endogenous peroxidase blocked (0.3% H_2O_2 in methanol), and permeabilized by incubation with a solution containing 0.1% Triton X-100. Samples were labeled with fluoresceinated nucleotides by terminal deoxynucleotidyl transferase. Apoptosis was observed under fluorescence microscopy.

Isolation of Liver Nuclear Extracts and Determination of Active NF-κB by Electrophoretic Mobility Shift Assay Analysis. Liver nuclear extracts were obtained as previously described.[20] Supernatants containing the protein nuclear extracts were collected and stored at $-80°C$. One small aliquot of nuclear extracts was used to determine protein concentration with the Bradford reagent (Bradford Bio-Rad protein assay; Bio-Rad, Hercules, CA). NF-κB binding activity was determined by electrophoretic mobility shift assay with a commercial oligonucleotide containing the κB consensus site (Promega, Madison, WI) labeled with [γ-^{32}P]–adenosine triphosphate as previously described[20] and using 5 μg of liver nuclear extracts. For competition experiments, a molar excess of the unlabeled κB oligonucleotide was added and

incubated on ice for 20 minutes before the addition of the labeled probe (not shown).

Ribonuclease Protection Assays. Liver tissue sections were processed for total RNA isolation by liver homogenization in 1 mL Ultraspect (Biotex, Houston, TX) with an Ultraturrax Driver T.25 (Janke & Kunkel, Ika-Labortechnik, Staufen, Germany). Total RNA was isolated following the manufacturer's instructions. The multiprobe ribonuclease protection assay (Riboquant; BD Biosciences Pharmingen) was performed according to the manufacturer's instructions using the multiprobe template set mCK-5b or specific probes for interferon-inducible protein 10 (IP-10) and for glyceraldehyde-3-phosphate dehydrogenase.

Immunostainings. Liver tissue was embedded in Tissue Tek compound (Miles Laboratories, Inc., Naperville, IL) and immediately frozen in liquid nitrogen. Tissue sections were fixed with 4% paraformaldehyde, endogenous peroxidase blocked (0.3% H_2O_2 in methanol), and permeabilized by incubation with a solution containing 0.1% Triton X-100. Samples were examined by indirect immunoperoxidase technique using an anti-core monoclonal antibody (kindly provided by Dr. Martinez-Anso, University of Navarra, Pamplona, Spain).

The identity of HCV core–expressing cells and apoptotic cells was analyzed by double staining using terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) reaction (green fluorescence) and indirect immunostaining with anti-core specific antibodies and anti–immunoglobulin G/Cy3 (Jackson ImmunoResearch, West Grove, PA) (red fluorescence). After performing the anti-core specific immunostaining, we performed TUNEL reaction as previously described. Analysis of double-labeling experiments was performed under a fluorescence microscope using different filters.

Western Blot Analysis. Liver pieces were homogenized in lysis buffer (1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L NaF, 20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L sodium vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, 0.5 μg/mL aprotinin, and 3 μg/mL leupeptin) using a glass-Teflon homogenizer. Extracts were cleared by centrifugation at 75,000 rpm at 4°C for 45 minutes. Twenty-five micrograms of protein from each sample was loaded onto 8% sodium dodecyl sulfate acrylamide gels followed by electrophoretic transfer to nitrocellulose membranes. Inducible nitric oxide synthase (iNOS) was detected using rabbit polyclonal anti-iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and secondary anti-rabbit horseradish peroxidase–conjugated antibody (Santa Cruz Biotechnology). Incubation of membranes with mouse immunoglobulin M monoclonal anti-actin antibody (Ab-1) followed by incubation with horseradish peroxidase–conjugated anti-mouse immunoglobulin M secondary antibody (Calbiochem, Darmstadt, Germany) was used for internal control. Protein bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany).

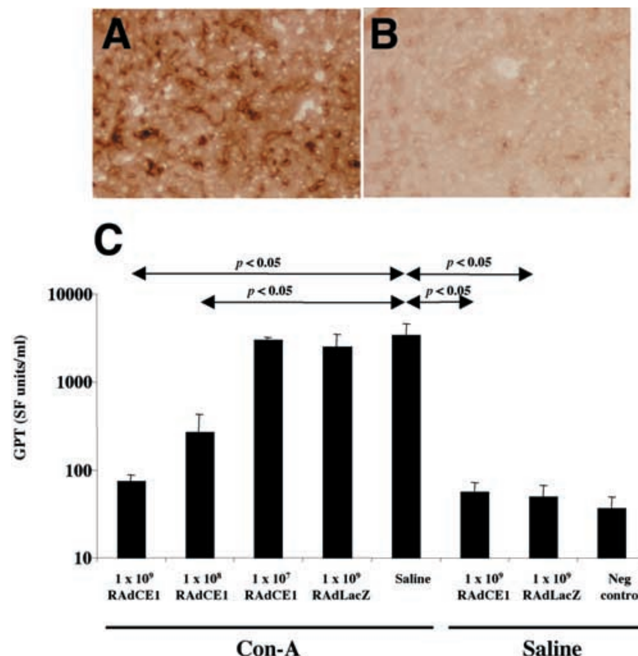

Fig. 1. Expression of HCV core in liver tissue of mice injected with RAdCE1 or RAdLacZ and protection against con A–induced liver damage in animals treated with RAdCE1. Indirect immunoperoxidase staining of HCV core protein in liver sections from mice injected intravenously with 1×10^9 pfu (A) RAdCE1 or (B) RAdLacZ. (Original magnification $\times$200.) (C) Groups of 4 mice were injected intravenously with different doses of RAdCE1, 1×10^9 pfu RAdLacZ, or saline and treated with con A or vehicle 24 hours after adenovirus injection. Serum transaminases (GPT) were measured 6 hours after administration of con A.

Results

Injection of Recombinant Adenovirus Expressing HCV Core and E1 Proteins Protects Mice From con A–Induced Acute Severe Hepatitis. Adenoviral vectors have strong hepatotropism. Doses of 10^9 plaque-forming units (pfu) RAdLacZ given intravenously resulted in transduction of more than 60% of liver cells with few occasional positive cells in other organs such as the kidneys, lungs, and spleen, whereas 10^7 pfu infected less than 10% of hepatocytes (data not shown). After intravenous administration of 10^9 pfu RAdCE1, HCV core protein could be detected by an indirect immunoperoxidase technique in more than 60% of liver cells, showing a diffuse cytoplasmic staining of hepatocytes and a more intense staining of nonparenchymal cells (Fig. 1A and B). As

shown in Fig. 1C, RAdCE1 or RAdLacZ given alone (at the highest dose used in this study) did not cause significant liver damage *per se*.

Intravenous administration of con A to BALB/c mice induces severe liver injury within 6 to 8 hours.[21] To study the effect of HCV structural proteins on con A–induced liver injury, groups of 6-week-old female BALB/c mice were injected intravenously with different doses of RAdCE1 (10^7, 10^8, and 10^9 pfu), 10^9 pfu RAdLacZ, or saline and 24 hours later received an intravenous injection of con A or vehicle. Parameters of liver damage were determined 6 hours after exposure to con A or vehicle.

We found that intravenous injection of RAdCE1 at the doses of 10^9 and 10^8 pfu (but not at 10^7 pfu) markedly reduced the increase in alanine aminotransferase levels induced by administration of con A, whereas 10^9 pfu RAdLacZ was ineffective (Fig. 1C). Similarly, serum bilirubin levels (concentration in normal mice, 0.16 ± 0.13 μmol/L) increased after administration of con A to 0.86 ± 0.14 and 0.82 ± 0.17 μmol/L in mice pretreated with 10^9 pfu RAdLacZ or saline, respectively, but showed values of 0.31 ± 0.06 μmol/L after con A challenge in those pretreated with 10^9 pfu RAdCE1 ($P < .05$ compared with RAdLacZ or saline).

Because administration of con A causes apoptosis of hepatocytes in a dose-dependent manner,[21,22] we analyzed the number of TUNEL-positive nuclei in the different groups of animals. In mice treated with RAdLacZ or saline, con A caused hepatocyte apoptosis with a patchy distribution within the liver parenchyma with areas with abundant TUNEL-positive nuclei (Fig. 2B and C) and areas with a lower rate of apoptosis. In sharp contrast, mice receiving 10^9 pfu RAdCE1 were fully protected against apoptosis of hepatocytes (Fig. 2A) in such a manner that liver sections were similar to those of animals that did not receive con A (Fig. 2D-F).

To determine the relationship between hepatocyte apoptosis and HCV core expression, we performed double staining for apoptosis (green fluorescence) and HCV core (red fluorescence) in liver specimens from mice treated with 10^7 pfu RAdCE1 that exhibited few transduced liver cells and a high rate of apoptosis after administration of con A. We found that the presence of red fluorescence in the cytoplasm of hepatocytes completely excluded TUNEL positivity in the cell nucleus in every instance (representative examples are shown in Fig. 2G-J), indicating protection of cells expressing HCV core against cytokine-induced apoptosis.

Administration of con A is accompanied by increased levels of several cytokines, with TNF-α and IFN-γ the main mediators of liver damage.[23,24] We measured the serum concentration of these 2 cytokines in con A–treated mice previously injected with RAdCE1 (10^9 pfu), RAdLacZ (10^9 pfu), or saline, and no significant differences were found for either IFN-γ ($5{,}998 \pm 520$, $5{,}800 \pm 248$, and $5{,}662 \pm 225$ pg/mL, respectively, in the 3 groups of mice) or TNF-α ($1{,}580 \pm 310$, $1{,}220 \pm 550$, and $1{,}210 \pm 280$ pg/mL, respectively) among groups. These results indicate that the protective effect of RAdCE1 is not due to an inhibition in the production of cytokines induced by con A but rather to a protection of hepatocytes against cytokine-mediated cell death.

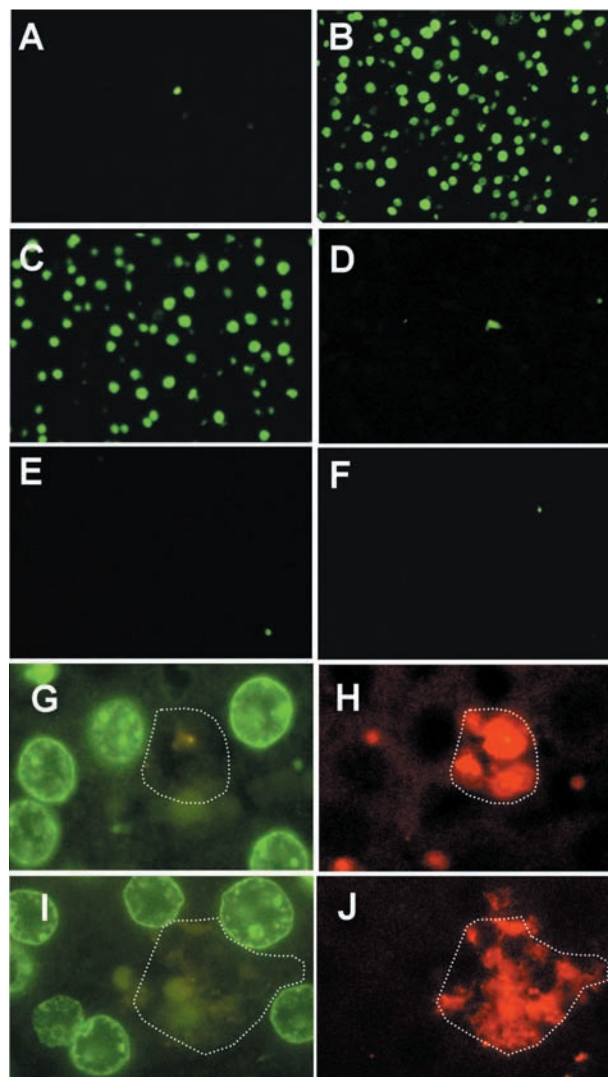

Fig. 2. Recombinant adenovirus RAdCE1 protects mice from apoptosis induced by administration of con A. Apoptosis was determined by TUNEL in liver sections from con A–treated mice previously injected with 1×10^9 pfu RAdCE1, RAdLacZ, or saline (A, B, and C, respectively) and from mice injected with 1×10^9 pfu RAdCE1, RAdLacZ, or saline that received vehicle instead of con A (D, E, and F, respectively). Double staining for apoptosis (G and J) (TUNEL reaction: green fluorescence) and for HCV core (H and I) (indirect immunostaining: red fluorescence) in liver sections from con A–treated mice previously injected with 1×10^7 pfu RadCE1. (Original magnification: A-F, ×400; G-J, ×1,000.) G and H as well as I and J correspond to the same fields under different light filters.

Treatment With RAdCE1 Prevents Acute Severe Hepatitis Caused by Administration of TNF-α/D-Galactosamine. TNF-α has been shown to play a crucial role in con A–induced hepatitis.[22,23] Because challenge with TNF-α after administration of D-galactosamine induces hepatocyte apoptosis in mice, we wanted to determine whether the cytoprotective effect of RAdCE1 also extended to this form of liver injury. To this aim, TNF-α/D-galactosamine was administered, as indicated in Materials and Methods, to mice treated 24 hours before with 10^9 pfu RAdCE1, 10^9 pfu RAdLacZ, or saline. Similar to what we observed in the con A model, RAdCE1 was able to exert strong protection of liver cells against TNF-α/D-galactosamine injury both as estimated by the levels of serum transaminases (Fig. 3A) and by the presence of TUNEL-positive nuclei in liver biopsy specimens (Fig. 3B-E).

Transduction of the Liver With RAdCE1 Inhibits Translocation of NF-κB to the Nucleus and iNOS Expression After con A Challenge. One of the downstream targets of TNF receptor is the transcription factor NF-κB. This factor is sequestered in the cytoplasm by IκB; on binding of TNF-α to its receptor, IκB is phosphorylated, ubiquitinated, and targeted to the proteasome for degradation. Free NF-κB then translocates to the nucleus and binds to consensus elements within the promoters of a variety of genes.[25] To analyze the mechanisms responsible for the protection afforded by RAdCE1 against con A–induced hepatitis, we determined the presence of nuclear NF-κB in the livers of the different experimental groups of mice. Thus, 6 hours after administration of con A, nuclear extracts from liver cells of animals treated with RAdCE1, RAdLacZ, or saline were obtained and NF-κB binding activity was assessed by means of electrophoretic mobility shift assay. We found that the translocation of NF-κB to nuclei was significantly reduced in mice given RAdCE1 compared with those receiving RAdLacZ or saline (Fig. 4). This finding indicates that the expression of HCV structural genes in liver cells causes a block in the signaling pathway of TNF-α, preventing nuclear translocation of NF-κB.

It has been shown that iNOS is strongly induced 4 to 8 hours after administration of con A and that this event is critical for immune-mediated liver injury induced by con A.[26] It has also been shown that activation of NF-κB is essential for the expression of iNOS in hepatocytes.[27,28] To gain insight into the mechanism of RAdCE1 protection against con A–induced hepatitis, we determined the levels of iNOS 6 hours after con A challenge in livers of mice treated with RAdCE1, RAdLacZ, or saline. Western blot analysis showed a complete absence of iNOS in livers from animals that received RAdCE1, whereas detectable

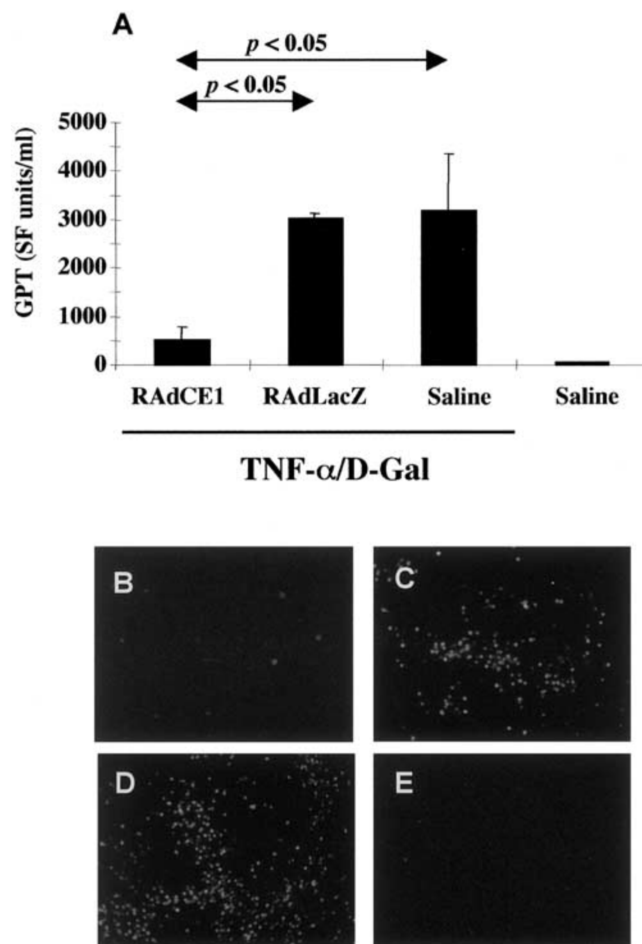

Fig. 3. Recombinant adenovirus RAdCE1 protects mice from acute severe hepatitis induced by administration of TNF-α plus D-galactosamine. Mice were injected intravenously with 1×10^9 pfu RAdCE1, RAdLacZ, or saline 24 hours before administration of TNF-α/D-galactosamine. A group of untreated mice was used as negative control. Six hours after administration of TNF-α/D-galactosamine, (A) serum transaminase levels and (B-E) liver cell apoptosis were measured. Apoptosis was determined by TUNEL in liver sections from mice treated with (B) RAdCE1, (C) RAdLacZ, or (D) saline before administration of TNF-α/D-galactosamine or from (E) untreated mice as negative control. (Original magnification $\times$200.)

levels were present in the livers of animals from the other 2 groups (Fig. 5).

Administration of RAdCE1 Reduces Macrophage Inflammatory Protein 2, Monocyte Chemoattractant Protein 1, and IP-10 Chemokine Expression and Inhibits Leukocyte Infiltration in the Livers of Mice That Received con A. The production of proinflammatory cytokines, notably TNF-α, during the first hours after con A challenge is followed by activation of transcription factors and mediators that induce the expression of chemokines that attract leukocytes to the liver.[29,30] These cells orchestrate an inflammatory response in the liver parenchyma contributing to the enhancement of

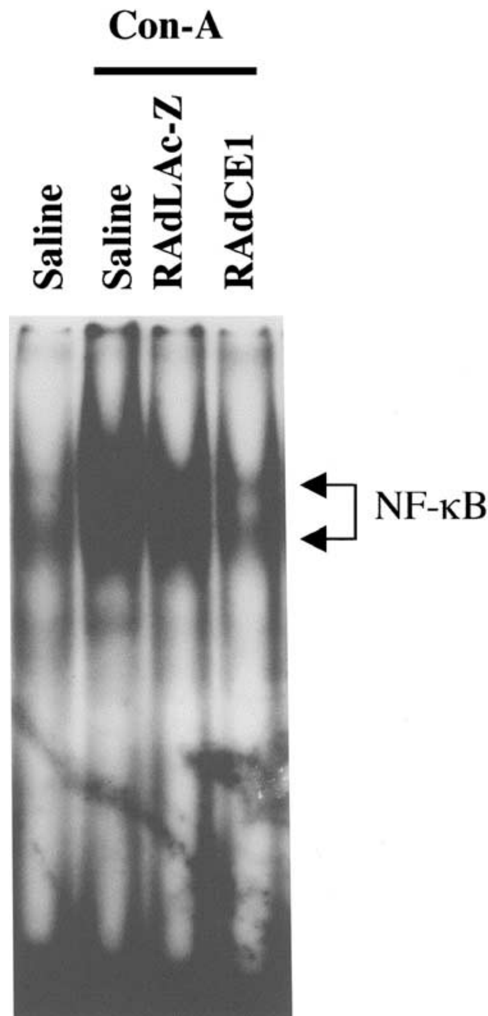

Fig. 4. Recombinant adenovirus RAdCE1 reduces NF-κB nuclear translocation induced by administration of con A. Mice were injected intravenously with 1×10^9 pfu RAdCE1, RAdLacZ, or saline 24 hours before administration of con A. A group of mice treated with saline was used as negative control. Six hours after administration of con A, liver nuclear extracts were obtained and NF-κB binding activity was determined by electrophoretic mobility shift assay using an oligonucleotide containing the κB consensus site labeled with [γ-^{32}P]-adenosine triphosphate. Separation of the complexes was performed in a 5% nondenaturing polyacrylamide gel electrophoresis and visualized by overnight exposition to x-ray film.

hepatocellular damage.[21] Using ribonuclease protection assay, we explored the expression of chemokines 6 hours after administration of con A in the livers of animals treated with RAdCE1, RAdLacZ, or saline. We found that administration of con A resulted in the induction of several chemokines, with macrophage inflammatory protein 2 (MIP-2), monocyte chemoattractant protein 1 (MCP-1), TCA-3, and IP-10 the most clearly overexpressed (Fig. 6). Interestingly, we observed that treatment with RAdCE1 influenced the expression of several chemokines in liver tissue. In particular, MIP-2 was reduced more than 15-fold and MCP-1 and IP-10 were decreased more than 3-fold in RAdCE1-treated mice compared with the control groups. No relevant changes were observed in the expression of TCA-3, Lnt, RANTES, Eotaxin, MIP-1β, or MIP-1α.

To determine whether the observed changes in chemokine expression had a reflection in the hepatic inflammatory cell infiltrate, we killed animals in the 3 experimental groups 24 hours after administration of con A and liver specimens were stained with hematoxylin-eosin (Fig. 7). We found that while animals from groups treated with RAdLacZ or saline exhibited apoptotic bodies, necrotic areas, and leukocyte infiltration in portal tracts (Fig. 7B and C), there was absence of portal leukocyte infiltration in those treated with RAdCE1 (Fig. 7A) and a liver histology similar to that found in mice that did not receive con A (Fig. 7F). Administration of 1×10^9 pfu RadCE1 or RAdLacZ in the absence of treatment with con A did not induce detectable leukocyte infiltration (Fig. 7D and E, respectively).

Discussion

There is no simple small animal model of HCV infection that could be used to analyze *in vivo* the influence of HCV structural proteins on the response of hepatocytes to proinflammatory cytokines. To investigate this point, we have used con A or TNF-α/D-galactosamine challenge in mice whose livers had been transduced with control adenovirus or with an adenovirus encoding HCV core and E1. In animals treated with 10^9 pfu RAdCE1, more than 60% of liver cells expressed the transgene. In patients with chronic HCV infection, HCV proteins can be detected immunohistochemically in 15% to 75% of hepa-

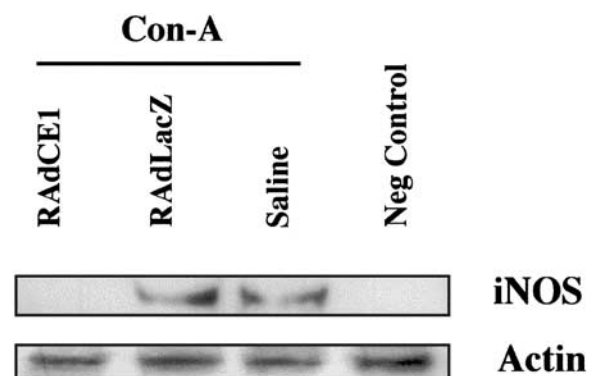

Fig. 5. Recombinant adenovirus RAdCE1 inhibits iNOS protein expression in the livers of con A–treated mice. Mice were injected with 1×10^9 pfu RAdCE1, RAdLacZ, or saline 24 hours before administration of con A. Six hours after injection of con A, 25 μg of liver protein extract was separated by electrophoresis on an 8% sodium dodecyl sulfate acrylamide gel. con A–induced iNOS expression was detected by western blotting. Actin was used as internal control. Liver protein extracts from nontreated mice served as negative control.

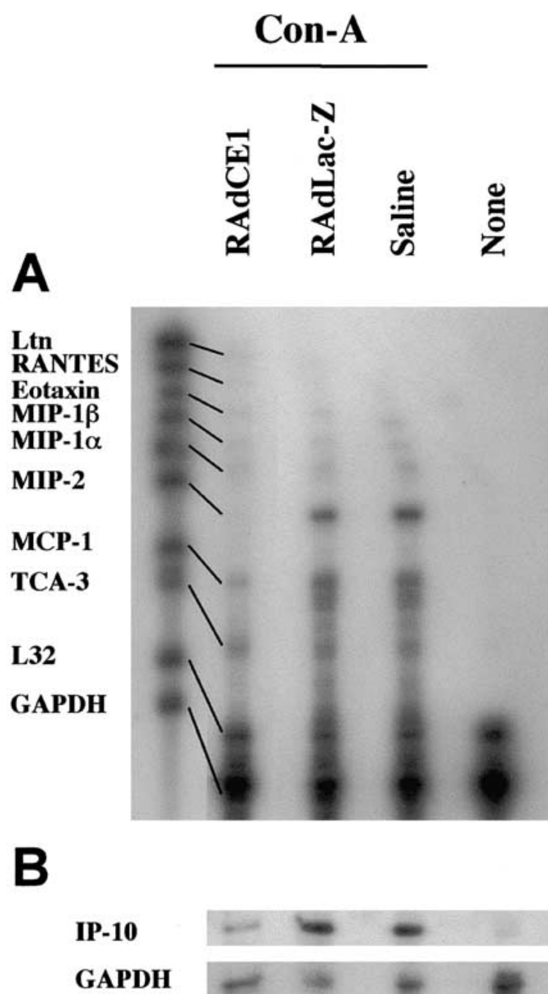

Fig. 6. Recombinant adenovirus RAdCE1 abrogated MIP-2 and reduced MCP-1 and IP-10 chemokine messenger RNA expression in the livers of mice treated with con A. Groups of 4 mice were injected intravenously with 1×10^9 pfu RAdCE1, RAdLacZ, or saline 24 hours before administration of con A. A group of untreated mice was used as negative control. Six hours after administration of con A, liver RNA was extracted and messenger RNA for different chemokines was quantified by using (A) mCK-5b multiprobe ribonuclease protection assay or (B) specific probes for IP-10 and glyceraldehyde-3-phosphate dehydrogenase.

tocytes.[31] Thus, although our model does not reproduce the immunopathobiological changes of chronic hepatitis C, it is a useful approach to investigate the effect of HCV proteins expressed inside liver cells on cytokine-induced hepatocellular damage.

In this study, we show that mice treated with RAdCE1 exhibit a marked suppression in the increase in serum transaminase and bilirubin levels as well as in the rate of apoptotic cells in the liver after con A challenge. con A is a T-cell mitogenic plant lectin that causes severe hepatitis within 4 to 8 hours.[21] Administration of con A is accompanied by increased levels of several cytokines, including interleukin 2, TNF-α, IFN-γ, interleukin 6, granulocyte-macrophage colony–stimulating factor, and interleukin 1.[21,23,24] Among these cytokines, TNF-α may play a major role in mediating apoptosis of hepatocytes.[22,23,32] In our study, treatment with RAdCE1 did not reduce the levels of TNF-α and IFN-γ after con A challenge, indicating that the observed protective effect was not due to a decrease in the release of cytokines but rather to an enhanced resistance to cytokine-induced apoptosis of liver cells. This concept is also supported by the finding that, in animals treated with 10^7 pfu RAdCE1 showing a low number of transduced cells and a high rate of apoptosis after con A challenge, the expression of core by hepatocytes was always found to exclude the presence of TUNEL positivity at the single-cell level.

Ligation of TNF-α receptor 1 (TNFR1) by TNF-α activates diverse intracellular signaling cascades that diverge at the inner cell membrane. TRADD, which binds to the cytoplasmic domain of TNFR1, is important for the activation of all of these pathways.[33,34] During TNFR1 activation, TRAF2 and RIP bind to TRADD, initiating downstream events leading to nuclear translocation of NF-κB. The inhibition of NF-κB translocation and liver cell apoptosis in the con A hepatitis model after

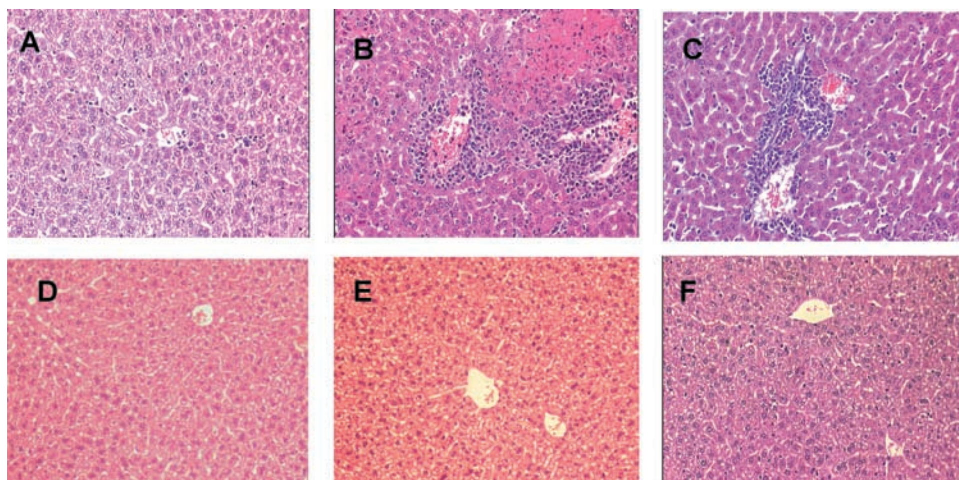

Fig. 7. Recombinant adenovirus RAdCE1 reduces leukocyte infiltration in the liver after administration of con A. Mice were injected intravenously with (A and D) 1×10^9 pfu RAdCE1, (B and E) RAdLacZ, or (C and F) saline 24 hours before administration of (A-C) con A or (D-F) vehicle. Twenty-four hours after administration of con A, liver sections were fixed in formol, included in paraffin, and stained with hematoxylin-eosin. (Original magnification ×200.)

administration of RAdCE1 seems to be related to the disruption of TNF-α signaling in hepatocytes because RAdCE1 also inhibits TNF-α/D-galactosamine–induced acute liver injury. This view is reinforced by recent data showing that HCV core protein can bind to the cytoplasmic domain of the TNFR1, forming a ternary complex with TNFR1 and TRADD.[35]

In con A–induced hepatitis, nuclear translocation of NF-κB is essential for generation of proinflammatory mediators and chemokines. In particular, NF-κB activation is necessary for hepatocellular expression of iNOS,[28] a key enzyme in mediating liver damage in con A–induced hepatitis.[26] In fact, serum transaminase levels and TUNEL-positive nuclei are markedly reduced after con A challenge in iNOS knockout mice compared with wild-type mice.[26] On the other hand, both NO and nuclear NF-κB are necessary for the induction of chemokine production. Thus MIP-2, MCP-1, and IP-10 are regulated by NF-κB[36-38] and NO is necessary for the expression of MIP-2[39] and MCP-1.[40] In our study, we found that while control mice showed marked nuclear translocation of NF-κB and production of iNOS in the liver after con A challenge, animals pretreated with RAdCE1 showed inhibition of NF-κB nuclear translocation and iNOS expression. This last group of mice also manifested reduced expression of chemokines MIP-2, MCP-1, and IP-10, which are regulated by NF-κB and/or NO. Therefore, it seems that blockade of NF-κB activation, decreased production of iNOS, and reduced chemokine expression in RAdCE1-treated mice are mechanistically connected events. However, although inhibition of iNOS production seems important in RAdCE1-mediated protection against con A challenge, this adenovirus seems to lessen cytokine-mediated liver injury by blocking other TNF-α death signals because it also protects against TNF-α/D-galactosamine, a model of liver injury that causes hepatocellular damage in the iNOS knockout mice.[26]

MCP-1 exhibits a chemoattractive activity for monocytes and T lymphocytes and triggers the adhesion of rolling monocytes to sites of inflammation. Similarly, IP-10 is a selective chemoattractant for activated T lymphocytes (reviewed by Baggiolini et al.[41]). MIP-2 is a chemoattractant for neutrophils[42] and dendritic cells.[43] The inhibition in the expression of chemokines, together with the inhibitory effect on hepatocyte apoptosis, may account for the lack of con A–induced inflammatory infiltrate in RAdCE1-treated livers. It also seems possible that the capacity of HCV core and E1 proteins to reduce the production of chemokines can interfere with the activation of antiviral immune responses, thereby suggesting a new mechanism by which HCV establishes a persistent infection.

TNF-α, along with other Th1 cytokines, is up-regulated in the liver of patients with chronic hepatitis C.[6,7,44] These cytokines have potent proapoptotic effects and could facilitate the elimination of infected liver cells. In addition, cytokines such as TNF-α can have a direct antiviral effect by a noncytolytic mechanism.[45] It is not clear how HCV can replicate actively and continuously in this adverse inflammatory environment. Our observations are relevant in this respect because they show that intracellular expression of HCV structural proteins may confer protection against cytokine-induced cytotoxicity. This ability of HCV core may represent a fundamental mechanism of chronicity of HCV infection because it provides a selective advantage for HCV replication, allowing for evasion of host antiviral defenses. Our data also point to the idea that, because HCV-infected hepatocytes have higher resistance to apoptosis than noninfected cells, the increase in serum transaminase levels and the histologic signs of hepatocellular injury in chronic hepatitis C would mainly reflect the bystander and nonspecific damage of noninfected cells in the liver inflammatory milieu.[46,47] In this context, it is important to consider that TNF-α is a potent inducer of the chemokine IP-10 in hepatocytes.[48] This chemokine selectively attracts activated liver infiltrating lymphocytes to cells that are producing the chemotactic factor.[29] In chronic hepatitis C, there is a marked up-regulation of IP-10,[49] which is an important player in the maintenance of the inflammatory reaction in the liver. Our data showing that HCV core/E1 blocks the cytokine-driven expression of IP-10 suggest that, in chronic hepatitis C, activated liver-infiltrating lymphocytes will be mainly attracted to noninfected hepatocytes with resulting diversion from infected cells and increased bystander damage to noninfected hepatocytes. Thus, our findings might favor the idea that, in chronic HCV infection, persistent liver inflammation might result in progression of liver damage without affecting viral replication.

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