Brief Definitive Report

CD40 Activation Induces Apoptosis in Cultured Human Hepatocytes via Induction of Cell Surface Fas Ligand Expression and Amplifies Fas-mediated Hepatocyte Death during Allograft Rejection

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Summary

We propose that a novel mechanism of hepatocyte apoptosis, involving a cooperative interaction between CD40 and Fas, is involved in the hepatocyte loss of chronic liver allograft rejection. We detected increased hepatocyte expression of Fas, Fas ligand (FasL), and CD40 associated with dropout of centrilobular (acinar zone 3) hepatocytes in chronic allograft rejection. Expression of CD40 ligand (CD40L) was also increased but was largely restricted to $CD68^+$ macrophages. A functional role for CD40 and Fas in hepatocyte apoptosis was demonstrated in vitro using primary human hepatocytes and the HepG2 cell line in both of which apoptosis was induced, not only by cross-linking Fas directly but also via CD40 activation. Our data suggest that CD40 activation induces apoptosis via Fas because (a) ligation of CD40 upregulated hepatocyte FasL expression, and (b) apoptosis induced via activation of CD40 was prevented by a neutralizing monoclonal antibody to FasL. Thus, CD40 engagement triggers apoptosis of human hepatocytes and might amplify Fas-dependent hepatocyte apoptosis in chronic rejection and other inflammatory liver diseases in which Fas-mediated apoptosis is involved.

Key words: CD40 activation • apoptosis • hepatocytes • allograft rejection

Chronic rejection affects up to 10% of patients after liver transplantation and is the major indication for retransplantation (1, 2). It fails to respond to increased immunosuppression and is defined histologically by a progressive loss of intrahepatic bile ducts, an occlusive arteriopathy, and severe cholestasis associated with dropout of centrilobular hepatocytes in acinar zone 3 (2). The pathogenesis of hepatocyte loss is unknown, although apoptosis of affected hepatocytes has been reported (3, 4). In the present study, we investigated the underlying mechanisms and propose that hepatocyte apoptosis occurs in response to a cooperative interaction between Fas (CD95/Apo-1) and CD40, two members of the TNF receptor superfamily that have important roles in regulating cell proliferation and death (5–8).

Ligation of cell membrane Fas triggers apoptosis in many cell types, including hepatocytes (5, 9), and FasL expression in tissue is therefore tightly regulated and largely restricted to immune-privileged sites such as the testis and eye (10). Fas is involved in regulating hepatocyte survival, and administration of anti-Fas Ab causes panacinar hepatocyte death and acute liver failure, which can be prevented by transfecting hepatocytes with the survival gene bcl-2 (9, 11). Inhibition of Fas on hepatocytes prevents fulminant hepatitis in murine models of hepatitis B, and Fas has also been implicated in hepatocyte apoptosis in alcoholic liver disease and Wilson's disease (12–15).

CD40 is expressed on subsets of leukocytes, endothelium, and some epithelial cell lines (7, 16). Its ligand, CD40L (CD154), is expressed on activated T cells, dendritic cells, and some endothelial cells and can engage CD40 as a membrane-bound or soluble ligand (16–18). CD40–CD40L interactions provide critical costimulatory signals for clonal T cell expansion and promote T cell–dependent activation of B cells, CTLs, and macrophages (6, 7, 17, 19). Ligation of endothelial CD40 increases adhesion molecule expression, suggesting a wider role in lymphocyte recruitment (18). It has been shown recently that ligation of CD40 on epithelial cell lines can inhibit growth (16, 20),

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/99/01/441/06 $2.00
Volume 189, Number 2, January 18, 1999 441–446
http://www.jem.org

although little is known about the expression or function of CD40/CD40L in the liver.

Materials and Methods

Liver Tissue. Needle biopsies were obtained from 24 liver transplant recipients of whom 6 had mild acute rejection (defined as a portal infiltrate on day 7 protocol biopsies in the absence of biochemical graft dysfunction); 6 had severe acute rejection defined histologically (2) and associated with biochemical dysfunction. Six had chronic rejection, defined by standard histological and biochemical criteria (1, 2) and a failure to respond to at least two cycles of high-dose corticosteroid. Six had stable graft function (normal liver function >1 yr after transplant). Maintenance immunosuppression was with corticosteroids, azathioprine, and either cyclosporin or tacrolimus. Acute rejection episodes were treated with three daily doses of 200 mg prednisolone or 1 g methylprednisolone. Surplus liver tissue from donor organs was used as nondisease controls ($n = 6$). Tissue was snap frozen in CRYO-M-BED mounting medium (Bright Scientific Instruments Ltd.) and stored at −70°C.

Immunohistochemistry. Standard techniques were used to stain acetone-fixed 4-μ cryostat tissue sections (21). Polyclonal rabbit Abs specific for Fas or FasL (Santa Cruz Biotechnology, Inc.) were used at 1:50 for 1 h at room temperature and detected using 1:100 alkaline phosphatase–labeled swine anti–rabbit Ab visualized with Fast red (Dako). CD40 was detected with an IgG1 mAb (16) and CD40L with an IgG2a mAb (a gift from Dr. R. Armitage, Immunex Corp.). Primary mAb was localized using an ABC detection kit (Vectastain; Novacastra Labs). The primary Ab was omitted in control sections. Dual immunofluorescence was used to characterize CD40L-bearing cells. CD40L was detected with FITC-labeled goat anti–mouse IgG2a secondary Ab at 1:50, and the cell type was determined by double staining for fibroblasts (IgG1 mAb; Dianova Labs); B cells (IgG1 mAb CD22; Dako), T cells (rabbit anti-CD3; Dako), NK cells (IgG1 mAb CD56; Dako), macrophages (IgG1 mAb anti-CD68; Dako), and endothelium (IgG1 mAb anti-CD31, clone NIH31.1; a gift from S. Shaw, National Institutes of Health) were detected with 1:50 TRITC-labeled goat anti–rabbit Ig or goat anti–mouse IgG1 (EuroPath, Cornwall, UK). Sections were blocked with 10% FCS before primary Ab. In control sections, the primary Ab was replaced by nonimmune serum. Dual staining cells were detected by confocal microscopy (21).

Histological Assessment. Staining was assessed blindly by S.G. Hubscher and S.C. Afford using a previously validated semiquantitative scale where − denotes no staining relative to control; + weak, equivocal positivity, and +++ maximum staining (21). The distribution of staining on liver cell types was recorded.

Hepatocyte Studies. Hepatocytes were isolated from human donor livers that were surplus to clinical requirements using collagenase perfusion and Percoll density gradient centrifugation. Immediately after isolation, cells were resuspended in Williams E medium containing hydrocortisone, insulin, glutamine (22) and allowed to adhere for 2 h. Medium was then replaced, and cells rested for 24 h before stimulation for 24 h with TNF-α (10 ng/ml), IL-1 (100 U/ml), anti-Fas Ab (1:25), 1–5 μg/ml purified soluble recombinant CD40L (a gift from Dr. R. Armitage), anti-CD40 mAb (1:100), or both anti-CD40 and TNF-α. In some experiments, 40 ng/ml mAb NOK-1 (PharMingen) was used to neutralize FasL. Cells were harvested by trypsinization for 5 min with 0.25% vol/vol bovine pancreatic trypsin neutralized by 10% FCS. In control experiments ($n = 9$), untreated hepatocytes were sampled at 2 h after plating and subsequently at 24-h time intervals for up to 4 d, and viability was determined by trypan blue dye exclusion. The HepG2 cell line also expresses CD40 (23), and the effects of CD40 activation and FasL neutralization were studied on HepG2 cells cultured in DMEM plus 10% FCS and 2 mM l-glutamine.

Assessment of Apoptosis. Apoptosis was quantified on hepatocyte and HepG2 cytospins using morphological criteria (cell shrinkage, chromatin condensation, Councilman bodies) and in situ DNA end labeling (ISEL) for detection of fragmented DNA (3, 24).

Flow Cytometry. Hepatocytes were assessed for Fas, FasL, CD40, or CD40L expression using a Coulter XL flow cytometer and the above Ab followed by 1:20 FITC-labeled rabbit anti–mouse Ig or FITC goat anti–rabbit Ig (Dako). Controls were isotype-matched Ig or nonimmune serum (25).

Results

Expression of CD40, CD40L, Fas, and FasL in Human Liver Allografts. In normal liver there was no detectable CD40, CD40L, or FasL expression and only very weak diffuse expression of Fas in hepatocytes. Stable allografts or those with mild rejection showed similar staining, with very weak hepatocyte Fas expression and no detectable FasL or CD40L. In mild rejection weak CD40 and moderate CD40L staining was confined to the portal infiltrate, whereas in severe rejection CD40 was also detected on vascular and sinusoidal endothelium (hepatocytes and biliary cells were largely negative) and strong CD40L staining was seen on the portal infiltrate (Table I, and Fig. 1). In chronic rejection CD40 stained strongly on centrilobular hepatocytes and sinusoidal endothelium, and both CD40 and

Table I. *Expression of CD40, CD40L, Fas, and FasL in Liver Allografts*

	CD40	CD40L	Fas	FasL
Severe Acute Rejection				
BD	+	−	+	+
VEC	++	−	++	++
SEC	+++	−	++	++
HEPS	−	−	++	++
INF	+++	+++	+++	+++
Chronic Rejection				
BD	+	−	+	+
VEC	−	−	−	−
SEC	+++	−	+++	+++
HEPS	+++	−	+++	+++
INF	+++	+++	++	+++

CD40, Fas, and their respective ligands were increased (compared with normal tissue or tissue from patients with mild rejection) during severe acute and chronic rejection. Strong expression of CD40 in centrilobular hepatocytes was only seen in chronic rejection, and CD40L expression was confined to the inflammatory infiltrate. Surviving intrahepatic bile ducts were weakly positive for CD40, Fas, and FasL during rejection. BD, bile ducts; VEC, vascular endothelium; SEC, sinusoids; HEPS, hepatocytes; INF, inflammatory cells.

CD40L were detected on the portal inflammatory infiltrate. In addition, centrilobular areas contained large numbers of $CD40L^+/CD68^+$ macrophages (Fig. 2). Fas staining in mild acute rejection was weak and restricted to hepatocytes, whereas in severe acute rejection it was also detected on vascular and sinusoidal endothelium and more strongly on hepatocytes throughout the lobule. FasL was absent in mild acute rejection but was detected on vascular and sinusoidal endothelium and hepatocytes in severe acute rejection when the portal infiltrate was also strongly positive. In patients who had progressed to chronic rejection, Fas expression persisted on sinusoidal endothelium and hepatocytes, with particularly intense staining associated with hepatocyte loss in centrilobular regions. FasL was detected on endothelium and on centrilobular hepatocytes (Table I, and Fig. 1).

Expression of Fas, FasL, CD40, and CD40L on Isolated Hepatocytes. In the absence of activation, cultured human hepatocytes expressed Fas (64.5 ± 3.7% positive), CD40 (33 ± 6.8%), and low levels of FasL (7.9 ± 2.6%) but not CD40L (1.1 ± 0.5%). Stimulation with TNF-α, IFN-γ, IL-1, TGF-β, or LPS or activation of CD40 did not change levels of cell surface Fas or CD40 and failed to induce CD40L (data not shown), whereas cell surface FasL expression increased after treatment with IL-1, anti-CD40, or anti-Fas. The largest increase in FasL was seen after CD40 ligation (Fig. 3).

The Effect of Activation of Fas or CD40 on Hepatocyte Apoptosis. The viability of the untreated hepatocytes at 2 h was 99.6 ± 0.4% and remained high beyond the duration of the experiment (viability at 4 d 98.1 ± 0.6%). Activation of either Fas or CD40 with cross-linking Ab induced hepatocyte apoptosis, and similar results were seen using soluble CD40L (Fig. 4). CD40-mediated apoptosis was prevented by addition of NOK-1, the neutralizing Ab to FasL. Comparable results were obtained using the HepG2 cell line (Fig. 4).

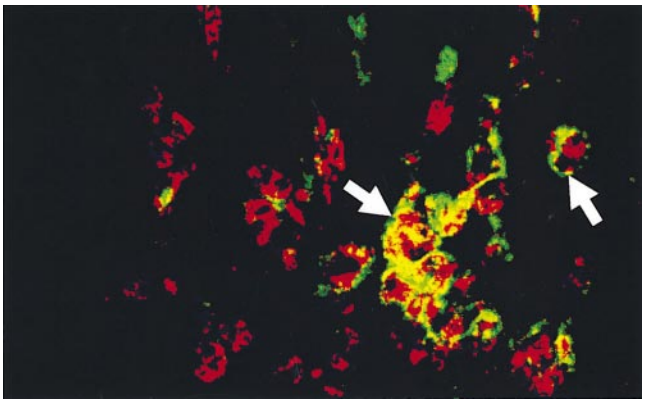

Figure 2. $CD40L^+$ cells in chronic rejection are $CD68^+$ macrophages. Dual staining was carried out using FITC-labeled anti-CD40L and TRITC-labeled anti-CD68 in chronic rejection. High-magnification sections of the centrilobular area are shown analyzed by confocal microscopy. Red, $CD40^-/CD68^+$ cells; green, $CD40L^+/CD68^-$ cells; yellow (marked with arrows), CD40L/CD68 double-positive cells.

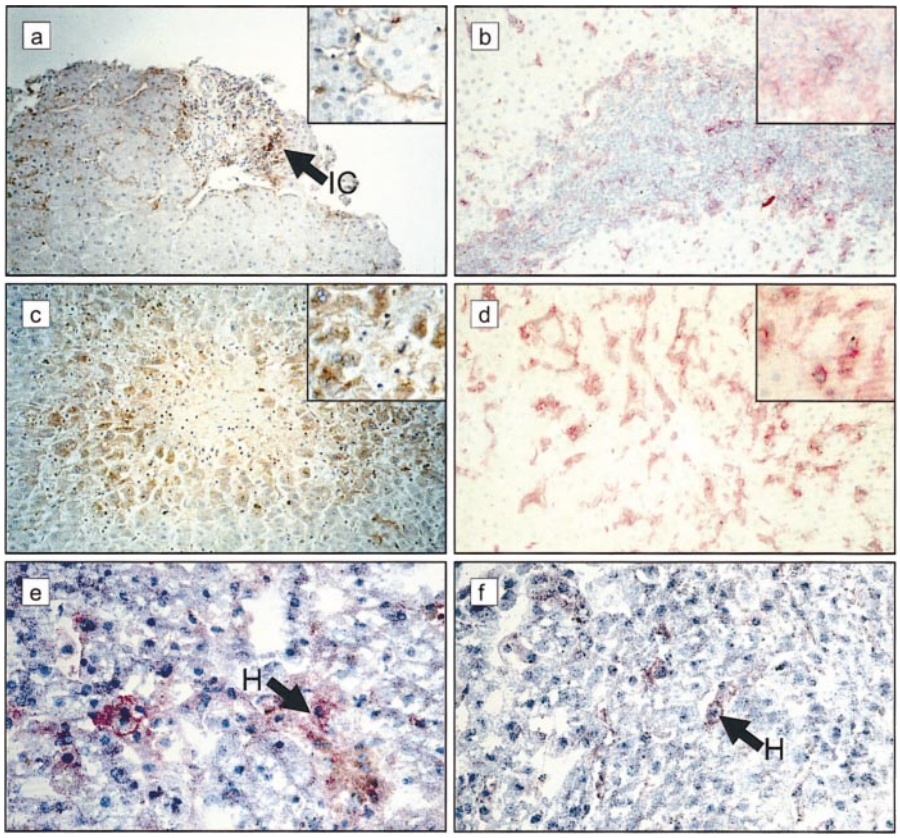

Figure 1. CD40, CD40L, Fas, and FasL are increased in severe acute and chronic rejection. (a) In severe acute rejection CD40 expression was strongest in inflammatory cells (IC) and in sinusoids, which are also shown in the higher magnification (inset). (b) CD40L was confined to inflammatory cells. In chronic rejection centrilobular hepatocytes (also shown in the inset at higher magnification) were strongly positive for CD40 (c), and surrounding inflammatory cells were $CD40L^+$ (d). Hepatocyte expression of Fas (e) and FasL (f) was concentrated in centrilobular areas in chronic rejection. H, positive stained hepatocytes.

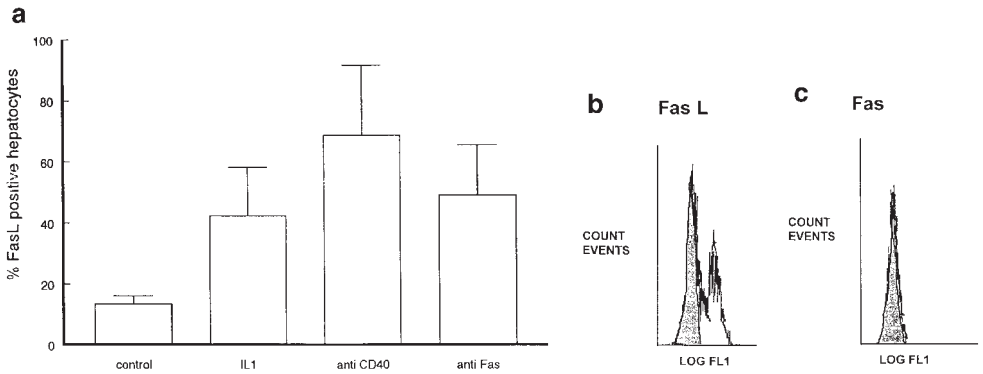

Figure 3. FasL expression is increased on primary human hepatocytes in vitro after CD40 activation. Expression of FasL in resting cells was low but increased significantly after activation by IL-1, anti-Fas, or anti-CD40. Data shown are percentage of cells positive by flow cytometry (a). CD40 activation did not affect Fas expression. Histograms from a representative experiment for FasL (b) and Fas (c) before (shaded) and after CD40 activation are shown.

Discussion

In this study we show that Fas/FasL and CD40/CD40L are increased in the human liver allograft during rejection, and provide evidence that these pathways cooperate in mediating hepatocyte death in chronic allograft rejection. We confirm the ability of Fas activation to induce apoptosis of human hepatocytes in vitro (9, 11, 12) and in addition show for the first time that engagement of hepatocyte CD40 by Ab or soluble CD40L is an equally potent inducer of apoptosis. The ability of CD40 ligation to induce hepatocyte death was surprising, given that the CD40 cytoplasmic tail does not contain a conventional death domain, and suggested to us that an indirect mechanism might be operating. We propose that this mechanism involves activation of Fas by autocrine or paracrine FasL because (a) CD40 ligation induces hepatocyte FasL expression, thereby providing the ligand to activate Fas on autologous or adjacent hepatocytes, and (b) CD40-induced apoptosis was blocked by a neutralizing anti-FasL Ab. Thus CD40 engagement can trigger Fas-dependent apoptosis of human hepatocytes and the HepG2 cell line, suggesting that this is an important mechanism for apoptotic death in cells of hepatocyte lineage.

The Fas pathway has been implicated in hepatocyte apoptosis in several diseases (12–15). In normal human liver we observed only weak Fas expression on hepatocytes and no FasL. Findings were similar in biopsies from stable transplants but not in those from patients with allograft rejection in whom striking upregulation of both Fas and FasL was observed. CD40 was not detected in normal liver, but in chronic allograft rejection it was upregulated on hepatic endothelium, bile ducts, and particularly on centrilobular hepatocytes in areas of hepatocyte loss. That hepatocyte CD40 expression was only seen in chronic rejection whereas Fas was detected in both acute and chronic rejection suggests that the coexpression of Fas and CD40 might be a factor in determining progression from acute to chronic rejection.

Fas and FasL expression was also detected on the portal infiltrate in acute rejection, reflecting the presence of activated lymphocytes within the graft. Although increased Fas expression by hepatocytes could increase their susceptibility to cytolysis by FasL-bearing CTLs, hepatocyte damage is not a conspicuous feature of acute rejection and our previous studies showed little hepatocyte apoptosis in acute rejection (3). Coexpression of CD40 and Fas on centrilobular hepatocytes was only seen in chronic rejection, and it is these patients who develop hepatocyte apoptosis. This suggests that these molecules cooperate to cause target cell damage and thus determine the severity of allograft rejection. The present study provides a molecular mechanism to explain this. Isolated primary human hepatocytes expressed both CD40 and Fas in culture, allowing us to determine the consequences of engaging hepatocyte cell surface Fas and CD40. The expression of CD40 and Fas on human hepatocytes in culture is at odds with the apparent absence of staining in normal tissue. This anomaly is probably due to activation induced by the isolation and culture procedures, because previous studies have shown CD40 upregulation on cultured epithelial cell lines (16, 20).

Cross-linking of hepatocyte Fas in vitro induced a high level of apoptosis, suggesting that FasL-bearing cells could promote hepatocyte apoptosis in chronic rejection. However, we detected relatively few $FasL^+$ lymphocytes in chronic rejection despite many apoptotic hepatocytes, which led us to look for an alternative source of FasL. Under some circumstances hepatocytes express FasL which can cause Fas-mediated suicide or fratricide (14, 15), and we thought a similar mechanism could be involved in chronic rejection where centrilobular hepatocytes coexpress Fas and FasL. Moreover, activation of hepatocytes in vitro with cross-linking Ab resulted in an increase in hepatocyte cell surface FasL. IL-1 also upregulated cell surface FasL (Fig. 3 a) and induced apoptosis in primary human hepatocytes (data not shown), and this has been previously reported in other types of epithelial cells (26). Centrilobular hepatocytes in chronic rejection also stained strongly for CD40, and cross-linking of CD40 caused hepatocyte apoptosis that was comparable to that seen with direct Fas activation (Fig. 4). Because the Fas pathway is known to be a potent inducer of hepatocyte apoptosis (9, 11, 12), we investigated the consequences of CD40 ligation on FasL expression in isolated human hepatocytes.

Engagement of CD40 led to increased expression of cell surface FasL and to induction of apoptosis that was comparable to that induced by Fas cross-linking. Furthermore, CD40-induced apoptosis could be prevented by a neutralizing Ab to FasL, providing evidence of a direct link between CD40 and Fas in the regulation of apoptosis. The

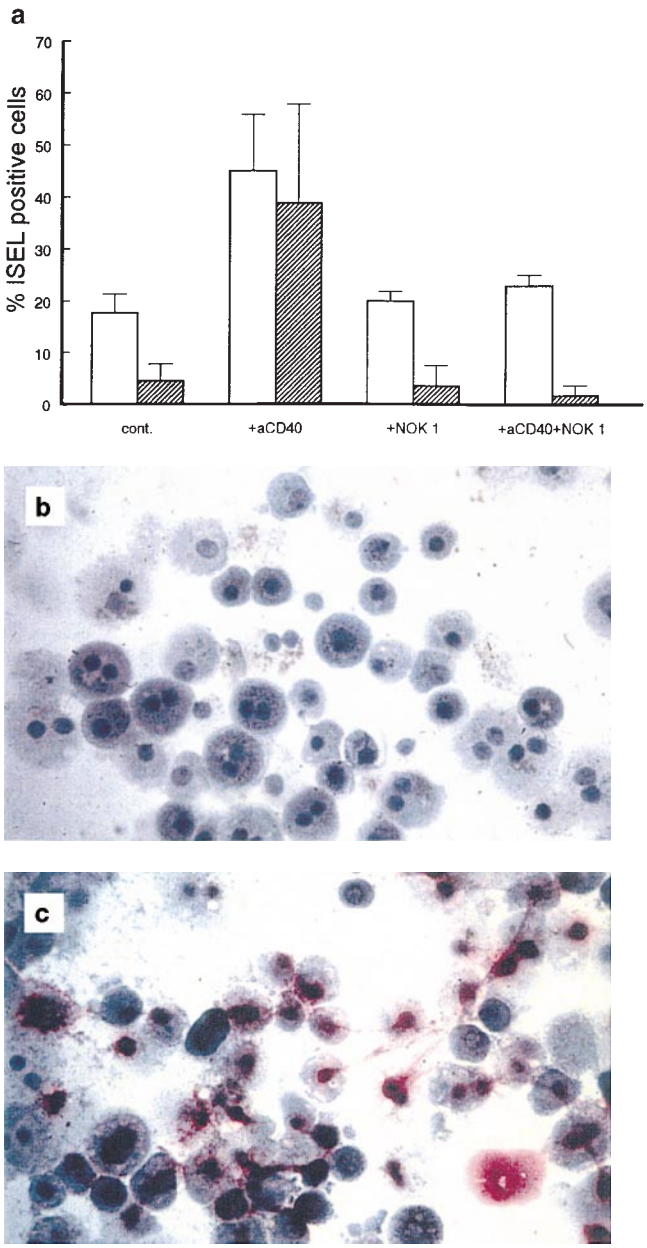

Figure 4. CD40 activation triggers FasL-dependent hepatocyte and HepG2 apoptosis. (a) Activation of CD40 with mAb induced apoptosis in hepatocytes and HepG2 cells. Treatment of unstimulated cells with NOK-1, a neutralizing Ab to FasL, alone had no effect, whereas NOK-1 inhibited apoptosis induced via CD40 activation. Results shown as percentage of cells positive by ISEL staining. White bars, results for primary hepatocytes; hatched bars, results for HepG2 cells. (b and c) ISEL staining of untreated hepatocytes or cells treated with 1 μg/ml soluble trimeric CD40L, respectively, demonstrating that soluble CD40L also induced hepatocyte apoptosis.

ability of hepatocytes to induce FasL-mediated apoptosis after engagement of CD40 suggests that CD40 promotes apoptosis via autocrine or fratricrine activation of Fas. It is notable that CD40 and Fas expression on centrilobular hepatocytes was seen in chronic rejection but not in severe acute rejection where the cells expressed Fas but not CD40. Hepatocyte apoptosis is not a conspicuous feature of acute rejection, and it is possible that Fas expression alone does not provide a potent enough signal.

Thus, CD40 may be a critical factor in the amplification of hepatocyte apoptosis in chronic rejection, and we subsequently looked for a cellular source of CD40L. Hepatocytes did not express CD40L protein or mRNA in vitro even after cytokine stimulation, and immunostaining of hepatocytes in tissue sections was consistently negative. However, CD40L was detected on $CD68^+$ macrophages/Kupffer cells in the centrilobular areas. In contrast very few infiltrating $CD3^+$ T cells expressed CD40L. Thus, CD40L/$CD68^+$ macrophages could provide the ligand for activation of hepatocyte CD40 in chronic rejection. This model, in which continuing hepatocyte damage is promoted by activated macrophages rather than by cytolytic T cells, could explain why centrilobular hepatocyte dropout continues in chronic rejection in the absence of a marked lymphocytic infiltrate, and why chronic rejection often fails to respond to antilymphocyte therapy (1).

In severe acute rejection CD40 was detected strongly on graft endothelium, whereas it was not detected in patients with mild self-limiting rejection, suggesting that CD40/CD40L might also be involved in amplifying and maintaining lymphocyte recruitment during graft rejection. In support of this, recent studies have shown that ligation of CD40 on cultured endothelial cells results in increased expression of adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1 [18]), that are expressed at high levels on endothelium in graft rejection. Thus, the induction and maintenance of these molecules might be a consequence of CD40 ligation on graft endothelium (27, 28).

Until now, little was known about the expression and function of CD40 in human liver. Our studies demonstrate a novel mechanism by which CD40L-bearing cells can amplify hepatocyte apoptosis through a Fas-dependent mechanism. A similar mechanism will likely apply to other diseases in which hepatocytes are destroyed by Fas activation, and in support of this we have recently detected CD40 on hepatocytes in viral hepatitis and alcoholic liver disease (12–15; Afford, S.C., P.L. Shields, and D.H. Adams, unpublished observations). We thus propose that this model is a mechanism of liver damage in a broad range of inflammatory liver diseases.

We thank Janine Fear, Ann Williams, and Adrian Keogh for excellent technical assistance; the Liver Unit Nursing and Clinical staff for procurement of human liver tissue (which was approved by the local ethics committee); and J. Neuberger and S. Adams for critical review of the manuscript.

This work was supported by grants from the Wellcome Trust, the Endowment Fund of the former United Birmingham Hospitals, and the Cancer Research Campaign.

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Received for publication 10 July 1998 and in revised form 14 October 1998.

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