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Hepatitis C-induced hepatocyte apoptosis following liver transplantation is enhanced by immunosuppressive agents

Running title: Immunosuppressant and HCV-induced cell death

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Abstract

In recurrent hepatitis C (HCV) post-liver transplantation (OLT), the combination of immunosuppressants and HCV is postulated to increase hepatocyte apoptosis and liver fibrosis. We evaluated hepatocyte apoptosis within the liver tissue of patients with post-OLT HCV recurrence compared to HCV-negative individuals and correlated these findings with the effects of immunosuppressants on HCV-induced cell death and its inhibition in primary mouse hepatocytes (PMoH). Liver biopsies from patients with and without HCV were evaluated by immunohistochemistry for markers of apoptosis M30 CytoDEATH (M30) and cleaved PARP (cIPARP). PMoH from C57BL/6 mice were infected with recombinant adenoviruses (rAdHCV) that expressed HCV proteins in hepatocytes. Infected cells were treated with cyclosporine, tacrolimus, sirolimus, and/or MMF with or without pan-caspase inhibitor Q-VD-Oph. Cell viability and apoptosis were evaluated using crystal violet assays and Western immunoblots probed for cleaved caspase 3 (cICas3) and cIPARP. Both M30 and cIPARP were increased in the liver biopsies of patients with post-OLT HCV recurrence compared to HCV-negative individuals. Treatment of rAdHCV-infected PMoH with cyclosporine, tacrolimus, or sirolimus reduced cell viability and increased cICas3 and cIPARP compared to rAdHCV infection alone. Addition of MMF to cyclosporine, tacrolimus or sirolimus further reduced cell viability and increased cICas3 and cIPARP. Q-VD-Oph improved cell viability in HCV-infected PMoH treated with immunosuppressants alone and in combination and reduced cICas3 and cIPARP by approximately 90%. Immunosuppressive agents, especially in combination, enhanced apoptosis in HCV-infected hepatocytes. The finding that Q-VD-Oph reversed hepatocyte death suggests that treatments utilizing apoptosis inhibition might reduce liver injury in post-OLT HCV recurrence.

Keywords:

Cyclosporine

Tacrolimus

Mycophenolate

Sirolimus

Liver allograft

Introduction

Hepatitis C virus (HCV) infection is a chronic disease affecting over 170 million individuals worldwide, accounting for approximately 476,000 deaths annually(1). HCV-related liver failure is now the commonest indication for liver transplantation in the US, Europe and Australia(2, 3). Despite significant advances in the treatment of chronic HCV with the advent of direct-acting antiviral agents, recurrent HCV disease in the HCV-infected liver transplant population remains a major challenge. Patients who develop HCV recurrence following liver transplantation have more aggressive liver disease compared to patients with chronic HCV in the non-transplant population. Indeed, more than 20% of patients transplanted for end-stage liver disease due to HCV develop cirrhosis again within 5 years of liver transplantation(4).

There is increasing evidence to suggest that liver damage in chronic HCV infection is associated with the induction of hepatocyte apoptosis(5, 6). We have previously shown that hepatocyte apoptosis is increased within the livers of patients with chronic HCV and that HCV infection *in vitro* is associated with enhanced hepatocyte cell death(7). Furthermore, we found that combinations of the immunosuppressive drugs most commonly used after liver transplantation promote hepatocyte apoptosis independently of HCV(8). These immunosuppressive agents have also been shown to induce apoptosis in numerous non-hepatocyte cell lines. Cyclosporine has been shown to induce endoplasmic reticulum stress (9), tacrolimus was noted to promote the generation of reactive oxygen species and induce mitochondrial dysfunction (10), sirolimus was found to inhibit anti-apoptotic NF- κ B (11), and MMF was found to promote epithelial apoptosis mimicking graft-versus-host-disease (12). It is therefore possible that these immunosuppressive agents may interact with HCV to enhance hepatocyte apoptosis and thereby contribute to the accelerated hepatic fibrosis that occurs in post-transplant HCV recurrence.

Here we evaluated hepatocyte apoptosis within the liver tissue of patients on immunosuppressive agents following liver transplantation in the presence or absence of hepatitis C infection and compared this to liver tissue of normal individuals without

liver disease. We correlated these findings with changes in primary mouse hepatocytes using replication-defective recombinant adenoviruses (rAdHCV) expressing the complete structural or non-structural proteins of HCV together with various combinations of immunosuppressive drugs to determine whether these drugs could enhance HCV-induced hepatocyte apoptosis. Finally, we investigated the effect of inhibition of apoptosis by the pan-caspase inhibitor Q-VD-Oph, and therefore the reversibility of the pro-apoptotic effects of HCV and immunosuppressive drugs.

Materials and methods

Immunohistochemistry of human liver specimens

Human liver tissue was stained for the markers of apoptosis cleaved cytokeratin 18 (M30 CytoDEATH, Enzo Life Sciences) and cleaved PARP (Cell Signaling Technology). Immunohistochemistry was performed as previously described(13). In brief, 4 µm sections of paraffin-embedded human liver tissue mounted on silane-coated glass slides were de-paraffinized in histolene and dehydrated in graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS. Non-specific proteins were blocked with Protein Block Serum-free (DakoCytomation) for 30 minutes at room temperature. Blocked tissues were incubated overnight at 4°C with either M30 CytoDEATH or cleaved PARP antibody, 1:100 in diluent as directed by the manufacturer. The following day, sections were incubated with their respective biotinylated-conjugated secondary antibody (1:200) for 1 hour at room temperature, followed by incubation with avidin–biotin Vectastain ABC system (Vector Laboratories) for 30 minutes. Diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich) was then added as a chromogen and sections counterstained in haematoxylin. The relative staining in each group was assessed by computerized image capture quantification using the MCID Analysis software (InterFocus Imaging) and the results expressed as the proportional area stained, which is the proportion of cells staining positive in the given area.

Preparation of Primary mouse Hepatocytes

Primary mouse hepatocytes (PMoH) were isolated from up to 12-week-old C57BL/6 mice, as previously described(14). In brief, PMoH were extracted via ex-vivo perfusion of the liver lobes with HEPES buffer (Invitrogen), followed by HEPES containing 500 mg/l collagenase IV (Sigma-Aldrich). Hepatocytes were separated on a 45% Percoll density gradient (Sigma-Aldrich) and seeded at a density of 50,000 cells/cm² in cell culture plates coated with 0.6 mg/ml rat-tail collagen (Sigma-Aldrich) and cultured overnight. Prior to infection with recombinant adenoviruses, non-adherent cells were washed off and adherent cells incubated with complete Williams E medium supplemented with 1% HEPES pH 7.4 (Invitrogen), 0.1% gentamicin (Invitrogen), 1% glutamine (Invitrogen), 1% linoleic acid (Sigma-Aldrich), 1% epidermal growth factor (EGF) (BD BioScience), 0.1% ITS (Sigma-Aldrich), 0.1% insulin (Sigma-Aldrich), 0.01% dexamethasone (Sigma-Aldrich) and 0.01% ethanolamine (Sigma-Aldrich).

Hepatocyte infections

Hepatocytes were infected with the recombinant adenoviruses expressing either the structural (rAdHCV-CoreE1E2) or non-structural (rAdHCV-NS3-5B) genes of HCV. Construction and characterization of these viruses has been described previously(7). Entry of recombinant adenoviruses into hepatocytes and production of HCV RNA and proteins was confirmed by green fluorescence protein (GFP) expression. A recombinant adenovirus expressing GFP alone (rAdGFP) was used as a negative control virus.

For mono-infections, hepatocytes were infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B or rAdGFP at a MOI of 1.0. For co-infection with both rAdHCV-CoreE1E2 and rAdHCV-NS3-5B (rAdHCV co-infection), a MOI of 0.5 for each rAdHCV-CoreE1E2 and rAdHCV-NS3-5B was used. PMoH were infected at 18 hr after harvesting and initial plating. In experiments examining inhibition of apoptosis, cells were treated at the time of viral infection with 50 μ M Q-VD-OPh (R&D Systems). Controls were treated with 0.5% DMSO. All experiments were performed in triplicate.

Hepatocyte treatment with immunosuppressive agents

PMoH were treated with immunosuppressive agents at 18 hr post-plating. Therapeutically relevant concentrations of cyclosporine (NeoralTM; a gift from

Novartis), tacrolimus (FK-506, F4679; Sigma), sirolimus (Rapamycin, R8781; Sigma), and MMF (CellceptTM; a gift from Roche) were employed. Combinations of drugs were selected to mimic immunosuppressant regimens commonly used in the post-liver transplant setting. Specifically, 1 µg/ml of cyclosporine, 0.005 µg/ml of tacrolimus, 0.01 µg/ml of sirolimus, and 5 µg/ml of MMF were employed.

Western immunoblot detection of cellular proteins

PMoH were harvested at 48 hr post-treatment in 200 µl of ice-cold cell lysis buffer (Cell Signaling Technology) supplemented with 1 mM sodium molybdate, 5 mM sodium fluoride, 1M DTT (Sigma-Aldrich) and 1x Complete Protease Inhibitor (Roche). Thirty micrograms of total cytoplasmic proteins were resolved by 12% denaturing SDS-PAGE gel, transferred into Hybond-C Extra membrane (GE Healthcare) and analyzed by immunoblotting. Induction of apoptosis was detected using anti-caspase 3 (Cell Signaling Technology) and anti-cleaved PARP (poly ADP-ribose polymerase) antibody (Abcam). Anti-pan-actin was used as a loading control. Immunoblots were analyzed with a BioRad GS800 densitometer using the Quantity One software.

Cell Viability assays

Cell viability post-treatment with immunosuppressive agents was determined using crystal violet assays. PMoH (2.5×10^5 cells/well) were seeded in 12-well plates and treated with various concentrations of the immunosuppressants. At 24, 48 and 72 hr post-treatment, wells were washed twice with sterile PBS and cells were fixed and stained with 0.1% crystal violet in 1M citric acid containing 20% methanol for 20 minutes at room temperature. Wells were washed thoroughly with sterile PBS to remove excess crystal violet and then air-dried. Bound dye was solubilized with 100 µl 100% DMSO for 20 minutes and the absorbance of the supernatants was measured at 544 nm using the FluoStar Optima (BMG LabTech) plate reader. Statistical analyses were performed using Prism 5.0 (GraphPad Software).

Statistical analysis

Statistical analysis was performed using the Prism 5.0 software (GraphPad). In all cases the mean ± standard error of the mean (SEM) is shown unless otherwise

stated. P values for statistical analysis were calculated using a one-tailed Mann-Whitney U test. Differences were considered statistically significant when p values were less than 0.05 ($p < 0.05$) with a 95% confidence level.

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The PMoH protocol was approved by the Animal Ethics Committee of La Trobe University (permit Number: 09-14 B). The study using human liver tissue was approved by the Austin Health Human Research Ethics Committee (HREC approval number H2010/03979). Written informed consent was obtained from the patients for the use of their liver samples in research.

Results

Hepatocyte apoptosis is increased in liver tissue of patients with post-liver transplant HCV recurrence

Liver biopsies collected 6 to 26 months after transplantation from 10 patients who underwent liver transplantation for end-stage HCV-associated cirrhosis were evaluated for the markers of apoptosis, M30 CytoDEATH and cleaved PARP. These were compared to liver biopsies from 3 patients who cleared HCV prior to undergoing liver transplantation and remained HCV-negative following transplantation, 10 patients who were transplanted for end-stage cirrhosis related to alcoholic liver disease, and 9 patients who were undergoing liver resection for colorectal metastasis (control group). The control patients did not have any underlying liver disease and the biopsies were taken from normal liver away from the site of metastatic tumour. Clinical and demographic data on these patients can be found in supplementary table 1.

The level of M30 CytoDEATH in liver tissue of patients with HCV-recurrence after transplantation was 8.3-fold higher than patients transplanted for end-stage cirrhosis who cleared HCV ($p = 0.004$), 6.9-fold higher than patients transplanted for end-stage alcoholic cirrhosis ($p < 0.0001$), and 33.7-fold higher than normal subjects ($p < 0.0001$) (Figure 1A). The levels of cleaved PARP were increased 6.5-fold in the livers of

patients with HCV-recurrence after transplantation (Figure 1B) compared to patients transplanted for end-stage cirrhosis who cleared HCV prior to transplantation ($p=0.004$), 6.8-fold higher than patients transplanted for end-stage alcoholic cirrhosis ($p<0.0001$), and 10.6-fold higher than normal subjects ($p<0.0001$).

Cyclosporine enhances HCV-induced cell death in hepatocytes

To understand whether the immunosuppressive agents used in our patients may have promoted HCV-induced hepatocyte cell death, we then investigated the effects of these agents on apoptosis in rAdHCV-infected primary mouse hepatocytes (PMoH) in culture. The effect of cyclosporine on the viability of hepatocytes infected with rAdHCV was studied using crystal violet assays. PMoH were co-infected with rAdHCV-CoreE1E2 and rAdHCV-NS3-5B to express both the structural and non-structural HCV proteins as would occur within the hepatocytes of an infected patient. Co-infected PMoH treated with cyclosporine had a 37% ($\pm$ SEM 0.88) reduction in cell viability at 48 hours compared to untreated hepatocytes, an effect significantly greater than rAdHCV co-infection alone ($p<0.05$) (Figure 2A).

We then investigated the relative contribution of structural and non-structural HCV proteins to the effects of HCV infection and cyclosporine on PMoH apoptosis (Figure 2B). Cleaved PARP levels were increased 4.2-fold ($\pm$ SEM 0.47), 4.1-fold ($\pm$ SEM 0.50), and 4.4-fold ($\pm$ SEM 0.50) ($p<0.05$ for all 3), and cleaved caspase 3 levels were increased by 2.1-fold ($\pm$ SEM 0.36), 2.0-fold ($\pm$ SEM 0.29), and 2.2-fold ($\pm$ SEM 0.39) ($p<0.05$ for all 3) respectively compared to rAdGFP infection. We found that there was no difference between rAdHCV-CoreE1E2, rAdHCV-NS3-5B or co-infection with regards to the combined effects of viral protein and cyclosporine.

MMF does not significantly enhance HCV-induced cell death in hepatocytes

In PMoH co-infected with rAdHCV-CoreE1E2 and rAdHCV-NS3-5B, and treated with 5 μ g/ml of MMF, a 30% ($\pm$ SEM 1.61) reduction in cell viability compared to untreated cells was observed at 48 hours (Figure 2C), but this did not achieve significance against rAdHCV co-infection alone ($p=0.10$). In MMF-treated PMoH that were infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B, or co-infected with both, cleaved PARP levels were 2.1-fold ($\pm$ SEM 0.29), 2.3-fold ($\pm$ SEM 0.26), and 2.4-fold ($\pm$ SEM

0.28) higher ($p < 0.05$ for all 3), and cleaved caspase 3 levels were 1.6-fold ($\pm$ SEM 0.07), 1.7-fold ($\pm$ SEM 0.06), and 1.6-fold ($\pm$ SEM 0.04) higher ($p < 0.05$ for all 3), compared to rAdGFP-infected hepatocytes treated with MMF at 48 hours (Figure 2D). We again found that there was no difference between rAdHCV-CoreE1E2, rAdHCV-NS3-5B or co-infection with regards to the combined effects of viral protein and MMF.

The combination of cyclosporine and MMF further enhances HCV-induced cell death in hepatocytes

Post-liver transplantation, the combination of cyclosporine and MMF is commonly used as an immunosuppressive regimen. To investigate the effect this combination has on cell death in HCV-infected hepatocytes, rAdHCV-infected cells were treated with cyclosporine and/or MMF, and compared to treated hepatocytes infected with the control virus rAdGFP, and untreated uninfected hepatocytes. Treatment of rAdHCV co-infected PMoH with both cyclosporine and MMF resulted in a 47% ($\pm$ SEM 1.65) decrease in cell viability at 48 hours compared to untreated uninfected PMoH ($p < 0.05$), an effect greater than either cyclosporine or MMF treatment alone ($p < 0.05$ for both) (Figure 3A). These results indicate that the combination of cyclosporine and MMF further enhanced HCV-induced reduction of cell viability in primary hepatocytes and this was greater than the effect seen with either cyclosporine or MMF alone.

In PMoH infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B, or co-infected with both viruses, treatment with cyclosporine plus MMF resulted in a 2.4-fold ($\pm$ SEM 0.40), 2.7-fold ($\pm$ SEM 0.41), and 3.5-fold ($\pm$ SEM 1.24) elevation in cleaved PARP ($p < 0.05$ for all 3), and a 3.0-fold ($\pm$ SEM 0.62), 3.6-fold ($\pm$ SEM 0.94), and 3.3-fold ($\pm$ SEM 0.61) elevation in cleaved caspase 3 ($p < 0.05$ for all 3) respectively compared to rAdGFP-infected hepatocytes treated with cyclosporine and MMF (Figure 3B). Again, no difference was noted between rAdHCV-CoreE1E2, rAdHCV-NS3-5B or co-infection with regards to the combined effects of viral protein, cyclosporine and MMF.

Tacrolimus combined with MMF promotes HCV-induced cell death in hepatocytes

Treatment of rAdHCV-co-infected PMoH with 0.005 μ g/ml of tacrolimus was found to reduce cell viability by 39% ($\pm$ SEM 1.2) compared to untreated hepatocytes at 48

hours, and this was significantly higher than the effect of rAdHCV co-infection alone ($p=0.02$) (Figure 4A). The effect of tacrolimus in enhancing the rAdHCV-induced reduction in cell viability was comparable to the effect of cyclosporine ($p=0.06$).

The combination of tacrolimus and MMF is another common immunosuppression regimen that is used after liver transplantation. This combination was found to further enhance the reduction in cell viability in rAdHCV-co-infected PMoH, resulting in a 64% ($\pm$ SEM 0.7) decrease in cell viability compared to untreated PMoH at 48 hours, an effect significantly greater than tacrolimus alone ($p=0.02$). The effect of the combination of tacrolimus/MMF on rAdHCV-co-infected PMoH was comparable to the cyclosporine/MMF combination ($p=0.34$).

In PMoH infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B, or with both viruses, treatment with 0.005 μ g/ml of tacrolimus resulted in a 3.6-fold ($\pm$ SEM 0.61), 11.8-fold ($\pm$ SEM 2.59) and 6.8-fold ($\pm$ SEM 0.22) increase in cleaved PARP ($p<0.05$ for all 3), and a 1.6-fold ($\pm$ SEM 0.12), 3.5-fold ($\pm$ SEM 0.35) and 2.8-fold ($\pm$ SEM 0.29) increase in cleaved caspase 3 ($p<0.05$ for all 3) respectively at 48 hours post-infection compared to rAdGFP-infected tacrolimus-treated cells (Figure 4B). In PMoH treated with tacrolimus, infection with rAdHCV-NS3-5B appeared to be more toxic than infection with rAdHCV-CoreE1E2 ($p<0.05$ for both cleaved PARP and cleaved caspase 3).

When PMoH infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B and co-infected with both viruses were treated with tacrolimus and MMF, a greater enhancement of apoptosis was seen compared to tacrolimus/MMF-treatment of rAdGFP infected hepatocytes, with cleaved PARP levels increased 5.3-fold ($\pm$ SEM 2.17), 9.3-fold ($\pm$ SEM 1.29) and 7.7-fold ($\pm$ SEM 0.14) ($p<0.05$ for all 3), and cleaved caspase 3 levels increased 1.7-fold ($\pm$ SEM 0.27), 2.3-fold ($\pm$ SEM 0.43) and 2.6-fold ($\pm$ SEM 0.38) ($p<0.05$ for all 3) respectively at 48 hours post-infection (Figure 4C). In PMoH treated with tacrolimus and MMF, infection with rAdHCV-NS3-5B appeared to be slightly more toxic than infection with rAdHCV-CoreE1E2, but this effect did not achieve significance ($p=0.10$ for cleaved PARP and $p=0.35$ for cleaved caspase 3).

The effect of tacrolimus with and without MMF on HCV-induced hepatocyte apoptosis was compared to cyclosporine with and without MMF, to determine which particular immunosuppressant regimen had the greatest effect on hepatocyte apoptosis (Figure 4D). Tacrolimus was found to enhance apoptosis in HCV-infected primary hepatocytes to approximately the same level as cyclosporine ($p=0.35$ for both cleaved PARP and cleaved caspase 3). MMF enhanced HCV-induced hepatocyte apoptosis but to a lesser extent than either cyclosporine or tacrolimus. However, the combination of MMF plus tacrolimus increased HCV-induced apoptosis in primary hepatocytes above the level seen with tacrolimus alone. No significant difference between the effect of the tacrolimus/MMF and cyclosporine/MMF combinations was observed ($p=0.50$ and $p=0.20$ for cleaved PARP and cleaved caspase 3 respectively).

Sirolimus promotes HCV-induced cell death in hepatocytes with further enhancement upon combination with MMF

Sirolimus is now being used more commonly following liver transplantation as a substitute for cyclosporine or tacrolimus in patients who develop calcineurin-related adverse effects. As such, the immunosuppressive regiment consisting of sirolimus and MMF may become commonplace in the future. Treatment of rAdHCV-co-infected PMoH with $0.01 \mu\text{g/ml}$ of sirolimus was found to reduce cell viability compared to rAdHCV co-infection alone ($p<0.05$), with cell viability decreased by 29% ($\pm\text{SEM } 2.2$) compared to untreated hepatocytes at 48 hours (Figure 5A). The effect of sirolimus in enhancing the rAdHCV-induced reduction in cell viability however, was not significantly different from the effect of tacrolimus ($p=0.50$).

The combination of sirolimus and MMF was found to further reduce cell viability in rAdHCV-co-infected PMoH compared to sirolimus treatment alone ($p<0.05$), resulting in a 40% ($\pm\text{SEM } 3.3$) decrease in cell viability compared to untreated PMoH at 48. The reduction of cell viability with the sirolimus/MMF combination was comparable to the tacrolimus/MMF combination ($p=0.50$).

In rAdHCV-CoreE1E2-, rAdHCV-NS3-5B-, and co-infected PMoH treated with $0.01 \mu\text{g/ml}$ of sirolimus, the levels of cleaved PARP were increased 2.1-fold ($\pm\text{SEM } 0.26$), 3.3-fold ($\pm\text{SEM } 0.36$) and 2.3-fold ($\pm\text{SEM } 0.20$) ($p<0.05$ for all 3), and cleaved caspase 3 levels increased 2.0-fold ($\pm\text{SEM } 0.29$), 4.0-fold ($\pm\text{SEM } 0.57$) and 3.0-fold

($\pm$ SEM 0.68) ($p < 0.05$ for all 3), respectively at 48 hours p.i. compared to rAdGFP-infected cells treated with sirolimus. (Figure 5B). In PMoH treated with sirolimus, infection with rAdHCV-NS3-5B appeared to be more toxic than infection with rAdHCV-CoreE1E2 ($p < 0.05$ for both cleaved PARP and cleaved caspase 3).

The enhancement of rAdHCV-induced apoptosis by sirolimus was comparable to tacrolimus, with no significant difference between sirolimus and tacrolimus treatment ($p = 0.50$ for cleaved PARP and $p = 0.35$ for cleaved caspase 3) (Figure 5C).

In PMoH infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B, and co-infected with both, treatment with 0.01 μ g/ml sirolimus and 5 μ g/ml MMF increased cleaved PARP levels by 8.9-fold ($\pm$ SEM 2.43), 11.3-fold ($\pm$ SEM 2.63) and 11.4-fold ($\pm$ SEM 2.63) ($p < 0.05$ for all 3), and increased cleaved caspase 3 levels by 5.9-fold ($\pm$ SEM 1.23), 6.6-fold ($\pm$ SEM 1.14) and 6.5-fold ($\pm$ SEM 1.15) ($p < 0.05$ for all 3) respectively at 48 hours p.i. compared to rAdGFP-infected sirolimus/MMF-treated hepatocytes (Figure 5D). We found no difference between rAdHCV-CoreE1E2, rAdHCV-NS3-5B or co-infection with regards to the combined effects of viral protein, sirolimus and MMF. There was no significant difference between the effect of the sirolimus/MMF combination compared with the tacrolimus/MMF combination ($p = 0.50$ for cleaved PARP and $p = 0.20$ for cleaved caspase 3) (Figure 5E).

Immunosuppressant-enhanced HCV-induced promotion of cell death was mitigated by pan-caspase inhibition

rAdHCV co-infected PMoH were treated with various immunosuppressants in the presence of Q-VD-Oph or DMSO as control, and compared to untreated hepatocytes. Cell viability in rAdHCV co-infected PMoH treated with cyclosporine or tacrolimus or sirolimus and/or MMF was previously shown to be significantly reduced at 48 hours post-infection compared to uninfected hepatocytes. Treatment with Q-VD-Oph significantly improved cell viability in rAdHCV co-infected PMoH treated with cyclosporine, tacrolimus, sirolimus, or MMF by 81% ($p = 0.01$), 80% ($p = 0.01$), 78% ($p = 0.03$), and 93% ($p = 0.03$) respectively at 48 hours post-infection (Figure 6A).

Improvements in cell viability were also noted with Q-VD-Oph treatment of rAdHCV co-infected PMoH treated with dual immunosuppressive agents, with cell viability

increased by 67% ($p=0.01$), 73% ($p=0.01$) and 81% ($p=0.03$) in rAdHCV-co-infected PMoH treated with the cyclosporine/MMF, tacrolimus/MMF, and sirolimus/MMF combinations respectively.

We next investigated whether Q-VD-OPh could also mitigate the immunosuppressant-enhanced HCV-induced hepatocyte apoptosis. Increased levels of apoptosis were previously shown in rAdHCV-co-infected primary hepatocytes treated with various immunosuppressive agents. Treatment with Q-VD-OPh reduced apoptosis in rAdHCV-infected PMoH treated with either cyclosporine or tacrolimus or sirolimus or MMF, with the cleaved PARP level decreased by 88%, 89%, 82%, and 82% ($p<0.05$ for all 4), and cleaved caspase 3 decreased by 86%, 89%, 81%, and 83% ($p<0.05$ for all 4) respectively (Figures 6B, 6C and 6D).

In rAdHCV-co-infected PMoH treated with the combinations of cyclosporine/MMF, tacrolimus/MMF, and sirolimus/MMF, treatment with Q-VD-OPh was also found to reduce apoptosis, with cleaved PARP reduced 93%, 92%, and 92% ($p<0.05$ for all 3), and cleaved caspase 3 reduced 87%, 89%, and 91% ($p<0.05$ for all 3) respectively (Figures 6D and 6E).

Discussion

Patients transplanted for HCV-related cirrhosis have a poorer outcome post-transplantation compared to patients transplanted for other causes of chronic liver disease (15). The development of more severe liver disease in these patients maybe the result of both HCV and the immunosuppressive agents used after transplantation contributing together to promote liver injury. An important mechanism by which HCV is thought to promote liver inflammation and fibrosis is by the induction of hepatocyte apoptosis(6). In the post-liver transplant setting, a high level of HCV replication has been correlated with increased hepatocyte apoptosis and this has been associated with the subsequent development of rapidly progressive graft injury and fibrosis(16).

Immunosuppressive drugs could contribute to increased hepatocyte apoptosis by impairing immune control of HCV replication, thereby allowing higher levels of

viraemia and a greater direct viral effect on hepatocyte apoptosis together with a more severe inflammatory response in the liver(17). Certainly, the use of heavy immunosuppression post-transplantation has been correlated with rapid HCV disease progression(18), as has the use of powerful immunosuppressive drugs to treat acute graft rejection(19). However, the immunosuppressive agents could also have a direct toxic effect on the liver that is independent of HCV. We have previously shown that both cyclosporine and tacrolimus, when combined with MMF, enhanced hepatocyte apoptosis(8). The combined effect of various immunosuppressive agents on HCV-induced hepatocyte cell death in post-liver transplantation HCV recurrence has not been well studied.

Our results show that when HCV infection is combined with cyclosporine, tacrolimus or sirolimus, hepatocyte cell death is enhanced to a greater level than seen with HCV infection alone. Indeed, others have also found that these immunosuppressive agents are capable of promoting apoptosis. Cyclosporine has been shown to induce apoptosis by inducing endoplasmic reticulum stress(9) and upregulating TGF- β expression(20). Tacrolimus was found to increase the generation of reactive oxygen species, promote mitochondrial dysfunction and enhance apoptosis(10). Sirolimus was noted to induce apoptosis by activating caspase-3 and inhibiting NF- κ B nuclear translocation(11).

We found that cyclosporine, tacrolimus or sirolimus promoted HCV-induced hepatocyte apoptosis to similar levels. Indeed, in patients undergoing liver transplantation for HCV disease, Berenguer and colleagues found no difference in graft survival and patient mortality between cyclosporine therapy and tacrolimus therapy(21). We noted that MMF alone did not significantly increase HCV-induced hepatocyte apoptosis. Mycophenolic acid, the active metabolite of MMF, has been found to inhibit HCV replication in Huh7 cells without inducing apoptosis(22), perhaps in part accounting for its reduced toxicity.

Importantly, although MMF alone did not significantly increase HCV-induced cell death, it enhanced the harmful effects of cyclosporine, tacrolimus, and sirolimus. The effect was similar for all 3 of these drugs. The finding that these commonly used immunosuppressive regimens interact with HCV to promote hepatocyte cell death

may in part explain the accelerated progression of hepatic fibrosis seen in post-liver transplant HCV recurrence. From our results, MMF monotherapy appears to be the least toxic regimen to use after liver transplantation for HCV disease.

Apoptosis is a highly regulated process mediated by the caspase family of intracellular proteases. Q-VD-Oph is a broad-spectrum inhibitor of caspases, effective against caspase 3, 8, 9, 10, and 12, while being non-toxic to cells even at high concentrations(23). To further emphasize the role of hepatocyte apoptosis induced by HCV and immunosuppressants, treatment with the pan-caspase inhibitor Q-VD-Oph was shown to largely inhibit cell death, regardless of the type of immunosuppressive agent or immunosuppressant combination involved.

In conclusion, these cell culture experiments showed that the treatment of HCV-infected hepatocytes with the therapeutically relevant concentrations of cyclosporine, tacrolimus, and sirolimus reduced cell viability and enhanced apoptosis compared to HCV infection alone, and the addition of MMF to this combination further augmented this toxic effect. This HCV and immunosuppressant-induced cell death was inhibited by Q-VD-Oph, suggesting a novel therapeutic approach by utilizing caspase inhibitors to block hepatocyte apoptosis, thereby reducing liver injury in post-liver transplant HCV recurrence.

Disclosures

We the authors state that we have no conflicts of interest to declare.

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Figure Legend

Figure 1. Increased hepatocyte apoptosis is seen in HCV positive patients after liver transplantation on immunohistochemistry staining of liver tissue.

Photomicrographs show immunohistochemical staining of human liver sections for M30 CytoDEATH and cleaved PARP at 200x magnification, indicating increased levels of these markers of apoptosis in HCV positive post-liver transplant patients compared to HCV negative patients. Graphs show the relative staining of these apoptotic markers in the liver of each patient assessed by computerized image capture quantification with (A) M30 CytoDEATH and (B) cleaved PARP results expressed as mean proportional area stained within the given area and error bars represent SEM.

Figure 2. Cyclosporine at therapeutic concentration increases cell death in HCV-infected hepatocytes, but not mycophenolate mofetil.

(A) Percentage reduction in cell viability from crystal violet assays of rAdHCV-infected PMoH with or without treatment with 1 µg/ml of cyclosporine compared to untreated cells.

(B) Western blots of cleaved PARP and cleaved caspase 3 levels in PMoH infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B, or both, or rAdGFP and treated with 1 µg/ml of cyclosporine compared to untreated cells at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to rAdGFP-infected cells. Each bar represents the average of 3 experiments and error bar represents SEM. P-values are compared to rAdGFP-infected cyclosporine-treated hepatocytes.

(C) Percentage decrease in cell viability from crystal violet assays of rAdHCV-infected PMoH with or without treatment with 5 µg/ml of MMF compared to untreated cells.

(D) Western blots of cleaved PARP and cleaved caspase 3 levels in PMoH infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B, or both, or rAdGFP and treated with 5 µg/ml of MMF at 48 hours compared to untreated cells. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to rAdGFP-infected cells. Each bar represents the average of 3 experiments and error bar represents SEM. P-values are compared to rAdGFP-infected MMF-treated hepatocytes.

Figure 3. The combination of cyclosporine and mycophenolate mofetil further enhances cell death in HCV-infected hepatocytes.

(A) Percentage reduction in cell viability from crystal violet assays of rAdHCV-infected PMoH treated with 1 µg/ml of cyclosporine and/or 5 µg/ml of MMF compared to untreated cells.

(B) Western blots of cleaved PARP and cleaved caspase 3 levels in PMoH infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B, or both, or rAdGFP and treated with 1 µg/ml of cyclosporine and 5 µg/ml of MMF compared to untreated cells at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels in cyclosporine/ MMF-treated PMoH at 48 hours relative to rAdGFP-infected cells. Each bar represents the average of 3 experiments and error bar represents SEM. P-values are compared to rAdGFP-infected cyclosporine/MMF-treated hepatocytes.

Figure 4. Tacrolimus at therapeutic concentration increases cell death in HCV-infected hepatocytes, and this effect is enhanced upon addition of mycophenolate mofetil.

(A) Percentage reduction in cell viability from crystal violet assays of rAdHCV-infected PMoH with or without treatment with 0.005 µg/ml of tacrolimus or 1 µg/ml of cyclosporine and/or 5 µg/ml of MMF compared to untreated cells.

(B) Western blots of cleaved PARP and cleaved caspase 3 levels in PMoH infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B, or both, or rAdGFP and treated with 0.005 µg/ml of tacrolimus compared to untreated cells at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to rAdGFP-infected cells. P-values are compared to rAdGFP-infected tacrolimus-treated hepatocytes.

(C) Western blots of cleaved PARP and cleaved caspase 3 levels in PMoH infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B, or both, or rAdGFP and treated with 0.005 µg/ml of tacrolimus and 5 µg/ml of MMF compared to untreated cells at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to rAdGFP-infected cells. P-values are compared to rAdGFP-infected tacrolimus/MMF-treated hepatocytes.

(D) Western blots of cleaved PARP and cleaved caspase 3 levels in PMoH infected with both rAdHCV-CoreE1E2 and rAdHCV-NS3-5B, and treated with 0.005 µg/ml of tacrolimus or 1 µg/ml of cyclosporine and/or 5 µg/ml of MMF compared to untreated cells at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to untreated cells. Each bar represents the average of 3 experiments and error bar represents SEM. P-values are compared to untreated hepatocytes.

Figure 5. Sirolimus at therapeutic concentration increases cell death in HCV-infected hepatocytes, and this effect is enhanced upon addition of mycophenolate mofetil.

(A) Percentage reduction in cell viability from crystal violet assays of rAdHCV-infected PMoH with or without treatment with 0.01 µg/ml of sirolimus or 0.005 µg/ml of tacrolimus and/or 5 µg/ml of MMF compared to untreated cells.

(B) Western blots of cleaved PARP and cleaved caspase 3 levels in PMoH infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B, or both, or rAdGFP and treated with 0.01 µg/ml of sirolimus compared to untreated cells at 48 hours. Graphs show fold change

in cleaved PARP and cleaved caspase 3 levels relative to rAdGFP-infected cells. P-values are compared to rAdGFP-infected sirolimus-treated hepatocytes.

(C) Western blots of cleaved PARP and cleaved caspase 3 levels in PMoH infected with both rAdHCV-CoreE1E2 and rAdHCV-NS3-5B, and treated with 0.01 µg/ml of sirolimus or 0.005 µg/ml of tacrolimus compared to untreated cells at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to untreated cells. P-values are compared to mock-treated hepatocytes.

(D) Western blots of cleaved PARP and cleaved caspase 3 levels in PMoH infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B, or both, or rAdGFP and treated with 0.01 µg/ml of sirolimus and 5 µg/ml of MMF compared to untreated cells at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to rAdGFP-infected cells. P-values are compared to rAdGFP-infected hepatocytes.

(E) Western blots of cleaved PARP and cleaved caspase 3 levels in PMoH infected with both rAdHCV-CoreE1E2 and rAdHCV-NS3-5B, and treated with 0.01 µg/ml of sirolimus or 0.005 µg/ml of tacrolimus and 5 µg/ml of MMF compared to untreated cells at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to mock-treated hepatocytes. Each bar represents the average of 3 experiments and error bar represents SEM. P-values are compared to untreated hepatocytes.

Figure 6. Caspase inhibition with Q-VD-Oph successfully mitigates HCV and immunosuppressant-induced cell death in hepatocytes.

(A) Percentage reduction in cell viability from crystal violet assays of rAdHCV co-infected PMoH treated with 1 µg/ml of cyclosporine, 0.005 µg/ml of tacrolimus, 0.01 µg/ml of sirolimus, and/or 5 µg/ml of MMF treated with Q-VD-Oph or DMSO as control at 48 hours compared to untreated hepatocytes.

(B) Western blots of cleaved PARP and cleaved caspase 3 levels in cyclosporine- or tacrolimus-treated PMoH infected with both rAdHCV-CoreE1E2 and rAdHCV-NS3-5B, and treated with either Q-VD-Oph or DMSO as control, compared to untreated cells at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to untreated cells.

(C) Western blots of cleaved PARP and cleaved caspase 3 levels in sirolimus- or tacrolimus-treated PMoH infected with both rAdHCV-CoreE1E2 and rAdHCV-NS3-5B, and treated with either Q-VD-Oph or DMSO as control, compared to untreated cells

at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to untreated cells.

(D) Western blots of cleaved PARP and cleaved caspase 3 levels in cyclosporine/MMF- or tacrolimus/MMF-treated PMoH infected with both rAdHCV-CoreE1E2 and rAdHCV-NS3-5B, and treated with either Q-VD-Oph or DMSO as control, compared to untreated cells at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to untreated cells.

(E) Western blots of cleaved PARP and cleaved caspase 3 levels in sirolimus/MMF- or tacrolimus/MMF-treated PMoH infected with both rAdHCV-CoreE1E2 and rAdHCV-NS3-5B, and treated with either Q-VD-Oph or DMSO as control, compared to untreated cells at 48 hours. Graphs show fold change in cleaved PARP and cleaved caspase 3 levels relative to untreated cells. Each bar represents the average of 3 experiments and error bar represents SEM.

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