Accelerated liver disease in aggressive hepatitis C recurrence post-liver transplantation may be due to enhanced apoptosis mediated by both virus and immunosuppressants

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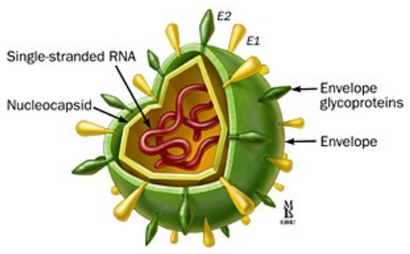
Introduction



Hepatitis C (HCV)-related liver failure is now the commonest indication for liver transplantation (OLT) in Australia.

These patients are noted to have a poorer survival compared to those transplanted for other indications.

This is because HCV recurrence in the allograft commonly follows an aggressive course, with at least 20% of patients developing cirrhosis within 5 years of transplantation.

Induction of hepatocyte apoptosis may be one mechanism by which HCV drives liver injury.

In post-liver transplant HCV recurrence, the combination of immunosuppressants and viral replication is postulated to increase hepatocyte apoptosis and accelerate liver fibrosis.

Aims

We investigated:

1) Hepatocyte apoptosis in liver biopsies of HCV-infected patients pre- and post-liver transplant,
2) The effects of HCV and immunosuppressants on cell death in - primary human hepatocytes (PHH),
   - primary murine hepatocytes (PMoH), and
   - human hepatoma cells (Huh7),
3) The effects of pan-caspase inhibition and RIP-kinase inhibition on HCV-induced hepatocyte cell death.

Materials and Methods

Human Liver Immunohistochemistry

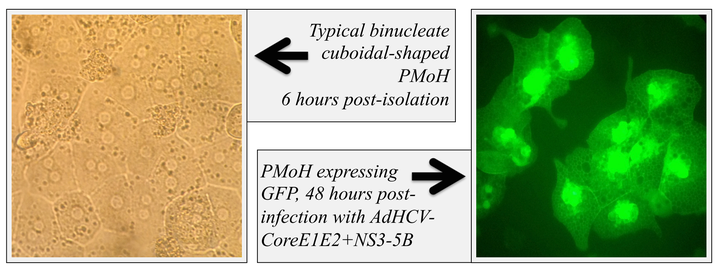
Hepatocyte apoptosis was assessed via immunohistochemistry in liver tissue of pre- and post-transplant liver biopsies of HCV-infected and HCV-negative patients for markers of apoptosis:

- M30 CytoDEATH (M30)
- Cleaved PARP (clPARP)

Cell Experiments

Huh7 cells, PHH (from Lonza), and PMoH (from C57BL/6 mice) were infected with **recombinant adenoviruses** encoding

- GFP (used as control viral infection)
- HCV-CoreE1E2 (structural proteins)
- HCV-NS3-5B (non-structural proteins)



in the presence or absence of physiologically relevant doses of

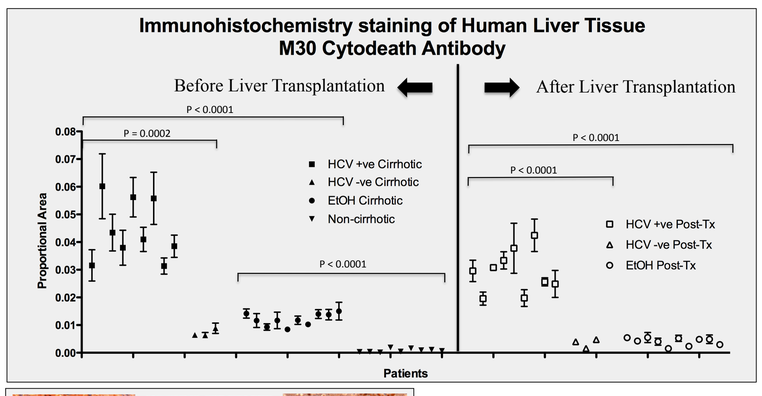
- **cyclosporine** (CyA, kind gift from Novartis) and/or
- **mycophenolate mofetil** (MMF, kind gift from Roche)
- Pan-caspase inhibitor **Q-VD-Oph** (Q-VD)
- RIP-kinase inhibitor **Necrostatin-1** (Nec-1)

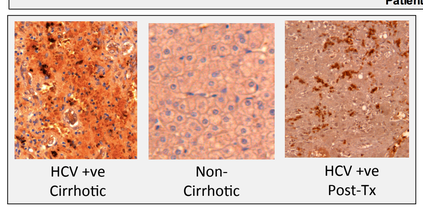
Treated cells evaluated at set time points, compared to mock or rAdGFP.

- Cell viability was evaluated using **crystal violet assays**.
- Cell apoptosis was evaluated using **Western immunoblots** performed on cell lysates probed for
  - cleaved Caspase 3 (clCasp3) and
  - cleaved PARP (clPARP).

Results

Increased Apoptosis in HCV-infected Liver





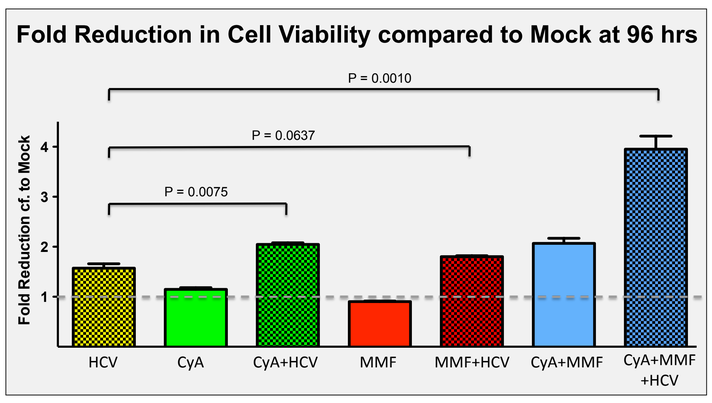
Patients with HCV cirrhosis had
- 2.7- and 3.5-fold ↑ in M30
- 7- and 8-fold ↑ in clPARP

compared to HCV-negative cirrhotics and non-cirrhotics respectively.

Post-liver transplant, patients with HCV recurrence had a
- 4-6-fold ↑ in M30 and clPARP

compared to HCV-negative post-OLT patients.

Effect of Immunosuppressants on Cell Viability

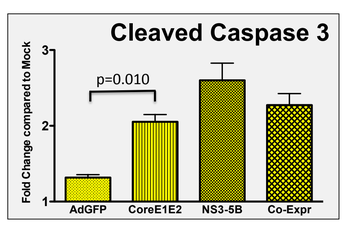


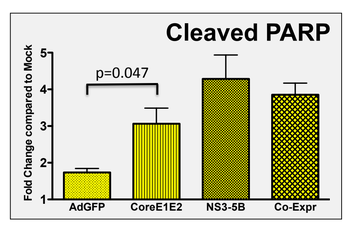
- rAdHCV infection ↓ hepatocyte cell viability by 1.6-fold
- Addition of various immunosuppressants to rAdHCV infection further ↓ cell viability
- The combination of CyA and MMF added to rAdHCV infection appeared to be the most toxic, ↓ cell viability by 4-fold

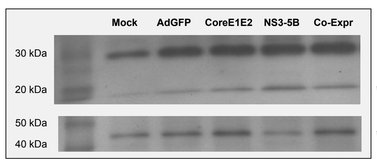
Effect of Immunosuppressants on Apoptosis

Effect of rAdHCV Infection on Apoptosis

- **PHH** harvested at 48 hours post-infection with rAdHCV.







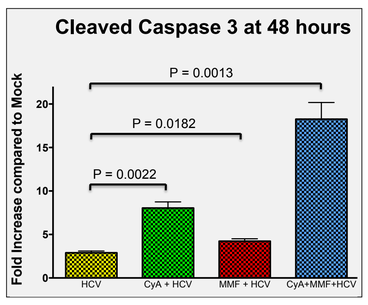
Western Immunoblot of **PHH** lysates harvested at 48 hours and probed for
- **Cleaved Caspase 3**,
- **Pan-Actin**

- Infection with rAdGFP
  - only ↑ cleaved PARP by 1.7 fold,
  - and ↑ cleaved caspase 3 by 1.3 fold.
- but infection with rAdHCV-CoreE1E2, rAdHCV-NS3-5B & both,
  - cleaved PARP was ↑ by 3.1, 4.2 and 3.9 fold,
  - and cleaved caspase 3 was ↑ by 2.1, 2.6 and 2.3 fold.

Effect of Immunosuppressants Alone on Apoptosis

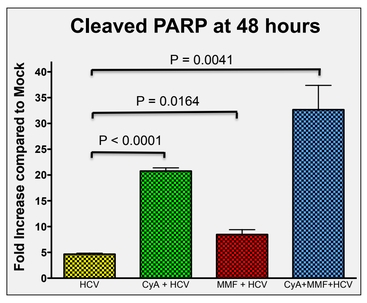
- **in PMoH, CyA** at 1 µg/mL had no effect on cleaved PARP or cleaved caspase 3 compared to mock
- In contrast, **MMF** at 5 µg/mL
  - ↓ cleaved PARP by 2.0 fold,
  - ↓ cleaved caspase 3 by 1.9 fold.
- The combination of **CyA** (1 µg/mL) and **MMF** (5 µg/mL)
  - ↑ cleaved PARP by 2.5 fold,
  - ↑ cleaved caspase 3 by 1.9 fold.

Effect of Immunosuppressants on rAdHCV-infected cells

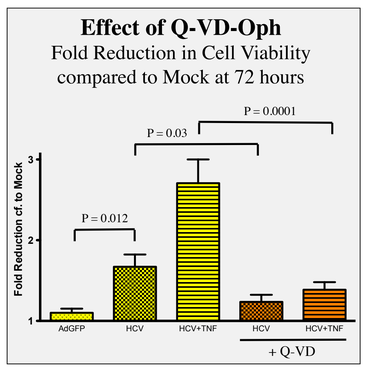


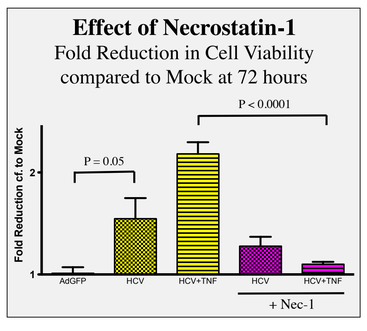
In **PMoH** harvested at 48 hours post-treatment

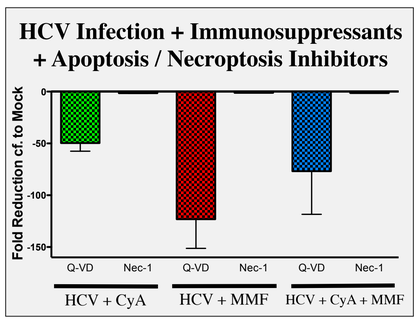
- Effect of **CyA**
  - ↑ clCasp3 by 2.8-fold
  - ↑ clPARP by 4.5-fold
- Effect of **MMF**
  - ↑ clCasp3 by 1.5-fold
  - ↑ clPARP by 1.9-fold
- Effect of the combination of **CyA** & **MMF**
  - ↑ clCasp3 by 6.3-fold
  - ↑ clPARP by 7.1-fold



Inhibition of Cell Death







Effect of Q-VD and Nec-1 on rAdHCV infection

In **Huh7 cells**,
- Infection with rAdHCV
  - ↓ cell viability by 1.7-fold
- In the presence of **Q-VD**,
  - rAdHCV ↓ in cell viability by only 1.2-fold (P = 0.03)
- In the presence of **Nec-1**,
  - rAdHCV ↓ cell viability by only 1.2-fold (P = 0.15)

In rAdHCV-infection sensitized with 10 ng/mL of TNF-α
- rAdHCV infection + TNF-α
  - ↓ cell viability by 2.7 fold
- This was improved significantly by the addition of both **Q-VD** and **Nec-1**

Effect of Q-VD and Nec-1 on Immunosuppressant-treated rAdHCV infection

- Addition of **Q-VD**
  - greatly reduced clPARP by 50- to 120-fold (P < 0.006).
- Addition of **Nec-1**
  - had no effect on clPARP (P = 0.753).

Conclusions

- Hepatocyte apoptosis was significantly increased in HCV-infected patients pre- and post-OLT compared to HCV-negative patients.
- HCV infection reduced cell viability and increased apoptosis.
- Immunosuppressive agents CyA and MMF further promoted cell death, and may explain the accelerate progression of liver disease in post-liver transplant HCV recurrence.
- Inhibition of apoptosis by Q-VD-Oph partially restored cell viability and reduced cell death in rAdHCV-infected hepatocytes.
- Partial reversal of cell death by Necrostatin-1 suggests a possible alternate pathway of cell death in HCV infection (ie. necroptosis).

These results provide an insight into the mechanisms responsible for accelerated liver fibrosis seen in HCV recurrence post-liver transplantation and possible novel therapeutic targets in this setting.

Acknowledgements

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