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<tr>
<td><strong>Author</strong></td>
<td>Tura, Benjamin James</td>
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<td><strong>Qualification</strong></td>
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IFN\textsubscript{\textgamma} induced apoptosis in the murine hepatocyte.

Benjamin James Tura

PhD
The University of Edinburgh
2003
Declaration

The work presented in this thesis has been performed and presented solely by myself. Contributions from others are indicated at the appropriate point in the text.

Ben Tura, September 2003.
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My thanks go out all who have helped me achieve this, for their patient assistance and assurance. In the liver group particularly Nick, Chris and Helen for being there all the way through from my very first perfusion, through all the contamination, sick hepatocytes and sick jokes. Also thanks to Sheona, Nicola and Sharon: all round support, blonde jokes, films and too many training sessions. My supervisor David Harrison gave support and direction and helped to remind me that there is a bigger picture than what you see down the microscope.

Thanks also to those who have shared this time with me in the lab, Dom, Kerry, Duane, Gareth, Stuart, Anca, and Nathalie. This could go on.

I would like to acknowledge the support of the Medical Research Council for funding the project.

My Mother always knows the right thing to say, when I need to be told to wake up and get on with it or when I need to take my time and think about things, invaluable support. Thanks also Dad and the girls.

I won’t forget how hard I can make things on other people, so I can’t forget how good other people can be to me, Andrea.
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Cell Culture Solutions

Media

Confocal timelapse imaging of mitochondrial depolarisation
### Abbreviations

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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>(d)ATP</td>
<td>(deoxyribose) Adenine Triphosphate</td>
</tr>
<tr>
<td>(s)ALT</td>
<td>(serum) Alanine amino transferase</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide transporter</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease activating factor I</td>
</tr>
<tr>
<td>BH</td>
<td>Bcl-2 homology</td>
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<tr>
<td>BIR</td>
<td>Bacculoviral IAP repeat</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>CAD</td>
<td>Caspase-activated DNase</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase Recruitment Domain</td>
</tr>
<tr>
<td>CED</td>
<td>cell death</td>
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<tr>
<td>cFLIP</td>
<td>cellular FLICE Inhibitory Protein</td>
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<tr>
<td>CHX</td>
<td>Cyclohexamind</td>
</tr>
<tr>
<td>CMXRos</td>
<td>Chloromethyl X Rosamine</td>
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<td>ConA</td>
<td>Conconavalin A</td>
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<td>CPAN</td>
<td>Caspase activated nuclease</td>
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<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>Cyto c</td>
<td>cytochrome c</td>
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<tr>
<td>DAP</td>
<td>Death associated protein</td>
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<tr>
<td>DD</td>
<td>Death Domain</td>
</tr>
<tr>
<td>DED</td>
<td>Death Effector Domain</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>DEVD-CHO</td>
<td>Caspase 3 inhibitor</td>
</tr>
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<td>DFF40</td>
<td>DNA Fragmentation factor 40</td>
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<tr>
<td>D-GalN</td>
<td>D-galactosamine</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Direct IAP binding protein with low pl</td>
</tr>
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<td>DISC</td>
<td>Death Inducing Signalling Complex</td>
</tr>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EF</td>
<td>Embryonic fibroblast</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>Fas-Associated Death Domain</td>
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<td>FK506 Binding Protein</td>
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<td>FLICE-inhibitory protein</td>
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<td>Gamma activated factor</td>
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<td>Gamma activated site</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
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<td>HAV</td>
<td>Hepatitis A Virus</td>
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<td>HBsAg</td>
<td>Hepatitis B surface antigen</td>
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<td>HBV</td>
<td>Hepatitis B virus</td>
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<td>Hepatitis C Virus</td>
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<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>IAP</td>
<td>Inhibitor of Apoptosis Protein</td>
</tr>
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<td>ICAD</td>
<td>Inhibitor of CAD</td>
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<td>IFNγ</td>
<td>Interferon gamma</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>IRF</td>
<td>Interferon regulatory factor</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
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<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>mCICCP</td>
<td>Cyanide m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MOPS</td>
<td>3(N-morpholino) propane sulphonic acid</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial Permeability Transition</td>
</tr>
<tr>
<td>MPTP</td>
<td>Mitochondria permeability transmission pore</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
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<tr>
<td>NCS</td>
<td>Normal Calf Serum</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor κB</td>
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<tr>
<td>NK</td>
<td>Natural Killer Cell</td>
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<tr>
<td>NPC</td>
<td>Non-Parenchymal Cells</td>
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<td>PARP</td>
<td>poly (ADP ribose) polymerase</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PKC</td>
<td>Protein Kinase C</td>
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<tr>
<td>PKR</td>
<td>Protein Kinase R</td>
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<td>Ribonucleic acid</td>
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<td>Description</td>
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<tr>
<td>RPA</td>
<td>Ribonuclease protection assay</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate – Polyacrylamide Gel Electrophoresis</td>
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<td>Smac</td>
<td>Second Mitochondria-derived activator of caspase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TGFα</td>
<td>Transforming growth factor alpha</td>
</tr>
<tr>
<td>TMRE</td>
<td>Tetramethylrhodamine ethyl ester</td>
</tr>
<tr>
<td>TMRM</td>
<td>Tetramethylrhodamine methyl ester</td>
</tr>
<tr>
<td>TNFR1</td>
<td>TNF receptor 1</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-Related Apoptosis Inducing Ligand</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
</tr>
<tr>
<td>z-DEVD-fmk</td>
<td>carboxbenzoxyl- aspartic acid-glutamic acid-valine-aspartic acid-fluoromethylketone. Caspase 3-like inhibitor.</td>
</tr>
<tr>
<td>z-IETD-fmk</td>
<td>carboxbenzoxyl- isoleucine-glutamic acid-threonine-aspartic acid-fluoromethylketone. Caspase 8 inhibitor</td>
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<td>z-LEHD-fmk</td>
<td>carboxbenzoxyl-leucine-glutamic acid-histidine-aspartic acid-fluoromethylketone. Caspase 9 inhibitor</td>
</tr>
<tr>
<td>Δψ&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Change in mitochondrial membrane potential</td>
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Abstract

Interferon gamma (IFNγ) is a key mediator of liver injury and hepatitis. The major fashion in which IFNγ brings about injury is through apoptosis of the parenchymal cells of the liver, the hepatocytes. In the primary murine hepatocyte IFNγ-induced hepatocyte apoptosis is dependent on the anti-tumourigenic transcription factor IRF1 and involves caspase 3 like enzymes. Liver injury leads to a pre-malignant state that can lead to carcinogenesis. Inappropriately controlled apoptosis is thought to be one of the factors that contribute to cancer formation.

The aim of these investigations was to investigate the pathway to apoptosis between the IRF-1 transcription factor and caspase 3. These areas may be affected by environment, viruses, drugs or by the cell itself in favour of life or death.

Specifically we set out to identify the upstream caspases that were activated by IFNγ and the role of the mitochondria in apoptosis.

We selected an *in vitro* primary murine hepatocyte system to investigate this pathway. Despite experimental difficulties and questions about the relevance of animal research to human disease, this system was chosen as it provided a practical model of cell injury. We demonstrate that apoptosis occurs in a significant proportion of the population 72hrs after administration of IFNγ and that this apoptosis is subject to modulation. Serum was shown to have an effect on the kinetics of apoptosis. An interaction of the IFNγ and Fas apoptosis pathways is demonstrated with the use of the Jo2 anti-Fas antibody. This pathway is sensitive to inhibition by Cyclosporin A (CsA) and media changes.
Using caspase inhibitors and western blot analysis it was demonstrated that both caspase 8 and caspase 9 are involved in hepatocyte apoptosis. Following 48 hours of IFN\(\gamma\) treatment evidence of Bid cleavage is seen. Following 72 hours of IFN\(\gamma\) treatment cleavage and activation of caspase 9 is seen. These results demonstrate for the first time that in IFN\(\gamma\) treated hepatocytes caspase 8 is activated followed by caspase 9. We hypothesised that a type II membrane death receptor pathway was operating. This hypothesis was tested with the FasL blocking antibody MFL3. IFN\(\gamma\)-induced apoptosis was successfully blocked with this agent. Our results lead us to conclude that IFN\(\gamma\) acts through the Fas pathway to achieve hepatocyte apoptosis. This finding suggests manipulation of components in the Fas pathway may be a beneficial treatment strategy in hepatitis.

To investigate the involvement of the mitochondria in the pathway we administered the drug CsA to IFN\(\gamma\) treated cells. CsA was effective in reducing the percentage of cells showing apoptotic morphology at 72 hours. Studies of the mitochondrial membrane potential revealed that a large proportion of the population (~80%) undergo mitochondrial depolarisation. Membrane depolarisation was found to occur despite the presence of apoptosis inhibitors. Both CsA and the caspase 8 inhibitor z-IETD-fmk, were unable to prevent mitochondrial depolarisation indicating that it is not dependent on the mitochondrial permeability transition pore or the presence of tBid. This model is relevant to hepatocarcinogenesis where both IFN\(\gamma\) and Fas play leading roles.

To summarise, the major findings of this thesis show for the first time activation of caspases 8 and 9 following IFN\(\gamma\) treatment, an effect of IFN\(\gamma\) on the hepatocyte mitochondria and a direct link between IFN\(\gamma\) and the Fas pathway in the hepatocyte.
Introduction

This introduction will discuss a variety of points that are salient to the topic of interferon gamma (IFNγ) induced hepatocyte apoptosis. Hepatitis will be discussed which will put into context the medical importance of the process. The biochemistry of apoptosis will then be discussed in detail, followed by the specific role of IFNγ in both hepatitis and apoptosis.

1.1 Hepatitis

The liver's everyday task of detoxifying the body has been further complicated by both recreational and therapeutic pharmaceuticals, such as alcohol and paracetamol. Added to these problems is the conundrum of viral hepatitis. Infection with one of the hepatitis viruses has a range of potential outcomes from acute infection to chronic inflammation. The type of injury to the liver varies according to the insult sustained. Drugs (such as alcohol and paracetamol) are metabolised into toxic species that can put the liver cell in danger, in the case of a chronic viral infection it is persistent futile immune mechanisms that cause injury.
Figure 1.1. A range of consequences reflects the range of responses to liver damage
Hepatitis A infection causes an acute inflammatory response that swiftly resolves the infection (Panel A). Chronic alcohol abuse causes changes in the hepatocyte that lead to fat deposition before hepatitis develops. Cirrhosis and loss of liver function may follow (Panel B).
Hepatitis C is sometimes resolved quickly like Hepatitis A infection but in the majority of cases the virus is not cleared. Chronic hepatitis develops which can lead to cirrhosis and, in some cases, hepatocellular carcinoma (Panel C).
The body’s mechanism for dealing with viral infection and to a certain extent with alcoholic insults is the immune system. The effectiveness of the immune response in dealing with viral hepatitis can be exemplified by looking at a common cause of chronic liver injury, Hepatitis C (Fig 1.2).

**Figure 1.2. Hepatitis C: a common cause of chronic liver disease in the USA**
The aetiology of chronic liver disease: Hepatitis C alone is responsible for 57% of chronic liver disease. Data taken from US sentinel counties between December 1998 and November 1999. The incidence of newly diagnosed cases of chronic liver disease is 72.3 per 100,000 people (Kim *et al*, 2002).

NAFLD Non Alcoholic Fatty Liver Disease.
1.1.1 Hepatitis C

The hepatitis viruses were first identified in 1947, the two types now known as Hepatitis A (HAV) and Hepatitis B (HBV) were then identified as "infective or epidemic hepatitis" and "serum hepatitis" respectively (Anon 1947). It wasn’t until 1989 that the agent responsible for non-A, non-B viral hepatitis was identified as Hepatitis C Virus (HCV) (Choo et al, 1989, Kuo et al, 1989). Now HCV has emerged as a major pathogen in human disease. In 1997 the WHO published an article concerning HCV indicating wide ranging occurrence and prevalence (WHO. 1997). Current data estimates about 3% of the world’s population to be infected with HCV (WHO. 2000).

The virus itself is exceedingly efficient, it has found a way to get around the human body’s defences with only 7 peptides encoded by a single stranded RNA genome. The transcript from the 9.5kb genome is a polyprotein precursor of about 3 010 amino acids which is then cleaved by host and viral proteases (Fig 1.3) (Major and Feinstone 1997).
**Enzyme cleavage**

**Host peptidase**  NS2 protease  NS3 protease

**Figure 1.3. The HCV polyprotein**
Proteins encoded by the HCV genome consist of the structural core and envelope (C, E1 and E2) proteins, the P7 protein, whose function is unknown, and the six non-structural proteins (NS2, 3, 4A, 4B, 5A and 5B). NS2 is a metalloproteinase and NS3 is a serine protease (Flajolet et al, 2000).

### 1.1.1.1 The immune response to HCV

The spectrum of injury in Hepatitis C infection is shown in Figure 1.1, in the minority of cases there is an acute, clinically apparent response. This acute hepatitis is due to the response that the immune system mounts against the viral challenge. The body recruits both the cellular and the humoral arms of the immune system to fight HCV infection. However resolution of an acute infection may depend on a weaker humoral response rather than a vigorous one as discussed below.

The cellular arm of the immune system is crucial in dealing with HCV infection adequately. Acute HCV infection is associated with a robust Th-cell response with a type 1 cytokine profile expressed (i.e. expression of interleukin 2 (IL-2), IFNγ) and an early, vigorous cytotoxic T lymphocyte (CTL) response. While chronic infection is associated with a type 2 response at the outset (IL-4, IL-10).
While the production of proinflammatory cytokines like IFN$\gamma$ is a beneficial strategy it can also be detrimental. The immune response can act both as a positive or negative force in the resolution of HCV infection.

1.1.1.2 Repair and Injury

Viral hepatitis can occur either as an acute episode or, if the virus persists, as a chronic disease. The hepatitis C virus has a tendency to occur as a chronic disease (Di Bisceglie et al, 1991, Alter et al, 1992). In those patients that clear the virus quickly a T helper 1 type (i.e. IFN$\gamma$, IL-2) cytokine response is seen, (Cerney and Chisari 1999). This type of profile activates the CD8$^+$ T cells. CD8$^+$ T cells recognise the major histocompatibility complex (MHC) type I antigen which is expressed by the majority of cells. CD4$^+$ cells recognise MHC II antigens, which are generally expressed only by antigen presenting cells such as dendritic cells and macrophages. Hepatocytes however can be induced by IFN$\gamma$ to express MHC II antigen. MHC II reactivity was seen on hepatocytes after 24hrs of exposure to IFN$\gamma$ at 100U/ml in vitro. Liver biopsies taken from patients with hepatitis of different aetiologies revealed the presence of MHC II antigens in 19 out of 65 samples. Expression of the MHC I antigen is also induced by IFN$\gamma$ in hepatocytes in a similar fashion (Franco et al, 1988). In this study, the control population of cells did not express MHC antigens. The upregulation of MHC antigens appears to be controlled by the transcription factor interferon regulatory factor 1 (IRF-1) (Hobart et al, 1996). Such upregulation of MHC I molecules is important in directing CD8$^+$ T cell activation. In a chimpanzee model of HCV infection, those animals capable of clearing the virus (resolvers) showed low circulating anti-HCV antibodies but a strong CD8$^+$ T cell response against multiple proteins. Regions of the HCV polyprotein were expressed in a
cell line that was lysed by CD8+ T cells specific for that region. CTLs were found to be specific for epitopes across 5 regions of the HCV polyprotein in resolvers, but in non-resolvers a weaker response (to 1-3 regions) or no response was observed. During the resolution of the infection CTLs were isolated from one chimpanzee and used to establish cell lines. The specificity of these cells to HCV epitopes displayed in the context of different MHC I epitopes was investigated. It was found that the CD8+ T cells were capable of recognising 4 different epitopes (Cooper et al., 1999). A lack of anti-HCV antibodies was also seen in human resolvers, while in patients that progressed to chronic HCV infection a strong antibody response was seen (Baumert et al., 2000). To resolve HCV infection quickly the appropriate response is needed from the immune system which includes co-ordination of the intrahepatic CTL population. MHC I specificity has been shown to be a limiting factor in acute HCV infection in humans. Clones of the CTLs from one patient were able to recognise a total of eight epitopes (from NS2, NS3, NS4 and NS5) in the context of a variety of MHC I epitopes (Lechner et al., 2000).

Studies of peripheral CD4+ T cells in humans at an early stage of HCV infection showed a variation in the response of these cells to viral epitopes in the context of MHC II molecules. Those patients that resolved infection were found to have stronger proliferative responses to four regions of the HCV polyprotein (E2, NS3, NS4, NS5) than patients who could not clear the infection. When challenged with the E1 region, responses from both groups were comparable (Missale et al., 1996).

Thus, IFNγ can potentiate the killing of the hepatocyte by CD8+ T cells through upregulation of MHC I. In addition, the inflammatory response is perpetuated by the CD4+ specific MHC II expression. A further contribution to the inflammatory response is made by the transcription of pro-inflammatory caspase 11. IFNγ has been reported to
induce caspase-1 (another pro-inflammatory caspase) in a leukaemia cell line (Tamura et al, 1996) this was not seen in primary hepatocytes (Kano et al, 1999) but has been observed in transgenic mouse livers overexpressing IFNγ (Okamoto et al, 1999).

The majority of HCV infections are chronic, the virus evade immune mechanisms, perhaps by mutations that can attenuate the recognition of the virus by CTLs (Erickson et al, 2001) and decrease the inflammatory response (Large et al, 1999). There may be supplementary mechanisms at work within hepatocytes such as subversion of apoptotic pathways (Gale et al, 1999, Marusawa et al, 1999) or other crucial signalling pathways (Heim et al, 1999). As discussed above the production of antibodies and a narrow range of T cell activation is detrimental to the resolution of HCV infection in the short term. In the long term however, the opposite may be true.

The antibody titres of chronic HCV sufferers were examined and it was found that a high anti-HCV titre was beneficial in the clearance of the virus following IFNα therapy (Baumert et al, 2000). Polymerase chain reaction (PCR) analysis was used to identify the cytokines present in chronic HCV infection and compared against disease severity. As liver injury got histologically worse the amount IFNγ and IL-2 increased with decreasing IL-10 (Napoli et al, 1996). IFNγ and IL-2 both have diverse effects on the cells of the immune system however IFNγ also has effects on hepatocytes. IFNγ mediates the inflammatory state of the hepatocyte as discussed above, the hepatocyte anti viral response via the 2-5 A system (Zhou et al, 1997) and PKR (Guidotti et al, 2002), the cell cycle, and also apoptosis (Tura et al, 2001). The role of IFNγ in the liver may be key in determining the outcome of hepatitis including the development of hepatocellular carcinoma (HCC).
1.1.2 HBV infection

HBV is responsible for 4.4% of chronic liver disease in US sentinel counties (Fig 1.2). The virus affects only humans and chimpanzees, with either an acute (95%) or chronic (5%) course. HBV has a 3.2Kb circular DNA genome encoding seven peptides. Like HCV the virus elicits an immune response, in resolvers there is a strong CTL response that is absent in chronic cases.

Guidotti and Chisari (1996) illustrated the enormity of the task faced by a CD8+ T cell when dealing with viral infection in the liver. There are $10^{11}$ hepatocytes in the human liver that are potentially infectible with the HBV. In an infection more than 90% of hepatocytes are regularly infected with HBV (Yoo et al, 1987). Some simple maths leaves you with a ratio of 1 CD8+ T cell to every 1000 infected hepatocytes. The authors continue to argue that for one effector cell to mediate the death of all of these target cells would be unfeasible. Indeed, the required level of hepatocyte apoptosis would be unsustainable.

A model of acute hepatitis utilises HBV transgenic mice. These transgenic mice overexpress a copy of the envelope-coding region. In HBV infection the gene product, hepatitis B surface antigen (HBsAg), is synthesised in the cytoplasm of infected hepatocytes and secreted into the blood. Transgenic mice expressing HBsAg in the majority of their hepatocytes were injected with HBsAg specific CD8+ T cells. This led to a period of cell death peaking at 3 days and returning to baseline at 7 days. It was estimated that a maximum of 24% of the hepatocytes died. Northern blot analysis revealed that the viral RNA was either down regulated or was no longer present 5 days after injection of the CD8+ lymphocytes (Guidotti et al, 1999).
Monoclonal antibodies to tumour necrosis factor alpha (TNFα) and IFNγ were administered intravenously to the mice. This treatment prevented the clearance of viral DNA from the hepatocytes, but not the cell death (Guidotti et al, 1999). Chimpanzees were also used to demonstrate viral purging without liver damage. 90% of viral DNA was cleared from the liver before T cell infiltration (Guidotti et al, 1999). The authors report that ribonuclease protection assays demonstrated the upregulation of IFNγ and TNFα prior to T cell infiltration (Guidotti et al, 1999). Therefore, with TNFα, IFNγ can assist the removal of viral DNA from hepatocytes.

HBsAg expression renders hepatocytes acutely sensitive to the cytopathic effects of IFNγ (Gilles et al, 1992). It could be argued that the cellular injury came about as a result of activation of the cellular immune response, however, this cytopathic effect is not mediated by the immune system. A group of HBsAg transgenic mice were sublethally irradiated to remove all T cells and the level of IFNγ-induced liver injury compared to an immunologically competent control group. No significant difference was found in the sALT profile of the two groups after 16hrs, hence demonstrating a direct effect of IFNγ on hepatocytes (Gilles et al, 1992).

In other backgrounds where the hepatocyte is not hypersensitive to IFNγ (i.e. ‘normal’ hepatocytes) the cytokine is not responsible for hepatocyte apoptosis in the short term. The executioners in this context are Fas and perforin/granzyme B (Nakamoto et al, 1997). IFNγ therefore has three roles in acute HBV infection, activation of CD8+ T cells, an anti-viral RNA purging effect and a direct cytotoxic effect on hypersensitive hepatocytes.
1.1.3 Hepatocellular Carcinoma

Hepatocellular carcinoma (Figure 1.4) has been extensively linked with both HCV and HBV infection (Di Bisceglie, 1997, Buendia, 1998). The pathogenic mechanisms of HCC are not well defined however. Both HBV and HCV are capable of over-riding signalling pathways in the hepatocyte that may contribute to oncogenesis. Integration of HBV genetic material into the host genome occurs and, it is postulated that integration can lead to mutagenesis. The HCV genome does not, however, incorporate itself with the host, thus insertional mutagenesis is not the sole mechanism. Further predisposing factors to HCC include diabetes mellitus (Hassan et al, 2002) genetic disorders such as haemochromatosis (Powell et al, 1996), and Wilson’s disease (Bermann, 1988). Environmental factors include exposure to aflatoxin B1, alcohol consumption and cigarette smoking (Chen et al, 1997). A synergy between viral infection and alcohol consumption as risk factors has been observed (Hassan et al, 2002).
Figure 1.4: Hepatocellular carcinoma

Sections from a normal (upper image, magnification x250) and cancerous (lower image, magnification x400) are displayed. Hepatocytes of the cancerous liver have a highly dysregulated morphology and may have nuclear abnormalities (arrow). A fibrotic area surrounds the mass (1). Images kindly provided by Dr C. Bellamy
An experimental system has demonstrated that an immune response to viral antigens is necessary and sufficient to cause HCC. HBsAg transgenic mice, described above, were thymectomised and irradiated to remove all T cells and reconstituted with bone marrow from either transgenic (group 1) or non-transgenic animals (group 2). One week later the group 1 animals were given splenocytes taken from wild type animals that had been infected with a recombinant vaccinia virus carrying the HBsAg and were thus immunogenic. Group 2 animals received splenocytes from immunologically tolerant transgenic littermates. Group 1 animals expressing HBsAg and with immunogenic T and B cells showed progressive liver disease (inflammation, necrosis, cirrhosis) culminating at 17 months in multiple liver tumours. The livers of group 2 animals showed far less severe damage in the liver and unlike group 1 these animals did not harbour HBsAg specific CTLs (Nakamoto et al, 1998). An immunological response therefore seems to be key in the generation of chronic liver disease, pathology and neoplasia.

It is now recognised that interface hepatitis, classically seen in chronic hepatitis, is caused by apoptosis of hepatocytes. In cirrhotic livers hepatocytes are regenerating to replace the loss of liver mass to apoptosis and necrosis (Fausto, 2000). Hepatocyte apoptosis is occurring throughout chronic liver disease, however, some cells avoid the apoptotic signals and potentially progress to neoplasia.
1.2 Apoptosis

One arm of biology could be justifiably criticised for being mislabelled as the study of apoptosis is the study of death rather than of life. The study of death is an integral part of scientific understanding of development, tumorigenesis, pathogenesis, neurology and immunology. Kerr's original observations on thymocytes gave us the word apoptosis and described the cellular morphology of the process (Kerr et al, 1972). The process has now been defined from initiation, through decision and execution to clearance. The morphological features described by Kerr included shrunken cells with mostly normal organelles, except for the nuclei which were condensed into aggregates at the nuclear membrane. These observations reflected profound changes in an apoptotic nucleus. Along with condensation of chromatin the DNA is cleaved into nucleosomal subunits of 180bp and changes occur in the nuclear membrane.

1.2.1 Apoptotic nuclear changes

The condensation of chromatin is one of the morphological hallmarks of apoptosis. It is thought that condensation (and cleavage of DNA) occurs to allow packaging and phagocytosis of potentially oncogenic material. Three cellular proteins have been identified that are important in the cleavage and condensation of DNA, lamins, Acinus and caspase activated DNase (CAD).

The lamins are a family of filamentous proteins important in chromatin organisation and DNA replication (Buendia et al, 2001). Mutations in lamins are implicated in a number of disorders such as cardiomyopathy, muscular dystrophy (Bonne et al, 1999) and
lipodystrophy (Shackleton et al, 2000). Lamins are cleaved during apoptosis at a conserved aspartic acid residue (residue 230) by caspase 6 (Orth et al, 1996) and are involved in chromatin condensation and nuclear shrinkage (Rao et al, 1996, Neamati et al, 1995). A substitution mutation of aspartic acid at the cleavage site on Lamin A or B delayed cell death by 12 hours with no chromatin condensation or nuclear shrinkage evident (Rao et al, 1996).

Many studies of apoptosis employ a cell-free experimental system, such a design was used to assess the morphological changes in isolated nuclei. Cytosolic extracts were taken from a chicken B cell lymphoma clone with the caspase 6 gene deleted (casp6⁻⁰). The extracts of apoptotic control cells, but not of casp6⁻⁰ apoptotic cells, were able to induce apoptotic changes in isolated nuclei. Further experiments revealed that the changes seen were due in part to cleavage of lamin A. Cleaved lamin A was detected following incubation of nuclei with apoptotic control extracts. However, when apoptotic casp6⁻⁰ extracts were used lamin A was not cleaved and nuclear changes were not observed (Ruchaud et al, 2002).

Acinus is a novel protein identified after fractionation of bovine thymus extracts, three fractions were found to cause chromatin condensation in permeablised HeLa cells. The first fraction contained caspase 6, the second factor was CAD, the function of which will be discussed below. The third was a protein that was named Acinus (after the Greek for berry or grape due to the 'bunch of grapes' appearance of condensed chromatin). The active form of the protein, from the bovine lysates, was truncated compared to the transcribed form of the human homologue. Recombinant proteins that were synthesised lacking the N terminal region could induce chromatin condensation only after cleavage by caspase 3 at Asp 1093. Extracts from Jurkat T cells immunodepleted of acinus were unable to induce chromatin condensation. Jurkat T cells do not express lamin A, so it is
possible that in these cells Acinus, after processing by caspase 3 and other proteins, is responsible for condensation while in HeLa cells it is by caspase 6 cleavage of lamin A that chromatin condensation occurs (Sahara et al, 1999).

A biochemical change, unique to apoptosis is the cleavage of DNA into internucleosomal fragments of 180bp (Wyllie, 1980). The protein responsible for these changes is a DNase identified as DFF40 or CAD, DFF40/CAD is able to induce chromatin condensation, but it was originally isolated by 3 separate groups on the merits of its DNA cleavage function. The protein was isolated from lymphoid cells (Enari et al, 1998), HeLa cells (Liu et al, 1997) and Jurkat cells (Halenbeck et al, 1998) and was identified as CAD, CPAN or DFF respectively. For ease, it will be identified here as CAD.

CAD is a cytosolic protein bound to its inhibitor (ICAD), following cleavage of ICAD by caspase 3 CAD translocates to the nucleus where it is able to cleave genomic DNA. Degradation by CAD appears to be a two-step process. Firstly 50-200 kbp fragments are generated, possibly by cleavage of AT rich regions at the nuclear scaffold. It is hypothesised that this allows unfolding of the DNA such that the spacer regions between nucleosomes are exposed to CAD, thus the 180bp fragments are produced.

Two typical features of apoptosis, DNA condensation and degradation to nucleosomal fragments are dependent on enzymes whose activation is bought about by a caspase. Lamins are cleaved by caspase 6, and caspase 3 is responsible for the activation of Acinus and CAD. The caspase family of proteins lie at the heart of apoptosis regulation.
1.2.2 Caspases

The caspase family of proteases can disassemble a cell quickly, they are important to the cell to prevent uncontrolled growth and division, as important to the cell are the protective mechanisms employed to keep the caspases sheathed. In this section caspases will be discussed within the paradigm of the caspase activation cascade.

Caspases were first identified by homology with the *Caenorhabditis elegans* CED-3 gene. The nematode worm *C. elegans* has been used as a model organism in genetic and development studies. Researchers noted the apoptosis of 131 cells from 1090 during its development (Ellis *et al.*, 1991) and this observation led to the biochemical investigation of cell death. Following a period of varied nomenclature the 14 known caspase genes are now generally identified as caspase 1-14. The word caspase is derived from the enzyme’s Cysteine protease mechanism, and "aspase" is from the distinctive ability to cleave after aspartic acid (Alnemri *et al.*, 1996). Some caspases were previously named ICE-like proteases after caspase 1 was originally identified as ICE-1 (Interleukin-1β Converting Enzyme), others were identified as Mch proteins with others not identified in either nomenclature (Earnshaw *et al.*, 1999). Caspase 1 itself was identified as part of the inflammatory response and maturation of cytokines, similarly, other caspases (4, 5 etc) are not involved in the apoptotic pathway (Nicholson and Thornberry 1997), and will not be discussed further.

1.2.2.1 The caspase cascade

The caspases necessary for apoptosis can be separated into initiator and effector caspases. Membrane receptor caspases (8 and 10) possess a death effector domain (DED) while intrinsic initiator caspases (2 and 9) contain a caspase recruitment domain.
(CARD). These pro-domains are important in the oligomerisation and activation of the caspases.

The nature of the caspase cascade was elucidated by a series of experiments in a cell free system (Slee et al, 1999, Slee et al, 2001). Using cytochrome c (cyto c) to induce apoptosis in Jurkat T cell extracts the authors demonstrated cleavage of apoptotic caspases but not pro-inflammatory caspases. Following activation by cyto c caspases 2,3,6,7 and 9 were found cleaved within 30min, but the membrane receptor caspases 8 and 10 were not cleaved until about 60mins, demonstrating a positive feedback mechanism in the caspase cascade. Initiator caspases may cleave effectors which in turn activate other initiators. In this system caspase 9 was shown to be the apical caspase by immunodepletion, extracts lacking caspase 9 were unable to process downstream caspases (Slee et al, 1999). In vitro this effect has been replicated in casepe 9~embryonic stem cells. These cells are resistant to many apoptotic stimuli and do not process downstream apoptotic targets, such as caspase 3 or poly ADP ribose polymerase (PARP), in response to ultraviolet (UV) irradiation. Requirement for caspase 9 in apoptosis is not absolute, α-Fas induced apoptosis was not affected by a lack of caspase 9 in Jurkat T cells and dexamethasone treated thymocytes show a limited amount of apoptosis with a limited amount of downstream processing (Hakem et al, 1998 and Kuida et al, 1998).

The second phase of cyto c induced caspase cleavage was investigated using the peptide inhibitor DEVD-CHO which inhibits caspase 3 and 7 activity. In the presence of this inhibitor maturation of caspases 2,6,8 and 10 does not occur, while caspase 3 and 7 are still processed into active enzymes. To extend these observations cell extracts were immunodepleted of caspase 3. Removal of caspase 3 had no effect on processing of caspase 7 but prevented activation of caspase 2,6,8 and 10 (Slee et al, 1999). This
evidence suggests that caspase 3 might be a caspase specific caspase while caspase 7 continues with the job of cleaving other cellular substrates. Further immunodepletion experiments did not bear out this theory. Depletion of caspase 7 does not affect cleavage of a wide variety of substrates investigated. Indeed it is caspase 3 that is a requirement for cleavage of many substrates while caspase 6 is required to cleave lamin A (Slee et al, 2001). Caspase 3 is however indirectly responsible for cleavage of caspase 8 and 10 as immunodepletion of caspase 6 prevents processing of these caspases. Thus a cascade of caspase activation can be extrapolated from these data. Following cyto c/dATP activation of caspase 9 caspase 3 and 7 are the next level of activation with caspase 3 feeding back upstream to caspase 9 and 2 and via caspase 6 activating caspase 8 and 10 (Figure 1.5).
After Slee et al, 1999

**Figure 1.5: The caspase activation cascade**
Using a cell free system the above cascade of caspase activation was identified. The triggering event is release of cyto c from the mitochondria, allowing formation of the apoptosome with the subsequent cleavage of caspase zymogens as illustrated and explained in the text.
Inhibition of caspase 3 by DEVD-CHO in the cyto c activated cell lysates resulted in an active enzyme but not a fully processed enzyme. The inactive zymogen is a 32kDa cytosolic protein which contains 2 caspase recognition sites, a caspase 3-like recognition site ESMDjS and a caspase 8 site IETDjS. Normal cleavage of the caspase releases a 3kDa pro-domain and the fully mature p17/p12 caspase. Competitive inhibition of the ESMDjS cleavage generated a p20/p12 product with no p17 fragment, conversely inhibition of the IETDjS cleavage site tended to maintain the p32 protein, at lower concentrations of the inhibitor the p17 fragment was observed (Han et al, 1997). These data suggest an initial cleavage by an initiator caspase at IETDjS to generate the p20/p12 product followed by autocatalysis and removal of the pro-domain to the p17/p12 enzyme. The pro-domain of caspase 3 functions to prevent spontaneous activity of the enzyme as demonstrated by truncated recombinant protein studies (Meergans et al, 2000). Generation of the p17/p12 product is inhibited by the X-linked inhibitor of apoptosis protein (XIAP), which inhibits caspase 9 but not caspase 8, suggesting that the initiator caspase in the cell free system used is caspase 9. Replacing the D residue with A in caspase 3 prevents recruitment of the enzyme to the apoptosome and subsequent processing by caspase 9 (Bratton et al, 2001).

1.2.2.2 Caspase activation

Effector caspases are activated by apical caspases such as caspase 8 and 9. Caspase 8, the apical caspase in the membrane death receptor pathways, auto-activates by homodimerisation. For caspase 9 the situation is slightly different, cleavage is not necessary for its activation.
The induced proximity model of caspase activation was examined using a dimer of FK506. The dimer was able to bind the FK binding proteins (FKBP) but lacked calcineurin binding activity. The prodomain of caspase 8 was replaced by the FKBP binding domain, thus mimicking the effect of Fas cross-linking by allowing two caspase 8 molecules to come into close proximity of each other. Following dimerisation, the constructs induced apoptosis as demonstrated by PARP and DNA cleavage (Muzio et al, 1998). The physiological relevance of over-expressed caspases forcibly, chemically co-localised is questionable. The exact orientation of the zymogens in the death inducing signalling complex (DISC) is also unknown and may not be reproduced in these experiments.

Caspase 8 is cleaved at 3 sites, two cleavages are required to remove a linker domain and one for the pro-domain (Figure 1.6). These cleavage sites exemplify caspase recognition motifs, four amino acids (P₁-P₂-P₃-P₄) with cleavage after P₁ (Thornberry et al, 1992). P₁ is almost invariably aspartic acid (D) with glutamate (E) the preferred residue in the P₃ position.
Figure 1.6: Cleavage sites of caspase 8

Caspase 8 exists as a zymogen until aggregation of the caspase by Fas crosslinking. Cleavage sites are indicated by arrows with the cleaved residues shown. The first cleavage is at the VETD\textsuperscript{316}S site. This generates a large (p18) and small (p11) subunit, with further cleavages at TISD\textsuperscript{168}S to remove the prodomain and LEMD\textsuperscript{385}S to remove the linker peptide (Watt et al., 1999).

The processing of the zymogen leads to the assembly of the mature caspase. Active caspases exist as a tetramer of two long and two short subunits. The caspase 8 p18/p11 dimer is cylindrical with one active site. Each p18/p11 dimer associates with a second, thus the active caspase contains two active sites. Residues in the active sites of caspase 1, 3 and 8 contain strongly conserved amino acids, especially in the pocket that binds P\textsubscript{1} of the substrate (S\textsubscript{1}). The S\textsubscript{1} region is composed of 2 arginine (R) residues, a glycine (G) residue and 2 serine (S) residues in caspase 3 and 1, in caspase 8 one serine is replaced by a threonine. This explains the high level of specificity for aspartic acid at this position. The preference for glutamine at P\textsubscript{3} is explained by a conserved arginine residue in the S\textsubscript{3} pocket that mediates this binding (Blanchard et al., 1999).

Data from caspase 7 has identified an N terminal section of the small subunit that flips through 180° to stabilise the catalytic domain on binding of the substrate. This conformational change is only allowed by cleavage of the large and small subunits, explaining why the zymogens are inactive (Chai et al., 2001). Recombinant caspases in
which the sequences of the large and small subunits are inverted have been generated, these enzymes are constitutively active (Srinivasula et al, 1998).

1.2.2.3 Inhibitor of Apoptosis Proteins

Maintaining caspases as inactivezymogens is one level of caspase control, the compartmentalisation of caspases is another level, further control can be exerted through phosphorylation of caspases. Human caspase 9 can be inactivated via Akt phosphorylation of Ser196 (Cardone et al, 1998) although this site is not present in murine caspase 9 (Fujita et al, 1999). The baculovirus p35 protein and the cowpox CrmA are viral inhibitors of caspase activity (Bump et al, 1995, Zhou et al, 1997). Baculoviruses complement the function of p35 with two inhibitors of apoptosis proteins (IAPs) (Crook et al, 1993, Clem et al, 1994) which are identified by their baculoviral IAP repeat domains (BIR). The BIRs are conserved and are found in endogenous mammalian IAPs.

The mammalian X linked IAP (XIAP) contains 3 BIR domains and an N terminal RING zinc finger (Takahashi et al, 1998). Recent studies on the interaction of XIAP and various caspases have helped elucidate the mechanism of caspases and IAPs. In a cell free system recombinant XIAP prevented apoptotic nuclear changes and caspase 3 activity. Similarly following transient transfection of XIAP into 293T cells apoptosis induced by Bax overexpression was inhibited. Processing of caspase 3 to the p17 fragment was also inhibited, demonstrating the role of XIAP in inhibition of caspase 9 and 3. The inhibition of caspase 7 activity was also demonstrated using purified recombinant enzymes (Deveraux et al, 1997).
It was found that the second BIR domain and a linker region were necessary for inhibition of caspase 3 and 7 (Sun et al, 1999) and using this fragment the structure of inhibitor bound caspase 7 was determined (Huang et al, 2001, Chai et al, 2001). Caspase 7 forms a substrate binding cleft using 4 peptide loops, this pocket is filled by part of the linker fragment of XIAP. Two residues Leu141 and Asp148 of XIAP are absolute requirements for caspase 3 inhibition (Sun et al, 1999), these residues fit snugly into the binding cleft. Many of the interactions of XIAP and caspase 7 were similar to the interactions of a tetrapeptide inhibitor (DEVD-CHO) and caspase 7 (Wei et al, 2000), despite the fact that the two substrates bind to the catalytic groove in opposite orientations. Riedl et al (2001) demonstrated that the actual BIR domain had limited connections with the caspase enzyme (using caspase 3) and confirmed that inhibition was effected by the linker domain. The BIR domain appears to stabilise the conformation of the linker domain in this situation, stabilisation and inhibition of caspase activity could be achieved by binding the linker region with GST (Chai et al, 2001).

1.2.3 Apoptotic pathways

1.2.3.1 The role of the mitochondria in apoptosis

Mitochondria have long been known for the respiratory chain, producing ATP, and having their own DNA, however as mediators of cell death (apoptosis and necrosis), these organelles have truly duplicitous characters. Exemplifying the dual nature of the mitochondria is cyto c. As a factor in the respiratory chain this molecule functions as an
electron carrier, but following pro-apoptotic signals, cyto c is released from its place in the mitochondrial membrane as serves as a vital part of the apoptotic machinery.

1.2.3.1a Structure of mitochondria

Advances in electron microscopy have led to the recognition that the conventional model of internal mitochondria structure is incorrect. The arrangement of the mitochondrial cristae was thought to be in a 'baffle' formation, with the inner mitochondrial membrane folded in on itself many times. Following the use of EM tomography an alternative model has been suggested. The cristae are distinct structures, not a continuation of the inner mitochondrial membrane. Individual cristae are tubular structures connected to the inner membrane by 28nm christa junction (Frey and Manella, 2000). Functionally this implies that the internal mitochondrial environment is more restricted than previously thought. This may then have important implications in the process of oxidative phosphorylation and transport of cyto c during apoptosis.

1.2.3.1b Bcl-2 protein family

The Bcl-2 protein family have helped to elucidate the ways in which mitochondria control the apoptotic process. The protein family is identified by Bcl-2 homology (BH) domains, however overall homology between proteins is low. Table 1 shows a (non-comprehensive) list of Bcl-2 family members and their apoptotic allegiance. There remains a large degree of speculation as to the actual function of individual Bcl-2
proteins, however given the heterogeneity of structure it is perhaps unsurprising that different family members appear to play very different roles.

Table 1 Pro- and anti-apoptotic Bcl-2 family molecules

<table>
<thead>
<tr>
<th>Pro-apoptotic</th>
<th>Anti-apoptotic</th>
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<tbody>
<tr>
<td>Bcl-Xs (Boise et al, 1993)</td>
<td>Bcl-Xl (Kharbanda et al, 1997)</td>
</tr>
<tr>
<td>Bid (Wang et al, 1996)</td>
<td>Bcl-w (Gibson et al, 1996)</td>
</tr>
<tr>
<td>Bok (Hsu et al, 1997)</td>
<td>Mcl-1 (Lomo et al, 1996)</td>
</tr>
<tr>
<td>Bim (O’Connor et al, 1998)</td>
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<tr>
<td>Bad (Yang et al, 1995)</td>
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<tr>
<td>Bik (Boyd et al, 1995)</td>
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<tr>
<td>Bmf (Puthalakath et al, 2001)</td>
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<tr>
<td>Hrk (Inohara et al, 1997)</td>
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<tr>
<td>Noxa (Oda et al, 2000)</td>
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<tr>
<td>Puma (Yu et al, 2001)</td>
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There are 4 BH domains, the function of which is to regulate protein interaction (Borner et al, 1994) but specific functions have not been assigned to any domain. The eponymous Bcl-2 was identified in a B cell lymphoma (Tsujimoto et al, 1985), upregulation of the gene and over-expression of the protein product protected cells from a wide variety of apoptotic stimuli (Rossé et al, 1998, Itoh et al, 1993, Yang et al, 1997). Bcl-2 and the C. elegans CED-9 protein share homology, indeed Bcl 2 can protect C. elegans embryonic cells from apoptosis (Vaux et al, 1992). The anti-apoptotic effects of
Bcl-2 are focused on the mitochondrial machinery, but its true function is not entirely clear. Pro-apoptotic Bax was identified by co-immunoprecipitation with Bcl-2 (Oltvai et al, 1993) immediately suggesting interaction of the two family members is responsible for swinging the balance between life and death. Further clues to the function of Bcl-2 were found at membranes. Unlike some family members Bcl-2 is localized solely in membranes (mitochondria, nucleus and endoplasmic reticulum) (Krajewski et al, 1993), studies in synthetic membranes demonstrate that Bcl-2 proteins can form pores (Schendel et al, 1997, Minn et al, 1997). These observations led to theories that pro-apoptotic Bcl-2 family members formed channels in the mitochondrial membrane allowing consequent apoptotic events. Anti-apoptotic Bcl-2 proteins may block pore formation (Antonsson et al, 1997). This theory was tested in HL-60 cells challenged with staurosporine and etoposide. It was found that cyto c was lost from the mitochondria of control cells to the cytosol (3-4hrs) but in cells overexpressing Bcl-2 the overwhelming majority of cyto c remained in the mitochondria (Yang et al, 1997). Similar results were obtained when the role of Bcl-X\textsubscript{L} was investigated in response to ionising radiation, indeed it was found that cyto c bound directly to Bcl-X\textsubscript{L} in transgenic cells overexpressing Bcl-X\textsubscript{L}. It was found that pro-apoptotic Bcl-X\textsubscript{S} did not bind cyto c but did bind to Bcl-X\textsubscript{L} and, in excess, prevented the association of Bcl-X\textsubscript{L} and cyto c (Kharbanda et al, 1997). These data support the hypothesis that pro-apoptotic Bcl-2 proteins form cyto c permeable pores at the mitochondrial membrane and these pores can be regulated by anti-apoptotic Bcl-2 proteins by their association with both Bcl-2 proteins and cyto c.

To investigate the function of Bcl-2 Yang et al (1997) overexpressed Bcl-2 in HL-60 cells and found release of cyto c and caspase activation blocked following apoptotic stimulation. Rossé et al (1998) overexpressed Bax in R6 and SK2 cells and found cyto c
was released. When Bcl-2 was overexpressed in conjunction with Bax it was expected that Bcl-2 would block the release of cyto c. However in this (non-physiological) situation cyto c release was not blocked by Bcl-2. Apoptosis was nonetheless prevented by overexpressed Bcl-2. Bcl-2 works on two levels, in the first instance Bcl-2 and Bax interact at the mitochondrial level and by this interaction regulate the release of cyto c. At a second level Bcl-2 functions downstream of cyto c release and prevents caspase activation and apoptosis. More recent research shed some light on this. Second Mitochondria-derived activator of caspase / Direct IAP binding protein with low pi (Smac/DIABLO) is a mitochondrial protein that, like cyto c, when released from the mitochondria can drive a cell to apoptosis (Du et al, 2000, Verhagen et al, 2000). It was found that Bcl-2 and Bcl-XL were capable of inhibiting the release of Smac/DIABLO from the mitochondria. Smac/DIABLO functions by interacting with endogenous inhibitors of caspases and regulating caspase activity (Ekert et al, 2001).

The liver is susceptible to apoptotic injury by the Fas pathway following injection of anti- Fas antibodies (Ogasawara et al, 1993). In the hepatocyte FasL induces a mitochondrial death pathway (Feldmann et al, 2000) that can be attenuated by manipulation of Bcl-2 family proteins. Bcl-2 and Bcl-XL transgenic mice are resistant to apoptosis induced by the Fas antibody Jo2. Bcl-XL overexpression appears to work in a dose dependent manner as, in the heterozygote population, 58% of mice survive whereas in the homozygote population 85% survive. In the hepatocyte D-galactosamine (D-GalN) leads to the depletion of uridine nucleotides and transcriptional arrest. When a non-lethal dose of Jo2 is administered with D-GalN in vivo Bcl-2 loses its protective effect. At 0.15mg/kg Jo2 is non-lethal to wild type mice in vivo, however in combination with D-GalN 88% wild type mice died, and 80% of a sample size of 10 Bcl-2 transgenics succumbed. On the other hand 80% of Bcl-XL transgenic mice survived the
same treatment (De La Coste et al, 1999). These results suggest Bcl-2 and Bcl-XL have different modes of action, with Bcl-2 requiring transcription to exert its effect.

A third Bcl-2 protein that influences the Fas apoptosis pathway in the hepatocyte is the "BH3-domain only" Bid (Wang et al, 1996, Yin et al, 1999). Bid exists in the cytoplasm as a 22kDa molecule that is cleaved following caspase 8 activation to a truncated 15kDa form (tBid) (Gross et al, 1999, Li et al, 1998, Luo et al, 1998) which translocates to the mitochondrial membrane where it allows the release of cyto c (Zha et al, 2000).

Mice generated with a non-functional Bid gene were treated with anti-Fas antibody. Nine of the eleven treated mice survived whereas 7 of 8 wild type controls died within 6hrs. The effect of Bid is not influenced by the loss of transcription. Cyclohexamide (CHX) and Jo2 were co-administered in cultured hepatocytes, after 8 hrs Bid"+" cells remained viable while wild type cultures had lost ~40% viability. In vivo Bid"+" mice were not wholly protected, 2 of the 11 mice treated with Jo2 died after 6.6 hours.

Analysis of the livers revealed that effector caspases 3 and 7 were activated, although largely found at the periphery of the cell, there was little evidence of apoptotic nuclear morphology, and cyto c was detectable in the cells. The authors suggest a modifier locus to be responsible for this injury (Yin et al, 1999).

Bid is a "BH3 domain only" protein and as such it does not have membrane-spanning domains (Wang et al, 1996). To exert its function Bid needs to interact with other Bcl-2 family proteins such as Bak and Bax (Korsmeyer et al, 2000, Wei et al, 2000). Using two models of receptor mediated apoptosis, D-GalN in conjunction with TNFalpha (induced by lipopolysaccharide (LPS) injection) and Fas antibodies, in vivo Zhao et al (2001) found cleavage of Bid and translocation to the mitochondrial membrane in murine hepatocytes. Bax was also found to translocate and insert into the mitochondrial membrane in wild type mice. Using Bid deficient mice it was found that Bax would
translocate to the mitochondria but, in the absence of Bid, would not insert into the membrane. Conversely, Bax deficient mice were not resistant to cyto c release, caspase activation and apoptosis implying that Bid interacts with a second protein to induce apoptosis. In receptor mediated apoptosis Bid is required but Bax plays a redundant role (Zhao et al, 2001). The second protein that Bid is known to be involved with is Bak. Mouse embryonic fibroblasts expressing tBid undergo spontaneous apoptosis, this is also seen in fibroblasts deficient for either Bak or Bax. However Bak and Bax double knockout fibroblasts do not undergo apoptosis. A wide variety of apoptotic stimuli including staurosporine, etoposide, and UV radiation cause apoptosis through the mitochondrial pathway as demonstrated by the fact that the double knockout embryonic fibroblasts are resistant to all these stimuli. Additionally this pathway can be exploited through at least 2 disparate routes as Bid deficient cells did not survive these challenges. The liver of both Bax and Bak single knockout mice were susceptible to Fas apoptosis in vivo. However, the double knockouts were resistant to this stimulus (only 3 double knockout mice reached adulthood in this study since a large number of these mice die at birth) (Wei et al, 2001).

The Bcl-2 family proteins have a variety of interactions with each other and are critical to regulation of apoptosis at the mitochondrial level. It has been hypothesised that part of the function of Bcl-2 family proteins is to form a pore permeable to pro-apoptotic mitochondrial proteins such as cyto c, and Smac/DIABLO. The existence and exact nature of this pore is the subject of some debate.
The mitochondria maintain an electrochemical gradient between the matrix and intermembrane space. This is achieved by a proton pump, sustained by the respiratory chain, which transports protons out of the inner membrane. The gradient is maintained by the impermeability of the inner mitochondrial membrane, the outer membrane is permeable to solutes of less than 5kDa due to the presence of the voltage dependent anion channel (VDAC). The chemiosmotic model was first postulated by Mitchell in the 1960s (Mitchell and Moyle, 1967). The inner membrane, however, can become permeable to solutes below 1.5kDa in size. This event characterises the mitochondrial permeability transition (MPT) or loss of membrane potential ($\Delta\psi_m$). MPT may be transient (Szalai et al, 1999) or permanent and lethal (Lemasters et al, 1998). Research on isolated mitochondria demonstrated a dazzling array of substrates that would induce MPT (reviewed by Zoratti and Szabo 1995).

Theories as to the origin of $-\Delta\psi_m$ focussed on formation of a pore, membrane disruption or some combination of both. Illustrating the evidence to support the membrane damage hypothesis is the work of Pfieffer's group who investigated the function of phospholipase A$_2$. Mitochondrial permeability would be increased by PA2 due to insertion of lysophospholipids in the inner membrane. Inhibiting this transition was acyl-CoA-lysophospholipid acyl transferase. The interaction between the two enzymes shared similarities with MPT, in that it was Ca$^{2+}$ dependent, and pharmacological inhibitors of MPT, were found to inhibit phospholipase A$_2$ with the same effectiveness (Beatrice et al, 1980).

More evidence, however, has accumulated that is consistent with MPT being due to a pore. The inner membrane is permeable to molecules up to 1.5kDa (Haworth and
Hunter 1979, Crompton et al, 1987), this strict cut off point suggests a pore rather than membrane damage. Other inhibitors of -ΔΨm and apoptosis, such as cyclosporin A (CsA) and bongkrekic acid, also lend weight to the pore theory. As discussed above the pro-apoptotic Bcl-2 family members can form pore-like structures at the mitochondrial membrane and may have some involvement.

In one model the Adenine nucleotide transporter (ANT) opens allowing osmotic swelling of the inner membrane. It is hypothesised that due to the larger surface of the inner membrane, the outer membrane ruptures and mitochondrial contents are released (Narita et al, 1998. Crompton 1999, Brenner et al, 2000). This mechanism may have to be reassessed in light of emerging data on the structure of the inner membrane.

Much experimental evidence, however, suggests the involvement of the ANT. CsA blocks MPT by binding cyclophilin D which interacts with ANT (Woodfield et al, 1998), bongkrekic acid maintains ANT in its native form (Zamzami et al, 1996), thus blocking MPT, conversely atractyloside and calcium keep the pore in the open position, thus encouraging MPT (Halestrap and Davidson, 1990). Bcl-2 family members may exert their pro- and anti-apoptotic influence by specific interaction with the ANT (Marzo et al, 1998, Jacotot et al, 2001).

A second model postulates the VDAC as a cyto c channel, causing permeabilisation of the outer membrane but not the inner membrane. Experiments describing Bcl-XL inhibiting the release of cyto c from VDAC containing liposomes and Bax and Bak enhancing cyto c release support this theory (Shimizu et al, 1999). Additionally, cyto c translocation is induced by Bax. Koenig’s polyanion, a VDAC inhibitor, inhibits cyto c translocation suggesting that Bax plays a secondary role to VDAC, and VDAC itself forms the pore (Saito et al, 2000). The evidence also casts doubt on another theory where Bcl-2 family members form an independent pore (Korsmeyer et al, 2000).
Bringing together the inner membrane and the outer membrane is an interaction between VDAC and ANT, a transient increase in $\Delta \psi_m$ would result in a voltage dependent closure of VDAC and loss of outer membrane permeability. The excess electrical gradient is proposed to be dissipated by a Bcl-2 family ion pore. Pro-apoptotic Bcl-2 members would block this pore leading to outer membrane rupture and release of cyto c from the inter membrane space.

1.2.3.1d Mechanism

Interest in mitochondrial involvement in apoptosis was sparked by experiments demonstrating the need for mitochondria to induce apoptotic morphology in isolated nuclei. Critical observations were made on cyto c translocation and since then many other factors crucial in control of apoptosis have been identified in the mitochondria. The apoptosome, a structure analogous to the membrane death receptor death inducing signalling complex (DISC), has been identified. Composed of both mitochondrial and cytosolic factors its successful induction is key to the mitochondrial apoptotic pathway. Using the MPT modulators atractyloside and bongkrekic acid (inducer and repressor respectively) Zamzami et al (1996) demonstrated that isolated mitochondria needed to be undergoing MPT to induce apoptotic nuclear morphology. This system utilised HeLa nuclei and murine hepatocyte mitochondria with an ATP regeneration system. Purified mitochondria were unable to induce nuclear changes until treated with atractyloside (the drug alone had no effect on isolated nuclei). However, atractyloside treated mitochondria in the presence of bongkrekic acid did not induce apoptotic nuclear changes. Similarly mitochondria isolated from control hepatocytes had no effect on
isolated nuclei but mitochondria from hepatocytes in which apoptosis had been induced were effective in inducing chromatin condensation.

The authors identified a mitochondrial, heat labile soluble factor >10kDa in size that was able to induce apoptotic changes in the absence of the entire organelle (Zamzami et al, 1996). Liu et al (1996) purified a pro-apoptotic 15kDa protein from HeLa cell extracts. By spectrophotometric absorbance and amino acid sequence analysis the protein was identified as cyto c. Immunodepletion experiments confirmed that cyto c was the active factor (Liu et al, 1996).

The release of cyto c from the mitochondria was studied in HeLa cells stably transfected with a cyto c/ green fluorescent protein (GFP) fusion protein. It was shown that, following a variety of apoptotic stimuli, cyto c release went from initiation to completion in approximately 5 min. The time from the initial challenge to initiation of cyto c release varied according to the insult, (e.g. initiation in staurosporine treated cells varied from 4 to 20 hours but once started cyto c was released from the mitochondria of an individual cell in 5 min) (Goldstein et al, 1997).

Cyto c required both ATP or dATP and the protein Apaf-1 (apoptotic protease activating factor) to induce apoptosis in the cell free system used by Zamzami et al (1996). Apaf-1 is a cytosolic protein (Zou, et al, 1997) with a region homologous to the C. elegans CED 4 protein. There is an N terminal CARD, which allows binding of caspases and C terminal WD-40 repeats mediating Apaf-1 oligomerisation (Srinvasula et al, 1998).

Cyto c in the presence of ATP or dATP allows oligomerisation of Apaf-1 via its WD-40 repeats. The uncleavable ATP-CP analogue of ATP allows apoptosome formation suggesting that the fate of ATP in the apoptosome is not a hydrolytic one, but binding (Jiang and Wang 2000).
A truncated Apaf-1 lacking 664 amino acids at the WD-40 containing C terminus was generated. Full length Apaf-1 recruited and activated caspase 9 in the presence of cyto c and ATP. Cyto c and ATP were not necessary for caspase 9 activation by the truncated Apaf-1, demonstrating that the WD-40 repeats, cyto c and ATP together regulate the activation of caspase 9 by Apaf-1. Further truncated forms of Apaf-1 were generated and it was shown that both the CARD domain and the CED 4 homology domain were necessary for Apaf-1 activity (Srinvasula et al, 1998).

Apaf-1 was found to form a biologically active multimer of ~700kDa in cell lysates. This complex was also identified in apoptotic cells. A proportion of Apaf-1 remains as monomers, and a small proportion was found to form an inactive complex of 1.4MDa. The significance of the two forms of apoptosome – one active, the other inactive, is unknown. The larger form may be a result of dysregulated binding and oligomerisation and may allow post-mitochondrial control of the caspase activation sequence (Cain et al, 2000).

The potential workings of the apoptosome have been elucidated by the recent publication of its three dimensional structure (Acehan et al, 2002). The apoptosome consists of 7 Apaf-1 molecules forming a wheel like structure. The C terminal CARDs form the hub of the structure with the CED-4 homology domain comprising part of the hub and an extension from the hub. The WD-40 repeats form a ‘Y’ shaped domain at the end of the spokes. It is proposed that the WD-40 Y domain sequesters the CARD in Apaf-1 monomers, cyto c may displace the CARD in a competitive manner, while ATP binding locks the monomer into the open configuration allowing oligomerisation. Pro-caspase 9 binds via CARD interactions to the hub in a 1:1 ratio with Apaf-1, this allows recruitment of further pro-caspase 9 molecules, dimerisation and activation. The observations of Cain et al (2000) that a ~700kDa apoptosome complex is biologically
active do not appear to be consistent with this model. Acehan et al (2002) eluted an active fraction at ~1.3MDa and isolated the apoptosome from Apaf-1 overexpressed in insect cells, unlike Cain et al (2000) who used human cells. However, one observation may help to explain the biologically inactive ~1.4MDa complex observed by Cain et al (2000). The apoptosome complex can form a dimer complex where supposedly the CARDs of caspase-9 molecules bound at the hub join 2 apoptosome complexes (Acehan et al, 2002). If a single apoptosome complex is active the active site may be blocked by the dimerisation corresponding to the 1.4MDa complex.

1.2.3.2 Membrane death receptors

In the cell free systems discussed cyto c has been used as the initiator of apoptosis, inducing oligomerisation of Apaf-1, activation of caspase 9 and subsequent activation of downstream caspases. This apoptotic pathway is termed the intrinsic pathway. An extrinsic pathway is initiated by membrane death receptors through caspases 8 and 10. Fas (CD95), TNFα and TNF-Related Apoptosis Inducing Ligand (TRAIL) are membrane bound death receptors which, when cross linked by their ligands can lead to assembly of a multi-protein death inducing signalling complex (DISC). Membrane death receptors base their activation on binding of ligands and receptor trimerisation allowing interaction of cytoplasmic domains and intracellular signalling. The DISC may be seen as analogous to the apoptosome on a certain level. Both DISC and apoptosome cause co-localisation of caspases which allows autoactivation, similarly inhibition of caspases can occur at the DISC and apoptosome.
Members of the TNF receptor superfamily have a variety of functions, only one of which is to induce apoptosis. The superfamily consists of 28 receptors with 18 ligands, of these 5 cause apoptosis. TNF-R1 was the first to be identified with Fas (CD95, APO1), DR3 (APO3/TRAMP/Wsl-1/LARD), DR4 (TRAIL-R1) and DR5 (TRAIL-R2) following. “Decoy” receptors exists for some of these ligands soluble Fas can “mop up” FasL on CTLs (Krams et al, 1998) while DcR1 and DcR2 lack crucial apoptotic signalling domains.

1.2.3.2a DISC composition

The intracellular domain of membrane death receptors contains a conserved region of 66 amino acids described as the death domain (DD) (Itoh et al, 1993). Through a homotypic DD interaction Fas associated death domain (FADD) binds to the receptor complex (Boldin et al, 1995 and Chinnaiyan et al, 1995). FADD binding then allows recruitment of caspase 8 (Medema et al, 1997) through the death effector domain, all of which is collectively known as the DISC (Kischkel et al, 1995). Decoy receptors lack the DD and hence cannot induce DISC formation (Sheridan et al, 1997 and Pan et al, 1997).

Receptor trimerisation is initially mediated by the receptor’s own pre-ligand association domain at the extracellular N terminal cystein rich domain (Siegel et al, 2000).

Following ligand binding a conformational change occurs to allow DD interactions.
One paradigm pathway to apoptosis is the Fas pathway. Two papers integrated the death receptor pathway (discussed above) and the mitochondrial pathway into one functional unit. The authors identified two groups of cell types that activate apoptosis through Fas by two different mechanisms. Type I cells demonstrated early activation of caspase 8 and 3 (within 10 min). Type II cells were identified by late activation of caspase 8 and 3 (60 min), despite high levels of Fas expression. The kinetics of apoptosis were however similar between the two types, as was the kinetics of mitochondrial membrane depolarisation. The difference in the two cell types lay in the reliance on the mitochondria. Type II cells such as hepatocytes (Yin et al., 1999) have a requirement for mitochondrial involvement. Overexpression of Bcl-2 did not affect the susceptibility of type I cells to Fas induced apoptosis but in Type II cells overexpression of Bcl-2 was protective and apoptosis was reduced (Scaffidi et al., 1998).

The significance of the existence of two pathways may be one of cellular control. Type II cells can introduce measures to affect the decision to die at a variety of levels. PKC activation can reduce the amount of tBid formed which would not affect apoptosis in type I cells but protects type II cells. At this level of control the mitochondrial step is protective, but the mitochondria can also sensitise to apoptosis. As an example ceramide can induce apoptosis only in type II cells not in type I cells (Scaffidi et al., 1999).
1.3 The role of IFNγ

1.3.1 IFNγ in hepatitis

IFNγ has been demonstrated to be present in hepatitis in various settings. The cytokine has been found to be a key player in models of hepatitis.

1.3.1.1 Conconavalin A

Conconavalin A (ConA) is a T cell mitogen that causes activation of T cells and acute hepatitis (Tiegs et al, 1992). A range of cytokines are detected in ConA induced hepatitis including TNFα, IFNγ, IL-2 and IL-6 (Mizuhara et al, 1994, Mizuhara et al, 1996). Following injection of ConA, serum IFNγ levels reached a peak at 8hrs. Monoclonal antibodies to IFNγ were administered to the mice 30mins before ConA injection. This treatment reduced the sALT levels at 24hrs approximately 10 fold (Mizuhara et al, 1996) i.e. there was a reduction in liver injury. The injury in this model may be mediated by the direct effect of IFNγ. However, the speed of the response suggests that CD8+ T cells are the agents of death. In addition to CD8+ T cells both eosinophils (Louis et al, 2002) and Natural Killer T cells have a pathological role (Takeda et al, 2000).

1.3.1.2 Overexpression of viral protein

A second model of acute hepatitis utilises HBV transgenic mice. The transgenic mice contain a copy of the envelope-coding region, which is overexpressed. In HBV infection
the gene product, (HBsAg), is synthesised in the cytoplasm of infected hepatocytes and secreted into the blood. HBsAg expression renders hepatocytes acutely sensitive to the cytopathic effects of IFNγ as discussed previously (Gilles et al, 1992).

1.3.1.3 Overexpression of IFNγ

A mouse model of chronic hepatitis exists that utilises an IFNγ gene linked to a liver specific promoter. Serum ALT levels in these mice increased gradually throughout their life and liver histology similar to chronic active hepatitis was seen. Liver damage was positively correlated to the amount of IFNγ mRNA transcribed (Toyonaga et al, 1994). The authors did not investigate whether the effects were due to CD8+ cytotoxicity or a direct effect on the hepatocyte. Sublethal irradiation of the mice would shed light and the question and provide further evidence of the action of IFNγ \textit{in vivo}.

1.3.1.4 Alcoholic hepatitis

Increases in IFNγ and other proinflammatory cytokines (TNFα, IL-1 and IL-12) were seen in an experimental model of alcoholic hepatitis. Rats were fed with different dietary fats and ethanol for 1 month before being sacrificed. It was found that fish oil with ethanol produced the most severe pathology and induced greatest expression of proinflammatory cytokines. Increased expression of proinflammatory cytokines was associated with larger necrotic foci and increased sALT levels (Nanji et al, 1999).
1.3.2 The role of IFNγ in apoptosis

IFNγ causes apoptosis in many cell types, it is reported to have effects on conjunctival cells (De Saint Jean et al., 2000), macrophages (Inagaki et al., 2002, Spanaus et al., 1998), cervical carcinoma cells (Um et al., 2000), ovarian epithelia (Quirk et al., 1997, Quirk et al., 1998), the vascular endothelium (Li et al., 2002) and various tumours (Lee et al., 2000, Ahn et al., 2002).

The anti-tumorigenic transcription factor through which IFNγ exerts its effects, IRF-1 has been exposed, along with co-operation with p53 to activate p21, downstream of these agents many of the mediators of IFNγ function are unknown (reviewed in Tura et al., 2001). IFNγ exerts its effects through Jak/STAT signalling (Lew et al., 1991, Muller et al., 1993, Watling et al., 1993, Meraz et al., 1996, Durbin et al., 1996) causing transcription of IRF-1 (Li et al., 1996). IRF-1 is essential for the apoptotic effect of IFNγ since IRF-1⁻/⁻ hepatocytes do not undergo apoptosis when challenged with IFNγ (Kano et al., 1999). A summary of current knowledge on IFNγ induced apoptosis in the hepatocyte is provided in figure 1.7.
Figure 1.7 Summary of consequences of IFNγ stimulation of hepatocyte. IFNγ signalling is well characterised up to a point. The anti-apoptotic transcription factor IRF-1 is upregulated following JAK/STAT activation, p53 and p21 contribute to cell cycle arrest. IRF-2, an oncogenic agonist to IRF-1 is also transcribed to allow negative feedback. Downstream apoptosis is induced by caspase 3-like proteases and is inhibited by HGF, EGF and 2% serum, however their site of action is unknown. For clarity positive modulators of apoptosis, such as TNFα are not included in this summary. After Tura et al, 2001.
were transcribed at a higher level in transgenic mice than in controls (Okamoto et al, 1998). IFNγ is capable of upregulating expression of Fas on primary murine microglia, increasing FasL induced apoptosis from about 20% to about 80% following 48 hours pre-incubation with IFNγ and 20hrs FasL treatment. IFNγ was also found to increase cFLIP and decrease expression of anti-apoptotic Bcl-2 (Spanaus et al, 1998). The murine cell line L929 is induced to express Fas by IFNγ (Mullbacher et al, 2002). An in vivo model of tumour progression using renal carcinoma cells reconstituted in to BALB/c mice has been published. The renal carcinoma cells were transfected with either Fas or, as a control, a truncated, inactive form of the Fas gene. When Fas overexpressing cells were injected into mice, the rate of tumour progression was slower than the in the controls. However in IFNγ−/− mice this effect was not seen, demonstrating a substantial interaction between IFNγ and the Fas pathway (Lee et al, 2000).

Primary murine hepatocytes express Fas constitutively, but FasL is not transcribed in cultures maintained with IFNγ (Kano et al, 1999). While primary hepatocytes are susceptible to both IFNγ and Fas induced apoptosis, human hepatoma cells are generally resistant to Fas induced apoptosis (Shin et al, 2001). Preincubation with IFNγ can sensitise cells to Fas induced apoptosis (Spanaus et al, 1998) however in hepatoma cells this course was ineffective (Shin et al, 2001). Further investigation of the system revealed that instead of a Fas dependent pathway, the hepatoma cells were susceptible to a TRAIL pathway (Shin et al, 2001a).
1.4 Aims

The experiments presented in this thesis attempt to investigate how IFNγ causes apoptosis in the hepatocyte. These experiments will establish a set of data that can be used to further study IFNγ induced apoptosis in the hepatocyte. Areas that could be investigated once a model of apoptosis has been established include how inhibition IFNγ induced apoptosis by HGF is achieved, or how synergy with other factors such as TNF is brought about.

Specifically I shall look at the caspase family in an attempt to determine which caspases and therefore what kind of pathway to apoptosis is operating. Once a pathway is determined this information can be used to further study the mechanics of IFNγ induced apoptosis in the hepatocyte. In order to do this an experimental system where apoptosis can be reliably induced by a given amount of IFNγ will be established. The first specific hypothesis to be tested will be that IFNγ leads to activation of caspase 9. The mitochondrial pathway will be implicated if caspase 9 is found to be active. The pathway to the mitochondria will then be examined using the hypothesis that a membrane death receptor is responsible for activation of caspase 9 through caspase 8. This model will allow further research into the mechanics of IFNγ induced apoptosis.
2 Materials and Methods

2.1 Primary hepatocyte isolation

Hepatocytes were obtained from male C3H mice between 8 and 12 weeks of age using a two-step collagenase perfusion method (Seglen, 1976) as described previously, with minor modifications (Bellamy et al, 1997). Prior to the perfusion procedure all equipment was sterilised using 70% ethanol and rinsed with sterile H2O. All instruments were autoclave sterilised. Animals were sacrificed by cervical dislocation and tested for loss of deep reflexes. The exterior of the abdomen was washed with 70% ethanol and the peritoneum dissected along the midline. The ribcage was removed and the intestines reflected to expose the hepatic portal vein. A polythene cannula (o.d. 1.0mm, i.d. 0.5mm(Portex)) was inserted through an incision in the right atrium into the thoracic inferior vena cava. Using a peristaltic pump liver perfusion media (Gibco) (See Appendix) was perfused into the liver at a low rate while the hepatic portal vein was incised. The flow rate was then increased to 8ml/min causing a blanching of the liver and 40ml of media was perfused through. The second perfusate was a 0.05% collagenase type IV solution in HBSS (Ca2+ and Mg2+ free) containing 50μg/ml DNasel (digestion media) (See Appendix). Digestion was observed by the spontaneous fissuring of the parenchyma and typically occurred after 7-12 minutes perfusion with digestion media.

The digested liver was removed from the abdomen, in its capsule, to a universal containing plating media (See Appendix) at 37°C. All subsequent steps were performed in a sterile class II laminar flow cabinet. The liver was then removed to a sterile petri dish previously chilled on ice, the gall bladder was discarded and the capsule released.
Hepatocytes were mechanically freed from the supporting fibrous tissue by a gentle brushing.

The hepatocyte suspension was passed through a wide bore pipette to disaggregate clumps of cells then filtered through a 100μm nylon sieve into a fresh chilled petri dish. The cells were then transferred to a chilled 50ml universal and the petri dish washed twice with fresh, ice cold plating media, which was added to the universal. The cells were centrifuged at 50 \times g for 5 minutes at 4°C. The supernatant was discarded. To remove non-parenchymal cells and dead cells the pellet was resuspended in 43% Percoll (Amersham Biosciences)/plating media and centrifuged at 50 \times g for 10 minutes at 4°C. The viability of the remaining cells was assessed by trypan blue exclusion. A 20μl aliquot of cells was diluted in 80μl of 0.25% trypan blue in PBS. The suspension was mixed well and one drop was allowed to spread under the cover slip of an improved Neubauer haemocytometer. Live and dead cells were counted across five 1mm squares on both sides of the haemocytometer and an average taken. The chamber depth of the haemocytometer is 0.1cm allowing cell yield to be calculated using the following formula.

\[
\text{Yield / ml} = \text{cell number in 5 squares} \times 10^4
\]

Viability was calculated as a percentage of total cells and only preparations above 90% viability were used in experiments.
2.2 Primary hepatocyte culture

Culture surfaces (either chamber slides or petri dishes) were coated with 10μg/ml fibronectin (Sigma) either overnight at 4°C or for at least 1 hour at 37°C. Fibronectin was used in the experiments as it was found that this substrate allowed for better hepatocyte survival than collagen. Hepatocytes were seeded at a density of 2 x 10^5 cells/ml in DMEM/F12 containing 10% foetal bovine serum (See Appendix) and gently agitated to ensure even distribution of cells across the culture surface. Following a 2-3 hour attachment period at 37°C under an atmosphere of 5% CO2 cells were cultured in modified Chee’s media or William’s E media (See Appendix). The culture media was changed every 24 hours. Appropriate reagents were added as indicated.

2.3 Assessment of apoptosis

Following culture under the appropriate conditions apoptosis was assessed by nuclear morphology using Feulgen’s stain. Briefly, cultured cells were fixed overnight in modified Bouin’s fixative (without picric acid) (See Appendix), treated with 5M HCl for 45min, washed thrice in tap water for 5min and incubated with Schiff’s reagent (Sigma) for 1hr. Following further washes the cytoplasm was counterstained with 1% light green. After drying in air the slides were mounted in Cedarwood oil (Sigma) and cover slipped. Apoptosis was assessed by nuclear morphology under a light microscope. Results are shown as percentage apoptotic cells from counts of at least 500 cells.
2.4 MTT Assay of Hepatocyte Viability

The MTT assay is dependent upon the reduction of the yellow (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) tetrazolium salt (MTT) (Sigma) to a purple formazan by mitochondrial succinate dehydrogenase in viable cells (Mossman et al., 1983; Carmichael et al., 1987). For the MTT assay cells were cultured on 24-well plates and incubated with MTT at a final concentration of 1mg/ml in medium at 37°C for 3 hours. Cells were washed twice with PBS after removal of medium and left to dry completely. 150μl DMSO (Sigma) was added to dissolve the formazan dye, 100μl was transferred to a 96-well ELISA plate, and the absorbance read at 490nm by an ELISA plate reader (MR 5000, Dynatech). The negative control omitted MTT.

2.5 Visualisation of mitochondrial membrane potential

Hepatocytes were isolated and cultured as described. Following culture in appropriate conditions the cells were stained using MitoTracker® Red (CMXRos) (Molecular Probes). CMXRos is a mitochondria selective dye that is sequestered by intact mitochondria but not a de-energised organelle (Scaduto and Grotyohann 1999). The dye is maintained in the organelle after fixing and has been used to study mitochondrial membrane permeability transitions in rodent hepatocytes (Isenberg and Klaunig 2000). CMXRos was added directly to the culture medium at a concentration of 100nM for 45 minutes. From this stage onwards cells were protected from light. Following incubation the cells were washed twice in PBS at 37°C before being fixed in 4% paraformaldehyde for 10 minutes. The cells were removed from paraformaldehyde and washed twice in PBS. Following fixing a nucleic acid dye ToPro 3 (Molecular Probes)
was used to stain nuclei. A concentration of 250nM was used diluted in PBS. The stained slides were mounted in Mowiol (See Appendix) and cover slipped before examination. Confocal microscopy was accomplished with a Zeiss LSN510 confocal microscope system and HeNe lasers. MitoTracker Red was detected using 453nm excitation filter and ToPro 3 was detected using a 633nm excitation wavelength, a longpass emission detection filter was used.

Fluorescent microscopy was carried out using an Axiovert S1000 (Zeiss) fitted with a UV lamp (HBO50) through a triple bandpass filter set (Excitation 540-570nm, Emission 580 – 630nm) (Chroma). Images were captured using a CoolSNAP Fx digital camera and analysed using MetaMorph imaging software.

2.6 Western analysis

Cultured cells were assayed for caspase activation and cleavage of Bid to tBid by SDS-polyacrylamide gel electrophoresis and immunoblotting. Cells were harvested in 70°C denaturing lysis buffer (See Appendix), scraped off using a cell scraper (Greiner) and stored at -70°C until use. Prior to use cells were passed through a 23G needle and protein concentration assessed by BCA assay (Pierce). The extract was diluted to 1mg.ml and 50µl was heated with an equal volume of LDS sample buffer (NuPAGE) (See Appendix) at 60°C for 10 min. Final amount of protein loaded was 10µg.

Samples were run alongside a set of markers of known molecular weight (Magic Mark, Invitrogen). For analysis of caspase cleavage, samples were run on a pre-cast 10% Bis-Tris gel (Novex) in 3(N-morpholino) propane sulphonic acid (MOPS) SDS running buffer (NuPAGE) (See Appendix). Electrophoresis of extracts for detection with anti-
Bid was achieved on 12% Bis-Tris pre-cast gel (Novex) in 2(N-morpholino) ethane sulphonlic acid (MES) SDS buffer (NuPAGE) (See Appendix). The gel was run at 200V, 120mA, for 45min in an XCell II Mini-Cell (NOVEX).

Proteins were transferred onto a HyBond ECL Nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane, blotting pads and filter paper were pre-soaked in transfer buffer and arranged, according to the manufacturer's instructions, in the blot module, with the gel. The proteins were transferred at 25V, 160mA for 90min.

Following transfer to the nitrocellulose membrane, the membrane was washed three times for 10min in TBST (See Appendix). Non-specific binding sites were blocked using 10% Marvel in TBST with shaking at room temperature for 1hr. The primary antibodies used and their corresponding secondary antibodies, concentration and incubation conditions are shown in table 1. Between each primary and secondary antibody incubation membranes were washed thrice in TBST for 5 minutes.

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Primary antibody and concentration</th>
<th>Incubation conditions</th>
<th>Secondary antibody and concentration</th>
<th>Incubation conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caspase 8</td>
<td>Monoclonal mouse IgG2b-κ against C terminal of caspase 8 (MBL) (aa180-440) at 2µg/ml</td>
<td>TBST/1%/Marvel, 24hrs, 4°C</td>
<td>Horseradish peroxidase (HRP) linked rabbit anti-mouse (Dako), 0.43µg/ml</td>
<td>TBST/2%/Marvel, room temperature, 1hr</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>Monoclonal mouse IgG1 against N terminal of caspase 9 (MBL) (aa1-250) at 2µg/ml</td>
<td>TBST/1%/Marvel, 24hrs, 4°C</td>
<td>HRP linked rabbit anti-mouse (Dako), 0.43µg/ml</td>
<td>TBST/2%/Marvel, room temperature, 1hr</td>
</tr>
<tr>
<td>Bid</td>
<td>Polyclonal goat antibody (MBL) at 1µg/ml</td>
<td>TBST/1%/BSA, 1hr, room temperature</td>
<td>HRP linked rabbit anti-goat (Dako), 0.25µg/ml</td>
<td>TBST/1%/BSA, room temperature, 1hr</td>
</tr>
</tbody>
</table>
Following a series of TBST washes, excess liquid was poured off and the membrane was exposed to ECL Western blotting detection system (Amersham Pharmacia Biotech) combined at the appropriate ratio for 1 minute according to the manufacturer's instructions. Excess chemiluminescence reagents were removed, the membrane was covered with a sheet of acetate and loaded into a developing cassette. Under safe light illumination the membrane was exposed to film for a few seconds to a few minutes, depending on signal intensity, before being developed using an Amersham Hyperprocessor.

2.7 Isolation of RNA from primary cells

The working area was cleaned using RNA Zap wipes (Ambion) to remove RNases and washed twice in sterile DEPC water (See Appendix). Cells were washed twice in PBS at 37°C before being lysed in a TRIzol (1 ml/cm²) (Life Technologies / Invitrogen). Samples were incubated for 5 minutes at room temperature before adding 0.2 ml chloroform per 1 ml TRIzol, tubes were mixed by inversion for 15 s before incubation at room temperature for 2-3 minutes. Samples were centrifuged for 15 min at 4°C and 12 000 x g. The RNA in the aqueous phase was removed to a clean eppendorf tube. To precipitate the RNA isopropanol was added at 50% volume of TRIzol used initially. Samples were incubated for 10 minutes at room temperature then centrifuged at room temperature for 10 minutes at 12 000 x g. The precipitated RNA forms a pellet at the base of the eppendorf tube. The supernatant was removed and the pellet washed in 1 ml of 75% ethanol in diethyl pyrocarbonate (DEPC) water per 1 ml TRIzol used. The pellet was washed by gentle pipetting and centrifuged for 5 minutes at 4°C and 7, 500 x g. Following centrifugation the ethanol was removed and the pellet air dried for 10 minutes.
before redissolving the RNA in DEPC water for 10 minutes at 59°C. RNA concentration was assessed using a BioMate 3 (Thermo Spectronic). RNA quality was assessed by visualisation under UV after separation on 2% agarose (49mA, 96V), and ethidium bromide staining.

2.8 Ribonuclease protection assay

The ribonuclease protection assay (RPA) uses specific DNA templates to generate RNA probes capable of binding and protecting transcribed RNA species, following RNase treatment single stranded RNA is degraded and the remaining protected, labelled fragments can be analysed. RPA was carried out using BD RiboQuant™ Multi-Probe System (BD Biosciences Pharmingen) according to the manufacturer’s instructions. To minimise the risk of RNase contamination the working area was cleaned using RNase Zap Wipes (Ambion) and washed twice with DEPC treated water. All plasticware, glassware, tips and Eppendorf tubes used were RNase free.

Radiolabelled antisense probes were synthesised overnight using the In Vitro Transcription kit (BD Biosciences Pharmingen), the mAPO3 set of templates (BD Biosciences Pharmingen) and $^{32}$P-dUTP (ICN). The cDNA fragments specific for various death receptor pathway molecules are cloned into plasmids containing bacteriophage promoters, labelled RNA templates are transcribed using T7 RNA polymerase in the presence of $^{32}$P-dUTP for 1 hour at 37°C. The mixture was then incubated with DNase (30 min 37°C) to removes the templates. Templates were purified by phenol : chloroform extraction and precipitation in 4M ammonium acetate and ice cold ethanol. The pellet was washed once in ice cold 90% ethanol and air dried, and dissolved in hybridisation buffer (BD Biosciences Pharmingen). Counts/min of probe
was assayed in a scintillation counter (LKB Wallac) to determine whether the activity was greater than $3 \times 10^5$ counts/min.

20µg hepatocyte RNA extract was vacuum dried and redissolved in 8µl hybridisation buffer. Probe was diluted in hybridisation buffer to $4 \times 10^5$ cpm/µl and 2µl added to the hepatocyte RNA extract. The samples were covered with RNase free mineral oil, centrifuged briefly and removed to a heated block at 90°C, immediately the temperature was reduced to 56°C and the hybridisation allowed to complete overnight.
3 IFNγ induced apoptosis in the hepatocyte

This chapter considers the phenomenon of IFNγ induced apoptosis. The kinetics of death are reported on and potential modulators, both positive and negative are investigated. Additionally, the establishment of appropriate cell culture conditions, media composition, serum content and exposure to serum are discussed. These initial, largely descriptive studies provide a number of insights toward further mechanistical studies that could be achieved. The ideas formulated in these investigations informed many of the hypotheses made in later experiments. The IFNγ death pathway is found to be slow, taking days to induce apoptosis in a significant proportion of the cell population, however this pathway can be accelerated with the addition of other factors.

3.1 Results

3.1.1 IFNγ induces apoptosis in hepatocytes

The cytokine IFNγ exerts its effects on the hepatocyte through a JAK/STAT signalling pathway inducing IRF-1 dependent transcription. In a variety of cell types IFNγ causes the cells to undergo apoptosis (see Introduction). To study the process of IFNγ-induced apoptosis in the murine hepatocyte a primary cell culture system was employed. Administration of 100U/ml IFNγ induces apoptosis in murine hepatocytes. At 24 and 48 hours no differences in the number of apoptotic cells are seen between treated and control populations. However at 72 hours IFNγ treatment, the apoptotic rate increases to 10% with approximately 50% after 96 hours. The background level of apoptotic cell
death in the control population remains approximately 1% throughout the culture period (Figure 3.1). As well as apoptosis, necrotic cell death is observed in the cultures.

This experiment forms the basis of the experimental system for investigation of IFNγ induced apoptosis. At 72 hours after IFNγ treatment there is a significant difference between the treated and control populations. This time point was taken as an end point for apoptosis. The remaining experiments in this chapter help to develop the experimental system and hypotheses tested in the thesis.
Figure 3.1 IFNγ induces hepatocyte apoptosis

A) Isolated hepatocytes were cultured in Chee's medium for the times indicated in the presence of 100U/ml IFNγ. Data is representative of 3 independent experiments and shows means ± SD. * represents significant difference to control p<0.05. B) Light micrographs of Feulgen stained hepatocytes. Viable hepatocytes are shown on the left with full nuclei (purple) and spread out cytoplasm (green). Two apoptotic hepatocytes (a) are shown on the right with a viable cell. The condensed nuclei indicative of apoptosis are visible.
3.1.2 The effect of serum on hepatocyte apoptosis

Serum is frequently used in primary cell culture to increase cell viability. However serum is undefined and does not help maintain differentiation (Reid et al, 1986). Additionally serum can aid the survival of non-parenchymal cells (Munthe-Kass et al 1975). Research from this laboratory has demonstrated the effect of serum on progression through the cell cycle (C.T. McCullough personal communication). Co-incubation of hepatocytes in 2% serum increases the percentage of proliferating cells as assessed by Bromodeoxyuridine (BrdU) incorporation. Additionally serum factors protect from IFNγ induced apoptosis. In light of the protective effect of serum upon the hepatocytes maintenance of the cultures in serum was not a viable option. However, maintaining cell viability remained a consideration, unhealthy cells are more likely to undergo necrotic cell death than apoptosis.

Following perfusion cells are allowed to attach to the culture dish for 3 hours. During this time the cultures are maintained in supplemented Dulbecco’s Modified Eagle Medium/F12 (DMEM/F12) (Plating Media see Appendix). Normal calf serum (NCS) was added to the plating media at 2% and 10% v/v for the period of attachment. Serum pre-incubation increases hepatocyte viability. Cultures maintained in serum free plating media appear more necrotic than those cultured in 2% or 10% NCS (Fig 3.2a), and by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) metabolism assay these cell are less viable at 72 hours than those pre-incubated with serum (Fig 3.2b).
Serum pre-incubation affects the apoptotic rate. Compared to serum free media, pre-incubation with 2% NCS protects, but in 10% NCS apoptotic cell death is statistically increased (p<0.05 by t-Test) (Figure 3.2c).

The effect of serum pre-incubation is two-fold. At both 2% and 10% the viability of cells is increased, the cells look healthier and there are less necrotic cells. Additionally following pre-incubation in 10% serum there is an increased level of apoptosis. Serum pre-incubation therefore increases the quality of cells while allowing death to occur. Serum is undefined and it could be one of many factors or combination of factors that is affecting the hepatocyte. HGF can also protect hepatocytes from IFNγ induced apoptosis. TNF is a hepatocyte mitogen but in combination with IFNγ causes hepatocyte apoptosis. The effect of another common agent in the liver, Fas, was investigated in combination with IFNγ in an attempt to provide further clues as to the nature of the pathway to apoptosis.
Figure 3.2 Effect of serum preincubation on hepatocyte viability and apoptosis.

Isolated hepatocytes were preincubated for 3 hours in the either 0%, 2% or 10% NCS /Plating media then maintained for 72 hours in Chee’s media. A) Serum preincubation affects hepatocyte viability at 72 hours. Cultures were assayed for viability at 72 hours by MTT incorporation. Data is expressed as % viability of cultures prepared in 0% serum. Means ± SEM, n=3) B) Light micrographs of Feulgen stained hepatocytes at 72 hours showing differences in quality of culture. After 0% preincubation frequent necrotic cells (n) are seen, with vacuolation in viable cells (v) and cell debris where cells have died (c.d.). With 2% and 10% serum preincubation necrotic cells and vacuolation is seen less often and cell debris is rare. C) Serum preincubation has consequences for the level of apoptosis seen at 72 hours after Feulgen stain. Data shown represents means ± SEM ** indicates significant differences from IFNγ treated cells with 0% serum preincubation p=0.005.
B

0% serum preincubation

2% serum preincubation

10% serum preincubation
3.1.3 IFNγ co-operates with Fas to induce hepatocyte apoptosis

Investigations into cytokine action heralded a new era in the area of immunology, but the true functions of cytokines were only realised when the interactions of cytokines were clarified. As with cells of the immune system, combinations of cytokines have differing effects on hepatocytes. Cytokine cocktails of IFNγ, IL1 and IL6 have effects on hepatocyte nitric oxide production. The interaction of IFNγ and Fas was chosen for investigation. The inflamed liver is characterised by the inflammatory T cell infiltrate. CD8+ T cells bring both IFNγ and FasL to the hepatocyte. FasL is recognised as a potent mediator of hepatocyte cell death both in vivo and in vitro, data also suggests that, like TNFα, Fas can also mediate a rescue pathway via nuclear factor-κB (NF-κB) (Ponton et al, 1996). IFNγ and Fas co-operate to induce apoptosis in a variety of cell types, additionally IFNγ co-operates with the related TNFα. To test the hypothesis that IFNγ and Fas would co-operate to increase hepatocyte death the anti-Fas antibody Jo2 was used.

Experiments were carried out in supplemented William’s E media (see Solutions) and a model system of IFNγ co-operating with Jo2 was established. IFNγ concentration was held constant at 100U/ml, while the concentration of the anti-Fas antibody was varied. No effect of Jo2 is observed at 10, or 50ng/ml at 48 hours. At 100ng/ml Jo2 and IFNγ synergistically increases hepatocyte apoptosis to 6% at 48 hours (p<0.05). These experiments were the result of collaboration between the author and M. Dollinger.
Research carried out by the author and other collaborators (C. T. McCullough and D. L. Guernsey) has demonstrated IFNγ has a synergistic effect on apoptosis with not only Jo2, but also thyroid hormone, TNFα, and UV irradiation.

Fas trimerisation in the hepatocyte leads to apoptosis through the mitochondrial Type II pathway. It was hypothesised that the mitochondrial pathway was involved when IFNγ co-operates with Fas. The drug CsA can inhibit apoptosis caused by the Type II pathway. This inhibitory effect was explored in the system.
Figure 3.3 IFNγ and Jo2 co-operate to increase apoptosis. Isolated murine hepatocytes were cultured in William’s E media for 48 hours. Apoptosis was assessed by nuclear morphology after Feulgen stain. With 100ng/ml Jo2 antibody alone there is no difference to controls. With the addition of 100U/ml IFNγ and 100ng/ml there is a statistically significant difference (p<0.005, indicated by *). Data shown is means ± SEM, n=3. representative of two independent experiments.
3.1.4 CsA inhibits IFNγ and Jo2 synergy

In the hepatocyte FasL causes apoptosis by trimerising Fas, inducing auto-activation of caspase 8, cleavage of Bid and release of cyto c from the mitochondria. It was hypothesised that IFNγ converges with the Fas pathway at the mitochondria. CsA is commonly used to prevent the MPT and consequent apoptosis. In this situation it was used to determine the involvement of mitochondria in the apoptotic pathway. The cytotoxic effect of CsA on isolated hepatocytes was tested using metabolism of MTT as an indicator of viability. At or below 1μg/ml CsA had no effect on viability, however at 5 and 10μg/ml the drug had an adverse effect on the quality of the cultures (Fig 3.4a and b).

Using the MTT metabolism assay the combination of 100U/ml IFNγ and 100ng/ml Jo2 reduces the viability of cultures to approximately 50% of controls at 48 hours in supplemented William’s E media. IFNγ alone has no effect on viability at this time, Jo2 alone, reduces viability to approximately 80% of controls. With the addition of 1μg/ml CsA there is no significant effect on controls, IFNγ or Jo2 treated cultures, however the viability of IFNγ and Jo2 treated cultures is significantly increased (p<0.05) to levels comparable to cells treated with Jo2 alone (Fig 3.4c).

The toxic and non-toxic levels of CsA were established in the system and its efficacy in inhibiting IFNγ/Jo2 induced apoptosis shown. Discrepancies exist between the apoptotic count and the viability of the populations treated with IFNγ. It was hypothesised that the media used was affecting viability. The experiments were therefore conducted in an alternative cell culture medium.
A

![Graph showing viability as % of control with CsA concentrations in ng/ml.](image)

B

![Images showing control culture and cultures with CsA concentrations of 1, 5, and 10 μg/ml at x400 magnification.](image)
Figure 3.4 CsA inhibits co-operation between IFNγ and Jo2

The effect of CsA on viability was tested by MTT incorporation. Cultures were assayed at 48 hours. A) The toxic effect of CsA is illustrated. Above 1μg/ml CsA is toxic to hepatocytes. Data represents means ± SD. B) Light micrographs showing the effect of high concentrations of CsA on the hepatocyte. Cells are healthy at 1μg/ml, at 5μg/ml the cytoplasm is sparse and nuclei. At 10μg/ml the vast majority of cells appear to be necrotic. C) Compared to controls IFNγ has no effect, 100ng/ml Jo2 reduces viability to approximately 80% (p<0.05). The combination of Jo2 and IFNγ reduces viability to approximately 50% of controls. CsA at 1μg/ml apparently increases viability in all treatments, however this increase is not significant except in the case of Jo2 and IFNγ (p<0.05, indicated by *). Data shown is of means ± SEM and is representative of three independent experiments.
3.1.5 Effect of media composition on synergism

A discrepancy exists between the morphological apoptotic cell death and viability of cultures by MTT assay when cells are treated with Jo2. While the apoptotic count is ~2%, the reduction in viability is 20%. It was hypothesised that the simple media used for these experiments (supplemented William’s E) was reducing the general viability of hepatocytes and their ability to withstand insults. Thus experiments were repeated using a richer media formulation. The modified Chee’s media developed by C. O. C. Bellamy in this laboratory is used for hepatocyte culture. The media contains high levels of amino acids and is known to prolong hepatocyte survival and differentiated functions (Waxman et al, 1990, Arterburn et al, 1995, Jauregui et al, 1986).

Experiments with IFNγ and Jo2 were repeated using the standard formulation of Chee’s media. Under these conditions there was no difference between controls and IFNγ treated cells. However the reduction in viability of Jo2 treated cells was ~70% (compared to 20% in William’s E media). Jo2 in combination with IFNγ caused no further decrease in viability. With the addition of 1μg/ml CsA no difference was observed between the control conditions and CsA treatment. IFNγ had no effect on Jo2 mediated death and the change in viability with Jo2 alone was greater than expected.

The synthetic glucocorticoid, dexamethasone, is present at 100nM in the standard formulation of Chee’s media. While dexamethasone aids maintenance of differentiation (Colbert et al, 1985), it also upregulates expression of Fas pathway components (Muschen et al, 1998). We hypothesised that the increased death was due to increased Fas. In an attempt to replicate the observations obtained in William’s E media the standard supplements were replaced with the supplements in William’s E (i.e. EGF,
glutamine and gentamycin). This adaptation returned Jo2 induced death at 48 hours to ~20% by MTT assay, however there was no increased death upon addition of IFN\(\gamma\). Co-incubation with CsA (1\(\mu\)g/ml) had no effect on controls, IFN\(\gamma\) or Jo2 treated cultures. A significant difference exists between Jo2 / IFN\(\gamma\) treated cells and Jo2 / IFN\(\gamma\) / CsA treated cells, however, it is not the expected increase but a decrease in viability.
Figure 3.5 No synergy between IFNγ and Jo2 in Chee's media. Hepatocytes cultured for 48 hours in Chee’s media were assayed for viability by MTT incorporation. A) Using the standard formulation of Chee’s media Jo2 antibody reduces viability to ~30% of controls. Jo2 and IFNγ in combination have the same effect. CsA has no effect on the viability. B) Using Chee’s supplemented with EGF, ITS, gentamycin and glutamine there is a reduction in loss of viability with Jo2, however, no synergism is seen between Jo2 and IFNγ. Again CsA does not rescue the cells. Significant differences from control viability is indicated by * p<0.05, and ** p<0.005. Data shown (means ± SEM) is representative of three independent experiments.
3.2 Discussion

This chapter shows the effect of IFNγ on primary murine hepatocytes. Apoptotic hepatocytes begin to appear 72 hours after addition of IFNγ. We have defined the primary cell culture system for the investigation of IFNγ induced apoptosis and identified some modulators of cell death, both exposure to serum and to the anti-Fas antibody Jo2 can modulate IFNγ induced apoptosis. That system consists of the isolation technique described in the Materials and Methods followed by a 2-3 hour pre-incubation period in DMEM/F12/10% FBS. Cells are maintained in Modified Chee’s Medium supplemented as described in the Appendix. Using a two-step retrograde perfusion method followed by a plating period in 10% serum, healthy cultures were obtained with a low level of background apoptosis (1%). The timing and level of apoptosis is in accordance with published data, i.e. ~10% at 72 hours. A synergy between IFNγ and the Fas pathway is observed, a novel finding in murine hepatocytes. The increase in apoptosis is inhibitable with CsA, presumably through its effects on the MPTP. However this synergy is not always detectable, in the presence of dexamethasone the anti-Fas antibody (Jo2) alone induces a high level of death such that no effect of IFNγ is observed. Additionally, the extracellular environment, in terms of the specific cell culture media used has profound effects on the properties of the system. The use of modified Chee’s media in the place of William’s E media prevents co-operation between the Fas pathway and IFNγ.
3.2.1 Kinetics of apoptosis

The range of effects of IFNγ on the hepatocyte is well documented, in addition to an anti-viral effect (Guidotti et al, 1996, Guidotti et al, 1999) the cytokine induces a G₁ arrest (Kano et al, 1999). It may be that IFNγ acts as a slow burning fuse in the liver, following an attempt to rescue the cell from viral infection there is a second chance to allow repair with cell cycle arrest, and when all other options are exhausted the signal for apoptosis is given (Tura et al, 2001). Although in vitro this process takes but 72 hours to come to completion in vivo the situation is different. Transgenic mice expressing IFNγ under a liver specific promotor do not die of fulminant hepatic failure, there is a rescue signal. The clue may be in the cell cycle. Serum co-incubation protects hepatocytes from cell death. Protection may be conferred by mitogens that force the cells to cycle (Morita et al, 1995). A connection between lack of cell cycle arrest and a reduction of apoptosis has previously been demonstrated (Roberts et al, 1995). Serum pre-incubation may have a similar effect on the cultures. Perfusion of the liver synchronises hepatocytes in G₁ (Loyer et al, 1996) and under appropriate conditions hepatocytes enter S phase (Hansen et al, 1994, Loyer et al, 1996). Entry into S phase is mediated by the substratum and mitogens that can push the cells through the restriction point in G₁. Plating hepatocytes in serum free media may reduce the proportion of cells that are primed to enter the S phase. The increase in viability and IFNγ dependent apoptosis at 72 hours following pre-incubation of cells in 10% serum may be due to a higher proportion of cells entering S phase and arresting in G₁ respectively. Therefore it is
possible that a greater number of cells are at a point in the cell cycle that would favour arrest following serum withdrawal and this may have consequences for apoptosis. IFNγ can cause transcription of p53, an important mediator of cell cycle arrest, through IRF-1 (Kano et al, 1999). IFNγ induced apoptosis is independent of p53 and in the presence of serum, G1 arrest is also p53 independent. The relationship between cell cycle arrest and apoptosis is controversial and is beyond the scope of these investigations.

3.2.2 Co-operation of IFNγ and Fas pathways

Many factors modulate IFNγ dependent apoptosis, serum, hepatocyte growth factor (HGF) and epidermal growth factor (EGF) can protect hepatocytes while other cytokines such as TNFα and Fas can increase the apoptotic stimulus. This study also demonstrates a novel interaction of the Fas pathway and IFNγ in the hepatocyte. A protective effect of CsA on the interaction of IFNγ and Jo2 anti-Fas antibody is also reported.

Fas and IFNγ would be expected to be present in a chronically inflamed liver. Fas antigen is constitutively expressed by hepatocytes and is upregulated in models of hepatitis. Immunohistochemical studies showed Fas antigen expression to be increased near areas of interface hepatitis. The source of FasL is debateable, it may be provided by infiltrating CD8+ T cells, NK cells, or there may be upregulation of FasL by the hepatocyte allowing autocrine/paracrine signalling. FasL is, under normal circumstances, not expressed in the hepatocyte in vivo, but is seen in areas of interface hepatitis in the livers of chronic hepatitis C patients by immunohistochemistry. In vitro
studies of IFNγ treated hepatocytes showed no upregulation of FasL at the mRNA level (Kano et al, 1999).

Given that Fas, FasL and IFNγ are present in the chronically inflamed liver it could be hypothesised that the interaction described is taking place. However media changes totally obliterate this interaction at the level of cell death. Replacing William’s E media with Chee’s media provides increased nutrient levels, amino acids, electrolytes and osmolality. Additionally Chee’s predisposes towards a more hepatocyte rich culture in that markers of differentiation are maintained and non-parenchymal cells (NPC’s) are selected against. Consequently NPC’s such as Kupffer cells and Ito cells may provide a stimulus for the synergistical increase in death that is observed in William’s E media where these cells persist. Alternatively, the hepatocyte specific functions maintained in Chee’s media may be responsible for the lack of increase in cell death seen when this media is used.

It was with these considerations that further research into the consequences of signalling was undertaken. It was hoped that by identifying the pathway responsible for IFNγ induced apoptosis, testable hypotheses could be formulated that would assist the understanding of this interaction.

In Chee’s media containing dexamethasone Fas induced death in such a large proportion of cells (80%) that no interaction with IFNγ was seen. This may be due to dexamethasone’s influence on Fas/FasL. Dexamethasone increases the expression of Fas and FasL in cultured hepatocytes.

CsA has a variety of effects in the cell, however its main effect on apoptosis is through inhibition of cyclophillin D binding to ANT at the mitochondria, thereby preventing formation of the mitochondrial permeability transition pore (MPTP). Inhibition of the synergistical increase in cell death by CsA indicates that a mitochondrial pathway
creates the synergy. Since IFNγ/Fas synergy is dependent on MPTP formation it is possible that the higher nutrient or Ca²⁺ levels in Chee’s media compared to William’s E media predisposed the MPTP to remain in a closed formation thereby blocking apoptosis.

The information presented in this chapter paves the way for continued research into the effects of IFNγ on the hepatocyte. The culture system developed here provides a healthy differentiated hepatocyte monoculture. With the addition of IFNγ apoptosis can be induced reliably. This chapter also discusses how the level of IFNγ induced apoptosis can be modulated. The experiments that follow will use this system to investigate the mechanics of IFNγ induced apoptosis in the murine hepatocyte. The IFNγ/Fas system of apoptosis described in this chapter provides interesting data that supports the possibility of death receptor involvement. Additionally the inhibition of IFNγ/Fas induced apoptosis by CsA suggests and involvement of the mitochondria in the pathway. The hypothesis to be tested was that IFNγ causes apoptosis through a mitochondrial pathway involving caspase 9, that is instigated by a membrane death receptor and caspase 8.
4 Identification of IFNγ induced apoptotic process

Having set up a primary cell culture system and identified a number of factors that could modulate IFNγ induced hepatocyte apoptosis an investigation into the mechanics of apoptosis began. The system used was a 2-step retrograde perfusion method with a 2-3 hour pre-incubation period in DMEM/F12/10% FBS. The primary hepatocytes were then maintained in supplemented modified Chee’s medium. Using 100U/ml IFNγ apoptosis can be reliably induced at 72 hours with no nuclear morphological evidence of apoptosis at 24 or 48 hours. It has been reported previously that a caspase 3-like protease was involved in IFNγ induced hepatocyte apoptosis, however the nature of the upstream signalling pathway was unknown. The aim of these studies was to identify the caspases involved and postulate a pathway to apoptosis. It was hoped that by identifying the upstream caspases a clearer idea of the cellular mechanisms necessary for apoptosis would be gained. Downstream mediators of apoptosis were not investigated here, a caspase 3-like enzyme is known to be active in IFNγ induced apoptosis (Kano et al, 1999) and the importance of the downstream caspases have been elucidated with respect to many further targets (Slee et al, 2001). As IFNγ has been reported to induce death through both membrane receptor and mitochondrial pathways the first aim of this chapter was to delineate which was being invoked in this situation.
4.1 Results

4.1.1 IFN\(\gamma\) induced apoptosis is caspase dependent

To confirm the assumption that apoptosis in the hepatocyte is dependent on caspase activity an inhibitor-based strategy was used. Before using the inhibitors in the IFN\(\gamma\) system a model of caspase induced hepatocyte apoptosis was tested. Fas induced apoptosis is well recognised as a paradigm of apoptosis. The inhibitor was tested in this system as it has been previously shown that caspases are active in hepatocytes undergoing Fas induced cell death (Jones et al, 1998). The pan-caspase inhibitor z-VAD-fmk was assayed for its cytotoxicity and an effective dosage tested using the Jo2 anti-Fas antibody to induce apoptosis.

Hepatocytes were incubated with the peptide inhibitor z-VAD-fmk and the Jo2 antibody for 48 hours, viability was assayed by MTT incorporation. No significant difference was seen between the viability of controls and cells treated with z-VAD-fmk at all the concentrations tested. The inhibitor successfully blocked apoptosis at all concentrations tested (Figure 4.1).

Once it had been established that caspases were at work and caspase inhibitors could be effective in the experimental system attention was turned to answering questions about the specific caspases responsible. The first hypothesis was that inhibition of caspase 9 with z-LEHD-fmk would inhibit apoptosis in the hepatocyte.
Figure 4.1 Caspase inhibitor z-VAD-fmk is non-toxic and effective in hepatocytes
Isolated hepatocytes were maintained for 48 hours in Chee's media with the addition of 100ng/ml Jo2 antibody and z-VAD-fmk as indicated. Cells were incubated with MTT, lysed in DMSO and assay for absorption at 550nm.
* indicates Jo2/z-VAD-fmk treated cultures significantly different to Jo2 treated cells alone. (p<0.05, by paired T-test)
Having established that z-VAD-fmk was both non-toxic and efficacious in our system the effect of the inhibitor was tested in the IFNγ model. Hepatocytes were cultured for 72hrs in the presence or absence of IFNγ with the addition of z-VAD-fmk and appropriate controls containing the vehicle, dimethylsulphoxide (DMSO). Following fixation in Bouin’s fixative the cultured cells were stained by Feulgen’s method and apoptosis was scored by nuclear morphology. A dose dependent decrease in apoptosis was seen with the addition of z-VAD-fmk. At the highest concentration tested (10µM) z-VAD-fmk totally inhibits IFNγ induced apoptosis in the hepatocyte (p<0.005). At intermediate doses there are statistically significant differences between controls and treated cells (p<0.005 at 0.1µM, p<0.05 at 1µM). At all concentrations tested there is a difference between z-VAD-fmk / IFNγ treated cells and the DMSO / IFNγ treated cells (p<0.05 by paired t-Test) (Figure 4.2).
Figure 4.2: z-VAD-fmk inhibits IFNγ induced apoptosis. Isolated hepatocytes were cultured for 72hrs with IFNγ at 100U/ml, Z-VAD-fmk was added to the cultures at 0hrs at the concentrations indicated. Apoptosis was assessed by nuclear morphology. The data is representative of 3 independent experiments. Values displayed indicate the means (n=3) ± SEM. ** indicates significant difference from IFNγ alone p< 0.005 * indicates significant difference from IFNγ alone p<0.05
4.1.2 Involvement of caspase 9

IFNγ has been shown to upregulate Bcl-2 family members and we have shown that CsA is effective in reducing apoptosis caused by IFNγ and the Fas pathway. We therefore hypothesised that the mitochondrial pathway may be involved. Caspase 9 is recruited to the apoptosome following invocation of the mitochondrial pathway, thus the role of caspase 9 was examined. To identify the importance of this enzyme a caspase 9-specific inhibitor was employed.

The cell permeable caspase inhibitor z-LEHD-fmk inhibits caspase 9 activity. When cultured hepatocytes were co-incubated with both IFNγ and z-LEHD-fmk for 72hrs, a significant decrease in apoptosis was seen (p<0.05) at 10μM z-LEHD-fmk, but not at any other concentration tested (Figure 4.3).

The involvement of caspase 9 was confirmed by Western blot. An antibody to caspase 9 that recognises both the full length and cleaved forms of caspase 9 was used. In the control population at 24, 48 and 72 hrs no cleavage of caspase 9 was observed. At 24 and 48 hrs incubation with IFNγ full-length caspase 9 was detected but no cleaved caspase. At 72 hrs IFNγ a decrease in full-length caspase 9 is seen, coincident with an increase in the 37kDa cleaved form of the enzyme.

The involvement of caspase 9 and therefore the Type II pathway has been established. The next stage of the hypothesis to be tested focussed on what was upstream of the mitochondria. It was hypothesised that caspase 8 was the upstream agent and inhibition of caspase 8 with z-IETD-fmk would inhibit apoptosis.
Figure 4.3 Caspase 9 is activated and cleaved during IFNγ induced hepatocyte apoptosis.

Cultures of primary murine hepatocytes were treated with 100U/ml IFNγ and maintained for 72 hours. A. Cells were co-incubated with z-LEHD-fmk at the concentrations indicated and apoptosis was scored by morphology at 72 hours. n=3 +/- SEM. B Western blot showing change in full length caspase 9. Lane 6 shows a decrease in full length caspase (45kDa). C After overexposure, an increase in cleaved caspase 9 can be seen at 72 hours IFNγ treatment (lane 6). Data is representative of 3 independent experiments. Lane 2 shows a shorter caspase 9, please refer to the discussion.
4.1.3 IFNγ initiates apoptosis through a caspase 8-like enzyme

Although caspase 9 was shown to be involved in the apoptotic process this observation does not rule out the possibility that caspase 9 is cleaved following cleavage of caspase 8. A number of reports suggest IFNγ causes apoptosis through death receptor pathways such as Fas, TRAIL and TNFR1. Thus the activity of caspase 8 was investigated.

Murine hepatocytes were isolated and cultured as described. The effect of the caspase 8 inhibitor (z-IETD-fmk) on apoptosis was determined. Apoptosis was assessed by nuclear morphology after Feulgen's staining. Inhibition of apoptosis at 72hrs was achieved with z-IETD-fmk. At 10μM inhibition was almost complete with a dose dependent effect observed at intermediate concentrations. To confirm activation of caspase 8 whole cell extracts were subjected to separation by Sodium dodecyl sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) and probed for the presence of full-length caspase 8. Untreated cultures did not demonstrate a change in the level of full-length caspase 8 at 24, 48 or 72hrs. Unexpectedly, however, no change was observed in caspase 8 levels in the IFNγ treated cultures at these times either.

The hypothesis that caspase 8 was activated before caspase 9 and inducing caspase 9 activity after formation of the apoptosome needed to be re-evaluated in light of the apparent lack of processing of caspase 8. As an upstream activator of the mitochondrial pathway caspase 8 cleaves the Bcl 2 family member Bid to tBid. Pro-apoptotic Bcl 2 family proteins are then thought to interact with Bid at the outer mitochondrial membrane allowing release of cyto c and other mitochondrial apoptogenic factors. Bid
cleavage was therefore utilised as a marker for caspase 8-like enzymatic activity. Investigation of Bid by Western blotting revealed that this protein was not cleaved in the control population at any of the time points indicated. However the 15kDa tBid was observed at 48hrs and at a higher level at 72hrs. At 72hrs a decrease in 22kDa Bid was observed in the IFNγ treated population. Although cleavage of Bid and inhibition by z-IETD-fmk are characteristic of caspase 8 activity it cannot be ruled out that another caspase such as caspase 10 may be responsible. In addition a non-caspase enzyme may cleave Bid.

As both caspase 9 and a second caspase, possibly caspase 8 had been shown to effect death in the hepatocyte it was hypothesised that a membrane death receptor may also be functioning. Since the liver is highly sensitive to Fas induced apoptosis and IFNγ can interact with the Fas pathway to increase apoptosis the Fas pathway was investigated. The hypothesis tested was that blocking FasL would prevent apoptosis in the system.
**Figure 4.4: IFNγ induces caspase 8-like activity.**

A) Primary murine hepatocytes were cultured for 72 hours in the presence of 100U/ml IFNγ caspase 8 inhibitor z-IETD-fmk. At a concentration of 10μM z-IETD-fmk IFNγ treated cells are significantly different to cells treated with IFNγ alone (p<0.05). B) Cells were cultured and lysed in denaturing lysis buffer before being separated by SDS-PAGE and probed for full length caspase 8. No decrease in caspase 8 was observed at either 48 or 72 hours (compare lanes 1 and 3 and lanes 2 and 4). C) As a marker of caspase 8-like activity Bid is cleaved at 48 hrs. Also shown, extracts from cells treated with Fas (F, lane 9) as a positive control for tBid with control cells in lane 8 (C).
4.1.4 IFNγ induced apoptosis is inhibited by blocking Fas signalling

The involvement of 9 and a caspase 8-like activity with apparent caspase 8-like activity before caspase 9 suggested a type II death receptor pathway was being induced by IFNγ. Reports from other cell types have shown that IFNγ causes upregulation of both TNF receptor and Fas pathways to cause apoptosis. Although upregulation of FasL mRNA has been demonstrated to be absent in primary murine hepatocytes treated with IFNγ it was hypothesised that the Fas pathway may be responsible for apoptosis. Pilot data from C. T. McCullough also suggested an involvement. TNFα does not cause apoptosis in the hepatocyte except when protein synthesis or transcription is inhibited. However IFNγ has been shown to synergise with TNFα to cause apoptosis. To investigate the involvement of the Fas pathway a Fas blocking antibody (MFL3) (Pharmingen) was used. MFL3 is an antibody to mouse Fas Ligand raised in hamsters. It binds FasL thereby preventing Fas/FasL induced apoptosis (Kayagaki et al, 1997)

At 10μg/ml MFL3 successfully inhibited apoptosis induced by IFNγ (p<0.005). At 2μg/ml however, there was no difference between IFNγ treated controls and MFL3 treated cultures. An isotype-matched antibody for CD3, a cell surface marker not expressed in the hepatocyte, did not inhibit apoptosis (Fig 4.5).

By blocking FasL and therefore the Fas pathway, apoptosis can be inhibited following treatment with IFNγ. It was hypothesised that IFNγ causes upregulation of FasL. Changes in FasL mRNA were investigated using a ribonuclease protection assay.
Figure 4.5 MFL3 inhibits IFNγ induced apoptosis.

Hepatocytes were maintained for 72 hours in modified Chee’s medium containing 100U/ml IFNγ and the MFL3 antibody at the concentrations indicated or an antibody to CD3. Treatment with αCD3 had no effect on apoptosis compared to controls. At 2μg/ml MFL3 there was no significant difference in apoptotic rate to controls. The highest concentration tested (10μg/ml) however inhibited apoptosis (p<0.005). Data shown is representative of 3 independent experiments, mean +/- SEM.
4.1.5 Effect of IFNγ on death receptor pathway

Fas signalling has a major contribution to IFNγ induced apoptosis in the cell culture system described here. Previous observations, however suggest that this is not the case. Although Fas is constitutively expressed by cultured murine hepatocytes FasL mRNA has not been detected after IFNγ treatment (Kano et al, 1999). Differences exist between the system described by Kano et al, and the one used here thus it is possible that different regulation of FasL could explain the apparent paradox. Additionally, downstream components of the death receptor pathway may be responsible for apoptotic signalling.

RNA was isolated from control and IFNγ treated cells, by Trizol extraction, at 24, 48 and 72 hours. The ribonuclease protection assay was performed to investigate changes in death receptor pathway molecules. The main observation from these experiments was that the FasL mRNA was not detectable in the cultures at any time point. Neither control nor IFNγ treated cells expressed detectable levels of FasL mRNA.

In the attempt to detect low levels of Fas mRNA the levels of control signal became saturated, thus extrapolating data from other probes would be unreliable.
Figure 4.6 Effect of IFNγ on death receptor mRNA.
RNA was extracted from hepatocytes at 24, 48 and 72 hours. No differences were seen between controls (lanes 1-3) and cells treated with IFNγ (lanes 4-6). Data shown is representative of 2 separate experiments.
4.2 Discussion

4.2.1 Caspase activity

Caspases have emerged as the first wave of effectors in a vast majority of apoptotic systems. Although some evidence of caspase independent apoptosis exists, the effector molecules are unclear. Peptidyl fluoromethyl ketone inhibitors of apoptosis are not 100% specific, z-IETD-fmk (caspase 8 inhibitor) can also inhibit the action of caspase 10, the caspase 3 inhibitor z-DEVD-fmk also has affinity for caspase 6 and 7. However the involvement of the caspases was confirmed by Western blot. No cleavage of caspase 8 was observed, this was thought to be due to a very low level of caspase cleavage that was below the limits of detection by this assay. Fluorogenic caspase substrates are commercially available and could have been utilised to confirm caspase 8 activity but the binding site is the IETD motif common to the inhibitor. Thus use of the fluorogenic substrate would not have added any further information. Published data show that cleavage of downstream caspase targets are detectable by Western blot before cleavage of the caspase responsible (Scaffidi et al, 1999). This strategy was therefore used in an attempt to confirm caspase 8 activity. While Bid cleavage is characteristic of caspase 8 it is possible that another z-IETD-fmk inhibitable enzyme is responsible for the cleavage. While inhibition with z-IETD-fmk completely abolished death, inhibition of caspase 9 did not. It is possible that a higher concentration of z-LEHD-fmk was required to bind all the active caspase 9. A low amount of active caspase 8 could easily be neutralised by lower concentrations of z-IETD-fmk, however following an amplification at the mitochondria a larger number of active caspase 9 molecules would be present. Alternatively all the caspase 9 may have been neutralised by the inhibitor and the
remaining proportion of apoptosis due to a redundancy system, for example caspase 2 may take the role of caspase 9, or a non-caspase effect may be involved.

Cleavage of caspase 9 was detected at 72 hours, although cleavage is not always necessary for caspase 9 activity. One band runs a little shorter than the other full length bands (Fig 4.3B, lane 2), this occurrence had been seen in other gels, not always at the 48 hour time point. Human caspase 9 contains a phosphorylation site (Cardone et al., 1998) but this is not present in the murine caspase (Fujita et al., 1999). A caspase 9 variant exists that lacks the catalytic domain, but is ~30kDa (Seol and Billiar 1999), thus neither of these reasons can satisfactorily explain the apparent anomaly. It is beyond the scope of these investigations to determine the reason for the shorter caspase band it could be due to removal of a localisation motif such as the mitochondrial localisation sequence (Roise and Schatz, 1988), nonetheless similar observations on caspase 9 have been published (Bratton et al, 2001, Costantini et al, 2002, Hakem et al, 1998).

4.2.2 Death Receptor Pathways

IFNγ has a number of links with the Fas pathway as described here and elsewhere cooperation of Fas and IFNγ pathways accelerates and increases apoptosis. Circumstantial links between IFNγ and Fas pathways in the ConA model of hepatitis exist (Mizuhara et al, 1994, 1996, Tagawa et al, 1998). In vivo FasL is upregulated in IFNγ transgenic mice (Okamoto et al, 1998) however this FasL may be originating in infiltrating T cells or NPC’s. Here we show directly that FasL is responsible for IFNγ induced apoptosis by inhibiting death using the FasL blocking antibody MFL3. To support this observation a RPA was performed to quantitatively assess any increase in FasL mRNA. No increase
was seen, indeed FasL mRNA was undetectable in this assay. Kano et al, (1999) also detected no FasL in primary murine hepatocyte culture following treatment with IFNγ.
5 The role of the mitochondria

Previous chapters have shown findings of factors affecting IFNγ induced apoptosis, such as serum pre-incubation and the presence of other cytokines. The process of apoptosis was then investigated, the initiator caspase 9 and a caspase 8-like activity were found. The Fas signalling pathway was also found to be important although no changes in Fasl transcription were detected.

In the hepatocyte Fas signals through caspase 8 to the mitochondria via Bid. As a BH3 domain only member of the Bcl-2 family Bid interacts with other Bcl-2 family members, hypothetically to induce assembly of the MPTP. CsA inhibits cyclophillin D binding to ANT, which in many systems can ablate the mitochondria – dependent apoptotic process as cyclophillin D and ANT are involved in the formation of MPTP. The relationship between MPTP, mitochondrial depolarisation and apoptosis is controversial. In some systems cytochrome c release from the mitochondria, indicative of early apoptosis, is seen before or independently of $\Delta \psi_m$. This chapter attempts to clarify the relationship of MPTP, mitochondrial depolarisation and apoptosis in the IFNγ hepatocyte system.

5.1 Results

5.1.1 CsA but not FK506 inhibits apoptosis induced by IFNγ.

The immunosuppressive CsA has a number of effects on the cell. In addition to its binding cyclophillin D the drug has transcriptional effects and modulates Ca$^{2+}$ by inhibiting calcineurin. In an attempt to control for these effects the CsA analogue FK506 was employed. FK506 possesses no cyclophillin D binding properties but does affect calcineurin and is an immunosuppressive.
Isolated primary murine hepatocytes were cultured for 72 hours in Chee’s medium. IFNγ was added at 100U/ml throughout the culture period. CsA was added at 0.1, 1 and 5μg/ml. As described in Chapter 3 CsA has some toxic effects in the hepatocytes at higher concentrations. At both 1 and 5μg/ml CsA has a significant effect on the level of apoptosis seen with a reduction of ~50% compared to controls (p<0.005 by T-test). The CsA analogue FK506 had no effect on hepatocyte apoptosis at any of the doses tested (0.1, 1 and 10μM) (Figure 5.1).

Since FK506 does not have an effect on cyclophilin D but share many of the other properties of CsA including binding calcineurin it is likely that the apoptosis inhibition effects seen when CsA is added are due to the cyclophilin D binding properties of CsA. As cyclophilin D is thought to be important in formation of the MPTP it was hypothesised that IFNγ leads to apoptosis in the hepatocyte through formation of the MPTP and consequent depolarisation of the mitochondria.
Figure 5.1 CsA inhibits IFNγ-induced hepatocyte apoptosis.
A) Murine hepatocytes were isolated and cultured in Chee’s media for 72 hours in the presence of 100U/ml IFNγ and CsA indicated. At 0.1μg/ml there was no difference to controls. However at 1 and 5 μg/ml CsA a significant difference was observed (** indicates p<0.005 by paired t-Test). B) As above hepatocytes were isolated and cultured for 72 hours with the addition of FK506 instead of its analogue CsA. CsA and FK506 share calcineurin inhibition properties, but FK506 lacks the ability to bind cyclophillin D. FK506 does not inhibit hepatocyte apoptosis at any of the doses tested. All data is representative of three independent experiments, and shows mean ± SEM, n=3
5.1.2 Staining of hepatocyte mitochondria.

To further elucidate the relationship between the mitochondria and apoptosis depolarisation of the organelle was investigated after IFNγ treatment. Depolarisation of the mitochondria is frequently seen in apoptotic cells. In some systems depolarisation is seen before cytochrome c release, in others cytochrome c is released before mitochondrial depolarisation.

Since observations are inconsistent across cell types and apoptotic stimuli, the importance of Δψm could not be predicted in our system. MitoTracker Red® is a mitochondria specific rhodamine dye that accumulates in organelles with an active membrane potential. Isolated hepatocytes have a high affinity for chloromethyl X Rosamine (CMXRos) and demonstrate staining following a 15 minute incubation at 100nM. When treated with the mitochondrial uncoupler cyanide m-chlorophenyl hydrazone (mClCCP) hepatocyte mitochondria lose their membrane potential and the dye diffused across the cytoplasm producing a weak even staining compared to the punctate intense pattern seen in hepatocytes containing healthy mitochondria (Fig 5.2). Hepatocytes treated with MitoTracker Red demonstrate strong fluorescence after short periods of incubation with the dye, however fluorescence is rapidly quenched when the cells are viewed under a fluorescent or confocal microscope. For this reason a longer period of staining using a higher concentration of dye was used in experiments. Hepatocytes incubated with MitoTracker Red for periods of up to 3 hours showed no signs of nuclear apoptosis (data not shown). Additionally a nuclear dye ToPro3 was used to help distinguish individual cells.
Figure 5.2 Staining of polarised mitochondria by MitoTracker Red

Isolated hepatocytes were cultured overnight. Mitochondrial uncoupler mCiCCP or an equal volume of DMSO (control) was administered for 30 minutes. Cells were stained with 100nM MitoTracker Red for either 15 or 45 minutes before fixation and analysis. A) Shows cells treated with 100nM MitoTracker Red for 15 minutes, visualised by epifluorescence. Upper panel shows control cells, lower panel shows cells depolarised using mCiCCP, also visible are intensely stained cells that have lifted off the substratum. Healthy mitochondria can be identified by punctate staining as the dye is retained by the mitochondrial membrane. B) Images obtained using a Leica TCS NT confocal microscope system. Shows detail of typical mitochondrial staining pattern in healthy cells (upper), the lower panel shows mCiCCP treated cells, one polarised cell (possibly NPC) is surrounded by depolarised cells exhibiting the diffuse pattern indicative of mitochondrial depolarisation.
5.1.3 Treatment of hepatocytes with 100U/ml IFNγ causes -$\Delta \psi_m$

Isolated hepatocytes were treated with IFNγ stained and fixed, control cultures were included. Following 24 hour incubation with IFNγ no difference was seen compared to controls. At 48 hours however a proportion of IFNγ treated cells appeared to contain healthy nuclei but depolarised mitochondria. The mitochondria of the control population maintained membrane potential. The majority of viable IFNγ treated cells at 72 hours contained depolarised mitochondria. Very few depolarised mitochondria were observed in the controls at this time (Figure 5.3). Throughout the culture period viable cells could be readily identified with the nuclear dye ToPro3. A large proportion, approximately 50% of viable IFNγ treated cells at 48 hours, and a between 70% and 80% of viable cells at 72 hours showed the diffuse MitoTracker Red staining indicative of -$\Delta \psi_m$.

IFNγ causes depolarisation of hepatocyte mitochondria. It was hypothesised that -$\Delta \psi_m$ and apoptosis were linked in this system. The point at which the apoptotic pathway feeds into the mitochondria was hypothesised to be through Bid. If Bid cleavage was inhibited this would prevent not only apoptosis but -$\Delta \psi_m$ also. To test this hypothesis the effects of known inhibitors of apoptosis were tested on -$\Delta \psi_m$. 
<table>
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**Figure 5.3 IFNγ causes mitochondrial depolarisation of hepatocyte.**
Isolated hepatocytes were cultured for the time indicated in Chee’s media with the addition of IFNγ. Cells were stained with 100nM MitoTracker Red for 45 minutes before fixation. Following fixation in 4% paraformaldehyde cells were stained with 250nM ToPro3 for 10 minutes, mounted in Mowiol and coverslipped. Images were obtained using a Zeiss LSM510 confocal microscope system. At 24 hours no depolarisation of hepatocyte mitochondria is seen in either control cells or IFNγ treated cells. At 48 hours depolarisation is seen in a number of cells treated with IFNγ. However at 72 hours the majority of cells treated with IFNγ are depolarised, in the control population a low number of cells containing depolarised mitochondria are seen. Bar = 10μM Arrow denotes cell containing depolarised mitochondria.
5.1.4 Mitochondrial depolarisation occurs independently of inhibition of apoptosis

Treating hepatocytes with IFNγ causes apoptosis at 72 hours and depolarisation of mitochondria at 48 hours. The relationship between $-\Delta \psi_m$ and apoptosis is unclear. The effect of inhibitors of apoptosis on mitochondrial depolarisation was considered. It was hypothesised that if mitochondrial depolarisation were dependent on cleavage of Bid, the caspase 8 inhibitor z-IETD-fmk would successfully inhibit the observed depolarisation. If assembly of the MPTP was responsible for the depolarisation it was hypothesised that CsA would be capable of inhibiting $-\Delta \psi_m$. As controls z-LEHD-fmk and FK506 were included. Figure 5.4 shows the results of one representative experiment from 3 repeats. Control cultures contained ~5% depolarised mitochondria at 72 hours, at this time 80% of viable IFNγ treated cells contained depolarised mitochondria. Treatment with the caspase inhibitors z-IETD-fmk and z-LEHD-fmk at 10μM inhibits apoptosis. These chemicals however had no effect on the levels of depolarisation induced by IFNγ as the mean number of cells containing depolarised mitochondria remained at ~80%. CsA inhibits IFNγ induced apoptosis but its analogue FK506 does not, as expected FK506 had no effect on depolarisation. CsA appears to reduce the percentage of hepatocytes containing depolarised mitochondria from 80% to ~65% however this is not a statistically significant decrease when the combined data from 3 experiments were analysed using the general linear model (GLM).
Figure 5.4 IFN\(\gamma\) causes mitochondrial depolarisation independently of inhibition of apoptosis. 

Hepatocytes were cultured in Chee's media with the addition of IFN\(\gamma\) for 72 hours. Cells were incubated with 100nM MitoTracker Red for 45 minutes, fixed in 4% paraformaldehyde, mounted in Mowiol, coverslipped and images obtained using an inverted fluorescent Zeiss microscope. Images were analysed using MetaMorph Image analysis software and data reported as % cells containing depolarised mitochondria. Data shown is representative of three independent experiments. Ten field were scored, error bars represent SD between fields. Using GLM there is no effect of any of the treatments used on depolarisation induced by IFN\(\gamma\).
To confirm that $-\Delta \psi_m$ is independent of Bid cleavage, IFN$\gamma$ treated hepatocyte extracts co-incubated with z-IETD-fmk or CsA were separated by SDS-PAGE and probed for the presence of Bid. As expected CsA was incapable of inhibiting Bid cleavage at 72 hours. However z-IETD-fmk does inhibit Bid cleavage, confirming that $-\Delta \psi_m$ is independent of Bid cleavage.
Figure 5.5 Bid cleavage is dependent on upstream inhibition of apoptosis.

Hepatocytes were cultured in Chee’s media with the addition of IFNγ and the agents indicated for 72 hours. Cells were lysed in denaturing lysis buffer, separated by SDS-PAGE and probed for the presence of full length or tBid. Lanes 1-3 contain extracts from control cultures treated with z-IETD-fmk (lane 2) or CsA (lane 1). Lanes 4-6 contain extracts from IFNγ treated cultures containing z-IETD-fmk (lane 5) or CsA (lane 6).
5.2 Discussion

The results shown in this chapter present evidence that IFNγ induces formation of the MPTP, inhibitable by CsA. Mitochondrial depolarisation in hepatocytes is observed from 48 hours with ~80% of cells containing depolarised mitochondria at 72 hours. Mitochondrial depolarisation is independent of both MPTP and Bid cleavage as neither CsA nor z-IETD-fmk have any effect on the proportion of cells containing depolarised mitochondria.

CsA was used here at a non-toxic dose that according to the literature and previous experiments is effective at inhibiting both MPTP formation and apoptosis. Apoptosis was reduced by ~50% of controls after treatment with CsA. While in isolated mitochondria CsA has a complete effect on the swelling indicative of MPTP in in vitro systems inhibition does not appear to be absolute. An alternative explanation for the remaining apoptosis is that the pathway may bypass the mitochondrial step in some cells. Scaffidi et al (1998) described 2 pathways to apoptosis after Fas stimulation, the type I pathway where there is an amplification step at the mitochondria appears to be the pathway induced by IFNγ as tBid and cleaved caspase 9 are observed, however some cells may be activating sufficient caspase 3 directly through caspase 8-like activity. Since this paradigm was described further research has shown the pathways can be altered and are flexible. Nonetheless hepatocytes of Bid−/− mice are resistant to Fas induced apoptosis, suggesting that Bid signalling to the mitochondria is a vital part of the pathway (Yin et al, 1999).
Caspase 8 may not be the only agent capable of cleaving Bid. An alternative caspase (such as caspase 10 or an as yet unidentified caspase) or a non-caspase protein may be responsible for Bid cleavage. The data shown in Figure 5.5 would suggest that the agent responsible for cleaving Bid is inhibitable by z-IETD-fmk but not CsA.

Detection of Δψₘ was accomplished using MitoTracker Red. It was necessary to make a number of assumptions using this reagent. The main assumption was that the differences observed were due solely to the effect of IFNγ and not a consequence of interaction between IFNγ and MitoTracker Red. There is evidence that rosamine derivatives suppress respiratory control (Scaduto and Grotyohann, 1999). In IFNγ treated cell a mitochondrial stressor may induce Δψₘ and this could be exacerbated by use of MitoTracker Red, although Scaduto and Grotyohann (1999) reported tetramethylrodamine ethyl ester (TMRE) and tetramethylrodamine methyl ester (TMRM) to have effects on respiratory control, not CMXRos. A second effect of CMXRos is that it can photosensitise mitochondria (Minamikawa et al, 1999), while this was not a problem for the fixed cells from which results have been reported here it made effective timelapse confocal microscopy to determine whether depolarisation was transient or long lasting very difficult. While other mitochondrial dyes are available MitoTracker Red was used as it is aldehyde-fixable (Macho et al, 1996) and had been used in primary hepatocyte cultures (Isenberg and Klaunig, 2000). One approach that may have been enlightening with respect to MPTP formation is the use of calcein in combination with Co²⁺ (Petronilli et al, 1999). Calcein is distributed throughout the cytoplasm, nucleus and mitochondria, Co²⁺ quenches the fluorescence in the cytoplasm and nucleus leaving only mitochondrial calcein fluorescence. When MPTP occurs the fluorescence is rapidly lost from the mitochondria.
The investigations presented here into ΔΨm with CsA and z-IETD-fmk demonstrated that -ΔΨm occurs regardless of the presence of potential inhibitors. Inhibition of mitochondrial respiration using azide or myxothioazol causes -ΔΨm in PC12 cells that is not inhibited by CsA (Bal-Price and Brown 2001). The protonophore mCICCP causes a CsA insensitive -ΔΨm in photoreceptors (Yang et al, 2001).

In IFNγ treated hepatocytes one potential cause of -ΔΨm may be free oxygen radicals. Cultured murine hepatocytes when treated with IFNγ show increased xanthine oxidase activity (Adamson and Billings, 1994), xanthine oxidase is the enzyme that converts xanthine into uric acid, creating oxygen free radicals in the process (McCord 1985). As a result of oxygen free radical formation damage to membranes may be sustained and evidence of damage to the mitochondrial membrane by xanthine oxidase has been shown to occur (Lee and Farrell, 2001).

Whatever the reason for -ΔΨm depolarised mitochondria in the cell would have consequences for cellular energy production. The synthesis of ATP by the mitochondria provides the cell with energy, this synthesis is driven by the mitochondrial membrane potential. ATP is needed not only for cellular metabolism but for completion of apoptosis, thus a cell containing depolarised mitochondria is living on borrowed time, it is surviving on the stocks of ATP it has accumulated or the cell is finding energy through glycosylation. In vitro the cells had not been provided with any sugars in vivo however sugars could be mobilised from the blood. In any case the fact that IFNγ causes long term -ΔΨm has consequences for the cell’s metabolism.

The next logical experiments would involve investigations of the localisation of cytochrome c, and the timing of its translocation from the mitochondrial matrix into the cytosol. In order to more accurately define the relationship between -ΔΨm and apoptosis a useful approach would be transient transfection of hepatocytes using a cytochrome c –
GFP plasmid. There are a number of practical problems associated with this approach. Firstly the proportion of transfectants in the hepatocyte system in our laboratory is unsatisfactory. A second major problem is that the time scale of hepatocyte apoptosis does not lend itself readily to combined transfection and IFNγ treatment. However should these problems be overcome it would provide the opportunity to study by confocal microscopy, on a cell by cell basis, the timing and importance of \(-\Delta\psi_{m}\) and cytochrome c release. It would be theoretically possible to study living transfectants stained with MitoTracker Red by timelapse confocal microscopy.

A method employing immunohistochemistry would not allow such precise dissection of the relationship. However if a protocol for immunohistochemistry using paraformaldehyde stained cell could be developed the hepatocytes could be stained with both MitoTracker Red and probed with a cytochrome c specific antibody.
6 Final Discussion

This thesis presents results demonstrating important novel findings in IFNγ induced apoptosis. We have shown upstream caspases are acting in IFNγ induced apoptosis in the hepatocyte. Caspase 9 is processed during apoptosis and evidence characteristic of caspase 8 activation found. Intriguingly the Fas pathway plays a pivotal role in the apoptotic process described. The death signal given by IFNγ is malleable and can be inhibited or augmented by the hepatocyte’s environment. Here we show that pre-incubation in serum can alter the proportion of apoptotic cells in the population. The results acquired, particularly the involvement of caspase 9 and inhibition of apoptosis by the cyclophillin D binding drug CsA, led us to hypothesise that the mitochondria were involved in apoptosis. Depolarisation of the mitochondria was observed in IFNγ treated cells. Depolarisation of mitochondria during apoptosis may be due to the formation of the mitochondrial permeability transition pore, however, in this system, CsA while inhibiting apoptosis, does not inhibit -$\Delta$$\psi_m$.

The aim of this research was to expand on the existing knowledge of IFNγ apoptotic pathways in the hepatocyte and provide potential targets for modulation of the hepatocyte’s responses to IFNγ. Following exploratory studies of the system, described in Chapter 3, a strategy was formulated to achieve these aims. Peptide based inhibitors of caspases were successfully utilised to established that IFNγ induced apoptosis in the hepatocyte is caspase dependent, specifically, caspase 9 and a z-IETD-fmk inhibitable agent such as caspase 8 or 10. Activation of these caspases was confirmed by Western blot. In the case of caspase 8-like activity Bid cleavage was assessed. Caspase 9 cleavage was demonstrated directly. Inhibition of IFNγ induced apoptosis was achieved
with the FasL blocking antibody MFL3, suggesting a critical role of the Fas signalling pathway. By RPA no upregulation of FasL mRNA was detected in the cultures. MPTP formation was blocked using CsA at a non-toxic dose and $-\Delta\psi_m$ observed in hepatocytes at 48 hours. Loss of mitochondrial membrane potential was not inhibited with either CsA nor the caspase inhibitor z-IETD-fmk, indicating $-\Delta\psi_m$ is independent of both MPTP formation and the action of Bid. These findings are summarised in Figure 6.1.
Figure 6.1. IFNγ induced apoptosis in the hepatocyte.

IFNγ induces a death pathway in the hepatocyte with a number of potential points of control. After transcriptional control at IRF-1, Fas/FasL interaction causes recruitment and activation of caspase 8. In our system it is possibly caspase 8 that leads to cleavage of Bid. Formation of the MPTP and activation of caspase 9 follow. A loss of mitochondrial membrane potential, independent of inhibition of apoptosis is seen. Inhibitors used and their points of action are shown in red. See text for detail.
6.1 The role of IFNγ in hepatitis and carcinogenesis

IFNγ plays an important role in the maintenance and resolution of the inflammatory state. The final outcome of hepatitis is variable, this inconsistency may be due to a number of factors that can affect the fate of the hepatocyte stimulated by IFNγ such as the presence of further cytokines and growth factors. One potential outcome of chronic hepatitis is hepatocellular carcinoma (HCC), the investigation of the action of IFNγ may be useful in the understanding of HCC development. HCC's show a decreased expression of the cyclin dependent kinase inhibitor p57 (KIP2) and IGF2, suggesting abnormal cell cycle regulation (Schweinbacher et al, 2000), another characteristic of HCC is that they are responsive to TRAIL, but not Fas. The opposite is true of normal hepatocytes, i.e. they are responsive to Fas but not TRAIL. Additionally, the majority of HCC lines and other transformed hepatocyte cell lines are unresponsive to IFNγ (Shin et al, 2001). HCC can be prevented in an animal model by blocking Fas signalling (Nakamoto et al, 2002). Here a potential mechanism is suggested. IFNγ sensitises to anti-Fas induced apoptosis and blocking FasL prevents IFNγ induced apoptosis. It is possible that IFNγ has transcriptional control over areas of the Fas pathway not yet investigated. It could be hypothesised that IFNγ exerts an apoptotic effect through IRF-1 and this is effective in hepatocytes that do not receive a rescue signal such as HGF or EGF, however when rescue occurs the oncogenic consequences of IRF-2 may be felt. A situation is postulated where a normal hepatocyte, through the consequences of IFNγ, will be stimulated to apoptosis through the Fas pathway and begin to upregulate TRAIL. HGF, EGF, an oncogenic mutation or a viral protein may then rescue the cell from Fas.
mediated apoptosis. A Fas/IFNγ insensitive, TRAIL sensitive hepatocyte remains that could develop further towards HCC.

The cell controls apoptosis in a variety of ways. In the course of this research a surrogate for cellular control has been provided in the use of caspase inhibitors, particularly z-LEHD-fmk mimics XIAP. Having shown that caspase inhibition is successful \textit{in vitro} can we presume that caspase inhibition will be successful \textit{in vivo}?

There are potential problems with this strategy. IFNγ causes a permanent -Δψₘ which has consequences for cellular metabolism and could lead to necrosis before apoptosis. It is generally held that interface hepatitis is due to secondary necrosis following a high level of apoptosis. Neighbouring cell and professional phagocytes cannot effectively dispose of the cellular debris, inflammatory cellular contents leak out and necrosis of surrounding cells follows. Based on the observation that IFNγ induces -Δψₘ before apoptosis occurs there is potential for a different or additional mechanism. Those cells that receive IFNγ stimulation, and coupled with TNFα or FasL or by high ATP levels, would die by apoptosis but bystander cells stimulated with IFNγ alone may undergo -Δψₘ and necrosis would follow due to this process, rather than a deficiency in phagocytosis of apoptotic bodies. Even if this process does not occur direct inhibition of caspase 9, while blocking apoptosis, would not resolve the problem of potential necrosis. Indeed inhibition of caspase 8, as demonstrated here would not be effective. These hypotheses could be tested \textit{in vitro} by assessing necrosis in inhibitor treated cultures.

By blocking FasL signalling with MFL3 the consequences of inhibiting Fas signalling by FLIP (FLICE Inhibitory Protein) may be replicated. FLIP binds to the DED of FADD in the DISC on ligation of Fas by FasL. The importance of FLIP in the hepatocyte is
emphasised by the experiments demonstrating Fas induced apoptosis is increased in livers of IL-6−/− mice, treatment of primary murine hepatocyte cultures with IL-6 protects from Fas induced apoptosis (Kovalovich et al, 2001), and in the mouse ConA model of hepatitis, which is mediated by IFNγ and Fas, IL-6 is protective (Mizuhara et al, 1994 Nishikage et al, 1999, Hong et al, 2002). Significantly IL-6 upregulates FLIP, Bcl-2 and Bcl-XL (Kovalovich et al, 2001, Hong et al, 2002). A second role for IL-6 is prevention of release of SMAC/DIABLO from mitochondria (Chauhan et al, 2001). Another relevant inhibitor of Fas signalling lies in the endogenous splice variants of Fas. The FasTMDel variant of Fas lacks the transmembrane domain and exists as a soluble decoy receptor (Cheng et al, 1994, Cascino et al, 1995). The HCV infected liver contains a higher ratio of Fas : FasTMDel than the healthy liver (Ferenbach et al, 1997). Both sFas and sFasL levels are higher in livers with HCC than in control healthy livers (Nagao et al, 1999) suggesting that this level of control is relevant both in hepatitis and HCC. The author was unable to demonstrate upregulation of FasL mRNA. Fas activation may be regulated by sFasL. In patients with chronic hepatitis without cirrhosis levels of sFasL significantly increase 12 hours after injection with IFN (Yoneyama et al, 2002). The possibility that other members of the apoptotic machinery are involved has been considered. Recent evidence suggests that factors outside the apoptotic pathway may have profound, direct effects on the control of cell death. HGF is a mitogen of hepatocytes and other cell types. In the liver HGF activity and protein levels increase rapidly following liver injury (Lindroos et al, 1991). The receptor for HGF, Met, was discovered and identified as a proto-oncogene before its ligand was known (Cooper et al, 1984). Met regulates life and death not only through the tyrosine kinase activity induced by HGF biding but also through sequestering and binding of Fas (Wang et al, 2002). Immunoprecipitation was used to show that Met associates with Fas in liver cell lines. At
low concentrations neither ligand could dissociate Met from Fas suggesting that Met can prevent apoptotic signalling by FasL. This mechanism is protective and could be part of the hepatocyte's response to IFNγ. As changes at the Fas and FasL level were not detected it is possible that a downregulation of Met could allow increased FasL signalling through the Fas liberated from Met. As a further progression of the multistep hypothesis Met is upregulated in many HCC (Ueki et al, 1997) and it may be that cells expressing high levels of Met survive IFNγ induced apoptosis and are predisposed to tumour formation.

6.2 The relevance of IFNγ induced apoptosis

The strategy taken throughout these investigations was to identify how the hepatocyte is affected by IFNγ in the process of apoptosis. The relevance of this model to wider research is dependent on a number of considerations. Do the observations recorded model a clinical situation? Is this pathway to apoptosis taken by other cell types undergoing IFNγ induced cell death? Have the results added any further weight to hypotheses on disease progression?

The first of these considerations is clearly not the case. In any clinical situation hepatocytes would be living side by side with non-parenchymal cells (NPC’s), which would be producing cytokines, ECM and growth factors. The ECM that the hepatocytes are grown on (fibronectin) is increased in cirrhotic livers, so healthy conditions are not replicated in our system. The substratum is an important consideration since ECM may contain protective growth factors such as EGF (Engel et al, 1989). In the hepatocyte monolayer conditions predispose towards the survival of hepatocytes but not NPC’s. The hepatocytes that survive the perfusion may be a selected population, not
representative of the true nature of hepatocytes. Hepatocytes from a particular lobe may be more likely to survive and be collected by the perfusion method. Considering that an average yield is approximately 15 million hepatocytes these considerations may not be trivial. Renton estimates that 40-50% of the theoretical maximum number of hepatocytes can be procured (Renton et al, 1978), this figure is probably misleading.

During the Percoll step ~50% of the cell pellet can be lost and while these cells do include NPC's a large number of hepatocytes are discarded here. Additionally the cells are synchronised to enter S phase, which is unlikely in vivo. A proportion of hepatocytes may be in cell cycle in the injured liver but not as large a proportion as in culture. The fact that the cells are exposed to a single cytokine is a limitation of the model, but also one of its strengths.

Despite these limitations, primary cell culture is an excellent way of getting reliable data. Individual cell types can be studied in monoculture in a defined environment. Primary cell cultures while a step removed from the in vivo situation, do not harbour mutations in the apoptotic programme like many cell lines do. The data generated from primary cell cultures are therefore more likely to represent an in vivo situation that data derived from cell lines. Under the non-physiological conditions of cell culture it is possible to get information on how cells may behave in vivo. These data can then be used to make predications and hypotheses about clinical situations.

In the model used here hepatocytes die by apoptosis after stimulation by IFNγ. In injured livers hepatocytes undergo apoptosis and IFNγ is a causal agent, nonetheless the question remains whether in vivo IFNγ is acting directly or in conjunction with FasL, TNF, T3 or a further stimulus.

The information collected in this thesis may help elucidate the mechanisms involved by demonstrating some of the ways in which IFNγ can cause or assist apoptosis. Future
investigations using co-culture of hepatocytes and non-parenchymal cells would provide an insight into the role of NPC’s and how hepatocytes could be rescued from IFNγ induced apoptosis. This experimental set up would be more like the in vivo situation replicating the cross talk between different cell types. The potential modulators of apoptosis such as other cytokines and growth factors could be examined using RNAi to block production of specific targets. To confirm that the NPC’s are responsible for production of the modulators transfection of reporter plasmids containing the promotor sequence of interest could be carried out.


This in vitro investigation has revealed that the pathway to apoptosis in the hepatocyte is malleable, caspase dependent, involves the mitochondria and the Fas pathway is necessary for its completion. These data may eventually provide opportunities for therapeutic interventions in the course of hepatitis, particularly, fulminant or acute hepatitis caused by alcohol, or HAV, HBV, or HCV where IFNγ is one of the causative agents. Disrupting apoptosis caused by IFNγ may have short term benefits in chronic hepatitis but the potential of developing more pre-cancerous cells may negate these benefits.
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Appendix

Solutions

*LDS sample buffer*

- 141 mM Tris-Base
- 106 mM Tris-HCl
- 10% glycerol
- 2% LDS
- 0.51 mM EDTA
- 0.22 mM bromophenol blue
- 0.175 mM Phenol Red

*MOPS SDS running buffer*

- 50 mM MOPS,
- 50 mM Tris base,
- 0.1% SDS,
- 1 mM EDTA

*MES SDS running buffer*

- 50 mM MES,
- 50 mM Tris base,
- 0.1% SDS,
- 1 mM EDTA

*Tris-Glycine Transfer buffer*

- 12 mM Tris-Base
- 96 mM Glycine
- 20% Methanol

*TBS Tween buffer*

- 10 mM Tris-Base
- 140 mM NaCl
- 0.1% Tween 20

*Modified Bouin’s fixative*

- 85% v/v Methanol
- 5% v/v Glacial acetic acid
- 10% v/v pure formalin solution (40% formaldehyde in water)
**Mowiol Mounting Medium**

Mowiol 4-88 is a high-quality mounting medium with good anti-fade characteristics. It hardens and matches the refractive index of immersion oil, and thus is particularly suited for this form of microscopy. Additional anti-fade (DABCO) is added to further retard photobleaching.

Mowiol 4-88 (Calbiochem; 475904)  
Glycerol (Fisher Chemicals; G/0650/08)  
Tris (Sigma; T-8524)  
DABCO (Sigma; D-2522)

1. Add 2.4g Mowiol to 6g glycerol and stir briefly with a pipette.  
2. Add 12ml dH2O and stir at room temp for several hours.  
3. Add 12ml 0.2M Tris (pH 8.5) and heat to 50°C for 1-2 hrs while stirring.  
4. When the Mowiol has dissolved, clarify by centrifugation at 500 x g for 15mins.  
5. Add DABCO to 2.5% (0.72g), aliquot and store at -20°C. Bubbles can be removed by centrifugation.  
   Aliquots can be stored for up to 2 weeks at 4°C or frozen to -20°C.

**Denaturing lysis buffer**

10mM Tris pH 7.5  
1% SDS

**DEPC treated water**

0.2% DEPC in distilled water  
Autoclave 120°C, 20 minutes.
<table>
<thead>
<tr>
<th><strong>Media</strong></th>
<th><strong>Supplement</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver Perfusion media</td>
<td>Gentamycin (50μg/ml)</td>
</tr>
<tr>
<td>Hank’s buffered Salt Solution (Digestion media)</td>
<td>Gentamycin (50μg/ml) 50μg/ml DNase1 0.05% collagenase</td>
</tr>
<tr>
<td>DMEM/F12 (Plating media)</td>
<td>Gentamycin (50μg/ml) L Glutamine (4mM) Dexamethasone (0.04μg/ml) Insulin (1μg/ml) Transferrin (0.67ng/ml) Sodium Selenite (0.55μg/ml) FBS as indicated</td>
</tr>
<tr>
<td>Modified Chee’s media</td>
<td>Gentamycin (50μg/ml) L Glutamine (4mM) Dexamethasone (0.04μg/ml) Insulin (1μg/ml) Transferrin (0.67ng/ml) Sodium Selenite (0.55μg/ml)</td>
</tr>
<tr>
<td>William’s E media with Glutamax</td>
<td>Gentamycin (50μg/ml) EGF (1ng/ml)</td>
</tr>
</tbody>
</table>
Confocal timelapse imaging of mitochondrial depolarisation

Live hepatocytes were plated onto glass chamber slides and examined by multi-photon confocal microscopy. Images displayed were acquired over a period of 25 minutes, treated cells had been exposed to IFNγ for 48 hours prior to observation. Non-treated control cells are also displayed.
Timelapse confocal images of live control and IFN-γ treated cells at 48 hours stained with MitoTracker. Control cells contain no depolarised mitochondria. Depolarised mitochondria are visible in IFN-γ treated cells at 48 hours alongside cells containing healthy mitochondria. Over the 25 minutes displayed, no cells are seen undergoing mitochondrial depolarisation. Bar = 50 µm.