

**LIVER DEATH AND REGENERATION: INDIRECT
MECHANISMS OF PARACETAMOL TOXICITY**

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Doctor of Medicine

The University of Edinburgh

2002



ABSTRACT

Background: Paracetamol overdose (POD) remains a pressing clinical problem as despite the availability of a safe and effective antidote, patients continue to die or require a liver transplant. Recent evidence suggests that toxicity after POD may be more than a simple direct toxic effect as has previously been accepted and that Kupffer cells and cytokines such as TNF- α are involved in the pathogenesis of liver injury.

Paracetamol and Hepatocyte Apoptosis Examination of liver in patients after POD revealed hepatocyte apoptosis occurring alongside striking regenerative activity. Apoptosis is important for several reasons. First, at this time paracetamol is undetectable in serum and paracetamol metabolites should be cleared from the liver, so apoptosis is not directly induced by paracetamol. Second, the rate of apoptosis represents a significant rate of cell loss from the liver. Third, the apoptosis occurs despite the background of regeneration.

Paracetamol and TNF- α : Some, but not all, studies support a role for TNF- α in inducing liver injury after paracetamol overdose. In a murine model, TNF- α was elevated in serum after POD but inhibition of TNF- α action did not alter survival or liver injury. However, TNF- α augmented paracetamol toxicity in vitro by increasing rates of both apoptosis and necrosis; TNF- α also lowered the threshold for toxicity. Paradoxically, while TNF- α had no apparent effect on hepatocytes in the absence of paracetamol, pretreatment with TNF- α protected against subsequent paracetamol toxicity.

Paracetamol and Kupffer Cells: Kupffer cells modulate toxic hepatic injury induced by several agents including paracetamol. Co-culture experiments comparing hepatocytes alone or in culture with Kupffer cells showed no differences in toxicity. However, production of TNF- α by macrophages was augmented by paracetamol and was further

significantly elevated in co-culture with hepatocytes. The levels of TNF- α in these experiments was similar to the concentration of recombinant TNF- α which augmented paracetamol toxicity in hepatocytes alone in previous studies but in this model no difference in toxicity was noted. This finding suggests that Kupffer cells produce TNF- α in response to high doses of paracetamol, that hepatocytes behave differently in co-culture than when grown alone and that the differences in response may relate to soluble factors produced by Kupffer cells.

Conclusions: These studies offer new insights into mechanisms of hepatocyte death by apoptosis and regeneration in the context of drug-induced liver injury. A model is presented whereby toxin-induced activation of Kupffer cells with resulting production of cytokines modulates hepatocyte responses and alters the course of disease.

ACKNOWLEDGEMENTS

I would like to thank many people for assistance in carrying out the research work and completing this thesis.

I am indebted to Professor David Harrison for help and advice from the beginning in setting up the project, for invaluable discussions when things went wrong and in support for travelling to various meetings to present the data. He also afforded me the opportunity to learn some liver pathology in parallel with this work, for which I am very grateful.

I would like to thank Dr Ken Simpson for support and encouragement, and for access to some of his previous data and expertise.

I am grateful to Nicola Martin, a good friend and co-worker in the laboratory, for her cheerful assistance on many occasions.

I would like to thank Mrs Frances Rae who provided a great deal of advice, support and help throughout. I would also like to thank Dr Sarah Howie for her invaluable experience and advice in many areas but particularly flow cytometry.

I thank Sharon White and Linda More, who worked closely with me in the laboratory with unfailing enthusiasm whether things went right or wrong and made the group a happy one to be in. I am also very grateful to Tim Andrews, who was an enthusiastic and hardworking member of the team during his BSc year.

I would like to thank all other staff from the 3rd floor laboratory, but in particular Matthias Dollinger, for advice and support on murine culture and co-culture; Alexey Glazyrin, the mad Russian, for teaching me how to perfuse liver with a method that only he has perfected; and Amanda Forsyth, research technician, an enthusiastic and hard-

working member of the team. I would also like to thank the other members of the liver research group.

I am grateful to Paul Fineron and Tim Squires for advice and support in working with the coproxamol overdose cases.

I thank John Verth and all the other staff in the old animal house for unfailing help and support in mouse perfusions.

I thank Dr Marilyn Moore for providing buffy coats and plasma for isolation of macrophages.

I am greatly indebted to PPP Healthcare / Royal College of Pathologists for providing the research fellowship that allowed me to carry out this work. I would also like to thank SHERT for financial support of the project.

I would like to dedicate this thesis to Rachel and Emma who will see a bit more of me from now on.

DECLARATION OF ORIGINALITY

I declare that the contents of this thesis have been composed exclusively by me, and that with the exceptions noted below, the work conducted and described herein is entirely my own.

For the data described in chapter 2 most of the immunohistochemistry was carried out by research technicians in the Department of Pathology, University of Edinburgh. All histological analysis was carried out by myself.

The cases of coproxamol and methadone overdose used in chapter 3 were identified by Tim Squires, who provided clinical and toxicological data.

The laboratory work for chapters 4-7 was carried out as part of a research group, with some work carried out by myself and some carried out by research technicians. This included cell culture work, ELISA assays, immunohistochemistry and molecular biology.

The animal experiments in chapter 5 were carried out in the University of Michigan, Ann Arbor, USA by Dr Ken Simpson, an MRC (UK) Travelling Fellow, supported by NIH Grants HL02401, HL31693, HL35276, HL50057. My contribution to this work was in analysis of the data and writing the paper that has subsequently been published (*J Pathol*; 190: 489-494, 2000).

I have not submitted this thesis in candidature for any other degree, diploma or professional qualification.

Angus H McGregor

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CHAPTER ONE

INTRODUCTION: THE PARACETAMOL PARADIGM

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INTRODUCTION: THE PARACETAMOL PARADIGM

Background: The problem of paracetamol overdose

Paracetamol (acetaminophen, N-acetyl-para-aminophenol, 4 acetamidophenol) is a mild analgesic with virtually no side effects at the recommended dosage (The Lancet, 1981). Toxicity after overdose was first described in the 1960's in animals (Eder, 1964; Boyd and Berezky, 1966) and then in humans (Davidson and Eastham, 1966; Thomson and Prescott, 1966). Paracetamol overdosage (POD) has become increasingly popular as a method of self-harm and has increased strikingly in incidence from the 1960's to the 1990's, with approximately 70,000 cases of paracetamol overdose occurring in the UK each year (Meredith et al, 1986; Litovitz et al, 1988; Fagan and Wannan, 1996). An increased understanding of the mechanisms of toxicity associated with depletion of glutathione led to the development of a safe, cheap and effective antidote (N-acetylcysteine: Prescott, 1981; Smilkstein et al, 1988; Jones, 1998). Hepatotoxicity can therefore be prevented in the vast majority of cases with a significant reduction in severe liver injury and huge improvement in survival after POD (Prescott, 1981; Prescott, 1983). However, despite this POD has become the commonest cause of fulminant liver failure and the commonest reason for emergency liver transplantation in the UK and the US (Makin et al, 1995; Schiodt et al, 1997; Bissell et al, 2001). Most patients who develop liver injury present late (after the period in which NAC has proven efficacy: [Meredith et al, 1986]) or have an unusual presentation (e.g. staggered overdose or therapeutic misadventure [Read et al, 1986; Fry and Seeff, 1995; Bridger et al, 1998]). Once liver failure has developed, current therapeutic options are limited as deterioration is relatively rapid and there is a narrow "window of opportunity" (O'Grady, 1991). In one series, only 35% of patients who fulfilled transplant criteria after POD actually underwent liver transplantation (Bernal et al, 1998b). Evidence has emerged over the last decade that additional mechanisms may be involved in paracetamol toxicity (e.g. activation of Kupffer cells and production of cytokines such as TNF- α). An understanding of these mechanisms of toxicity may lead to the development of new therapies to address what is a major clinical challenge.

Mechanisms of paracetamol toxicity

1. Bioactivation

Paracetamol itself is non-toxic and toxicity requires bioactivation i.e. production of reactive metabolites during liver metabolism: this area has been extensively studied (reviewed in Nelson, 1990; Thomas, 1993; Nelson, 1995) and is summarised in Figure 1.1. The major metabolic pathways for paracetamol involve glucuronidation or sulphation (1), forming non-toxic, soluble metabolites (2) that are excreted in urine (3). At therapeutic doses, approximately 5% of ingested paracetamol is metabolised by the cytochrome p450 system (CYP450: 4). This second pathway results in formation of a highly toxic electrophile (N-acetyl-*p*-benzoquinoneimine – NAPQI; 5). This metabolite is rapidly conjugated to non-toxic cysteine or mercapturate metabolites in a glutathione-dependent manner and excreted in urine and in bile (6). After paracetamol overdose, glucuronide and sulphate become depleted, with a concomitant increase in CYP450-dependent metabolism leading to increased production of NAPQI. As glutathione is depleted to 20-30% of normal or less (Mitchell et al, 1973: 7), intracellular accumulation of NAPQI occurs and is a critical event in paracetamol toxicity (Holme et al, 1984; Albano et al, 1985) resulting in cellular injury by a variety of mechanisms (8).

2. Metabolic factors influencing hepatic injury

Paracetamol toxicity is enhanced by factors that deplete glutathione (e.g. low protein diet) or increase formation of NAPQI (e.g. induction of CYP450 enzymes) within the liver (Thomas, 1993). The opposite also holds true where repletion of glutathione (e.g. administration of cysteine or methionine) or reduction in NAPQI formation (e.g. inhibition of CYP450 enzymes) and can reduce paracetamol toxicity (Thomas, 1993). CYP1A2 and CYP2E1 are the most important cytochrome isoforms in paracetamol metabolism in humans with CYP2E1 the major form (Raucy et al, 1989; Anundi et al, 1993). The importance of these enzymes is supported by the fact that CYP1A2 and CYP2E1 double knockout mice are protected from paracetamol toxicity (Zaher et al, 1998). CYP450 expression may vary up to tenfold between individuals, which may influence toxicity (Seddon et al, 1987); CYP2E1 is inducible

Figure 1.1

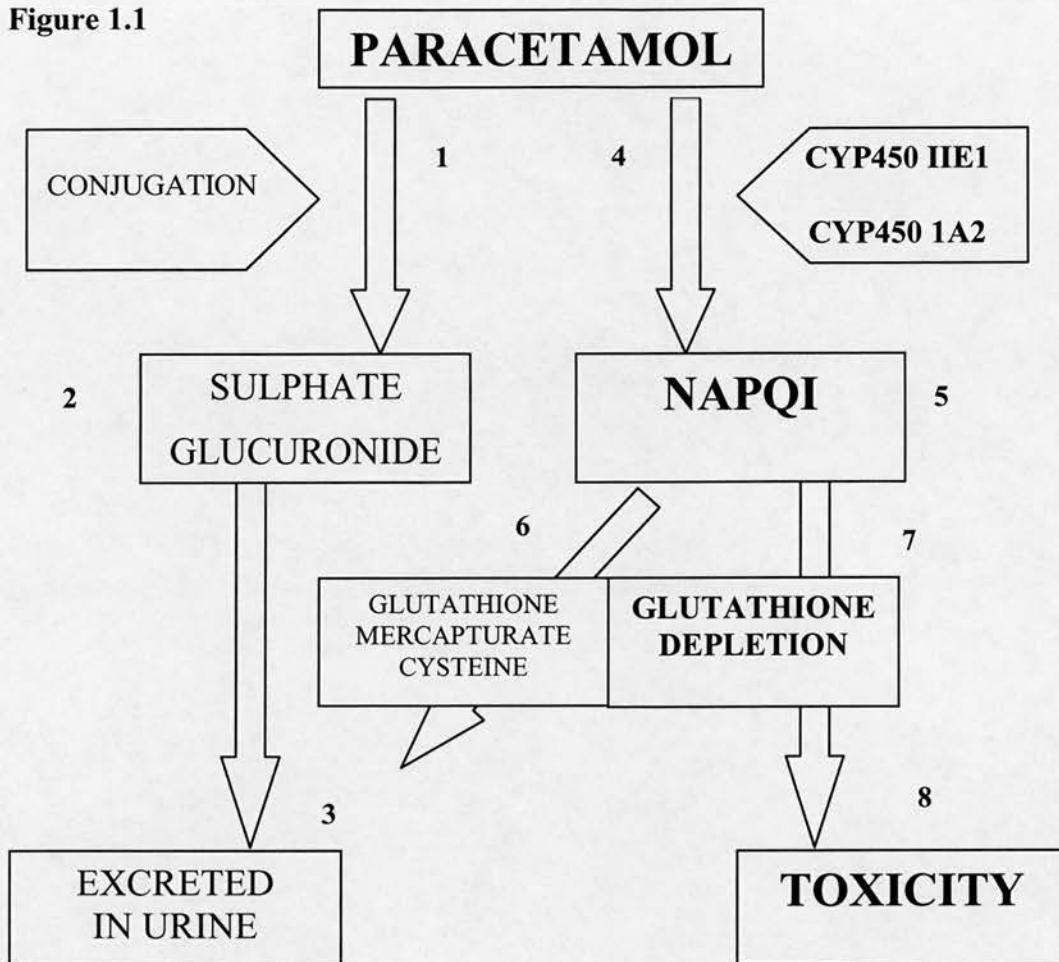


Figure 1.1 Paracetamol metabolism

Numbers refer to the description in the text. NAPQI = N-acetyl-*p*-aminobenzoquinone imine. Adapted from Thomas, 1993.

by ethanol and isoniazid and therefore users of either of these drugs may be at higher risk of toxicity (Ryan et al, 1986; Hall et al, 1986). The situation in alcoholics is more complex, however, as glutathione levels are usually also low and acute administration of alcohol appears to protect from paracetamol toxicity (reviewed in Nelson, 1990). Several other factors influence paracetamol toxicity including species, age and pregnancy (Nelson, 1990) but these largely relate to differences in metabolic pathways (Green et al, 1984; Holme and Soderlund, 1986; Smolarek et al, 1990; Nelson, 1990).

As therapeutic administration of N-acetylcysteine can completely prevent liver injury in clinical practice, glutathione depletion is an important factor in the development of

liver injury. However, glutathione depletion alone can injure hepatocytes (Anundi et al, 1979) and glutathione depletion is not the only determinant of liver injury in paracetamol toxicity (Larrauri et al, 1987). Evidence exists that more than one pathway to hepatic injury exists (Dai and Cederbaum, 1995) and that different pathways may be important at different drug concentrations (Farber et al, 1988).

3. Mechanisms of cellular injury and death

Sufficient evidence exists that metabolites of paracetamol (NAPQI) directly injure hepatocytes as the major feature of paracetamol overdose. However, conflicting data exist as to the precise mechanisms of cell injury and death (reviewed in Nelson, 1995). A strong correlation has been shown between paracetamol toxicity and the degree of covalent binding (and hence inactivation) of intracellular proteins (Jollow et al, 1973; Hinson and Pumford, 1995; Pumford and Halmes, 1997; Cohen et al, 1997) as a major mechanism of cell loss. However, additional mechanisms of cytotoxicity exist including oxidative stress with associated oxidation of thiols and lipid peroxidation (Kyle et al, 1987; Arnaiz et al, 1995); disruption of calcium homeostasis with associated disruption of the cytoskeleton and DNA (Nelson, 1990; Orrenius et al, 1991); and activation of lysosomal enzymes (Khandkar et al, 1996). The precise mechanism of direct cellular injury death is probably a complex combination of several of these mechanisms.

Indirect mechanisms of paracetamol toxicity

Increasing evidence has emerged that in addition to direct injury by toxic metabolites, indirect mechanisms are important in the pathogenesis of liver injury by several xenobiotics including paracetamol. Several lines of evidence support this concept:

1. interaction of various toxic agents with **Kupffer cells** can reduce or augment liver injury
2. cytokines such as **TNF- α** are produced during toxic liver injury and may alter the outcome
3. **apoptosis** has been observed as a mechanism of cell loss during toxic liver injury in addition to necrosis

1. Kupffer cells and toxic liver injury

Kupffer cells are resident liver macrophages, making up approximately 15% of cells within the liver (Kuiper et al, 1994). Kupffer cells have a variety of functions including phagocytosis, immune regulation, production of bioactive (proinflammatory) substances and cytotoxicity: Kupffer cells also have a role in hepatic regeneration (reviewed in Winwood and Arthur, 1998). Compelling evidence now exists from observation of macrophage accumulation and activation and experimental modulation of macrophage function that Kupffer cells are an important element in the pathogenesis of hepatic injury by various toxic agents (reviewed in Laskin and Pendino, 1995; Laskin, 1996; Tsukamoto and Lin, 1997; Bissell et al, 2001).

a. Kupffer cell accumulation and activation: A typical response to many xenobiotics is accumulation of activated macrophages. Macrophage recruitment has been shown following liver injury by various toxic agents including endotoxin (Pilaro and Laskin, 1986), carbon tetrachloride (Johnson et al, 1992), phenobarbital (Laskin et al, 1988), allyl alcohol (Przybocki et al, 1992), galactosamine (MacDonald et al, 1987) and paracetamol (Laskin and Pilaro, 1986). Macrophages accumulate in areas of tissue injury, the pattern of macrophage infiltration varying with different agents e.g. zone 3 for paracetamol and carbon tetrachloride, zone 1 for allyl alcohol, scattered for other toxicants such as galactosamine (Laskin, 1996). The accumulation of macrophages is explained by a combination of proliferation of Kupffer cells and recruitment from circulating monocytes (Johnson et al, 1992; Geerts et al, 1988; Jonker et al, 1990). Macrophages that accumulate exhibit an “activated” phenotype with enhanced phagocytic, cytotoxic and metabolic activity and enhanced production of inflammatory mediators (reviewed in Laskin and Pendino, 1995). However, the situation is more complex than simple activation, as Kupffer cell accumulation and activation induced by *C. parvum* protects against carbon tetrachloride and paracetamol injury by reducing expression of cytochrome P450 enzymes (Raiford and Thigpen, 1994). In some models of hepatotoxicity endotoxin elevation in portal blood is associated with Kupffer cell activation and

may also alter ICAM-1 expression on endothelium, facilitating accumulation of inflammatory cells (Nolan, 1989; Ahmad et al, 1999)

b. Kupffer cell modification: The most convincing evidence that Kupffer cells have an important role in toxic liver injury comes from studies where modification of Kupffer cells function results in a reduction in liver injury. Administration of anti-inflammatory agents such as hydrocortisone reduces hepatic injury secondary to carbon tetrachloride (Sudhir and Budhiraja, 1992). More specifically, administration of macrophage-depleting agents such as gadolinium chloride, dextran sulphate or diphosphonate has resulted in protection against ischaemia / reperfusion injury (Shiratori et al, 1994) and liver injury caused by several toxins including carbon tetrachloride (Chamulitrat et al, 1995), ethanol (Imuro et al, 1997), galactosamine (Stachlewitz et al, 1999) and paracetamol (Laskin et al, 1995; Goldin et al, 1996; Michael et al, 1999). Conversely, studies that have experimentally induced activation of macrophages (e.g. administration of LPS or poly I:C) have shown augmentation of hepatotoxicity secondary to carbon tetrachloride, galactosamine, *C. parvum* and paracetamol (Laskin and Pendino, 1995). The mechanism whereby Kupffer cells act is thought mainly to relate to production of bioactive inflammatory mediators.

c. Other cell types in paracetamol toxicity: Other cell types may also have a role in paracetamol toxicity. Conflicting evidence exists regarding the role of polymorphs in paracetamol toxicity (Jaeschke and Mitchell, 1989; Welty et al, 1993; Smith et al, 1998) and endothelial cell injury may contribute to necrosis in paracetamol-induced injury (DeLeve et al, 1997).

2. Production of bioactive substances in toxic injury

The production of various biologically active substances including reactive oxygen and nitrogen intermediates, lipid mediators and cytokines and chemokines has been reported following hepatic injury by many toxins; the major source of most of these agents is presumed to be Kupffer cells.

a. Reactive oxygen and nitrogen intermediates

Reactive oxygen species (ROS) are produced in many settings of cellular stress and injury and are produced both intracellularly (within hepatocytes) and extracellularly

(from Kupffer cells and neutrophils). ROS have been shown to contribute to ischaemia/reperfusion injury, endotoxaemia and toxin-induced injury in the liver where activated Kupffer cells predominantly release them as a critical element mediating hepatocyte injury (DelMaestro et al, 1980; Shiratori et al, 1990; Wang et al, 1992). ROS, including superoxide, hydrogen peroxide and hydroxyl, injure cells by a variety of mechanisms including lipid peroxidation, inactivation of anti-proteases, induction of apoptosis and modulation of gene transcription (Jaeschke et al, 1999; Jaeschke, 1999). Administration of antioxidants may be protective against ROS (Thomas, 1993; Gunawardhana et al, 1993; Laskin and Pendino, 1995) and in the setting of paracetamol poisoning, hepatocyte injury has been reduced by administration of superoxide dismutase (Nakae et al, 1990) and toxicity is reduced in metallothionein-null mice (Rofe et al, 1998).

Nitric oxide (NO) is produced by activated macrophages and has been implicated in various models of liver injury although its role in paracetamol toxicity is not clear. Paracetamol toxicity causes oxidative stress resulting in production of NO, which protects against injury (Kuo et al, 1997). However, conflicting studies have shown that paracetamol toxicity is reduced when NO formation is inhibited (Gardner et al, 1998). One mechanism whereby NO may be toxic is that superoxide can react with NO to form peroxynitrite, a highly reactive cytotoxic species that may contribute to hepatocyte death after paracetamol poisoning (Michael et al, 1999).

b. Cytokines

Good evidence exists that production of TNF- α is a common pathway to hepatic injury by various toxins (reviewed in Schumann and Tiegs, 1999) including galactosamine (Stachlewitz et al, 1999), ethanol (Iimuro et al, 1997), carbon tetrachloride (Czaja et al, 1995), actinomycin D (Leist et al, 1997) and α -amanitin (Leist et al, 1997). TNF- α is a pleiotropic cytokine with important roles in hepatocyte death by necrosis and apoptosis (Hill et al, 1992), hepatic regeneration (Michalopoulos and DeFrances, 1997) and protecting against subsequent hepatic injury (Takehara et al, 1998). TNF- α is not directly toxic to hepatocyte, the cytotoxic effect requiring sensitisation by inhibition of RNA or protein synthesis (Hill et al, 1992). The role of TNF- α in paracetamol toxicity remains controversial

as a range of conflicting reports exist (DeVictor et al, 1992; Blazka et al, 1995; Blazka et al, 1996; Boess et al, 1998).

Production of IL-1 α has also been described in paracetamol toxicity (Blazka et al, 1995) and IL-1 α protected against paracetamol toxicity in mice (Renic et al, 1993). Chemokines such as CINC/gro (rat) and MIP-2 (mouse) are also produced in paracetamol toxicity (Takada et al, 1995; Horbach et al, 1997). Chemokines are best characterised as chemotactic mediators of polymorph infiltration and mediate liver injury during ischaemia/reperfusion (Lentsch et al, 1998). However, administration of CXC chemokines including MIP-2 has resulted in protection against paracetamol toxicity, by facilitating hepatic regeneration (Hogaboam et al, 1999). The role of different cytokines and chemokines and how they interact in paracetamol toxicity is not known.

c. Other mediators

Lipid mediators (prostaglandins and leukotrienes) are produced by activated macrophages and in models of lung injury may modulate expression of cytokines (e.g. TNF- α [Christman et al, 1991]). Activated macrophages also release hydrolytic enzymes that may contribute to liver injury or alter hepatocyte functioning (Tanner et al, 1980; Decker, 1980).

3. Apoptosis and toxic liver injury

Several good reviews of apoptosis in the liver exist (Alison and Sarraf, 1994; Galle, 1997; Feldman, 1997; Patel et al, 1998). Apoptosis is an important mechanism to eliminate senescent, injured or deleterious cells, is the major mechanism of cell loss in liver atrophy and is an important contributor to liver homeostasis (Galle, 1997). Recent evidence has suggested that apoptosis is also an important mechanism of cell loss during a range of disparate liver diseases including viral hepatitis, cholestatic and biliary disease, hepatocarcinogenesis, allograft rejection, metabolic disease and toxin or drug-induced disease both in human liver disease and in animal models (see Table 1.1). Interest in apoptosis during toxic liver injury has increased for several reasons.

Table 1.1: Apoptosis and Liver Disease (Human and Experimental)

Liver Disease		Reference
Viral Hepatitis	Yellow fever	Feldman, 1997 [rev]
	Hepatitis B,C,D	Fang et al, 1994 Kondo et al, 1997 Lau et al, 1998 [rev]
Immune Injury	Allograft Rejection	Afford et al, 1995 Krams and Martinez, 1998
Hepatocarcinogenesis		Schulte-Hermann et al, 1997 [rev] Pitot, 1998 [rev] Thorgeirsson et al, 1998 [rev]
Cholestatic Disease	Cholestasis	Patel et al, 1994
	PBC	Patel and Gores, 1995 [rev] Kuroki et al, 1996
Ischaemia	Ischaemia/reperfusion injury	Cursio et al, 1999
Metabolic Disease	Wilson's disease	Strand et al, 1998
	Iron overload	Kato et al, 1996
Drugs	Ethanol	Nanji, 1998 [rev]
	Cocaine	Cascales et al, 1994
	Cytostatic drugs	Muller et al, 1997 Jiang et al, 1999
	Galactosamine	Medline et al, 1970*
	Carbon tetrachloride	Shi et al, 1998
	Dimethylnitrosamine	Pritchard and Butler, 1989
	Thioacetamide	Ledda-Columbano et al, 1991
Paracetamol	Ray et al, 1996	

Table 1.1

rev = review article. *published before first description of apoptosis but probably represents apoptosis.

i. Detection of apoptosis

Many methods have been used to measure apoptosis. There is currently no consensus as to the most appropriate method and it is accepted that different methods are better in different experimental models (reviewed in Goldsworthy et al, 1996; Patel et al, 1998). Further problems in measuring apoptosis in that apoptotic bodies are small and may be relatively infrequent and that the duration of apoptosis is short (Grassl-Kraupp et al, 1995; Galle and Krammer, 1998). Choosing an appropriate method to detect and quantify apoptosis is important.

ii. Apoptosis and necrosis

Many toxic agents produce hepatic necrosis as the major form of cell loss but the liver also shows evidence of apoptosis, the significance of which is unknown (e.g. thioacetamide [Lecda-Columbano et al, 1991], galactosamine [Medline et al, 1970] carbon tetrachloride [Shi et al, 1998], dimethylnitrosamine [Pritchard and Butler, 1989] and paracetamol [Ray et al, 1996]). In many of these models apoptosis precedes necrosis (Pritchard and Butler, 1989; Shi et al, 1998) but the contribution of apoptosis to total liver injury is not known. Several problems are noted in distinguishing apoptosis and necrosis (Columbano, 1995). Problems exist in identification and measurement of apoptosis and necrosis as some markers may be found during both processes (e.g TUNEL staining [Grasl-Kraupp et al, 1995] and DNA laddering [Bohlinger et al, 1996]). The end-stage of apoptosis may also be indistinguishable from necrosis (especially in tissue culture) and if insufficient tissue phagocytic capacity is present in vivo apoptosis may result in secondary necrosis with little inflammation (Oberhammer et al, 1996). As some features of apoptosis and necrosis overlap, the concept of “necrapoptosis” has been suggested (LeMasters, 1999). This concept was proposed to avoid confusion and to explain some of the common pathways to apoptosis and necrosis, the type of death depending on mitochondrial permeability and cellular ATP stores (Leist et al, 1997; LeMasters, 1999). Conceptual difficulties remain, however, as the presence of shared pathways does not necessarily merit separation of a third mechanism of cell death and there is no reason why different mechanisms of cell death should not co-exist.

iii. Apoptosis and inflammation

Apoptosis does not induce an inflammatory reaction and therefore is thought to be biologically beneficial compared with necrosis in which secondary inflammation is a *sine quo non* (Allison and Sarraf, 1994; Columbano 1995). However, recent evidence suggests that in certain situations apoptosis may also cause inflammation (Miwa et al, 1998; Lawson et al, 1998).

iv. Different pathways to signal apoptosis

Apoptosis is a genetically regulated, tightly controlled process of cell elimination, often signalled by specific receptor pathways; the occurrence of apoptosis raises important questions as to pathogenic pathways of disease. However, in several models of liver injury, induction of apoptosis by specific signal-receptor pathways has been confirmed. The CD95 (Fas) / CD95 ligand (FasL) pathway is well-characterised and, as hepatocytes express Fas, has a role in liver homeostasis (reviewed in Krammer, 1998; Feldmann et al, 1998). FasL is expressed mainly on T cells, mediating T cell-induced apoptosis in various physiological and pathological states (Vignaux and Golstein, 1994), but may also be expressed on hepatocytes and therefore may mediate both immune and nonimmune hepatocyte apoptosis (Galle et al, 1995). Fas / FasL signalling has been implicated in the pathogenesis of many important liver diseases (reviewed in Galle and Krammer, 1998), including viral hepatitis (Galle et al, 1995; Kondo et al, 1997), chronic CMV infection (Fleck et al, 1998), adenovirus infection (with consequences for gene therapy studies: Liu et al, 2000), alcoholic hepatitis (Galle and Krammer, 1998), primary biliary cirrhosis (Kuroki et al, 1996), Wilson's disease (Strand et al, 1998) and allograft rejection (Krams et al, 1998). The TNF- α / TNFR pathway is also well-defined and contributes to liver injury and apoptosis in many models including viral hepatitis (Bradham et al, 1998), adenovirus infection (Liu et al, 2000), fulminant liver failure (Muto et al, 1988), endotoxin administration (Beutler et al, 1985) and liver injury induced by several drugs and toxins including alcohol (reviewed in McLain et al, 1999), galactosamine (Stachlewitz et al, 1999), carbon tetrachloride (Czaja et al, 1995) and after paracetamol overdose (reviewed in Schumann and Tiegs, 1999). Other apoptosis-signalling pathways of less importance include TGF- β / TGF receptor, as is involved in regression after mitogenesis (Oberhammer et al, 1992; Grasl-Kraupp et al, 1998), perforin / granzyme in T cell-mediated liver injury (Galle and Krammer, 1998) and ceramide (Hoekstra, 1999). In some systems apoptosis may be signalled by more than one pathway (Jiang et al, 1999; Liu et al, 2000).

While some preliminary studies suggested that the Fas / FasL pathway may be involved in paracetamol toxicity (Braithwaite et al, 1998), this data has not proved reproducible (personal observation). However, a role for cytokine-induced

apoptosis, with TNF- α the main candidate (Leist et al, 1998) remains feasible in paracetamol toxicity. A mechanistic understanding of apoptosis may help elucidate appropriate pathways to allow intervention to increase or reduce apoptosis as appropriate (see below; Corcoran et al, 1994).

v. Inhibition of apoptosis

In certain situations apoptosis can be specifically inhibited, offering a novel approach to reducing hepatic injury (reviewed in Patel et al, 1998). Blocking apoptosis in hepatocytes in inflammatory / toxic diseases may improve outcome by reducing hepatocyte loss. For example, reperfusion injury can be blocked by a caspase inhibitor (Cursio et al, 1999) and Fas-induced apoptosis can be inhibited by clenbuterol (Andre et al, 1999). This represents the opposite approach to that espoused in hepatocarcinogenesis, where apoptosis may be reduced and augmentation of apoptosis may be beneficial (Grassl-Kraupp et al, 1998).

vi. Apoptosis and non-parenchymal cells

Apoptosis in hepatocytes has been extensively studied but non-parenchymal cells have received little attention. However, non-parenchymal cells are important in causing and perhaps also in treating liver disease. Stellate cells have an important role in causing liver fibrosis and inducing apoptosis in stellate cells may be a useful therapeutic option (Cales, 1998). Kupffer cells may promote liver injury induced by various toxins (Laskin, 1996) and induction of apoptosis in Kupffer cells may reduce injury.

4. Summary

Good evidence now exists that indirect mechanisms contribute to the toxicity of many xenobiotic drugs. Accumulation and activation of Kupffer cells is associated with increased production of various bioactive substances including cytokines such as TNF- α and chemokines such as MIP-2. These agents may have a central role in hepatocyte death by apoptosis and necrosis and may also be involved in regeneration after toxic injury. Paracetamol overdose is an important clinical problem to which many of these mechanisms may apply.

AIMS

The purpose of this study is to investigate hepatic injury and regeneration after paracetamol overdose by studying the role of Kupffer cells, cytokines such as TNF- α and apoptosis in the pathogenesis of paracetamol toxicity. Several questions will be specifically addressed:

1. Does apoptosis occur in human liver after paracetamol overdose?
2. Do Kupffer cells have a role in toxicity after paracetamol overdose?
3. Does TNF- α have a role in paracetamol toxicity?
4. Are other cytokines or chemokines involved in paracetamol toxicity?
5. Can any mechanisms be identified which may lead to the development of new approaches to therapy after paracetamol poisoning?

CHAPTER TWO

PARACETAMOL TOXICITY IN HUMAN LIVER

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PARACETAMOL TOXICITY IN HUMAN LIVER

SUMMARY

This study of paracetamol toxicity in human liver after POD revealed hepatocyte apoptosis at time of liver transplantation or death associated with striking regenerative activity. The biological significance and control of this is not known, but Kupffer cell activation with cytokine production is highlighted as a potential mechanism to link hepatocyte apoptosis and proliferation.

INTRODUCTION

Although apoptosis has been recognised to occur in the liver for many years, only recently has evidence begun to accumulate that hepatocyte apoptosis is important in the pathogenesis of many liver diseases including viral, cholestatic and toxin-induced hepatic injury (Patel et al, 1998). This is particularly interesting for toxic injury (e.g. following paracetamol overdose), where necrosis is the major mechanism of hepatocyte loss and induction of apoptosis not only offers new insights into the pathogenesis of liver injury but also offers new therapeutic opportunities (Thompson, 1995; Patel et al, 1999).

Paracetamol overdose is a major clinical problem as it represents the commonest cause of fulminant hepatic failure in the UK and the USA and is subsequently one of the commonest reasons for emergency liver transplantation (Makin et al, 1995; Fagan and Wannan, 1996; Schiodt et al, 1997). The availability of an effective antidote (N-acetylcysteine) has meant that the majority of patients do not develop severe hepatic injury. However, severe liver injury remains a major clinical challenge, as management options are limited for those patients who develop hepatic failure (reviewed in O'Grady, 1997). While the development of reliable prognostic criteria has allowed identification of a group with a poor predicted prognosis who will benefit from liver transplantation

(O'Grady et al, 1989; Donaldson et al, 1993; Bernal et al, 1998b) the overall death rate for this group is significant at 25-40% (Schiodt et al, 1999; Ostapowicz and Lee, 2000).

Patients who die or undergo liver transplantation following paracetamol overdose (POD) invariably show severe zonal liver necrosis (Portmann et al, 1975; Lesna et al, 1996) as the result of direct hepatic injury from toxic paracetamol metabolites (Jollow et al, 1973); recovery is associated with rapid and efficient regeneration (Lesna et al, 1976). However, good evidence exists that paracetamol toxicity is more complex than simple direct injury from the toxic metabolites and that additional mechanisms may modulate liver injury following POD such as Kupffer cell activation (reviewed in Laskin and Pendino, 1995) or production of TNF- α (Blazka et al, 1995). Hepatocyte apoptosis has been reported in paracetamol toxicity in murine liver (Ray et al, 1996) but has not been identified in human liver (Afford et al, 1995); the significance of apoptosis in paracetamol toxicity is not known.

The aims of this study were to investigate hepatocyte apoptosis and regeneration and Kupffer cell populations in human liver after POD.

MATERIALS AND METHODS

Patient Characteristics and Tissue Studied

This study was carried out with the approval of the local ethical committee. All patients presenting to the Scottish Liver Transplant Unit (SLTU) with fulminant hepatic failure (FHF) from 1992 – 1997 were identified (Table 2.1). Patients were included in the study when formalin-fixed, paraffin-embedded tissue blocks were available for study i.e. patients who had either received a liver transplant, or had died and an autopsy had been carried out. At least 6 blocks of liver were available for transplant cases; 1 to 5 blocks were available in autopsy cases. A proportion of the autopsy cases were excluded where significant autolysis precluded accurate histological analysis (POD 20 and non-POD 5 autopsy cases). The remaining cases were then separated into two groups into those secondary to POD and those of any other aetiology (non-POD: Table 2.1) for

comparison. Four-micron sections were stained with haematoxylin and eosin.

Table 2.1

FHF	Total	Survived	OLT	Died	No Path	Autolysis	Study cases
All Cases	295	191	35	71*	13	25	66
POD	241	167	20	56*	10	20	44
Non-POD	54	24	15	15	3	5	22

Table 2.1 FHF = Fulminant Hepatic Failure; Total = total number of cases presenting to SLTU in FHF from 1992-1997; Survived = patients who survived without liver transplant; OLT = Liver transplant carried out (*2 patients died after OLT); Died = patients who died (* 2 patients died after OLT); No Path = patients for whom pathological material was unavailable (POD: 5 patients had an autopsy elsewhere and blocks were not available, 3 cases had an autopsy limited to the brain, 2 patients did not have an autopsy: non-POD 1 patient had limited autopsy, 2 patients did not have an autopsy); POD – Paracetamol Overdose; non-POD = FHF of any other aetiology (see Table 2.2 for list of aetiologies).

Immunohistochemistry

Immunohistochemistry was carried out using the EnvisionTM+ system (Dako) following the standard manufacturers protocol after antigen retrieval by microwaving for 15 minutes in EDTA (1mM:pH8). Antibodies used were Ki-67 (clone MIB-1: Immunotech) to detect proliferating cells and CD68 (clone PGM-1: Dako) to detect Kupffer cells.

Quantitative Analysis

Necrosis was assessed on H&E stained sections, dividing cases into two groups: those showing either massive or sub-massive necrosis. Massive necrosis was defined as when no surviving hepatocytes were present on light microscopy. All remaining cases showed extensive (sub-massive) necrosis but with surviving hepatocytes in zone 1; in these cases hepatocyte injury was scored as present or absent, based on the presence of nuclear enlargement and cytoplasmic vacuolation. One representative section from the cases with submassive necrosis was then subsequently assessed to determine the rate of

hepatocyte apoptosis and proliferation in the areas of surviving parenchyma. Apoptosis was quantitated on H&E-stained sections using morphological criteria as previously used in our laboratory (Howie et al, 1994) counting only hepatocytes showing unequivocal apoptotic morphology in areas of non-necrotic hepatic parenchyma. Mitotic figures were counted in a similar manner. Proliferation was further quantitated by calculating the proportion of hepatocytes that expressed Ki-67, an antigen expressed in all phases of the cell cycle except G0 (Key et al, 1993). Kupffer cells were quantitated by counting the number of cells expressing CD68 present in areas of surviving hepatic parenchyma. For all quantitative counts, multiple fields were assessed and at least 1000 cells were counted in each case.

Statistical analysis

Comparison between the POD and non-POD groups was carried out for the quantitative data by simple graph plotting (MedCalc™, Belgium). Using this method, when the shoulders in the box-plots do not overlap then the difference is likely to be significant ($p < 0.05$).

RESULTS

Apoptosis and proliferation after paracetamol overdose

As expected all POD cases showed significant hepatic necrosis with 13 cases (30%) showing massive necrosis and the other 31 cases (70%) showing sub-massive necrosis (Figure 2.1). In some of the cases with submassive necrosis the necrosis was variable and in areas was “massive”; this group basically represented patients with sufficient parenchyma on which to score apoptosis and proliferation. Most of these cases showed evidence of hepatocyte injury (29/32 cases: Figure 2.2). Hepatocyte apoptosis was then counted in all livers with sub-massive necrosis. Hepatocyte apoptosis was identified in all 32 scoreable cases (with submassive, not massive necrosis) at the time of transplantation or post mortem (Figure 2.3) with a median apoptotic index of 0.6%. Striking proliferative activity was also identified in resected livers after POD (Figure 2.4) with a median mitotic index of 0.35% and Ki-67 positivity index of 4.2%.

Figure 2.1

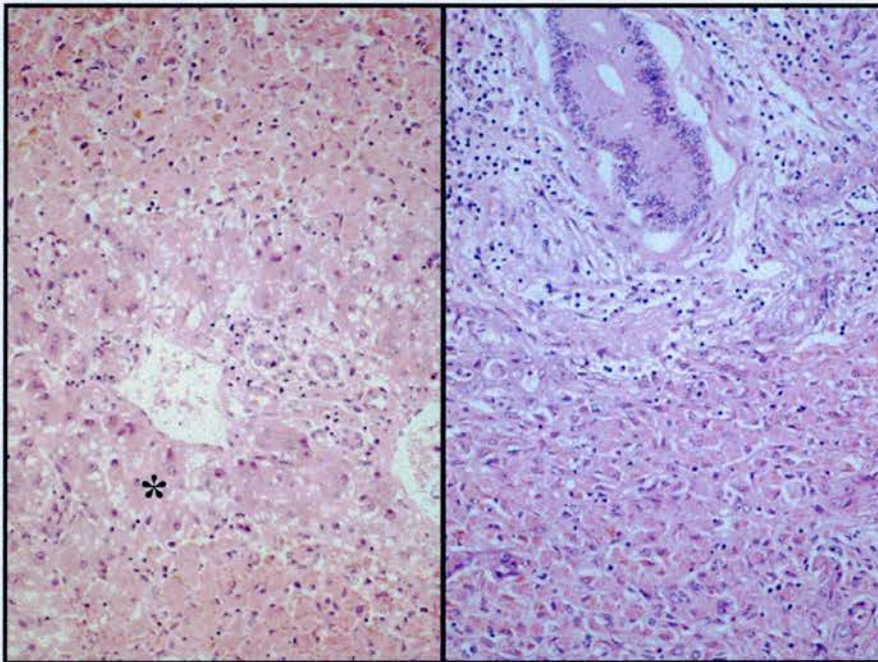


Figure 2.1
Photomicrographs showing (i) severe hepatic necrosis after POD [*indicates rim of surviving parenchyma in zone 1] and (ii) panacinar hepatic necrosis.

Figure 2.2

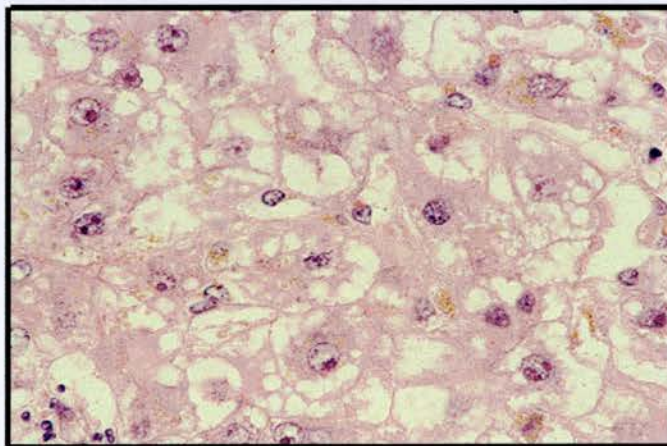


Figure 2.2
Photomicrograph showing hepatocyte injury in a case of POD. The most notable feature of injury was striking cytoplasmic clearing and vacuolation. There was also a degree of variability in nuclear size.

Figure 2.3

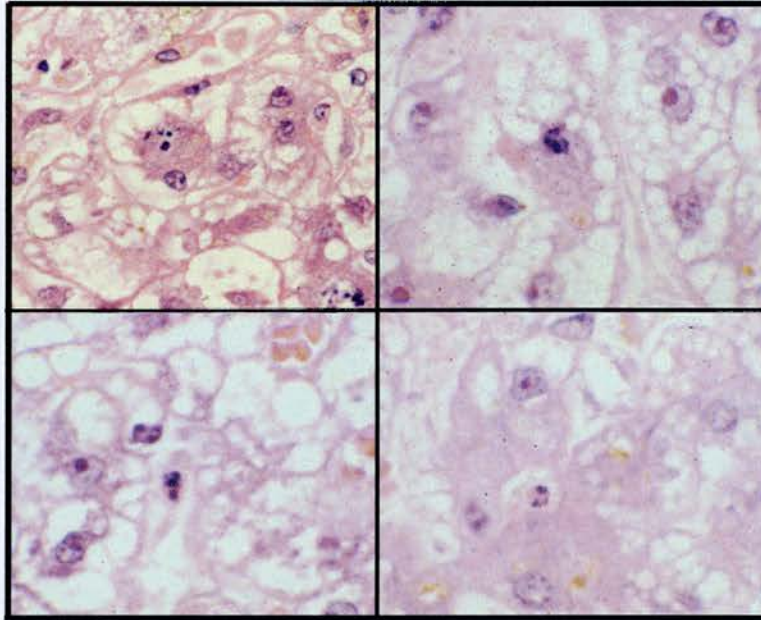


Figure 2.3

Photomicrographs showing hepatocyte apoptosis. Apoptotic hepatocytes were only counted when cells showed apoptotic morphology (cell shrinkage, chromatin condensation and nuclear separation into haematoxyphilic balls) and were considered to be hepatocytes (present in liver cell plate adjacent to other hepatocytes). Many apoptotic polymorphs were seen.

Figure 2.4

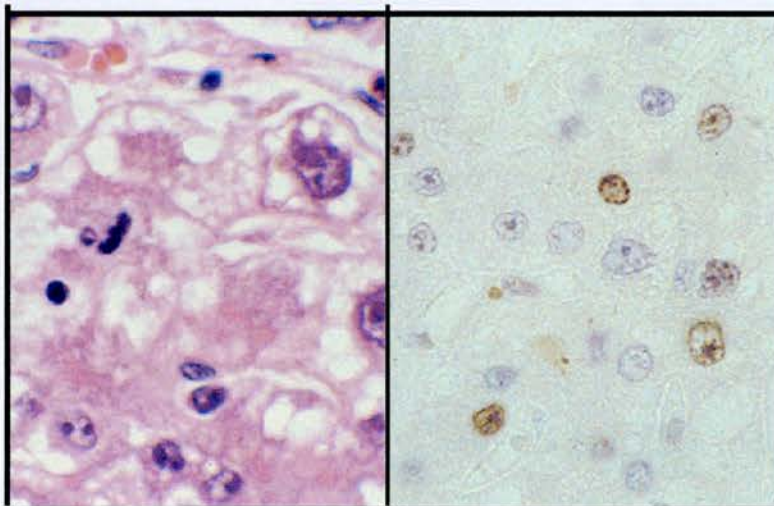


Figure 2.4

Photomicrographs showing typical mitotic and Ki-67 positive hepatocytes after POD.

Comparison of POD and non-POD cases

In the non-POD group massive necrosis was identified in 9 cases (41%) with sub-massive necrosis in 15 cases (69%). Hepatocyte injury was noted in the minority of these cases (5/15). In contrast to POD hepatocyte apoptosis was only seen in 9/15 of non-POD cases and apoptotic indices were much higher in POD (median 0.6, range 0.2-2.4) than non-POD cases (median 0.1, range 0-0.7: Figure 2.5). Much lower rates of mitosis and Ki-67 positivity were also seen in the non-POD group than had been noted after POD (Mitotic rate: POD [median 0.35 range 0-3.5] and non-POD [median 0.1, range 0-1.2]: Figure 2.6. Ki-67 positivity: POD [median 4.2, range 0.8-15.5] and non-POD [median 0.2, range 0-2.5]: Figure 2.7). No association between rates of apoptosis and proliferation was identified (mitosis or KI-67 positivity).

Figure 2.5

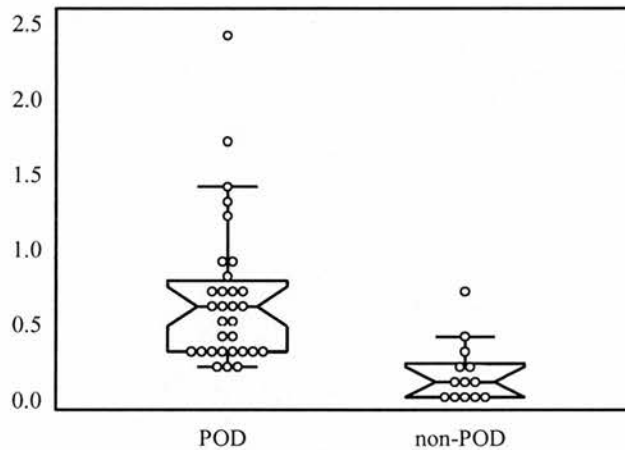
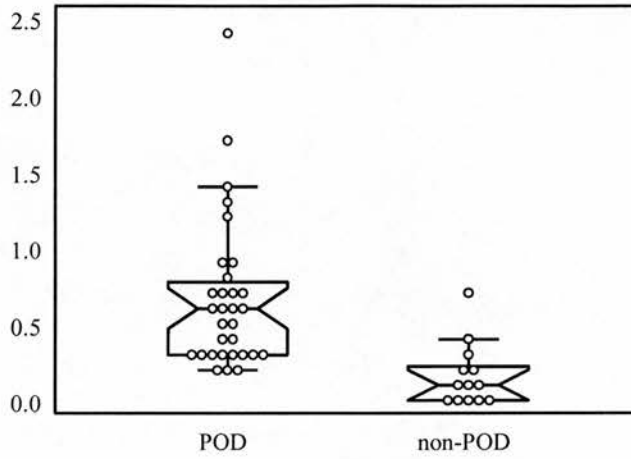
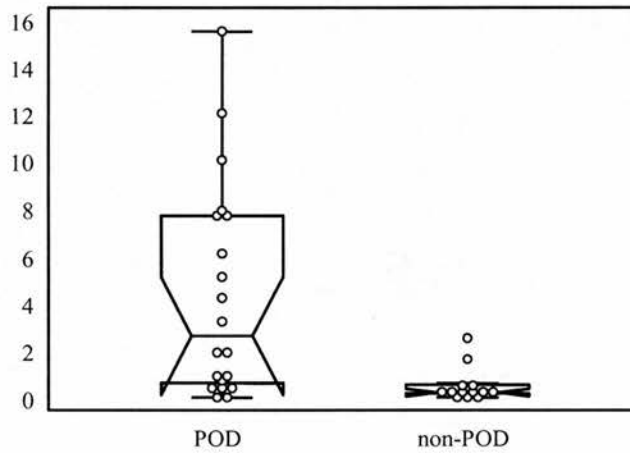


Figure 2.5

Apoptotic Index (%apoptotic hepatocytes) for POD and non-POD groups. Apoptotic index. Box-and-whisker plot (box represents 25 to 75 percentile with median; whiskers represent 95% CI of median). Shoulders on box-plot do not overlap indicating that the two groups are different with a 95% CI.

Figure 2.6**Figure 2.6**

Mitotic Index (% mitotic hepatocytes) for POD and non-POD groups
 Box-and-whisker plots (box represents 25 to 75 percentile with median; whiskers represent 95% CI of median).

Figure 2.7**Figure 2.7**

Proliferative Index (% Ki-67 positive hepatocytes) for POD and non-POD groups
 Box-and-whisker plots (box represents 25 to 75 percentile with median; whiskers represent 95% CI of median). Shoulders on box-plot do not overlap indicating that the two groups are different with a 95% CI.

Kupffer Cell Number

Zones of necrosis showed striking, confluent infiltration by CD-68-positive cells that comprised the majority of the total cell population in these areas (Figure 2.8). However,

within viable hepatic parenchyma the proportion of CD-68 positive cells was the same in POD (median 13.8%, range 8-20.7) and non-POD cases (median 12.6%, range 7.2-22.6) and was similar to that seen in normal liver (median 15.6%, range 13.2-18.2%; Kuiper et al, 1994). Double-staining was not carried out Ki-67 positivity was noted in a small proportion of the apparent CD-68 positive cells.

Figure 2.8

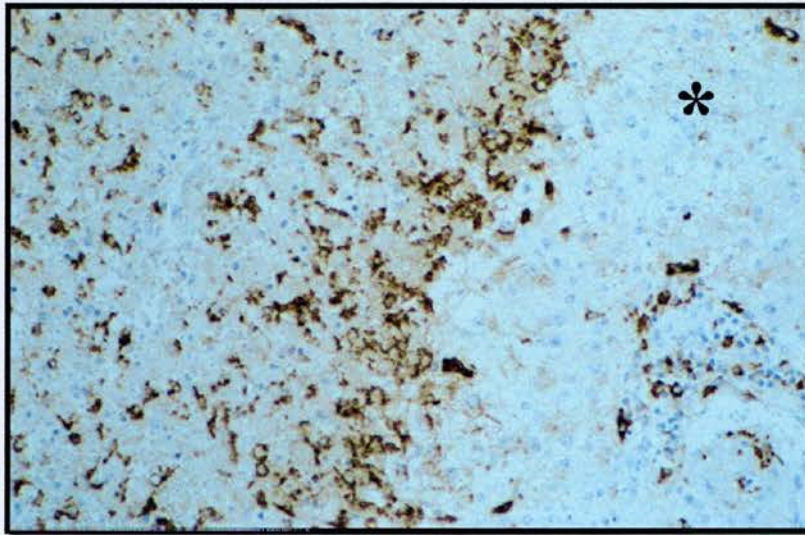


Figure 2.8

Photomicrograph showing accumulation of CD-68 positive cells in areas of zone 2-3 necrosis and few CD-68 positive cells present in surviving zone 1 parenchyma (asterix).

Clinical parameters of liver failure

The POD and non-POD groups represent patients with liver disease of similar clinical severity as measured by the King's College prognostic criteria (O'Grady et al, 1989: poor criteria met in 39/44 POD and 21/22 non-POD cases; Table 2.2) and also had a similar age and sex distribution. There were no differences between the groups in biochemical parameters of liver injury (maximum bilirubin, ALT, AST), maximum creatinine, mode of death or severity of encephalopathy. A prolonged prothrombin time (PT) was noted in POD compared with non-POD patients (maximum PT 95s for POD

and 59s for non-POD), an expected feature of paracetamol overdose. The major difference between the two groups was in the duration of illness with a mean disease length of 89.8 hours for POD (time from overdose to liver transplantation) and 20.9 days for non-POD (time from jaundice to encephalopathy).

Separating POD cases into those with massive versus sub-massive necrosis revealed no difference between these groups in terms of paracetamol level (maximum or at presentation), time to presentation at the SLTU, biochemical parameters of liver injury, prothrombin time or severity of encephalopathy.

Table 2.2

	Total Cases	Age	Sex	PT	Score	Duration
POD	44	36.5 (16-63)	13:31	97	39	89.8 hours*
Non-POD	22	42 (15-62)	7:15	56	21	20.9 days**

Table 2.2 Total cases – cases included in study; Age – median, range; Sex (m:f); PT – Maximum prothrombin time in seconds; Score = number of patients who reached poor prognostic criteria (O’Grady et al, 1989). Duration - measured from overdose to transplant in POD cases* and from onset of jaundice to development of encephalopathy for non-POD cases**. Aetiology of non-POD Cases: Unknown (nonAnonBnonC) hepatitis – 14; Drug-Induced (not paracetamol) – 3; Budd-Chiari – 3; Alcohol – 2; Wilson’s – 1; Autoimmune Hepatitis – 1; Hepatitis A – 1; Ischaemia – 1.

DISCUSSION

Hepatocyte necrosis and apoptosis after paracetamol overdose

Extensive and severe hepatic necrosis is well-recognised as a characteristic finding in liver failure after paracetamol overdose (Lesna et al, 1976; Portmann et al, 1975). Although the maximum extent of necrosis after which recovery is not possible is not clear, greater than 60% hepatic necrosis in human liver is associated with a poor outcome (Portmann et al, 1975; Gazzard et al, 1975). While formal quantification of the extent of necrosis was not carried out, all cases in this study probably had greater than 60% necrosis. In addition, this is the first definite description of hepatocyte apoptosis

after POD.

Apoptosis has been increasingly recognised to be an important mechanism of cell loss in toxic injury caused by a variety of drugs and toxins including alcohol, carbon tetrachloride, galactosamine, amanitin and bile salts (Leist et al, 1997; Patel et al, 1998). From this study, apoptosis has been identified after paracetamol overdose in human liver. Apoptosis has previously been described paracetamol-induced liver injury in murine liver (Ray et al, 1996) and although both early studies identified acidophil bodies in some cases (Portmann et al, 1975; Lesna et al, 1976), it has been suggested that apoptosis does not occur after paracetamol overdose in human liver (Afford et al, 1995). The latter study used an in situ end-labelling method to detect apoptosis and concluded that significant hepatocyte apoptosis does not occur after paracetamol overdose in a limited series of 4 cases (Afford et al, 1995). However, the rate of 1-2% apoptosis suggested (Afford et al, 1995) is in keeping with the rates of apoptosis in this study made by morphological assessment, which is generally considered to be a better method for quantitation of apoptosis (Goldsworthy et al, 1996; Galle, 1997). Given the known difficulties of quantifying apoptosis in vivo and short duration of visible changes (Grassl-Kraupp et al, 1995; Galle and Krammer, 1998), low rates of apoptosis are not surprising. In the rat, a rate of 0.5% hepatocyte apoptosis has been estimated to result in 25% reduction in liver mass in a few days (Bursch et al, 1990), suggesting the rates of apoptosis noted in this study would account for a rapid loss of a significant proportion of cells and may contribute to the liver failure in these patients.

Liver regeneration after paracetamol overdose

The regenerative response after paracetamol-induced liver injury has been studied previously, using different methodology but with broadly comparable data to this study (Koukoulis et al, 1992; Donaldson et al, 1993). The first study used PCNA staining to assess proliferation and identified rates of hepatocyte proliferation of 50-60% in cases at a comparable time to this study. While these rates are higher than the levels of Ki-67 positivity in this study (up to 15%), PCNA has a long half-life and may be induced in

quiescent cells (Hall et al, 1990; Ottavio et al, 1990). Furthermore while Ki-67 is cell cycle-specific PCNA is not as it also stains cells that have suffered DNA damage, which may occur after paracetamol poisoning (Holme and Soderlund, 1986). The second study found mitotic figures in hepatocytes in a greater proportion of cases of POD than cases of other aetiology, similar to this study, although mitotic counts were not carried out. The lower level of mitosis than Ki-67 positivity noted in this study can be explained by the short duration of mitosis and staining of cells in all phases of the cell cycle by Ki-67. Furthermore, this is the first study to identify hepatocyte apoptosis in the context of regeneration after toxic injury (see below).

Liver pathology in the absence of paracetamol

This study investigates “late” changes after paracetamol overdose i.e. those occurring at time of liver transplantation or death. At this time paracetamol is absent from serum (Thomas, 1993), raising interesting questions about the pathogenesis of the pathological changes. There are several possibilities:

1. *Elevated rates of apoptosis and proliferation are directly attributable to paracetamol, despite its absence from serum.* Paracetamol-protein adducts can be identified immunohistochemically and by Western blot and have been identified in regenerating hepatocytes in mouse-liver 72 hours after overdose (Roberts et al, 1991). However, paracetamol adducts can also be identified with non-toxic doses of paracetamol and are a good marker of covalent binding (Bartolone et al, 1987; Hinson et al, 1998) but may be long-lived and therefore may not represent an accurate indicator of ongoing injury.
2. *Elevated rates of apoptosis and proliferation are a generic feature of regeneration after any acute, massive liver injury.* The comparison between POD and non-POD patients noted striking differences in rates of apoptosis, mitosis and Ki-67 positivity. The non-POD patients are recognised to be an imperfect control group for several reasons: (i) they represent a heterogeneous group of patients with different liver diseases; (ii) different prognostic criteria are used for POD and non-POD patients although both sets of criteria accurately predict outcome (O’Grady, 1989); and (iii) the

non-POD cases have a much longer clinical duration than POD. However, biopsies are not available from “survivors” of POD, which would be ideal controls, and the non-POD group has been used as the best available group for comparison. Patients with non-paracetamol-drug-induced liver failure may represent a much better control group but the 3 patients in the non-POD group with drug-induced liver failure unfortunately all showed massive hepatic necrosis. The reason for the striking differences in apoptosis and proliferation between POD and non-POD cases is not known. While it may be due to paracetamol itself (see above), it seems likely that in paracetamol overdose there is a single massive insult and stimulus to regenerate and a short clinical duration, whereas in other cases of liver failure the duration is longer with ongoing injury. The close association of apoptosis and regeneration is recognised (Fan et al, 1998; Paulovich et al, 1997) and apoptosis has been suggested to be a mechanism of “fine-tuning” liver mass during regeneration (Sakamoto et al, 1999; Hasegawa et al, 1999). After partial hepatectomy in mice and rats apoptosis occurs up to 96 hours after hepatectomy and follows a peak of BrdU/thymidine positivity (Sakamoto et al, 1999; Sigal et al, 1999). IL-6, a critical part of the regenerative response may also be an important regulator of apoptosis during proliferation; after partial hepatectomy IL-6 knockout mice restore liver mass more slowly than wild type mice but have a lower net rate of proliferation and a lower rate of hepatocyte apoptosis (Sakamoto et al, 1999). The intriguing possibility is raised that an efficient regenerative process may contribute to liver injury by inducing concurrent cell loss by apoptosis, and suggests that intervening to alter the balance of cytokines may alter regeneration and improve the outcome.

3. *Elevated rates of apoptosis and proliferation are induced by cytokines produced by activated macrophages.* Extensive experimental evidence suggests that modulation of Kupffer cells can alter hepatotoxicity (Laskin et al, 1995; Goldin et al, 1996) associated with production of bioactive substances including reactive oxygen and nitrogen species (Michael et al, 1999) and cytokines such as TNF- α (Blazka et al, 1995). TNF- α in particular is implicated in toxic liver injury induced by various chemicals and can cause hepatocyte death by apoptosis and also signal hepatocyte proliferation (Bradham et al, 1998; Taub, 1998). This study of Kupffer cells confirm previously published

observations in human liver, namely that there is striking accumulation of Kupffer cells within areas of necrosis but there is no increase in Kupffer cells in surviving parenchyma (Mathew et al, 1994) and that Kupffer cells are proliferating (Koukoulis et al, 1990). While the production of cytokines is a feasible explanation for the elevated rates of apoptosis and regeneration, this observational study offers little in resolving the role of Kupffer cells and cytokines.

4. *Elevated rates of apoptosis and proliferation occur as the result of secondary ischaemic damage adjacent to necrotic liver.* The suggestion that ischaemia may occur adjacent to necrosis has been previously proposed to explain the hepatocyte injury noted in surviving parenchyma (Portmann et al, 1975). The injury noted in that study was characterised by nuclear pleomorphism, cell swelling and cytoplasmic vacuolation and seen mainly in patients who survived without liver transplantation (Portmann et al, 1975). This study confirmed hepatocyte injury in patients who died or underwent liver transplantation and was seen in most POD cases and few non-POD cases. Microvascular changes in zone 3 and polymorph plugging of sinusoids may contribute to early liver injury but conflicting evidence exists (Jaeschke and Mitchell, 1989; Welty et al, 1993; Lim et al, 1995). The situation after paracetamol overdose may be analogous to that occurring after myocardial infarction or cerebrovascular accident, where additional cell loss by apoptosis occurs outwith an area of ischaemic necrosis (Cohen, 1993; Gottlieb et al, 1994; Uyama et al, 1994). Of particular interest is the observation that inhibition of apoptosis in these situations can improve the outcome, raising the possibility of specifically inhibiting apoptosis after paracetamol overdose to improve the outcome.

5. *Elevated rates of apoptosis and proliferation occur by a combination of some or all the above mechanisms.* Evidence exists to support all of the above mechanisms to explain elevated rates of hepatocyte apoptosis and proliferation after paracetamol overdose. Kupffer cells and cytokines such as TNF- α remain promising candidates for a major role in paracetamol toxicity as they are involved in liver regeneration, toxic injury and also ischaemic injury. Further mechanistic studies of Kupffer cells and their secreted products are required to unravel the mechanisms of liver injury and

regeneration.

Liver death and regeneration in paracetamol toxicity

The major concern in paracetamol toxicity is the considerable clinical challenge of improving the outcome for patients who develop severe liver injury where mortality is high and therapeutic options are limited (Peleman et al, 1988; O'Grady et al, 1989). This observational study raises many important issues regarding paracetamol toxicity in human liver. Apoptosis is confirmed to occur during paracetamol toxicity in human liver at a late time and is associated with brisk hepatocyte regeneration. While the precise pathogenesis is not known and further research is required to understand the mechanisms of injury and regeneration in the liver, certain targets for potential therapeutic intervention have been identified. In particular specific inhibition of apoptosis and cytokines modulation of regeneration are appealing new approaches to improve outcome after paracetamol overdose in the future.

CHAPTER THREE

EARLY EVENTS IN PARACETAMOL TOXICITY

CHAPTER THREE

EARLY EVENTS IN PARACETAMOL TOXICITY

SUMMARY

Patients dying after coproxamol (combination of dextropropoxyphene and paracetamol) overdose represents a novel approach to investigate liver pathology caused by paracetamol. In this study, pathological changes in the liver attributable to paracetamol including necrosis, apoptosis, lipofuscin accumulation and hepatocyte injury were identified after coproxamol overdose. These features should be borne in mind as possible markers of paracetamol toxicity when a good history or toxicology is not available.

INTRODUCTION

Good evidence now exists that hepatocyte apoptosis occurs in addition to necrosis in murine and human liver but further studies are required to elucidate the role of apoptosis in paracetamol toxicity (Ray et al, 1996; chapter 2). The pathogenesis of paracetamol toxicity is complex. In addition to direct hepatocyte injury from toxic paracetamol metabolites, evidence has emerged from several experimental models that indirect factors such as Kupffer cell activation and cytokine production may be involved in paracetamol hepatotoxicity (Laskin 1996). However, species variation in paracetamol toxicity is well-described (Green et al, 1984; Holme and Soderlund, 1986; Smolarek et al, 1990) and the relevance of this new data in human liver is not clear. A major problem in studying paracetamol toxicity in humans is the lack of human liver tissue available in the early stages of paracetamol toxicity: there is no indication for biopsy after paracetamol overdose as clotting is invariably deranged and the risk of serious haemorrhage is significant. Some early studies of paracetamol toxicity in human liver identified early changes of toxicity including lipofuscin accumulation (Portmann et al, 1975; Lesna et al, 1976).

An alternative approach to study paracetamol toxicity in human liver is patients who die after overdosage of coproxamol. Coproxamol is a proprietary combination of dextropropoxyphene and paracetamol which when taken in overdose results in rapid

death due to dextropropoxyphene (opiate overdose) with no derangement in liver function (Young and Lawson, 1980; Lawson and Northridge, 1987; Spooner and Harvey, 1993). A minority of patients (5/27) develop liver failure secondary to the paracetamol component (Read et al, 1986) although the liver pathology after coproxamol overdose has not been studied.

The **aims** of this study were to investigate the pathological changes in human liver following coproxamol overdose, to address whether or not features of paracetamol toxicity including hepatocyte apoptosis were evident.

MATERIALS AND METHODS

Patient characteristics and tissue studied

A consecutive series of patients dying of coproxamol overdose were identified from the files of the Forensic Medicine Unit, University of Edinburgh over a three-year period from 1996 to 1999 as part of an ethically approved study of drug-related death (Prof A Busuttill, Dr P Fineron, Prof DJ Harrison). A similar series of age and sex-matched patients dying secondary to methadone overdose with no history of ingestion of paracetamol were identified for the same period (see Table 3.3). For each case 1-5 blocks of liver were available for study from which H&E sections were available.

Morphological Analysis

Several pathological features that have been previously described in paracetamol toxicity were studied (Table 3.1).

H&E-stained sections were examined to assess these features: lipofuscin deposition, hepatocyte injury and hepatic necrosis were scored as present or absent and hepatocyte apoptosis was scored by counting the number of apoptotic hepatocytes identified in 10 high power fields (x400). Autolysis was present in 5/23 cases after coproxamol overdose and 3/18 cases after methadone overdose; owing to the poor preservation these cases were excluded from the assessment of apoptosis.

Table 3.1

Pathological features of paracetamol toxicity	Reference
Lipofuscin deposition in hepatocytes	Portmann et al, 1975; Lesna et al, 1976
Hepatocyte injury*	Portmann et al, 1975; Chapter 2
Hepatocyte necrosis	Portmann et al, 1975; Lesna et al, 1976
Hepatic apoptosis	Afford et al, 1995; Chapter 2

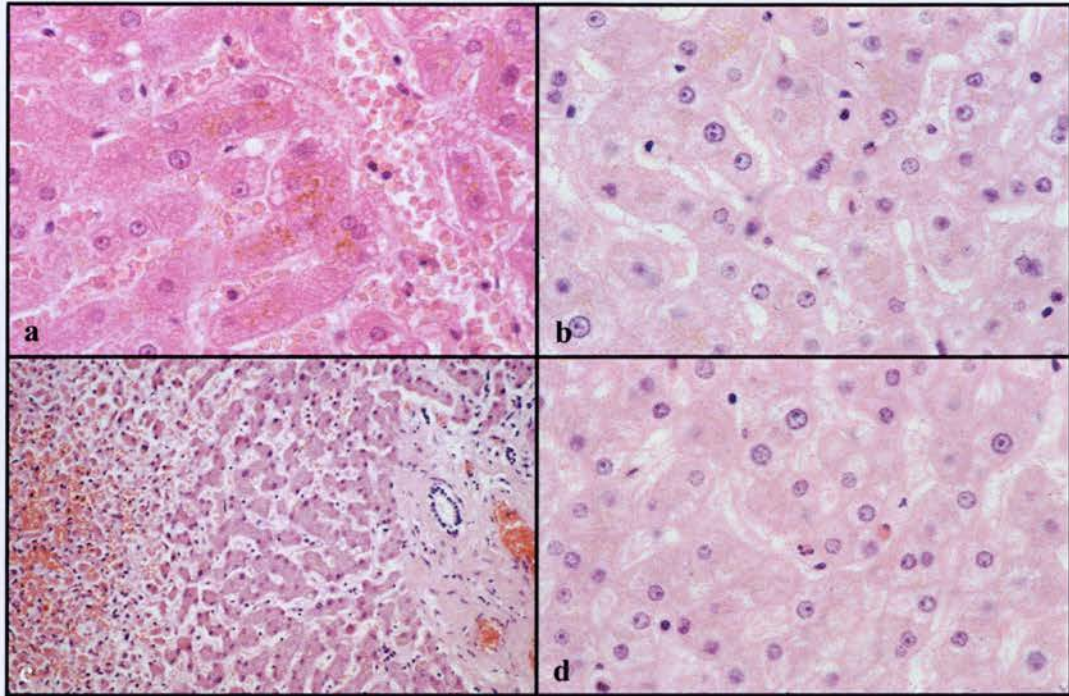
Table 3.1 Morphological assessment of liver pathology after paracetamol overdose.

*hepatocyte injury is characterised by nuclear enlargement and cytoplasmic vacuolation

RESULTS

Liver pathology after coproxamol overdose

After coproxamol overdose there was accumulation of lipofuscin in zone 3 hepatocytes (13% of cases), hepatocyte injury (61%), hepatic necrosis (9%) and hepatocyte apoptosis (26% of cases; Figure 3.1 and Table 3.2). In cases where apoptosis was identified a formal count was made and a mean of 1.8 ± 1.4 apoptotic bodies was identified per 10 high power fields. All of these pathological features were present only in zone 3 of the liver in coproxamol overdose cases and were not noted in any case of methadone overdose, suggesting that they are attributable to the paracetamol component of coproxamol. Notable hepatic congestion (expansion of sinusoids that were filled with blood) was present in 43% of case of coproxamol overdose and 77% of cases of methadone overdose. Three methadone cases also showed features suggestive of hepatitis C virus infection (lymphocytic infiltrate in portal tracts with lymphoid aggregates, mild increase of lymphocytes in the hepatic parenchyma) although no information was available regarding hepatitis virus serology.

Figure 3.1**Figure 3.1**

Histopathological changes in zone 3 of the liver after coproxamol overdose.

- A. Lipofuscin accumulation in hepatocytes in zone 3
- B. Hepatocyte injury
- C. Necrosis
- D. Apoptosis

Table 3.2

Aetiology	Coproxamol (23 cases)	Methadone (18 cases)
Lipofuscin accumulation	3 (13%)	0
Hepatocyte injury	14 (61%)	0
Hepatic necrosis	2 (9%)	0
Hepatocyte apoptosis	6* (26%)	0

Pathological Changes in Liver after Coproxamol or Methadone Overdose

Aetiology: Coproxamol = death after coproxamol overdose; Methadone = death after methadone overdose. * = assessed in 18 cases only – see methods.

Clinical Parameters

The patient groups in this study all died acutely of opiate poisoning, and none had any clinical evidence of liver disease. The cases were age and sex matched and had a similar time to death (Median age 34 years; female:male ratio 1.3:1).

DISCUSSION**Paracetamol toxicity after coproxamol overdose**

This study of patients dying after coproxamol overdose represents a novel approach to investigate paracetamol toxicity in human liver. The dextropropoxyphene component of coproxamol is associated with a risk of rapid death due to opiate toxicity (Whittington and Barclay, 1981). Patients dying after methadone overdose represent a very useful comparison group as although methadone and dextropropoxyphene are different agents, both have similar pharmacological action, have a similar risk of rapid death due to respiratory depression and there is no evidence that either drug causes liver injury. Several potential problems exist as several patients may have consumed alcohol or other drugs as well as coproxamol (8 cases alcohol, 3 cases benzodiazepine, 2 cases amitrypriline, 2 cases morphine). Occasional cases showed mild macrovesicular steatosis but features suggestive of alcoholic hepatitis were not noted in any case. A further potential problem was that an accurate time from overdose to death was not available in all cases and in some was estimated.

Less than 20% of patients who take an overdose of coproxamol develop liver failure at some time (Read et al, 1986) and in this study group none of the patients had any clinical evidence of liver disease. Despite this, over 60% of the patients had pathological changes in the liver attributable to paracetamol. This figure may be artefactually high as the group under study is biased towards those who may have developed liver injury, as opiate-induced death, followed by autopsy, was the major selection criterion, and any patients who survived with or without treatment were not included.

This study provides further evidence that apoptosis occurs as a feature of paracetamol toxicity in human liver, occurring at an early time before necrosis may develop. The two early studies of paracetamol toxicity both identified acidophil bodies in some cases after paracetamol overdose (Portmann et al, 1975; Lesna et al, 1976) and probably represent the first description of paracetamol-induced hepatocyte apoptosis but would require further verification. The rates of apoptosis are extremely low, but are comparable with those described in the previous chapter after paracetamol overdose (there are approximately 1000 – 1500 hepatocytes in 10 high power fields). As previously discussed low rates of apoptosis may nevertheless result in significant cell loss from the liver.

Several studies have addressed the pathological changes in human liver following paracetamol overdose (summarised in Table 3.4). All previous studies have been of patients who have taken an overdose of paracetamol. However, this observational study has identified several pathological changes in the liver after coproxamol overdose, which are attributable as early effects in paracetamol toxicity as they are not present in a similar group of patients dying after methadone overdose. There is evidence of hepatocyte death by necrosis in a minority of cases, an interesting finding as most patients who overdose on coproxamol do not develop “toxic” levels of paracetamol as the paracetamol dose is relatively low (Young and Lawson, 1980). These patients often die early, frequently prior to admission to hospital, and precise details of dose and time since overdose are often not available (Young, 1983; Whittington, 1984). Cautionary literature suggests that paracetamol toxicity may be overdiagnosed as a cause of death (Spooner and Harvey, 1993), however, this study suggests that the opposite should also be borne in mind and that paracetamol toxicity should not be forgotten where death occurs because of toxic effects of another drug.

While zone 3 hepatic necrosis is the main feature of established paracetamol toxicity, early features of toxicity including lipofuscin accumulation (Portmann et al, 1975; Lesna et al, 1976) related to the effects of lipid peroxidation, and hepatocyte injury as indicated by cytoplasmic vacuolation and swelling (Portmann et al, 1975; chapter

Table 3.4

Reference	Histopathology after POD
Portmann et al, 1975	Grading of injury and zone 3 necrosis Hepatocyte injury adjacent to necrosis Lipofuscin deposition in hepatocytes Evidence of regeneration Quantitation of necrosis
Lesna et al, 1976	Lipofuscin deposition, prominent Kupffer cells Spotty necrosis Zone 3 necrosis Acidophil bodies Regeneration
Koukoulis et al, 1992	Zone 3 necrosis Regeneration (PCNA positivity)
Mathew et al, 1994	Grading of hepatic injury and necrosis Quantitation of Kupffer cells and stellate cells

Table 3.4 Histopathology of the liver after POD (paracetamol overdose)

2) have been previously described and are confirmed in this study. While intuitively one might expect zone 3 congestion as an early feature of paracetamol toxicity, this was noted in more cases where paracetamol was absent than present. Lipofuscin accumulation was present only after coproxamol overdose but was only present in 13% of cases. Hepatocyte injury characterised by vacuolation and swelling was present in over 60% of cases of coproxamol overdose and was not present where paracetamol was absent. This may be a useful marker to suggest the possibility of paracetamol toxicity in cases where a good history or toxicology is not available.

CHAPTER FOUR

CHARACTERISATION OF PARACETAMOL TOXICITY IN VITRO

CHAPTER FOUR

CHARACTERISATION OF PARACETAMOL TOXICITY IN VITRO

SUMMARY

The aims of this study were to develop and characterise a model of paracetamol hepatotoxicity in vitro. Primary murine hepatocytes and two human hepatocyte cell lines were used. Paracetamol caused dose-dependent hepatotoxicity above a threshold level, with the human cell lines more sensitive to the effects of paracetamol and showing a more predictable pattern of toxicity than primary murine hepatocytes. Paracetamol caused significant rates of hepatocyte apoptosis by morphological assessment and annexin V staining, confirmed by electron microscopy. Necrosis was measured by propidium iodide uptake and flow cytometric detection. Paracetamol also blocked cell cycle activity by reducing rates of mitosis and BrdU incorporation. The study describes a well-characterised model of paracetamol toxicity, confirming that apoptosis occurs as a significant mechanism of cell loss.

INTRODUCTION

The major mechanism of cell death in paracetamol toxicity is necrosis, however, increasing evidence has suggested that apoptosis may also occur. Apoptosis represents a novel pathway during paracetamol toxicity and sufficient evidence exists from other models of liver injury to suggest that apoptosis may be an important mechanism of cell loss (Patel et al, 1998). Moreover, apoptosis is a tightly regulated process and specific inhibitors of apoptosis exist, raising the possibility of therapeutic intervention to reduce liver injury (Patel et al, 1999). Several groups have studied the occurrence of apoptosis in paracetamol toxicity using a wide range of doses and methods to study apoptosis (summarised in table 4.1). Apoptosis has been convincingly demonstrated in murine liver in vivo (Ray et al, 1996) and in various non-hepatic cell lines (Holownia et al, 1997; Wiger et al, 1999) and now in human liver (personal observations, chapters 2 and 3). However, the role of apoptosis in contributing to loss of liver mass in paracetamol toxicity is not known.

Table 4.1

Species	In Vivo?	Conclusions of Study	Paracetamol Dose	Apoptosis	Reference
Mouse	yes	Protection of toxicity by grape seed extract; reduced Bcl-XL expression with toxicity	400-500mg/kg	Sedimentation/ ladder Morphology	Ray et al, 1999
	yes	Caspase 3 is not involved in liver injury	100-500mg/kg	TUNEL Cell death ELISA Caspase activity	Lawson et al, 1999
	yes	Apoptosis precedes necrosis and contributes to toxicity	300-500mg/kg	DNA fragmentation Morphology	Ray et al, 1996
	no	Nuclear accumulation of calcium and DNA fragmentation correlates directly with cell death (necrosis/apoptosis)	1-25mM	DNA fragmentation	Ray et al, 1991 (in vivo); Shen et al, 1991; Shen et al, 1992; Ray et al, 1993
Trout	no	Paracetamol inhibits vitellogenin synthesis at non-toxic doses (≤ 1 mM); at toxic doses (≤ 10 mM) DNA fragmentation is not induced	0.1-10mM	DNA fragmentation	Miller et al, 1999; Miller et al, 1993
Rat PC12*	no	Toxicity involves apoptosis, necrosis and growth retardation	2-5mM	DNA fragmentation Morphology	Holownia et al, 1997
Human HL60#	no	Paracetamol causes cell cycle arrest and apoptosis	0-3mM	Morphology DNA fragmentation	Wiger et al, 1999

Table 4.1 Studies of Paracetamol-induced apoptosis

Apoptosis – method(s) used to study apoptosis. * PC12 cells are a rat adrenal pheochromocytoma cell line. # HL60 cells are a promyelocytic leukaemia cell line

Hepatocytes in culture are a useful model of liver injury. Most specific liver functions are retained by hepatocytes in culture (Ichiara et al, 1982; Castell et al, 1985; Guillouzo et al, 1986; Gomez-Lechon et al, 1990); in addition most drugs and toxins injure cultured hepatocytes in a similar manner to that seen in hepatocytes in vivo (Ekwall and Acosta, 1982; Jover et al, 1992). The main benefit of an in vitro approach is simplicity, removing many of the complexities that occur in vivo. Paracetamol poisoning is suited to an in vitro approach because paracetamol toxicity occurs by similar mechanisms in vivo and in vitro i.e. hepatotoxicity is dose-dependent above a threshold (Shear et al, 1995); toxicity results in glutathione depletion (Larrauri et al, 1987; Adamson and Harman, 1993); and rescue by administration of N-acetyl cysteine prevents injury (Massey and Racz, 1981). Many different in vitro models of paracetamol toxicity have been described using human or murine hepatocytes.

The aim of this study was to develop and characterise paracetamol hepatotoxicity in murine and human hepatocytes in vitro.

MATERIALS AND METHODS

Isolation of murine hepatocytes

Hepatocytes were isolated using a modified two-step collagenase perfusion method developed in this laboratory. The composition of the media is given in the appendix. 10-12 week old male C3H mice were killed by cervical dislocation and the inferior vena cava was cannulated via the right heart using 1mm plastic tubing attached to a peristaltic pump (Watson Marlow). Infusion of perfusion medium was commenced at a flow rate of 10ml/minute for a few seconds until the liver blanched and expanded slightly at which point the portal vein was incised. Perfusion medium was infused for 5 minutes. Digestion medium was then infused for a further 6-10 minutes at the same flow rate. The liver was carefully removed, placed in a sterile petri dish in 2-3ml digestion medium and carefully broken into small pieces using two pairs of sterile forceps. Plating medium was added and the resulting fragments and cell suspension were passed through a 100nm sterile filter (Nunc). The resulting cell

suspension was centrifuged at 300g for 10 minutes. The cell pellet was resuspended in 8ml plating medium and 5.25ml Percoll/Hanks buffered saline (Sigma Aldrich, Poole) and centrifuged at 300g for a further 10 minutes. The top layer of dead cells was discarded and the cell pellet resuspended in 5ml plating medium. Cells were counted and viability was assessed using trypan blue exclusion: cells were only used if viability was greater than 85%. Cells were plated on cultureware (Gibco-BRL, Paisley) previously coated with 1% fibronectin (Sigma; Appendix 1) in sterile PBS at 4°C overnight and placed in a 37°C incubator for 2 hours to allow the hepatocytes to adhere. The medium was changed to culture medium and the cells were incubated overnight prior to commencing each experiment. Several pilot experiments were carried out to establish optimal culture media and appropriate culture supplements, detailed in Appendix 2.

Human hepatocyte cell lines

Two human hepatocyte cell lines (HepG2 and WRL 68) were chosen for study. HepG2 cells, a human hepatoma cell line (Knowles et al, 1980; Darlington et al, 1987), were maintained in DMEM (Gibco) supplemented with 200mM L-glutamine and antibiotics (penicillin/streptomycin). WRL 68 cells, a normal human embryonal hepatocyte cell line (ECACC, Cambridge), were maintained in MEM (Sigma) supplemented with L-glutamine and antibiotics. Both cell lines express phenotypic characteristics of hepatocytes and have been used previously in drug toxicology studies (Darlington et al, 1987; Roe et al, 1993; Gutierrez-Ruiz et al, 1994). Serum-free culture conditions were developed to allow characterisation of cytokine production in subsequent experiments at a cell density that allowed logarithmic growth of untreated cells and therefore eliminate effects relating to cell confluence. All experiments were carried out on cultureware (Gibco) previously coated with 1% fibronectin (Sigma) in sterile PBS at 4°C overnight. Cells were plated at 2×10^4 cells per well in 24 well plates or at 1×10^4 cells per well in eight well glass chamber slides. Cells were allowed to settle for two hours (WRL 68) or overnight (HepG2) at which time non-adherent cells were washed off, the media was changed and experiments were begun. Several pilot experiments were carried out to determine

optimal culture conditions in terms of a serum-free system with hepatocyte growth supplements added (Appendix 3).

Measuring paracetamol hepatotoxicity

Paracetamol (Sigma) was dissolved directly in the appropriate culture media. BrdU (proliferation labelling agent, Boehringer: 1:100) was added three hours before ending each experiment. Cytotoxicity was assessed using the MTT assay as a measure of cell number. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Mosmann, 1983; Denizot and Lang, 1986; Carmichael et al, 1987) is a simple and reliable colorimetric method to assess cell number in drug toxicology studies (Carmichael et al, 1987). The culture supernatant was removed and the cells were washed gently with sterile PBS. MTT working solution (MTT stock 10mg/ml in PBS: working solution 1.6ml stock added to 5.4ml culture media) was added and the cells incubated at 37°C for four hours. The MTT working solution was carefully removed and the cells washed gently with PBS. Dimethyl sulphoxide (DMSO) was added to dissolve the formazan salt and the plates were read on an ELISA plate reader (Dynatech) within 15 minutes. Pilot studies were carried out to validate the use of MTT as a measure of cell number in the cell lines (Appendix 4).

Morphological assessment of injury and apoptosis

For morphological assessment, culture slides were fixed in 85% methanol / 10% formaldehyde [40%] / 5% glacial acetic acid at 4°C overnight and stored for up to one week. Slides were stained with Feulgen's method (Appendix 5) and counterstained with light green. The slides were examined to identify any morphological evidence of cytotoxicity as previously described (Hayes and Pickering, 1985). Counting of apoptotic and mitotic cells was carried out based on morphological criteria. In pilot studies running means were carried out to determine the minimum number of cells required to detect a significant difference between samples (Appendix 6). Subsequently, at least 1000 cells were counted in each case.

Immunohistochemistry

For immunohistochemistry, culture slides were fixed in acetone/methanol (1:1) at 4°C for 10 minutes and then stored in TBS at 4°C for up to 2 weeks prior to staining. Immunohistochemistry was carried out using the Envision™+ system (Dako) using the standard manufacturers protocol. For human cells, immunohistochemistry was carried out for BrdU and Ki-67. For murine cells, immunohistochemistry was carried out for BrdU only because antibodies against Ki-67 do not work in murine tissue (data not shown). For BrdU immunohistochemistry slides were pre-treated with 2N hydrochloric acid for 10 minutes and the anti-BrdU antibody (clone M0744, Dako; 1:500 dilution) was incubated for 1 hour at room temperature. No pretreatment was required for Ki-67 immunohistochemistry; the antibody (clone MIB-1, Dako; 1:100) was incubated for 1 hour at room temperature. Slides were assessed calculating the proportion of positive and negative cells after calculation of a running mean to determine how many cells had to be counted (Appendix 6).

Annexin V Staining

Cells were grown on fibronectin-coated petri dishes and supernatants were retained and mixed with adherent cells harvested using 0.02mM EDTA. Samples were centrifuged at 300g for 10 minutes, washed in cold PBS and resuspended in binding buffer (10mM HEPES/NaOH [pH7.4], 140mM NaCl, 2.5mM CaCl₂) at a concentration of 10⁶ cells per ml. 10µl of FITC-labelled Annexin V (R&D Systems) was added to 100µl of the sample (10⁵ cells) and after gentle mixing it was incubated at room temperature for 15 minutes in the dark. 10µl of propidium iodide solution (50µg/ml in PBS) and 400µl of binding buffer were added and samples were assessed by flow cytometry (Coulter EPICS™) within 1 hour.

Electron Microscopy

Samples of control and paracetamol-treated cells (72 hours) were harvested using 0.02mM EDTA / 1xtrypsin and fixed in glutaraldehyde. Grids for electron microscopy were prepared using standard techniques.

Statistical Analysis

Differences were assessed using a one-way analysis of variance (MedCalc[®], Belgium), with a value of $p < 0.05$ chosen as significant.

RESULTS

Paracetamol toxicity in murine and human hepatocytes

Administration of paracetamol to primary murine hepatocytes resulted in a reduction in cell number with 20mM paracetamol at 24, 48 and 72 hours and 10mM paracetamol at 48 and 72 hours ($p < 0.05$; Figure 4.1). Lower doses of paracetamol did not significantly alter cell number at any time. As a general comment, the cell number data showed marked variability in the murine model, with wide variability in most experiments.

Figure 4.1 Paracetamol Toxicity in Primary Murine Hepatocytes

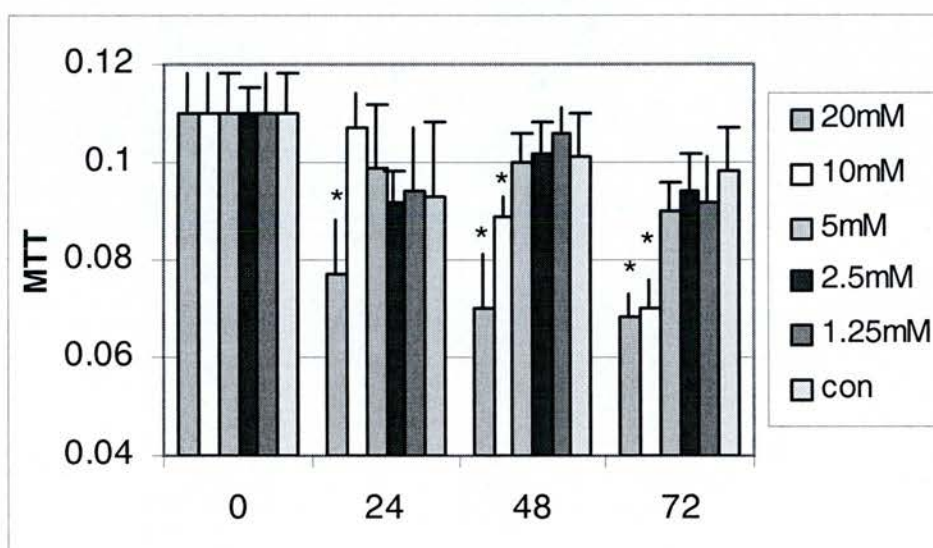


Figure 4.1

Cell number measured by MTT assay for primary murine hepatocytes. The control represents cells with media only; other groups have the dose of paracetamol noted. * reduction in cell number compared with untreated controls ($p < 0.05$). Results are the mean of four repeat experiments.

In human hepatocytes, paracetamol caused a dose-dependent reduction in cell number in both cell lines after 48 and 72 hours, however, HepG2 cells were less

sensitive to paracetamol than WRL 68 cells (Figure 4.2 and 4.3). The threshold for toxicity was at 2.5mM paracetamol for both cell lines. At this dose there was a significant reduction in cell number compared with untreated controls but the cell number still increased with time. At higher doses of paracetamol (>10mM for WRL 68 cells, >20mM for HepG2 cells) there was a significant reduction in cell number compared with controls and also compared with the starting number of cells at all time points ($p < 0.05$). A “balance point” was noted for both cell lines, where there was significant toxicity compared with controls but the actual cell number did not change over the 72 hour period (5mM for WRL 68, 10mM for HepG2). These doses were utilised for further studies on apoptosis and proliferation as there was significant toxicity, but the cell number remained high enough to allow study of surviving cells.

Figure 4.2 Paracetamol toxicity in HepG2 cells

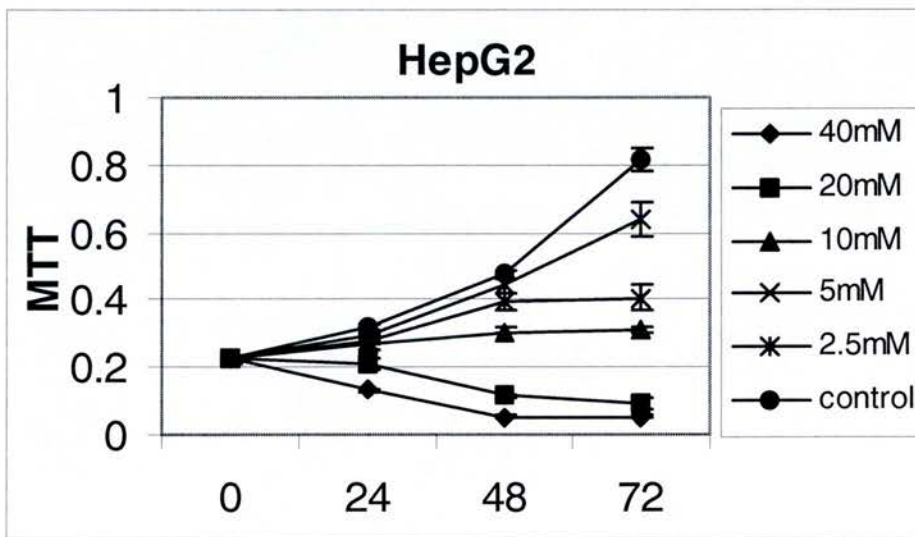
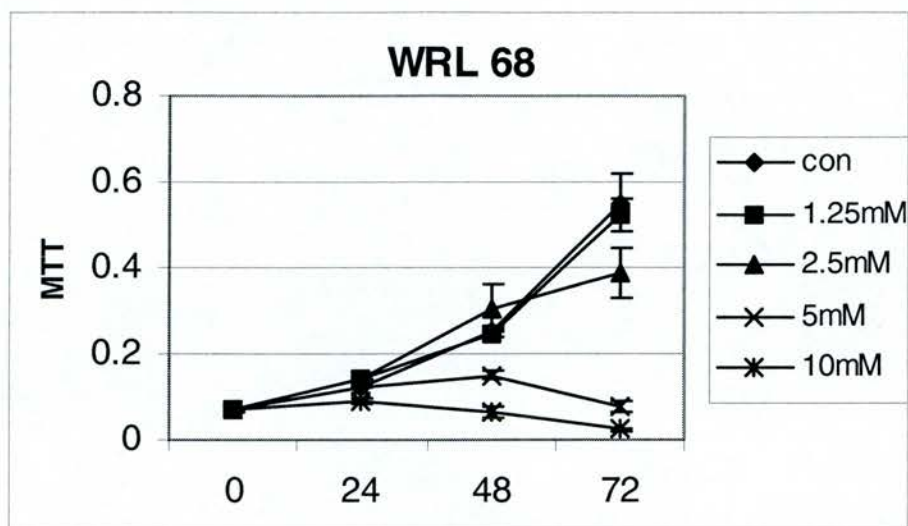


Figure 4.2.

Cell number, measured by MTT assay, for HepG2 cells up to 72 hours. Control represents cells with media only, the other groups have the dose of paracetamol noted. See text for significant differences ($p < 0.05$).

Figure 4.3 Paracetamol toxicity in WRL 68 cells**Figure 4.3.**

Cell number, measured by MTT assay, for WRL 68 cells up to 72 hours. Control represents cells with media only; the other groups have the dose of paracetamol noted. See text for significant differences ($p < 0.05$).

Paracetamol causes hepatocyte apoptosis and necrosis

Electron microscopy confirmed the presence of many cells with classic morphology of apoptosis (Figure 4.4), namely cell shrinkage, condensation of chromatin into compact masses, nuclear fragmentation, cytoplasmic vacuolation and membrane blebbing (Wyllie et al, 1980). Feulgen-stained culture slides similarly showed many cells with classical apoptotic morphology (Figure 4.5) and allowed quantitation of apoptotic and viable cells. Rates of apoptosis were significantly greater in paracetamol-treated than control groups at 24, 48 and 72 hours and also increased significantly with time ($p < 0.05$; Table 4.2).

Figure 4.4 Paracetamol induces hepatocyte apoptosis

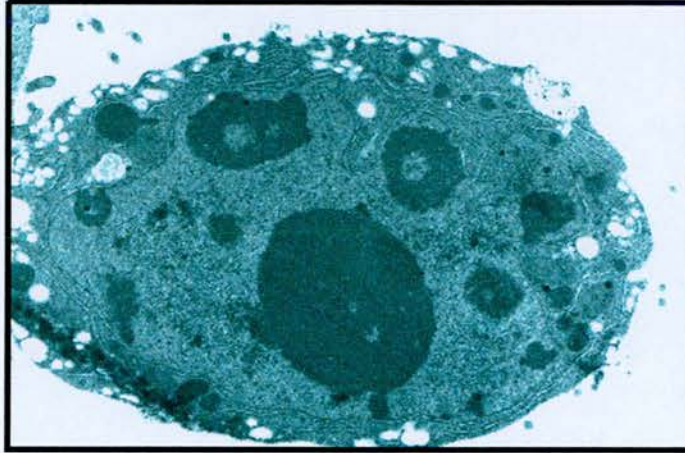


Figure 4.4 Electron micrograph showing typical apoptotic morphology in a hepatocyte (WRL 68) incubated with 10mM paracetamol for 48 hours.

Figure 4.5 Paracetamol induces hepatocyte apoptosis

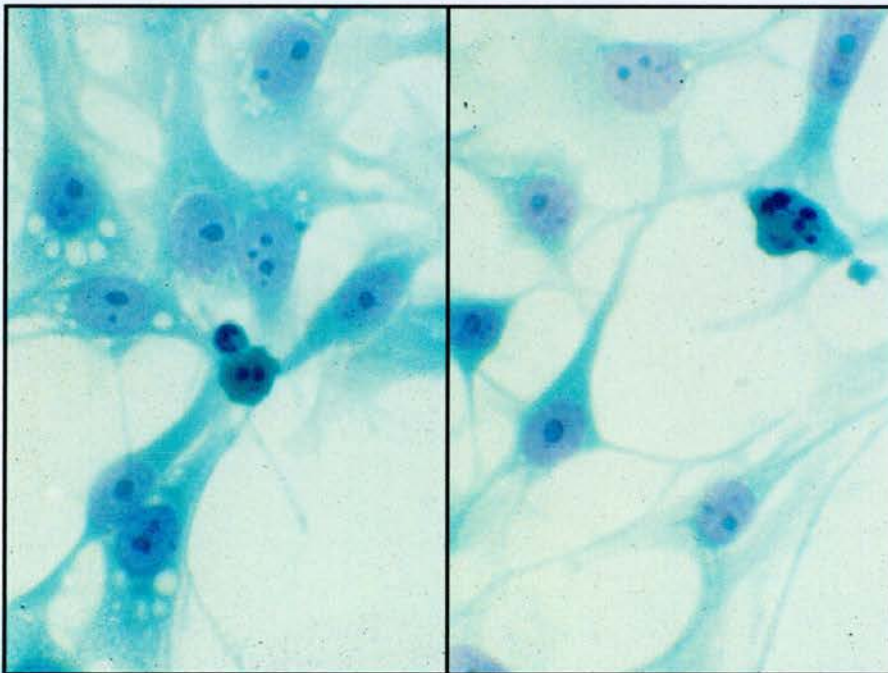


Figure 4.5 Photomicrographs of hepatocytes (WRL 68) showing apoptotic morphology. The apoptotic cells are shrunken, with condensation of the nucleus into tight balls of condensed chromatin and formation of multiple small apoptotic bodies.

Table 4.2 Paracetamol-induced hepatocyte apoptosis

Cells		24	48	72
WRL 68	control	0 ± 0.11	0.8 ± 1.18	0.6 ± 0.51
	paracetamol	1.1 ± 1.71*	5.8 ± 1.13*	14.6 ± 4.36*
HepG2	control	1.4 ± 0.89	0.4 ± 0.37	0.4 ± 0.52
	paracetamol	3.7 ± 1.38*	8.9 ± 2.55*	11.0 ± 3.38*

Table 4.2 Percentage of apoptotic hepatocytes on Feulgen-stained culture slides at 24, 48 and 72 hours. Data are mean ± standard deviation. Control = cells in serum-free culture media. Paracetamol = 5mM paracetamol for WRL 68, 10mM for HepG2. * = greater than control ($p < 0.05$).

Annexin V staining showed increased annexin positivity, indicating early apoptosis, in hepatocytes treated with paracetamol (Figure 4.6). Quantitation of apoptosis was possible comparing the proportion of annexin positive, PI negative cells (apoptosis) with cells which were PI positive (necrosis); the necrotic population by definition included cells undergoing secondary necrosis as the end-stage of apoptosis. Paracetamol caused striking necrosis, associated with an increase in the rate of apoptosis (Figure 4.7). Comparison of rates of apoptosis derived from culture slides and annexin showed a good correlation ($r = 0.9$ [0.36-0.99; $p = 0.013$]; Table 4.3).

Figure 4.6 Paracetamol increases annexin V staining

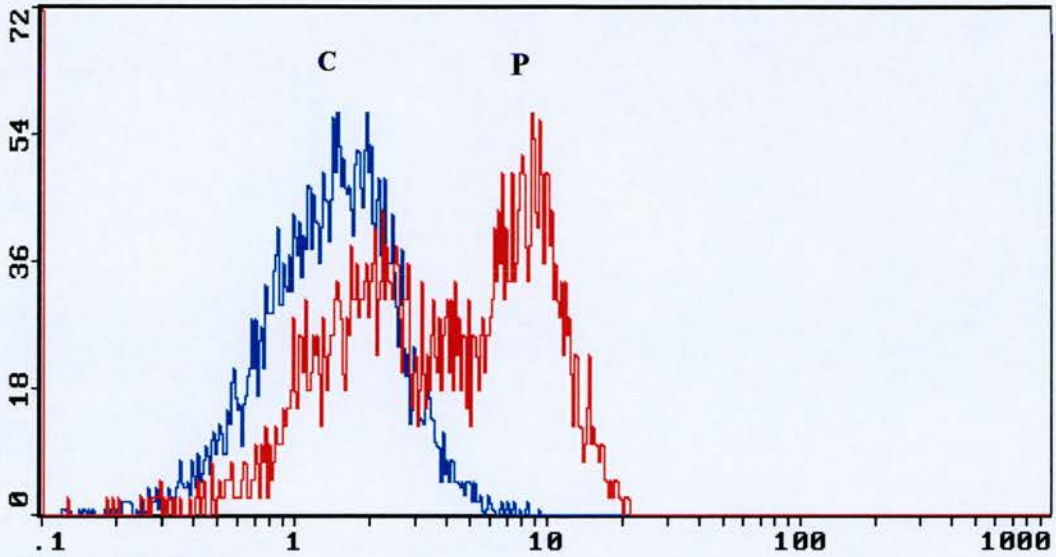


Figure 4.6

Flow histogram showing an increase in annexin V staining in WRL 68 cells treated with paracetamol (P) versus control (C).

Figure 4.7

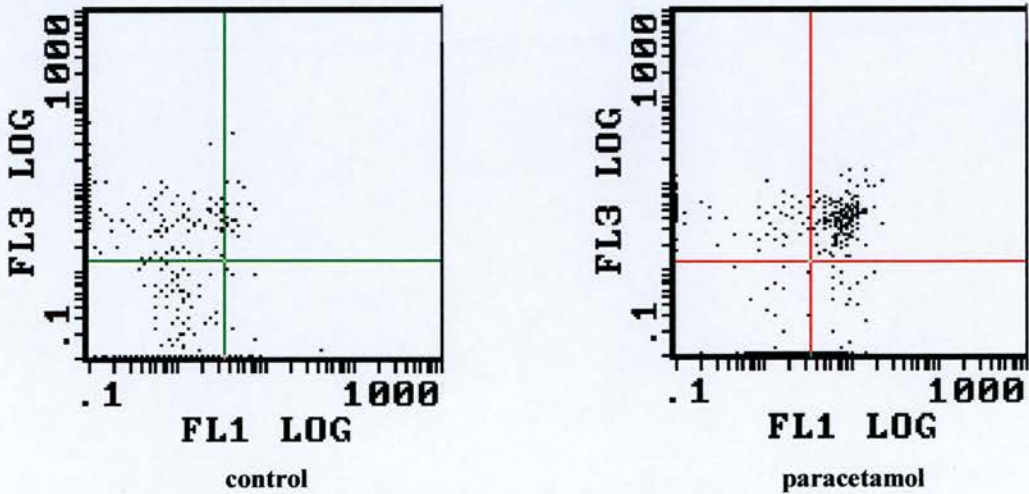


Figure 4.7

Flow histograms comparing WRL 68 cells with paracetamol and without (control) after 72 hours. The graphs are scattergrams showing the number of events (cells) that are positive for PI (FL3 log) and for annexin V (FL1 log). The bottom right quadrant represents apoptosis (annexin positive, PI negative). The two top boxes represent necrosis (PI positive).



Table 4.3 Rates of apoptosis and necrosis by flow cytometry

WRL 68 cells		24	48	72
Apoptosis	control	2.68	1.93	2.3
	paracetamol	6.43	9.3	11.28
Necrosis	control	7.9	8.58	12.4
	paracetamol	20.07	33.90	53.00

Table 4.3

Flow cytometric analysis of annexin V staining and PI incorporation (total of 3×10^5 cells per count. Apoptosis = percentage of annexin V positive cells; Necrosis = percentage of PI positive cells. control = untreated. paracetamol = 5mM paracetamol.

Paracetamol induces hepatocyte injury

Examination of human livers after paracetamol overdose has identified evidence of injury in non-necrotic hepatocytes characterised by swelling and vacuolation (Portmann et al, 1975). Hepatocytes exposed to paracetamol in vitro likewise showed morphological differences from untreated cells. The cells appeared smaller and showed marked cytoplasmic vacuolation with striking formation of cytoplasmic processes (Figure 4.8). This morphological change was first noted after six hours incubation with toxic doses of paracetamol and was then present at all subsequent time points studied. Notably, the appearance was seen human cells treated with doses of paracetamol at which cell death did not occur (1.25mM paracetamol for both cell lines). In pilot experiments, WRL 68 cells were incubated with paracetamol doses of low toxicity (2.5-5mM) for 6-12 hours and the media was then changed after which time the morphological changes regressed, supporting the concept that they represent reversible (sub-lethal) hepatocyte injury.

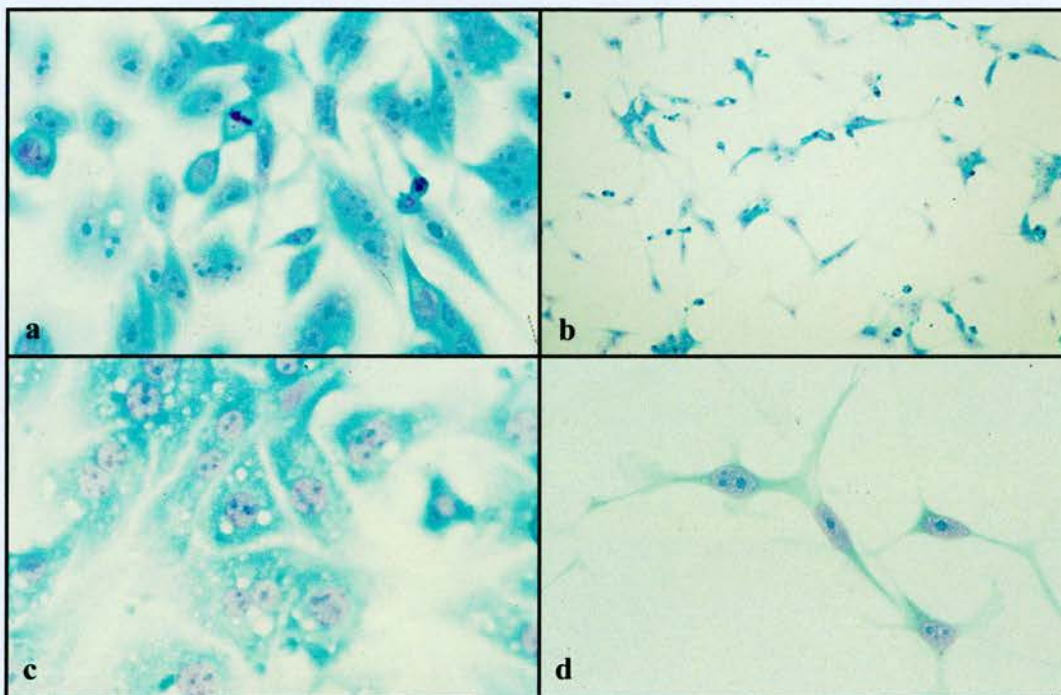
Figure 4.8. Paracetamol induces hepatocyte injury

Figure 4.8 Photomicrographs of control hepatocytes (WRL 68: a and c) and hepatocytes incubated with 5mM paracetamol, showing morphological evidence of injury (b and d). The injured cells are smaller than control cells with striking formation of fine cytoplasmic processes.

Paracetamol blocks hepatocyte proliferation

The reduction in hepatocyte numbers noted with paracetamol could be explained simply by an induction of cell death (apoptosis and/or necrosis). However, when proliferation was measured, paracetamol also caused a dose-dependent reduction in mitosis at 24, 48 and 72 hours ($p < 0.05$; Table 4.4). Paracetamol also caused a significant increase in BrdU positivity at 24 hours followed by a reduction to low levels at 48 and 72 hours ($p < 0.05$; Figure 4.9, Table 4.4). One problem in the interpretation of BrdU staining was that the intensity of staining was relatively low in many cells; any definite nuclear staining was scored as positive. Immunohistochemistry for Ki-67, an antigen expressed in all phases of the cell cycle (Key et al, 1993), revealed strong expression in $99 \pm 1.5\%$ of cells at all times, with and without paracetamol (Figure 4.9). These findings suggest that paracetamol inhibits proliferation and that the reduction in hepatocyte numbers occurs caused by

paracetamol occurs by a combination of cell death and reduction in proliferative activity.

Table 4.4

MITOSIS		24	48	72
WRL 68	control	6.1 ± 3.96	4.6 ± 2.84	5.8 ± 1.33
	paracetamol	0.3 ± 0.72*	0*	0.2 ± 0.49*
HepG2	control	4.6 ± 4.08	7.7 ± 4.41	5.3 ± 3.35
	paracetamol	0.6 ± 0.62*	0*	0 ± 0.15*
BrdU POSITIVITY				
WRL 68	control	32.88 ± 3.83	36.22 ± 5.5	32.29 ± 2.08
	paracetamol	68.03 ± 14.25**	18.6 ± 16.95*	1.2 ± 1.8*
HepG2	control	33.3 ± 7.77	29.85 ± 5.02	34.4 ± 3.08
	paracetamol	56.15 ± 18.9**	33.08 ± 9.85	9.9 ± 3.56*

Table 4.4 Hepatocyte mitosis and BrdU –positivity in paracetamol toxicity

MITOSIS = Percentage of mitotic hepatocytes on Feulgen-stained culture slides at 24, 48 and 72 hours. BrdU = Percentage of mitotic hepatocytes on Feulgen-stained culture slides at 24, 48 and 72 hours. Data are mean ± standard deviation. Control = cells in serum-free culture media. Paracetamol = 5mM paracetamol for WRL 68, 10mM for HepG2. * = less than control (p<0.05); ** = greater than controls (p<0.05).

Figure 4.9 BrdU incorporation and Ki-67 expression

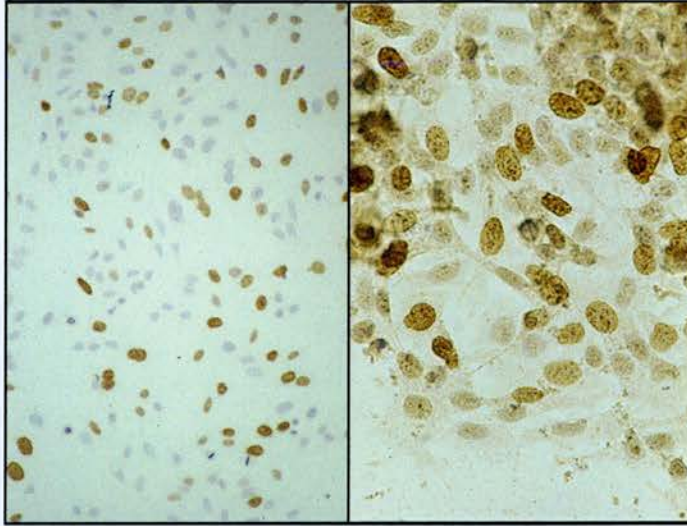


Figure 4.9 Photomicrograph of hepatocytes showing BrdU positivity (left) and Ki-67 expression (right). Almost all cells showed strong nuclear positivity for Ki-67. In contrast BrdU staining is of still strong but stain many fewer cells.

DISCUSSION

Although necrosis is recognised to be the major mechanism of cell loss after paracetamol overdose, recent evidence has indicated that apoptosis may also occur in murine liver (Ray et al, 1996) and various non-liver cell lines (Holownia et al, 1997; Wiger et al, 1999) but the evidence in human liver is not clear (Afford et al, 1995; personal observations in chapters 2 and 3). This study describes an in vitro model of paracetamol toxicity, using primary murine hepatocytes or human hepatocyte cell lines, in which apoptosis occurred in addition to necrosis and proliferation was inhibited.

Paracetamol toxicity and apoptosis

There are considerable problems in the study of apoptosis in the liver as the process of apoptosis is rapid and characteristic changes have a short duration (Collucci, 1996; Patel et al, 1998). The number of apoptotic cell may therefore be low, however, apparently low rates of apoptosis may nevertheless cause significant loss of liver mass (Bursch et al, 1990).

Confirmation of hepatocyte apoptosis in paracetamol toxicity: Early studies in mice found a strong association between nuclear accumulation of calcium with cell death, and while DNA ladders were shown differentiation between necrosis and apoptosis was not made (Ray et al, 1991; Shen et al, 1991; Shen et al, 1992; Ray et al, 1993). The same group provided the first convincing evidence that apoptosis occurs in addition to and preceding necrosis (Ray et al, 1996) by showing DNA laddering and morphological changes of apoptosis although the validity of using morphology to separate apoptotic and necrotic cells in areas of injury is questionable. Paracetamol also causes apoptosis in non-hepatic cell lines (Holownia et al, 1997; Wiger et al, 1999). However, species differences in toxicity may exist as DNA fragmentation does not occur in trout hepatocytes although a non-toxic dose of paracetamol inhibits protein synthesis (Miller et al, 1993; Miller et al, 1999). This study confirms that paracetamol-induced apoptosis occurs in murine and human hepatocytes in vitro, using electron microscopy as the gold standard method to confirm apoptosis based on well-documented and characteristic morphological appearances (Wyllie et al, 1980; Goldsworthy et al, 1996).

The mechanisms whereby paracetamol causes apoptosis require further study as conflicting data exists: paracetamol toxicity is associated with a reduction in bcl-XL expression (an anti-apoptotic protein; Ray et al, 1999) but other groups have shown that paracetamol does not activate classical caspase pathways and in certain situations may actually inhibit apoptosis (Lawson et al, 1999).

Quantitation of Hepatocyte Apoptosis: Morphological assessment of apoptosis by light microscopy was chosen for this study as a reliable and accurate method to confirm and quantitate apoptosis (Howie et al, 1994; Arends et al, 1994; Galle, 1997; Sloop et al, 1999). Problems with this method include that it is cumbersome and time-consuming to count numerous samples, possible errors may occur in identification of apoptotic cells and the number of cells to be counted is usually high as the duration of apoptosis is short and hence apoptotic rates may be low. These problems were addressed by using a running mean to determine the minimum number of cells that had to be counted to identify a 2% difference in apoptotic rate,

with sessions at a multiheader microscope with an experienced observer in the early stages of analysis to clearly denote morphological parameters considered to be apoptosis.

Detection of annexin V staining was chosen as a second method to study apoptosis. Annexin V has a high affinity for phosphatidylserine, normally found on the inner surface of the plasma membrane but externalised as an early event in apoptosis (Vermes et al, 1995; Lowin et al, 1996). Annexin V has been used to detect apoptosis in several cell types (Martin et al, 1995), correlating well with other methods of cell loss (Goldberg et al, 1999) and detectable by either fluorescence microscopy or by flow cytometry, with no definite benefit gained by either method (Plasier et al, 1999). Flow cytometry was chosen because the addition of PI allowed simultaneous evaluation of necrosis (Vermes et al, 1995). Both adherent and detached cells were studied to improve accuracy as increased floating cells (possibly early apoptosis) have been noted in paracetamol toxicity in vitro (Dai and Cederbaum, 1995) and cells may detach early in the apoptotic process (Arends et al, 1993). The main weakness of the annexin method is that it uses unfixed cells and requires immediate analysis, limiting the number of simultaneous experiments. A further weakness is that cross-reaction with necrotic cells may occur where membrane damage has occurred and phosphatidylserine becomes accessible (Plasier et al, 1999). However, an excellent correlation was noted comparing morphological scoring and measurement of annexin V staining by flow cytometry in quantitation of apoptosis ($r = 0.9$, $p=0.013$). This suggests that these techniques are both useful and reliable methods to quantify apoptosis.

Other methods have been developed which can identify characteristic (but not necessarily specific) features of apoptosis such as DNA fragmentation, caspase activation, PARP cleavage, detection of TGF- β , formation of neocytokeratin 18 (Leers et al, 1999) or eosin autofluorescence (Goldsworthy et al, 1996; Patel, 1998). In situ detection of DNA fragmentation (e.g. in situ end-labelling [ISEL] or TDT uridine triphosphate nick end-labelling [TUNEL]) has been used to detect apoptosis in many studies and has been proposed to be more sensitive than conventional

morphological examination (Wheeldon et al, 1995). However, problems exist, most notably that the method is not specific for apoptosis as necrotic and autolytic cells or cells undergoing DNA repair may also be positive (Grassl-Kraup et al, 1995; Mangili et al, 1999; Orita et al, 1999; Kanoh, et al, 1999; Lawson et al, 1999). Marked variation in staining may also be seen depending on length and type of fixation (Tateyama et al, 1998; Kiyozuka et al, 1999), tissue sectioning (Sloop et al, 1999) and proteinase K digestion (Mangili et al, 1999). Furthermore, while apparently normal cells may be positive, clearly apoptotic cells on morphological grounds may be negative in the same sample (Cuello-Carrion and Ciocca, 1999; personal observation). It has been suggested that the main use of this method probably requires simultaneous simple morphological analysis (Mangili et al, 1999) and several pilot experiments during this study found TUNEL to be unreliable.

Apoptosis and necrosis in paracetamol toxicity

The extent of necrosis in vivo is not the only factor determining the outcome in paracetamol toxicity. However, separating apoptosis and necrosis as mechanisms of cell loss is problematic. From the in vivo studies in human liver described previously rates of apoptosis are very low (chapter 2), but this does not exclude a significant reduction in cell number attributable to apoptosis (Bursch et al, 1990). In this in vitro model, apoptosis occurs at a much higher rate than in vivo (up to 12%), suggesting that apoptosis contributes significantly to hepatocyte loss during paracetamol poisoning. The data for primary murine hepatocytes is less clear as apoptotic rates are lower.

In the flow cytometry studies, control cells had a low level of necrosis (PI positivity: 11.8%) although it seems likely that the PI positivity relates to membrane damage induced when the cells were detached from the culture dish, with 7-12% of cells positive in control samples. Administration of paracetamol caused a significant increase in PI positivity, presumably related to induction of necrosis, with values of positivity up to 53% after 72 hours.

Hepatocyte injury: a measure of reversible hepatotoxicity?

Morphological changes induced by high doses of paracetamol have been previously noted, including plasma membrane damage and blebbing, probably the same as the elongated cytoplasmic processes noted in this study (George et al, 1981; Koo et al, 1987). Furthermore, it has been suggested that morphological assessment (cellular granularity, rounding up and detachment) may be a sensitive measurement of paracetamol toxicity, especially after chronic exposure (Hall et al, 1993). However, others did not identify significant morphological changes of injury (Hayes and Pickering, 1985; Hayes et al, 1986). The injury noted in this study clearly appeared to be a reversible phenomenon as it was seen not only at toxic but also at sub-toxic doses of paracetamol, that is where cell loss did not occur (e.g. 1.25 and 2.5mM paracetamol in human hepatocyte cell lines). This injury is presumably the in vitro equivalent of the hepatocyte injury noted after paracetamol overdose in vivo (chapters 2 and 3).

Paracetamol inhibits proliferation

Paracetamol toxicity has been shown to cause hepatocyte death by both necrosis and apoptosis. However, paracetamol also inhibits hepatocyte proliferation, a factor that may contribute to the toxicity noted. The first report of antiproliferative effects of paracetamol was noted in lymphocytes (Timson, 1968). In this study paracetamol caused a significant inhibition of proliferation with a reduction in mitotic rate at all time-points, even before significant cell loss occurred, and a reduction in BrdU positivity at 48 and 72 hours. An unexpected finding was the increase in BrdU positivity at 24 hours, a consistent feature of paracetamol toxicity (see below). One problem in interpreting this data is the very high intrinsic rate of proliferation in the human cell lines, and direct comparison with primary hepatocytes, or the liver in vivo, requires some caution. Nevertheless, if paracetamol inhibits hepatocyte proliferation this may have a profound effect as enhanced proliferation may protect against injury (Calabrese et al, 1993; Chanda and Mehendale, 1996).

The mechanism whereby paracetamol inhibits proliferation is not known although it may block DNA replication and impair DNA repair by inhibiting dNTP synthesis

(Brunborg et al, 1995). Paracetamol has been shown to inhibit DNA synthesis in several tissues in the rat but paradoxically not the liver (Lister and McLean, 1997). It has been suggested that the antiproliferative effect of paracetamol occurs via a different pathway to that causing cell death (Wiger et al, 1999). In the early stages of toxicity in this study paracetamol is associated with increased BrdU positivity, the BrdU positivity then dropping to low levels. This supports the concept suggested by Wiger et al (1999) that paracetamol induces a cell cycle arrest, with the arrested cells subsequently dying. After paracetamol poisoning there is a transient reduction in NF- κ B activity with inhibition of G0/G1 cell cycle transition and activation of *c-myc* and Raf-1 kinase (Blazka et al, 1995/1996; Boulares et al, 1999). Further studies are required to elucidate the intracellular pathways by which paracetamol causes these effects.

The usefulness of in vitro models of paracetamol toxicity

In vitro models of paracetamol toxicity are extremely useful because of similarities to toxicity in vivo: metabolism of paracetamol is similar (Kane et al, 1980), hepatotoxicity is dose-dependent (Shear et al, 1995), glutathione depletion occurs (Harman and Fischer 1983; Larrauri et al, 1987; Adamson and Harman, 1993) and administration of N-acetyl cysteine prevents injury (Massey and Racz, 1981). However, potential differences have been noted between toxicity in vitro and in vivo. First, a significant consideration is the pharmacokinetics of paracetamol, where the concentration in vitro remains the same throughout but in vivo it is rapidly eliminated from the liver (Fischer et al, 1981). Second, paracetamol is bioactivated to NAPQI, the toxic metabolite in vivo, but administration of NAPQI directly has a different effect than paracetamol (Harman et al, 1991). Third, the association of glutathione depletion with toxicity is not as strong as in vivo and protection with N-acetyl cysteine is not as effective in vitro (Larrauri et al, 1987). Fourth, several authors have proposed that the various toxic effects of paracetamol may be caused by different pathways (Wiger et al, 1999) where in vivo depletion of glutathione and rescue by N-acetyl cysteine probably prevents most toxicity. Nevertheless, despite these problems, in vitro studies have an important role in addressing specific questions regarding drug toxicity.

Paracetamol toxicity has been studied in many species, revealing striking inter-species differences (Green et al, 1984; Holme and Soderlund, 1986; Smolarek et al, 1990). Murine hepatocytes appear to be most similar to human hepatocytes and may offer useful models of paracetamol toxicity although conflicting evidence exists as paracetamol bioactivation in humans may be closer to rat than mouse liver (Seddon et al, 1987): a summary of studies of paracetamol in human and murine cells is given in Table 4.6. In general, the data for HepG2 cells show similar toxicity in the 4 studies including this one, the differences possibly relating to the different methodologies used to quantitate cell death (see below and Table 4.6). Furthermore, primary human hepatocytes show comparable levels of toxicity to cell lines. However, for murine hepatocytes the situation is less clear. The data from this study showed lower levels of toxicity than that previously published, with a wide range of toxicity. However, many differences exist between the different studies including strain, dose, culture-method and method used to measure toxicity. The primary murine cells also showed notably wider variation in toxicity than human cell lines. This variability is likely to be real, as the primary mouse cells represent a heterogeneous population of hepatocytes derived from all zones of the hepatic acinus, which may have differing sensitivity to paracetamol. In contrast the cell lines are likely to be very homogeneous with regard to drug sensitivity. The balance of evidence suggests that murine hepatocytes show more variation in toxicity than human hepatocytes and suggest that where possible human cells should be used to study paracetamol toxicity.

HepG2 cells have been used in several studies of paracetamol toxicity (Table 4.6) with similar toxicity found in this study. HepG2 cells are a well defined and widely used hepatic cell line known to express many functions of differentiated hepatocytes (Morris et al, 1982; Darlington et al, 1987) and proposed to represent a suitable in vitro model for assessment of both acute and chronic exposure to toxins (Hall et al, 1993; Nicolini et al, 1995). Furthermore HepG2 cells express CYP450 enzymes

Table 4.6 Paracetamol Toxicity: Human and Murine Cells In Vitro

Cells Studied	Measurement of Cell Death	Paracetamol Dose	% Cytotoxicity ^a	Reference
Primary liver (human)	Total protein ^b	5mM	30% (12 hours)	Larrauri et al, 1987
Primary liver (human)	MTT ^c	2.21mM 6.6mM	10% (24-48 hours) 50%	Jover et al, 1992
HepG2 (human)	SRB^d Morphology^e	7mM	50% (24 hours)	Hall et al, 1993
HepG2 (human)	MTT^c NR^f	5mM 10mM 20mM	Not toxic 21.5% (24 hours) 50%	Shear et al, 1995
HepG2 subline (human)	LDH^g Morphology^e	5mM 10mM 20mM	50% (96 hours) 67%	Dai and Cederbaum, 1995
HepG2 (human)	MTT^c	5mM 10mM 20mM	24 hours 72 hours 12% 50% 16% 62% 23% 89%	This study
HeLa, Chang (human)	LDH ^g	0.05-0.1mM	Not given	Ekwall and Acosta, 1982
HepG2 (human)	None	1mM	Not given	Roe et al, 1993
Primary liver (Swiss mice)	LDH ^g	1mM	50% (4 hours)	Massey and Racz, 1981
Primary liver (Swiss mice)	Trypan blue ^h	3mM 10mM	50% (18 hours) 100%	Holme et al, 1991
Primary liver (Swiss mice)	LDH ^g	1mM 5mM 10mM	45% (4 hours) 75% 85%	Harman et al, 1992
Primary liver (Swiss mice)	LDH ^g	1mM	60% (24 hours)	Adamson and Harman, 1993
Primary liver (mice ⁱ)	GPT / GOT ^j	15-25mM	2-3-fold ↑ (12 hours)	Wang and Zhang, 1994
Primary liver (Swiss, C3H)	MTT ^c	10mM	not given	DeLeve et al, 1997
Primary liver (C3H)	MTT ^c	10mM 20mM	24 hours 72 hours 10% 54% 49% 57%	This study

Table 4.6 a) % Cytotoxicity data presented as a reduction compared with untreated controls. b) Total protein: total protein content of attached cells compared with untreated controls. c) MTT assay. d) SRB (sulphorhodamine B): binds to fixed cellular protein and is measured colorimetrically after solubilisation. e) Morphology: Several authors have used morphological changes as a measure of cytotoxicity (see discussion, chapter 5). f) NR = neutral red, a vital dye. Cytotoxicity measured by NR uptake gives results consistently lower than measured by MTT. g) LDH = lactate dehydrogenase. LDH is released after membrane damage. h) Trypan blue is excluded by viable cells. i) Mouse strain not identified (paper in Chinese). j) GPT / GOT are liver enzymes released when cells are injured. Data in **bold** are studies of paracetamol toxicity in HepG2 cells.

albeit at a lower level than is seen in vivo (Gugen-Guillouzo et al, 1988). Importantly, HepG2 cells express CYP1B1 and CYP1A2, two enzymes known to be involved in paracetamol metabolism and activation (Roe et al, 1993). However, they only contain 50% of the glutathione of primary hepatocytes (Grant et al, 1987). HepG2 cells also express many cytokine genes (Stonans et al, 1999). While these cells have been extensively used as models of liver function, the line is derived from a hepatoma rather than normal liver and may not represent the best model of toxicity in normal liver.

WRL-68 cells are an embryonal cell line, derived from non-neoplastic liver, which express many functions of normal liver and have been used previously in toxicological studies (Gutierrez-Ruiz et al, 1994; Bucio et al, 1995). These cells were chosen for study because they were derived from normal liver and may represent a better model to study toxicity than HepG2 cells. It has been reported that WRL 68 cells do not express CYP450 enzymes (Olivares et al, 1997). If that were the case then it would be likely that these cells would be relatively resistant to paracetamol toxicity. However, the findings of this study suggest that the two cell lines show very similar paracetamol toxicity, both qualitatively and quantitatively, with similar threshold and dose-dependent toxicity with inhibition of proliferation. Two possible explanations are that WRL 68 cells do in fact express CYP450 enzymes or that paracetamol toxicity in vitro is not dependent on CYP450 activity. The latter possibility is intriguing given suggestions that some manifestations of paracetamol toxicity occur independently of CYP450 enzymes and may explain some of the differences between toxicity in vivo and in vitro.

Conclusions

This study describes a model of paracetamol toxicity that has been well characterised in terms of hepatocyte loss, apoptosis and proliferation. Comparison of primary murine hepatocytes with human hepatocyte cell lines and a review of the literature suggest that human cells offer a better and more reproducible model of paracetamol toxicity than murine hepatocytes.

CHAPTER FIVE

THE ROLE OF TNF- α IN PARACETAMOL TOXICITY

CHAPTER FIVE**THE ROLE OF TNF- α IN PARACETAMOL TOXICITY****SUMMARY**

The role of TNF- α in paracetamol overdose is controversial. The balance of evidence from in vivo studies is that TNF- α is produced during paracetamol toxicity but that inhibition of it does not affect the outcome. Conversely, in vitro studies show that TNF- α synergises with paracetamol when added simultaneously to augment hepatocyte death by apoptosis but when TNF- α is administered before paracetamol, it protects against toxicity. TNF- α has diverse effects on paracetamol toxicity. Its role is not clear but it may represent a signal for hepatic regeneration.

INTRODUCTION**TNF- α and the Liver**

Tumour necrosis factor-alpha (TNF- α) is a member of a superfamily of pleiotropic cytokines that is a paracrine and endocrine mediator of inflammation and immunity and also regulates growth and differentiation in many tissue types. Monocytes and macrophages are the major producers of TNF- α , but most cell types in the liver including hepatocytes, biliary epithelial cells and sinusoidal endothelial cells can produce TNF- α (Nagano et al, 1992; Gonzalez-Amaro et al, 1994; Hoffmann et al, 1994). TNF- α interacts with two receptors, TNFR1 (p55) and TNFR2 (p75), which may be functionally associated. TNFR1 is expressed ubiquitously and is important in cytotoxic TNF signalling (Tartaglia et al, 1991). TNFR2 is involved in signalling via membrane-bound TNF- α and may cooperate with TNFR1 in signalling effects of soluble TNF- α (Tartaglia et al, 1993a; Tartaglia et al, 1993b; Grell et al, 1996). TNF receptors lack catalytic kinase domains and rely on associated proteins to function as signal transducers to determine which intracellular signalling pathway is activated. Death signalling occurs via the death domain, a conserved motif found in a number of death-signalling proteins (reviewed in Faubion and Gores, 1999). One such receptor, FADD, interacts with TNFR1 via TRADD and also links directly with the apoptotic cascade via caspase 8/FLICE/MACH (Boldin, et al, 1996; Hsu et al, 1996;

Pan et al, 1997; Bradham, 1998). However, TNF receptors can also activate survival factors such as NF- κ B and jun kinase (JNK) and signal proliferation rather than death (Rothe et al, 1995; Reinhard et al, 1997).

TNF- α is implicated in many important pathological and physiological processes in the liver and can contribute to liver injury and regeneration. TNF- α mediates hepatocyte death, signalling both necrosis and apoptosis in toxic liver injury (Laster et al, 1988; Bradham et al, 1998). However, triggering cell death requires prior inhibition of mRNA or protein synthesis (Larrick and Wright, 1990; Hill et al, 1995). TNF- α is also an important element in hepatic regeneration, being up-regulated after partial hepatectomy (PH) and inducing a variety of other genes involved in proliferation and cellular survival (Bruccoleri et al, 1997). Furthermore, regeneration is defective in the absence of TNF- α or TNF R1 or following inhibition of TNF- α by neutralising antibodies (Akerman et al, 1992). In regeneration after PH, TNF- α signals via TNFR1 (Yamada et al, 1998), activating downstream proteins including NF- κ B in a well characterised pathway also involving IL-6 (Fitzgerald et al, 1995; Cressman et al, 1996; Yamada et al, 1998). NF- κ B activation probably represents a critical element in determining whether survival or proliferation occurs rather than apoptosis (Bellas et al, 1997).

TNF- α and other Cytokines in Paracetamol Toxicity

TNF- α is implicated in several experimental and important human liver diseases including viral hepatitis (Daniels et al, 1990; Bradham et al, 1998), fulminant liver failure (Muto et al, 1988), endotoxin administration (Beutler et al, 1985) and liver injury induced by several drugs and toxins including alcohol (reviewed in McLain et al, 1999), galactosamine (Gantner et al, 1995; Pfeffer et al, 1993; Stachlewitz et al, 1999), carbon tetrachloride (Czaja et al, 1995; Bruccoleri et al, 1997) and after paracetamol overdose (reviewed in Schumann and Tiegs, 1999). Paracetamol-induced liver injury occurs as a direct consequence of formation of a toxic metabolite (NAPQI) that injures cells as an essential event in hepatotoxicity (Nelson, 1995; Zaher et al, 1998). However, there is good evidence that liver injury may be modulated by factors such as activation of Kupffer cells (Laskin et al, 1995; Goldin

et al, 1996) associated with production of reactive oxygen and nitrogen intermediates (Michael et al, 1999), cytokines including TNF- α and IL-1 α (Blazka et al, 1995) and chemokines including CINC/gro and MIP-2 (Takada et al, 1995; Horbach et al, 1997; Hogaboam et al, 1999). The role of TNF- α in paracetamol toxicity is controversial (DeVictor et al, 1982; Blazka et al, 1995; Blazka et al, 1996; Boess et al, 1998).

IL-1 α is a cytokine that mediates the acute phase response (Dinarello, 1989; Blazka et al, 1995) and is involved in liver growth and regeneration (Ramadori and Armbrust, 1998). IL-1 α is produced after partial hepatectomy and may stimulate proliferation *in vivo*, although it has an anti-proliferative effect *in vitro* (Koga and Ogasawara, 1991; Higashitsuji et al, 1995; Nakamura et al, 1988). The major source of IL-1 α in the liver is Kupffer cells (Higashitsuji et al, 1995) although endothelial cells may also produce it (Feder et al, 1993). IL-1 α has been shown to protect against paracetamol toxicity in rats (Renic et al, 1997).

MIP-2 is a CXC chemokine that is chemotactic for neutrophils. Recent evidence suggests that TNF- α and IL-6 induce production of the CXC chemokines ENA-78 and MIP-2 (Colletti et al, 1996; Colletti et al, 1998). These chemokines can contribute to the inflammatory process in acute liver injury (Lentsch et al, 1998; Sprenger et al, 1997) but may also play a role in liver regeneration (Hogaboam et al, 1999). Administration of CXC chemokines MIP-2, IL-9 and ENA-78 protected against liver injury after paracetamol overdose in mice (Hogaboam et al, 1999). IL-1 α and MIP-2 represent good candidates, along with TNF- α , to be involved in paracetamol toxicity.

The **aims** of this study were to investigate the role of TNF- α in paracetamol toxicity:

1. using an *in vitro* model using human hepatocyte cell lines to investigate the role of TNF- α and other cytokines in hepatocyte death and regeneration.
2. using an *in vivo* murine model to investigate the production of TNF- α and effects of inhibition of TNF- α .

MATERIALS AND METHODS

Cell lines and experimental model

The human hepatic cell lines (HepG2 and WRL 68) were used in the same in vitro model of paracetamol toxicity as described in detail in chapter four. Briefly, cells were seeded in fibronectin-coated culture dishes overnight in serum-free media. The media was then replaced with fresh media or media containing paracetamol. Unless otherwise stated, WRL 68 cells and HepG2 cells were incubated with 5mM or 10mM paracetamol respectively for 24, 48 and 72 hours. These doses were selected as they were toxic but cell numbers remained sufficiently high to facilitate study of apoptosis and proliferation. Recombinant TNF- α (R&D Systems), IL-1 α (R&D Systems) or GRO- β (Peprotech) was dissolved directly in culture media, with or without paracetamol, as detailed below. For pretreatment experiments cells were incubated with media containing TNF- α for 24 hours after which the media was replaced. Cell number was assessed by MTT assay. Rates of apoptosis, mitosis and BrdU incorporation scored on culture slides. Flow cytometry after staining with annexin V and PI was also carried out. All methods are given in detail in chapter 4.

Murine Model of Paracetamol Overdose

Six week old female CBA/J mice were fasted with free access to water for eight hours prior to intraperitoneal (ip) injection of paracetamol (300mg/kg) dissolved in normal saline. Controls were sham-treated by injection of saline only. Mice were killed by cervical dislocation at various time-points up to 24 hours and samples of serum and liver were taken as detailed below. N-acetyl cysteine (300mg/kg by ip injection, control injected with saline: Sigma, St Louis, Missouri, USA) was used to prevent liver injury. Rabbit anti-murine-TNF- α antibodies were produced by multiple site immunisation of New Zealand white rabbits with recombinant murine TNF- α (R&D Systems) followed by purification of IgG using a protein A column. 0.5ml of immune serum was injected ip 1 hour prior to administration of paracetamol, with non-immune serum used as a control; this immune serum has been previously shown to inhibit the effects of TNF- α in vivo (Colletti et al, 1996; Remick et al, 1990; Eskandari et al, 1992; Hewett et al, 1993). Other mice were injected with IL-10 (5 μ g), soluble TNF receptor-chimera (200 μ g), dexamethasone

(4 μ g) or saline as a control 1 hour before administration of paracetamol. These treatments have also been shown to inhibit effects of TNF- α in vivo (Walley et al, 1996; Lukacs et al, 1995; Standiford et al, 1995; Chensue et al, 1991).

TNF- α Expression

Samples of liver were weighed and homogenised in 1ml of PBS containing Nonidet P40 (0.5%) and the supernatant recovered following centrifugation. Measurement of TNF- α from serum and liver homogenate were made using ELISA specific for murine TNF- α as previously described (Walley et al, 1996). RNA was extracted from liver snap-frozen in liquid nitrogen and homogenised in 1ml of 4.2M guanidine isothiocyanate, 25mM Tris (pH 8.0), 0.5% sarkosyl and 0.1M 2-mercaptoethanol. An equal volume of 100mM Tris (pH8.0), 10mM EDTA and 1% SDS was added and RNA was prepared following extraction in phenol-chloroform and chloroform-isoamyl alcohol. The isolated RNA was quantitated by spectrophotometric analysis at 260nm. 5 μ g of RNA was reverse transcribed into cDNA using oligo (dT) primers and AMV reverse transcriptase as previously described (Lukacs et al, 1995). The cDNA was amplified using specific primers for TNF- α and cyclophylin as a control (see table 5.1). The PCR amplification reaction was incubated at 94°C for 5 minutes followed by 35 cycles at 93°C for 45 seconds, 52°C for 45 seconds and 72°C for 90 seconds. Amplification products were visualised under UV light following separation in a 2% agarose gel containing ethidium bromide.

Table 5.1

Primer Sequences	
TNF- α sense	CCTGTAGCCCACGTCGTAGC
TNF- α antisense	TTGACCTCAGCGCTGGAGTTG
Cyclophylin sense	CATCTGCACTGCCAAGAC
Cyclophylin antisense	CTGCAATCCAGCTAGGCATG

Table 5.1 Primer sequences for TNF- α and cyclophylin

Assessment of Hepatic Injury

Hepatic injury was assessed by two methods. Serum aspartate aminotransferase (AST) activity was assessed using a standard colorimetric assay (Sigma, St Louis, Missouri, USA) and a blinded histological assessment was made scoring hepatic necrosis in a semi-quantitative manner (+ <30% total parenchymal necrosis, ++ 30-60% necrosis, +++ >60% necrosis).

Statistical analysis

Differences were assessed using a one-way analysis of variance, with a value of $p < 0.05$ chosen as significant.

RESULTS**Paracetamol hepatotoxicity in vitro is augmented by TNF- α**

The results were similar for both cell lines, hence data are presented for WRL-68 cells only for simplicity. When recombinant TNF- α was added over a wide range of doses (0.6-100ng/ml) paracetamol toxicity in the human hepatocyte cell lines was augmented at 24, 48 and 72 hours ($p < 0.05$, Figure 5.1). The effect was dose-dependent, with higher doses of TNF- α causing greater cell loss than lower doses. The threshold for the TNF- α effect was at 0.6ng/ml. The same effects were noted when TNF- α was added 12 hours after administration of paracetamol (data not shown). Rates of apoptosis by morphological counting and also by annexin V staining were higher with the combination of TNF- α and paracetamol than with paracetamol alone ($p < 0.05$; Table 5.2). The rate of necrosis, assessed by propidium iodide uptake, was also higher in cells treated with TNF- α and paracetamol than with paracetamol alone (Table 5.3).

Figure 5.1

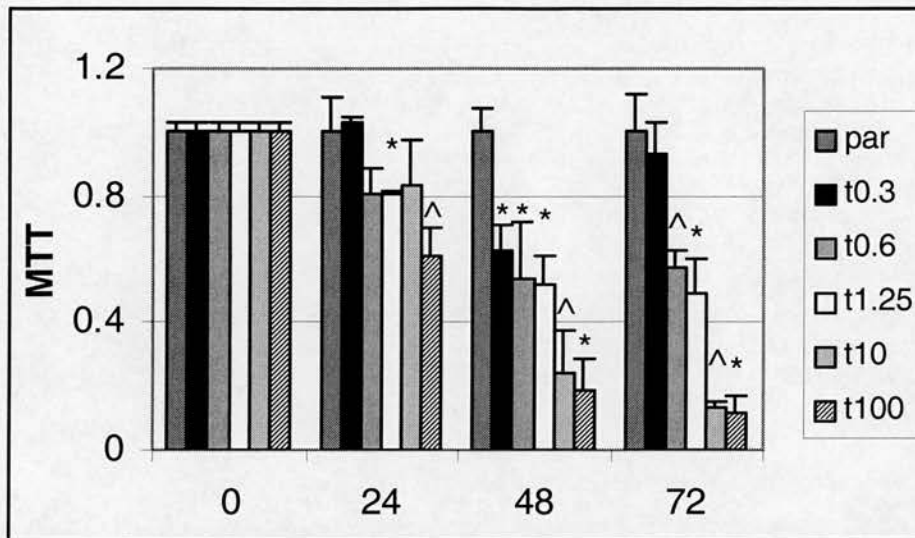


Figure 5.1

Effect on cell number of addition of TNF- α with paracetamol up to 72 hours. Data are expressed as a percentage of the data for paracetamol only. Par = 5mM paracetamol; t0.3 = 0.3ng/ml TNF- α ; t0.6 = 0.6ng/ml TNF- α ; t1.25 = 1.25ng/ml TNF- α ; t10 = 10ng/ml TNF- α ; t100 = 100ng/ml TNF- α . * = significant reduction in cell number compared with paracetamol only. ^ = significant reduction in cell number compared with control and previous dose of TNF- α .

Table 5.2

	24	48	72
APOPTOSIS			
Control	0 \pm 0.11	0.76 \pm 1.18	0.56 \pm 0.51
TNF- α	0.12 \pm 0.27	0.62 \pm 0.58	0.36 \pm 0.33
Paracetamol	1.1 \pm 1.71*	5.76 \pm 1.13*	14.62 \pm 4.36*
TNF- α / Paracetamol	4.34 \pm 3.88**	11.36 \pm 5.38**	18.6 \pm 5.53**
Pretreatment	0	3.88 \pm 1.57#	11.13 \pm 5.1*
NECROSIS			
Control	7.90	8.58	12.40
TNF- α	4.30	7.57	12.37
Paracetamol	20.07	33.9	53.00
TNF- α / Paracetamol	19.44	39.22	41.18

Table 5.2 Rates of apoptosis and necrosis in paracetamol toxicity

Control = media only; TNF- α = 0.6ng/ml; paracetamol = 5mM; TNF- α /paracetamol = both added simultaneously; pretreatment = 24 hour incubation with 0.6ng/ml TNF- α then 5mM paracetamol only. Results for apoptosis are three replicate counts \pm standard deviation. * = greater than control or TNF- α alone ($p < 0.05$). ** greater than paracetamol, TNF- α alone and control ($p < 0.05$). # = greater than control or TNF- α alone but less than paracetamol-treated ($p < 0.05$). Results for necrosis are flow cytometric count of 30000 cells and represent the proportion of PI positive cells.

TNF- α reduces the threshold for paracetamol toxicity

Addition of a dose of TNF- α known to augment toxicity (0.6ng/ml) also lowered the threshold for hepatotoxicity: 1.25mM paracetamol toxicity was cytotoxic at 72 hours and 2.5mM paracetamol was cytotoxic at 48 and 72 hours ($p < 0.05$; Figure 5.2).

Figure 5.2

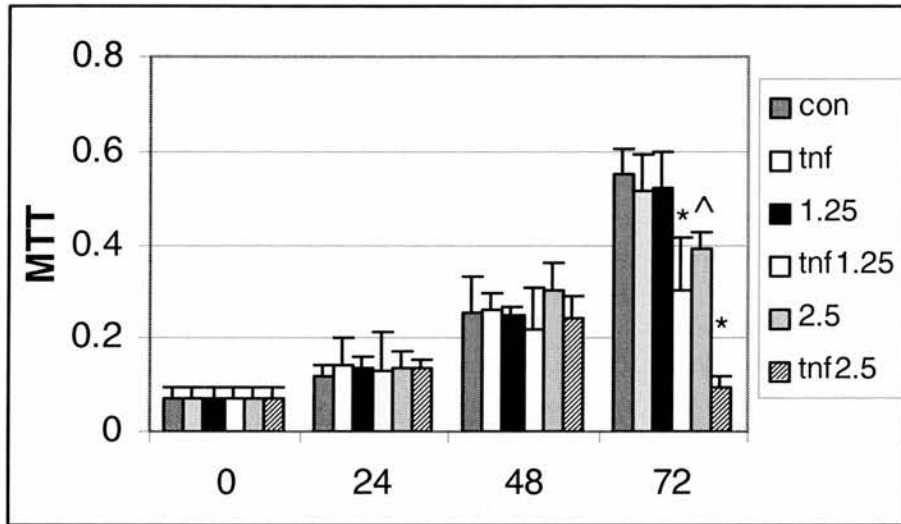


Figure 5.2

Administration of TNF- α augments paracetamol toxicity at low doses: the threshold for toxicity is reduced to 1.25mM when TNF- α is also present. Con = control; tnf = TNF- α (0.6ng/ml); 1.25 / 2.5 = 1.25 or 2.5mM paracetamol; tnf 1.25/2.5 = TNF- α plus 1.25 or 2.5mM paracetamol. ^ = significant reduction in cell number compared with control; * = significant reduction compared with paracetamol alone.

TNF- α has no discernible effect on cells when paracetamol is absent

When no paracetamol was present, addition of TNF- α over a range of doses (0.3-10ng/ml) had no effect on cell number or apoptosis at 24, 48 and 72 hours.

Pretreatment with TNF- α protects against paracetamol toxicity

Pretreatment of hepatocytes with TNF- α (0.6ng/ml) for 24 hours prior to addition of paracetamol resulted in protection against subsequent paracetamol hepatotoxicity after 24, 48 and 72 hours exposure to paracetamol ($p < 0.05$; Figure 5.3). At 48 hours, the pretreated group showed no reduction in cell number compared with controls; at 72 hours the cell number was reduced but remained significantly higher than with

paracetamol alone. The pretreated group showed a rate of apoptosis higher than controls but lower than paracetamol-treated cells at 48 hours and the same rate of apoptosis as paracetamol only at 72 hours ($p < 0.05$; Table 5.2).

Figure 5.3

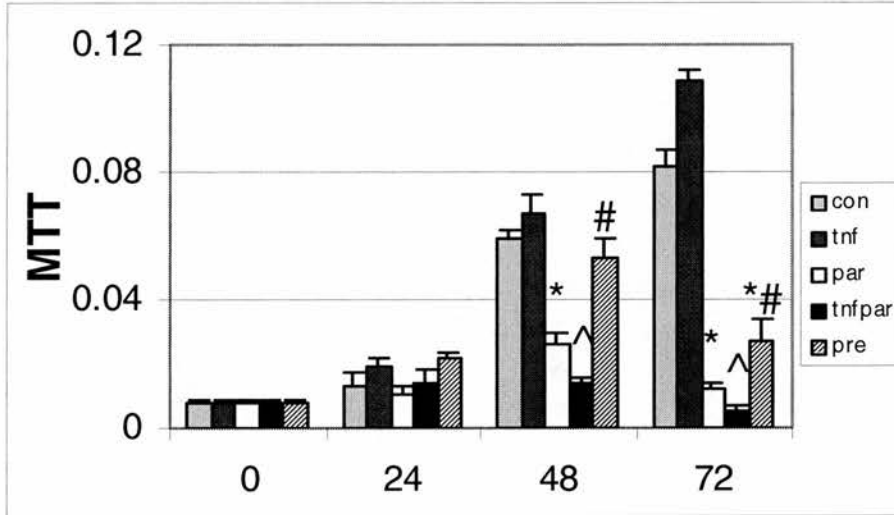


Figure 5.3

Pretreatment with TNF- α (0.6ng/ml) for 24 hours prior to exposure to paracetamol protects against toxicity. con = media only; par = 5mM paracetamol; tnfpar = TNF- α (0.6ng/ml) and paracetamol administered simultaneously; pre = pretreatment with TNF- α then exposure to paracetamol only. * = significant reduction in cell number compared with untreated controls. # significantly greater cell number than paracetamol only; ^ significantly lower cell number than paracetamol only ($p < 0.05$).

#

TNF- α alters hepatocyte proliferation

Paracetamol alone caused a significant reduction in mitosis at 24, 48 and 72 hours and an increase and subsequent reduction in BrdU labelling index as previously described in chapter 4. Addition of TNF- α with paracetamol resulted in a similar reduction in mitosis but the BrdU labelling index was the same as untreated controls i.e. it remained unchanged for the duration of the experiments with no increase and subsequent decrease as seen with paracetamol alone (Table 5.3).

Table 5.3

MITOSIS	24	48	72
Control	5.76 \pm 3.7	4.5 \pm 2.08	6.82 \pm 2.9
TNF- α	3.1 \pm 1.439	3.46 \pm 1.71	4.5 \pm 1.59
Paracetamol	0.32 \pm 0.72*	0*	0.22 \pm 0.49*
TNF- α / Paracetamol	0.34 \pm 0.76*	1.06 \pm 1.45*	0*
Pretreatment	1.81 \pm 1.82*	2.18 \pm 1.85**	0.14 \pm 0.31*
BrdU POSITIVITY			
Control	35.42 \pm 7.73	34.36 \pm 6.93	29.32 \pm 4.80
TNF- α	28.72 \pm 2.59	36.8 \pm 4.07	29.36 \pm 4.84
Paracetamol	72.48 \pm 13.57*	13.22 \pm 15.5**	0.3 \pm 0.47**
TNF- α / Paracetamol	28.88 \pm 9.78	42.02 \pm 26.08	31.13 \pm 10.7
Pretreatment	44.4 \pm 8.4*	15.5 \pm 6.2**	11.38 \pm 6.23**

Table 5.3 Rates of mitosis and BrdU positivity

Control = media only; TNF- α = 0.6ng/ml; paracetamol = 5mM; TNF- α /paracetamol = both added simultaneously; pretreatment = 24 hour incubation with 0.6ng/ml TNF- α then 5mM paracetamol only. Results are three replicate counts \pm standard deviation.

MITOSIS: * = greater than control or TNF- α alone ($p < 0.05$). ** less than control and TNF- α alone but greater than paracetamol treated ($p < 0.05$).

BrdU POSITIVITY: * = greater than control or TNF- α alone ($p < 0.05$). ** less than control and TNF- α alone ($p < 0.05$).

Other cytokines and chemokines have no effect on paracetamol toxicity

Addition of IL-1 α or GRO- β , with and without paracetamol, had no effect on cell number or apoptosis (Figure 5.4 and 5.5).

Figure 5.4

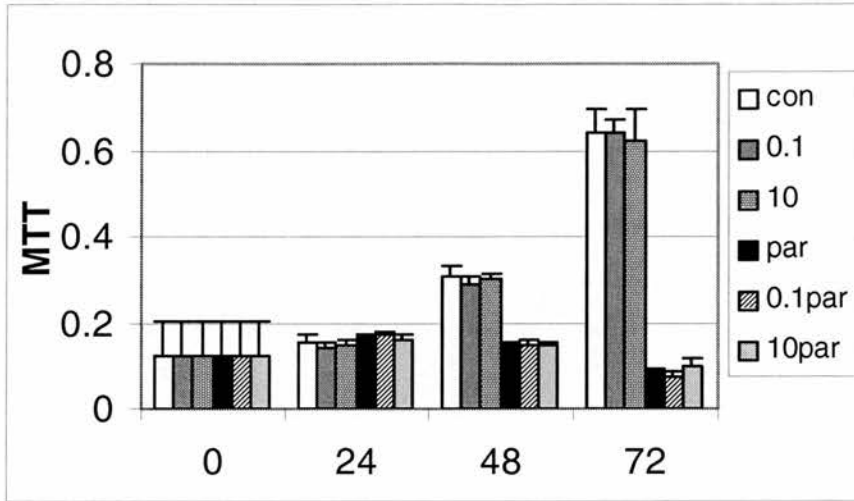


Figure 5.4

IL-1 α and paracetamol toxicity. con = control; 0.1 = 01ng/ml recombinant IL-1 α added; 10 = 10ng/ml IL-1 α added; par = 5mM paracetamol. IL-1 α alone shows no difference from control. IL-1 α and paracetamol show no difference from paracetamol alone.

Figure 5.5

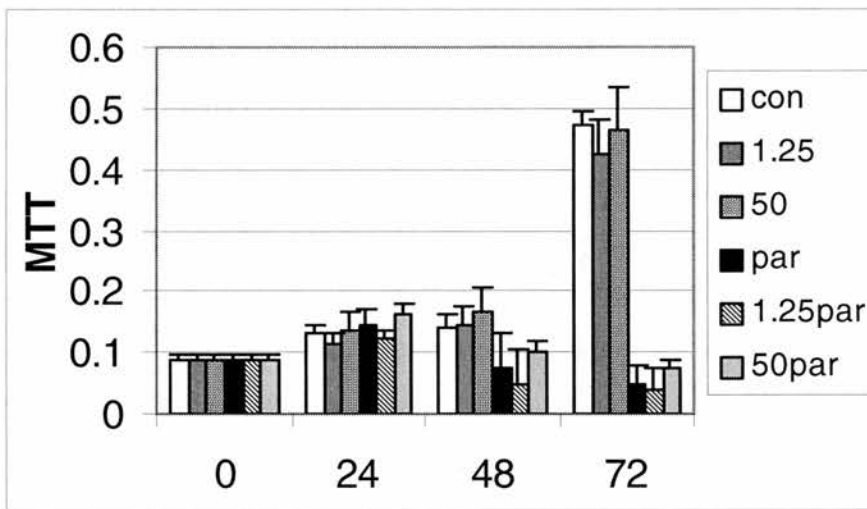


Figure 5.5

GRO- β and paracetamol toxicity. con = control; 1.25 = 1.25ng/ml recombinant gro- β ; 50 = 50ng/ml gro- β , par = 5mM paracetamol. No differences between gro- α only and control or gro- β plus paracetamol and paracetamol alone.

Paracetamol Toxicity In Vivo – the murine model

Administration of paracetamol resulted in liver injury after 3 hours with an increase in serum AST (paracetamol-treated 2726 ± 629 , control 22.5 ± 2.6 IU/l; $p < 0.05$) and

the occurrence of zone 3 hepatic necrosis in all cases compared with controls, which showed no necrosis at any time. The liver injury was completely prevented by administration of N-acetyl cysteine (not shown), confirming that this model is analogous to human paracetamol overdose.

TNF- α Expression after Paracetamol Overdose

Serum TNF- α was increased 3 hours after paracetamol injection (20.4 ± 0.6 pg/ml, $p < 0.05$; Table 5.4) and remained elevated for 24 hours compared with controls (2.4 ± 2.4 pg/ml). Hepatic TNF- α was similar at all time-points after paracetamol injection and did not increase compared with controls (Table 5.4). Hepatic TNF- α RNA expression likewise showed no increase compared with controls (Figure 5.6).

Table 5.4

	Serum TNF- α ¹	Hepatic TNF- α ²
1 hour	0	1.3 ± 0.5
3 hours	$20 \pm 7.1^*$	2.45 ± 0.85
6 hours	$20 \pm 6.8^*$	0.75 ± 0.05
12 hours	12.25 ± 5.5	0.8 ± 0.1
24 hours	$20.5 \pm 2^*$	0.85 ± 0.15
control	2 ± 2	0.8 ± 0.05

Table 5.4 Serum and hepatic TNF- α after paracetamol overdose. The data represent serum or hepatic TNF- α measured by ELISA at the given time-points after injection of paracetamol. ¹ serum TNF- in pg ml. ² hepatic TNF- α in ng/g liver weight. * = significant increase compared with sham-treated controls ($p < 0.05$); $n = 5$ in each group except controls where $n = 7$.

Inhibition of TNF- α after Paracetamol Overdose

The biological effects of TNF- α were inhibited by several different regimes including anti-TNF- α antibodies, soluble TNF receptors, IL-10 and dexamethasone. There were no differences in mortality, serum AST or extent of hepatic necrosis (Table 5.5) by any of the TNF- α inhibition regimes compared with controls given paracetamol only.

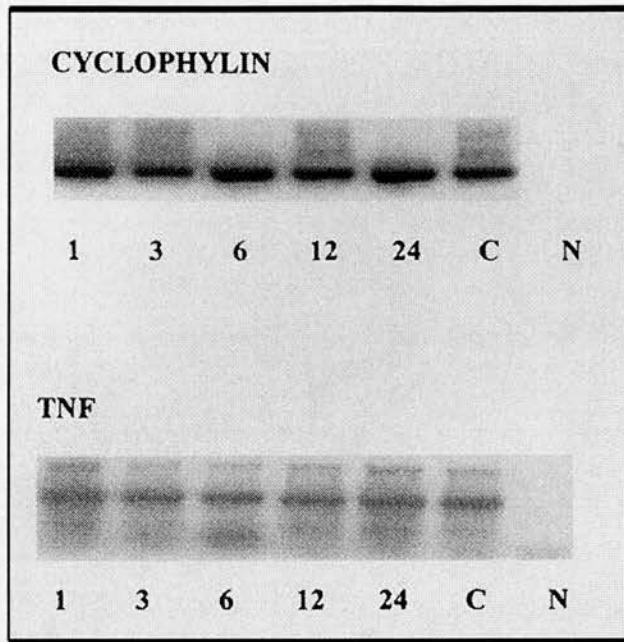
Figure 5.6

Figure 5.6 Hepatic TNF- α mRNA expression following paracetamol poisoning. Total mRNA was extracted from liver tissue and cDNA synthesised to determine mRNA expression. Cyclophylin was used as a control. The times given are hours after paracetamol injection. C= saline-injected controls. N= negative PCR control. No differences were noted in TNF mRNA expression.

Table 5.5

Treatment	No of cases	Mortality	Serum AST	Hepatic Necrosis		
				+	++	+++
control	16	0	6611 \pm 858	4	8	4
anti-TNF antibody	8	1	9684 \pm 762	0	2	5*
soluble TNFR	12	1	5770 \pm 772	1	3	6*
IL-10	8	1	6137 \pm 884	0	4	4
dexamethasone	10	0	5532 \pm 772	1	5	3*

Table 5.5 Mortality, serum AST and hepatic necrosis after paracetamol overdose. Control = paracetamol only; other treatments as detailed in methods. Serum AST is in IU/l, \pm SEM. * The mice that died all showed >60% hepatic necrosis. No statistically significant differences were noted.

DISCUSSION

In an *in vitro* study of paracetamol toxicity using human hepatocyte cell lines, TNF- α was shown to have paradoxical effects. When administered with paracetamol, TNF- α augmented paracetamol toxicity by increasing apoptosis and necrosis, and also altered hepatocyte proliferation. However, when administered before paracetamol, TNF- α protected against subsequent toxicity. Using an *in vivo* murine model of paracetamol overdose, TNF- α was elevated in serum but not liver and inhibition of TNF- α did not alter liver injury or survival. These experiments offer new insights into the role of TNF- α in liver injury after paracetamol overdose.

Paracetamol Toxicity In Vitro: (1) TNF- α augments hepatocyte death

Most studies of TNF- α in liver injury caused by various toxins have supported a role for TNF- α in causing hepatocyte death by apoptosis and necrosis (reviewed in Bradham et al, 1998; Schumann and Tiegs, 1999). Consequently TNF- α has been implicated in toxic liver injury induced by ethanol, carbon tetrachloride, endotoxin, galactosamine and concanavalin A, where TNF- α contributes directly to liver injury and inhibition of TNF- α reduces injury (Table 5.5) although the situation in paracetamol toxicity is less clear. In this study, TNF- α has a synergistic effect when administered with paracetamol, augmenting toxicity by increasing hepatocyte loss by apoptosis. TNF- α also lowers the threshold for paracetamol toxicity, a TNF-induced effect also noted with other toxins (Pfeffer et al, 1993; Leist et al, 1995). TNF- α can induce cell death by both necrosis and apoptosis in a variety of settings but TNF- α only induces hepatocyte apoptosis after protein or mRNA synthesis has been inhibited (Hill et al, 1992; Leist et al, 1995). However, a complete block of protein synthesis may have an opposing effect, as production of TNF- α would be inhibited and apoptosis may be prevented (Leist et al, 1995; Leist et al, 1997). The synergism of TNF- α and paracetamol may be explained by the ability of paracetamol to inhibit protein synthesis (Miller et al, 1999) thereby sensitising hepatocytes to the toxic effects of TNF- α . In this study, TNF- α had no effect on the rate of necrosis (PI uptake) compared with samples with no TNF- α present.

A unifying pathway has been proposed whereby different toxins may cause liver damage both directly and synergistically with endogenous TNF- α (Figure 5.7). The key element of this hypothesis is that several different pathways may contribute to hepatocyte death and that exogenous toxins may interact with endogenous factors to augment injury. A hepatotoxin may cause direct injury to hepatocytes (1) or sensitise hepatocyte to injury by another agent or cytokine (2). Conversely a toxin that causes hepatic necrosis may induce a brisk inflammatory reaction, with consequent production of TNF- α by macrophages (3). Finally, a toxin may substantially increase translocation of endotoxin from the intestine into the portal blood (4), a strong stimulus to TNF- α production by Kupffer cells (Chensue et al, 1991). However, many of the elements in this model remain unproven as precise mechanisms of sensitisation and the contribution of direct toxicity versus TNF- α -dependent toxicity is not known. The role of endotoxin in hepatotoxicity is discussed further in chapter 6.

Figure 5.7

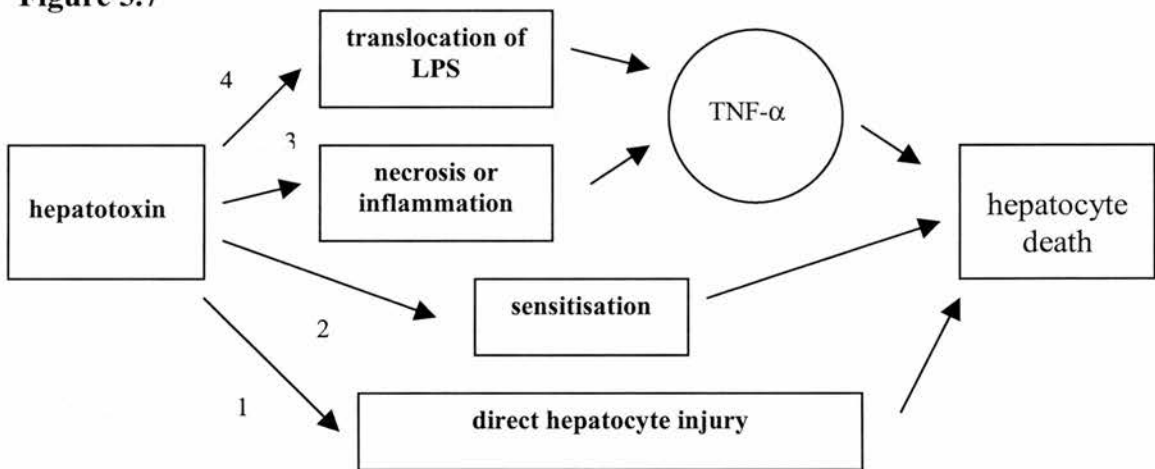


Figure 5.7 Putative pathways of TNF- α involvement in xenobiotic-induced hepatocyte death. (Adapted from Leist et al, 1997). LPS = lipopolysaccharide (endotoxin).

Paracetamol Toxicity In Vitro: (2) TNF- α protects against cell death

Using the in vitro model of paracetamol toxicity, pretreatment with TNF- α had a striking protective effect, with a reduction in cell loss and hepatocyte apoptosis up to

72 hours. However, in the absence of paracetamol, TNF- α treatment had no discernible effect. Pretreatment with TNF- α can protect against subsequent TNF-induced injury and protect against fas-induced caspase activation (Jacob et al, 1990; Hill et al, 1995; Takehara et al, 1998) but this first description of protection against the effects of a toxic drug by TNF- α . Two alternative explanations for the protective effect of TNF- α exist. First, TNF- α may alter CYP450 expression, reducing paracetamol bioactivation and hence decreasing hepatotoxicity. Second, TNF- α may signal cell survival and proliferation after injury, promoting efficient regeneration.

Conflicting data exist regarding alterations in CYP-450 activity induced by TNF- α as a mechanism of modulation of hepatotoxicity after paracetamol overdose. TNF- α may reduce CYP450 activity in mice (Ghezzi et al, 1986) although TNF- α / lymphotoxin- α knockout mice show no difference in CYP2E1 and CYP1A2 activity and no difference in paracetamol bioactivation compared with wild type mice (Boess et al, 1998). Conversely, increased expression of CYP2E1 may sensitise hepatocytes to the cytotoxic effects of TNF- α (Bissell et al, 2001). Several groups have studied the interaction of Kupffer cells and hepatocytes in paracetamol metabolism and toxicity. One problem is that while TNF- α is a major effector molecule, the precise role of TNF- α in these studies is not entirely clear and other factors may also be involved. Two groups have shown a reduction in CYP450-dependent paracetamol metabolism using coculture of Kupffer cells and hepatocytes (Raiford and Thigpen, 1994; Milosevic et al 1999). However, two other groups have clearly demonstrated that the Kupffer cell effect occurs with no alteration in paracetamol metabolism (Laskin et al, 1995; Michael et al, 1999). The balance of evidence suggests that alterations in CYP450 activity and paracetamol bioactivation is unlikely to be an explanation for the effects of TNF- α in paracetamol toxicity but further studies would be helpful in confirming this.

Alternatively, the mechanism whereby TNF- α induces protection may relate to the activation of intracellular survival or proliferation pathways rather than death pathways. TNF- α can induce both NF- κ B (Rothe et al, 1995) and jun kinase (JNK;

Reinhard et al, 1997), both pathways favouring regeneration over apoptosis (Yamada et al, 1997; Taub, 1998) and contributing to regeneration after hepatectomy or toxic injury (Diehl et al, 1994; Taub 1996; Bruccoleri et al, 1997). NF- κ B is of particular interest as inhibition of it converts a TNF- α -induced proliferative response to apoptosis (Xu et al, 1998). NF- κ B functions as an inhibitor of apoptosis (Bellas et al, 1997), suppressing caspase-8 activation (Wang et al, 1998). NF- κ B is normally retained in the cytoplasm bound to its inhibitor I κ B and is activated by a kinase cascade (Israel, 1997); the activating kinase binds TRAF2 and activates I κ B kinase- α which phosphorylates I κ B resulting in its ubiquitination and rapid degradation. NF- κ B then translocates to the nucleus, transcriptionally activating genes which protect against apoptosis (including TRAF1, TRAF2, c-IAP1, c-IAP2, IEX-1L; Wang et al, 1998; Wu et al, 1998). This transcriptional activation may explain why inhibitors of RNA or protein synthesis or dominant negative I κ B (loss of NF- κ B inducibility [van Antwerp et al, 1996]) augment killing by inhibiting the NF- κ B survival signal. This may be one specific pathway interrupted by the inhibition of RNA or protein synthesis in TNF- α -induced cell death. In paracetamol toxicity in mice, a reduction in NF- κ B and NF-IL6 DNA binding was noted associated with an inhibition of hepatocyte proliferation (Blazka et al, 1996; Boulares et al, 1999). Strategies to manipulate NF- κ B may be a valuable approach to therapy as augmentation of NF- κ B may allow survival of cells that would otherwise die.

The protective effect of TNF- α in paracetamol toxicity may be similar to the well-established effects of TNF- α in the regeneration cascade, whereby TNF- α induces transcription of immediate early genes but is not mitogenic itself, allowing proliferation signalled by various growth factors (reviewed in Michalopoulos, 1990; Fausto and Webber, 1994; Fausto et al, 1995; Michalopoulos and DeFrances, 1997). Moreover, a high proliferative capacity may be an important factor in determining survival after liver injury and may correlate with outcome (Calabrese et al, 1993; Mangipudy et al, 1995; Chanda and Mehendale, 1996; Horn et al, 1999). In this model, TNF- α had no effect on mitotic rate or BrdU positivity when added to hepatocytes alone but it altered hepatocyte proliferation when administered with

paracetamol. BrdU positivity remained similar to untreated controls, rather than increasing and subsequently decreasing as noted with paracetamol alone. The mitotic rate remained very low throughout, as with paracetamol alone. Several potential problems in interpretation include the difficulty in scoring BrdU positivity when staining may be weak (discussed in chapter 4) and the high innate proliferative capacity of the cell lines (approximately 98% of cells positive for Ki-67) compared with normal liver, where Ki-67 positivity is less than 1%. The significance of the difference in proliferation with and without TNF- α is not clear, but a tenable suggestion is of a cell cycle arrest in or after S phase with the cycle-arrested cells subsequently dying. The mechanism whereby co-administration of TNF- α and paracetamol abolishes the increase and subsequent decrease of BrdU positivity seen with paracetamol alone is not known. TNF- α may act as a signal to regenerate but requiring other growth factors and cytokines to affect a successful regenerative response. This may also explain some of the apparently contradictory data regarding TNF- α as although it has a central role in liver regeneration, for example, downstream factors such as IL-6 can replace TNF- α in the regeneration cascade after partial hepatectomy (Cressman et al, 1996).

Paracetamol Toxicity In Vitro: (3) Other cytokines and chemokines

IL-1 α and GRO- β had no detectable effect on hepatocyte death or proliferation in the in vitro model of paracetamol toxicity, despite evidence from in vivo studies that both may act as protective factors (Renic et al, 1997; Hogaboam et al, 1999). As with TNF- α , conflicting evidence exists regarding the role of IL-1 α in paracetamol toxicity, where in some studies IL-1 α protected (Renic et al, 1997) and in other studies IL-1 receptor antagonist protected against hepatic injury after POD (Blazka et al, 1995; Blazka et al, 1996). IL-1 β may protect against TNF- α toxicity after sensitisation with galactosamine, associated with production of nitric oxide (Bohlinger et al, 1995). Conflicting evidence exists regarding the role of nitric oxide in paracetamol toxicity, where production of nitric oxide has been shown to either mediate or protect against hepatocyte injury (Kuo et al, 1997; Gardner et al, 1998).

MIP-2, the mouse analogue of human GRO- β , is an important regulator of neutrophil infiltration and abscess formation (Ebe et al, 1999). Evidence has also emerged that the CXC chemokines including MIP-2 may also be important regulators of liver regeneration (Colletti et al, 1996; Colletti et al, 1998; Hogaboam et al, 1999). The striking protective effect of protection against paracetamol toxicity by these agents (Hogaboam et al, 1999) was not replicated using GRO- β in vitro.

Paracetamol toxicity in vivo: (1) the murine model

Administration of an overdose of paracetamol by intraperitoneal injection has been used widely in studies of paracetamol toxicity in mice, as the nature and extent of injury, high mortality and prevention of injury by N-acetyl cysteine is analogous to paracetamol overdose in humans. Mice, hamsters and human are relatively sensitive to paracetamol toxicity, whereas rats are relatively resistant to toxicity (Davis et al, 1974; Coldwell et al, 1976). The variation in species sensitivity to paracetamol relates mainly to the kinetics of glutathione depletion rather than a fundamental difference in toxicity (Davis et al, 1974). One problem with the murine model of hepatotoxicity is the much shorter time-course in mice than in humans (24 hours compared with 72-96 hours in humans), which gives a narrow window of opportunity for therapeutic intervention. While animal models are extremely useful to study drug toxicity, problems will always exist in accurately recapitulating human disease. For example, virtually all human patients will have received N-acetylcysteine in therapy, which has been reported to reduce production of TNF- α by Kupffer cells (Fox et al, 1997).

Paracetamol toxicity in vivo: (2) the role of TNF- α

TNF- α has been shown to have an important role in the pathogenesis of liver injury caused by several toxins including carbon tetrachloride, galactosamine and ethanol (Chamilutrat et al, 1995; Edwards et al, 1993; Mizuhara et al, 1994; Leist et al, 1995; Iimuro et al, 1997; Table 5.6). The role of TNF- α in paracetamol overdose remains controversial with conflicting data published in several studies. Elevation of TNF- α in has been shown in both liver and serum after paracetamol overdose (Blazka et al,

1995; Blazka et al, 1996). However, other groups have reported no elevation in TNF- α in serum (DeVictor et al, 1992) or serum and liver (Boess et al, 1998) after paracetamol poisoning in wild-type mice. The results of this study fall between these other studies, demonstrating elevation of TNF- α in serum but not liver in paracetamol toxicity. The reason for the disparate results is not clear but may relate at least in part to methodological differences in the different studies.

Table 5.6

Toxic agent	Inhibition of TNF	Reference
α -amanitin	Anti-TNF, TNFR-KO	Leist et al, 1997
actinomycin D	Anti-TNF, TNFR-KO	Leist et al, 1997
carbon tetrachloride	sTNFR	Czaja et al, 1995
concanavalin-A	Anti-TNF	Gantner et al, 1995
Endotoxin	Anti-TNF	Beutler et al, 1985
Ethanol	Anti-TNF	Iimuro et al, 1997
galactosamine	TNFR-KO	Leist et al, 1995
TCDD ¹	Anti-TNF	Fan et al, 1997

Table 5.6 Agents for which inhibition of TNF- α reduces liver injury

Inhibition of TNF- α by neutralising antibodies (Anti-TNF), TNF receptor knockout mice (TNFR-KO) or soluble TNF receptor (sTNFR).

¹2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Strategies to investigate the role of TNF- α have included using knockout mice or blocking TNF- α (eg with neutralising antibodies) to study the effect of the absence of TNF- α (Bradham et al, 1998). Blocking TNF- α expression or using anti-TNF antibodies reduces hepatotoxicity in many experimental settings (summarised in table 5.6) although the evidence that inhibition of TNF- α reduces liver injury in paracetamol toxicity is controversial. The methods used in this study have all been previously shown to effectively inhibit TNF- α . The antibody regime used to inhibit TNF- α has been shown to effectively reduce the high levels of TNF- α produced after

injection of lipopolysaccharide (80000pg/ml) to undetectable levels and reduce circulating TNF- α for 16 hours after caecal ligation and puncture (Eskandari et al, 1992). Likewise the soluble TNF receptor chimera and dexamethasone regime inhibit TNF- α after LPS injection (Chensue et al, 1991; Lukacs et al, 1997). All methods therefore should have been highly effective in inhibiting the low levels of TNF- α (30pg/ml) produced in this study.

Several studies have tried to elucidate the role of TNF- α in paracetamol toxicity with widely conflicting results. This study identified no difference in mortality or liver injury when TNF- α was inhibited by several different methods, supporting the findings of Boess et al (1998) that there was no difference in liver injury comparing TNF- α / lymphotoxin- α knockout and wild type mice (Boess et al, 1998), and suggesting that the absence of TNF does not affect liver injury after paracetamol overdose. However, two studies by the same group using neutralising antibodies to inhibit TNF- α have demonstrated a significant reduction in liver injury at 4, 8 and 24 but not 12 hours, 12 hours coinciding with maximal injury (Blazka et al, 1995, 1996). The explanation for the variation in reported data relating both to production of TNF- α and to inhibition / absence of it is not known. Comparison of the different studies is problematic since the studies have utilised different strain, sex and age of mice, different paracetamol administration regimes, different methods of killing of mice, different methods to measure liver injury and different methods to detect TNF- α (summarised in Table 5.7). These factors may explain why widely conflicting data have been published.

All of the in vivo studies have concentrated on the first 24 hours after injury, and no study has looked at later times for example to ascertain how the liver regenerates in the presence or absence of TNF- α . This may be of interest because in the regenerative response after partial hepatectomy in IL-6 knockout mice initial reports suggested *defective* regeneration but later studies confirmed *delayed* regeneration (Cressman et al, 1996; Sakamoto et al, 1999).

Table 5.7

Reference	Mouse Model	Paracetamol Dose	Method of Killing	Parameter of Injury	Detection of TNF- α
DeVictor et al, 1992	Swiss sex ? 10-12 w	1.5g/kg in PBS	cervical dislocation	ALT	Toxicity assay (s)
Boess et al, 1998	C57BL/6 Male 8-12w	400mg/kg in saline pH9.5	methoxy-fluorane	AST, ALT histology	ELISA (s) RT-PCR (l)
Blazka et al, 1995, 1996	B6C3F1 Female 22-28g	500mg/kg in saline pH7.4	CO ₂	AST, ALT, LDH, SDH histology	Toxicity assay (s) RT-PCR (l)
This study	CBA/J Female 6 w	300mg/kg in saline pH7.4	cervical dislocation	AST histology	ELISA (s,l) RT-PCR (l)

Table 5.7 Murine In Vivo Studies of TNF- α in Paracetamol Overdose

? = sex not given in paper, w = weeks, g = grams, s = serum, l = liver.

The balance of evidence suggests that inhibition of TNF- α does not reduce injury or mortality after paracetamol overdose and is therefore unlikely to be useful in therapy to reduce injury. However, this does not exclude a role for TNF- α in paracetamol overdose. TNF- α is not simply a pro-injurious cytokine and is an important factor in priming for regeneration, with little known about early signalling events after severe toxic injury. In this study TNF- α is elevated in serum but not liver, suggesting that the source of TNF- α is extra-hepatic (eg splenic or intestinal macrophages (Suzuki et al, 1996; Bathe et al, 1997)). However, subtle changes in TNF- α expression and production in the liver cannot be excluded. The source of TNF- α has not been identified in most studies of liver injury and regeneration and while it is generally presumed that Kupffer cells are the major source of most cytokines in liver injury, every cell-type within the liver is capable of producing TNF- α .

Given the substantial conflict in the literature regarding the role of TNF- α in paracetamol toxicity, the possibility that TNF- α has no effect on the liver must also be considered. Paracetamol overdose is associated with a hypothermic response

(Walker et al, 1981) closely linked with production of cytokines such as TNF- α and IL-1 α (Fey and Gauldie, 1990; Blazka et al, 1995; Blazka et al, 1996) and TNF- α may signal a systemic response rather than a local one.

Conclusions: What is the role of TNF- α in paracetamol toxicity?

Conflicting evidence exists for the role of TNF- α in paracetamol toxicity. The *in vivo* studies show that TNF- α is elevated in serum after paracetamol overdose but that inhibition of TNF- α does not reduce liver injury. Despite this, evidence from the *in vitro* studies suggests that TNF- α synergises with paracetamol to augment hepatocyte death but protects against toxicity when given before paracetamol. The profound effects on hepatocytes noted *in vitro* may be inhibited or antagonised by the cytokine milieu *in vivo*; neutralising TNF- α represents a crude attempt to eliminate TNF- α *in vivo* and offers little mechanistic insight into how TNF- α might contribute to liver injury or regeneration. Nevertheless it seems unlikely that TNF- α contributes significantly to cell loss after paracetamol overdose. However, a role for TNF- α in hepatic regeneration after injury remains feasible. For example, after carbon tetrachloride poisoning, TNF- α has no direct effect on injury but inhibition of TNF- α delays the time to recovery (Bruccoleri et al, 1997), supporting the suggestion that TNF- α may act as a stimulus to regenerate, rather than as a death-inducing factor, after liver injury. Further evidence that the functional role of TNF- α may be in signalling regeneration is the ability of TNF- α to protect against paracetamol toxicity; NF- κ B is a good candidate to be the central element in this TNF- α pathway and the possibility of manipulating NF- κ B to promote survival over death is intriguing (Xu et al, 1998). A better understanding of the effects of TNF- α in the liver are extremely important in designing therapeutic interventions such as inhibition or augmentation of cytokines and their intracellular signalling pathways.

CHAPTER SIX

THE ROLE OF KUPFFER CELLS IN PARACETAMOL TOXICITY

CHAPTER SIX**THE ROLE OF KUPFFER CELLS IN PARACETAMOL TOXICITY****SUMMARY**

Compelling evidence exists for an active role for Kupffer cells in toxic liver injury, including after paracetamol overdose. This study describes an *in vitro* model of co-culture of a human hepatocyte cell line with primary human macrophages as a surrogate for Kupffer cells. Co-culture had no apparent effect on hepatocyte number, apoptosis or proliferation, with or without paracetamol. However, TNF- α production by macrophages was augmented by paracetamol and was further augmented by co-culture with hepatocytes. The concentration of TNF- α produced was comparable with that of recombinant TNF- α known to augment paracetamol toxicity from previous studies. This suggests that hepatocytes behave differently in co-culture than when grown alone, and that macrophages may condition how hepatocytes respond to exogenous stimuli by production of soluble factors. Kupffer cells are important regulators of toxic injury to hepatocytes.

INTRODUCTION

Kupffer cells represent 80-90% of tissue macrophages in the body and make up 15% of cells within the liver (Kuiper et al, 1994). They are situated within hepatic sinusoids, anchored to the endothelium by cytoplasmic processes and probably have no direct contact with hepatocytes (Bouwens et al, 1986). Kupffer cells have a variety of functions including phagocytosis of particulate foreign material (Pilaro and Laskin, 1986), detoxification of LPS in the portal blood (Mathison and Ulevitch, 1979), acting as antigen-presenting cells (Rogoff and Lipsky, 1980) and releasing a large number of cytokines and inflammatory mediators (Decker, 1990; Laskin, 1990; Laskin and Pendino, 1995). Compelling evidence exists that Kupffer cells have an active role in toxic liver injury (Laskin and Pendino, 1995; Laskin, 1996; Tsukamoto and Lin, 1997).

Administration of xenobiotic agents such as carbon tetrachloride, ethanol, galactosamine and paracetamol results in accumulation of activated macrophages

within the liver (summarised in table 6.1), the site of macrophage accumulation depending on the toxic agent and the site of hepatic injury (Laskin and Pendino, 1995). Macrophage accumulation occurs by a combination of proliferation and recruitment from circulating monocytes (Geerts et al, 1988; Jonker et al, 1990; Johnson et al, 1992) and the macrophages have an activated phenotype (Laskin and Pendino, 1995). Moreover, studies in which Kupffer cells are depleted have shown modulation of hepatic injury: depletion of macrophages by gadolinium chloride, dextran sulphate or diphosphonate reduces hepatic injury secondary to various agents including carbon tetrachloride, ethanol, galactosamine and paracetamol (see table 6.1). Conversely, experimental activation of Kupffer cells has augmented injury in some models (Laskin and Pendino, 1995; Table 6.1).

Table 6.1

Agent	Kupffer cell activation	Activation increases toxicity	Depletion reduces toxicity	Reference
allyl alcohol			yes	Przybocki et al, 1992
ethanol	yes		yes	Adachi et al, 1994; Iimuro et al, 1997
carbon tetrachloride	yes	yes	yes	Johnson et al, 1992; Chamulitrat et al, 1995
dimethyl-nitrosamine	yes		yes	Dobbs et al, 1997
galactosamine	yes	yes	yes	Chojkier and Fierer, 1985; Shiratori et al, 1988; Stachlewitz et al, 1999
lead nitrate			yes	Dini et al, 1997
paracetamol	yes	yes	yes	Laskin and Pilaro, 1986; Pilaro and Laskin, 1986; Laskin, 1990; Raiford and Thigpen, 1994; Laskin et al, 1995; Goldin et al, 1996; Michael et al, 1999; Milosevic et al, 1999

Table 6.1 The role of Kupffer cells in toxic liver injury

Kupffer cell activation - Kupffer cells have an activated phenotype. Activation increases toxicity – Kupffer cells activation increase toxicity caused by the named agent. Depletion reduces toxicity – pharmacological depletion of Kupffer cells decreases toxicity of the agent. The gaps in the table indicate that no data is available. All agents were studied in rats except paracetamol, which has been studied in rats and mice.

In this study an *in vitro* model system has been used to investigate the interactions between Kupffer cells and hepatocytes in paracetamol toxicity. Hepatocytes in culture offer a very useful method of hepatotoxicity as they maintain many normal phenotypic features (Guillouzo et al, 1986). Several co-culture systems have been described using a variety of cell types along with hepatocytes (Corlu et al, 1997; Bhatia et al, 1999). It is evident that cellular interactions are extremely important in maintaining hepatocyte phenotype and may also influence the way in which hepatocytes respond to toxic stimuli (Maher and Friedman, 1993; Auth and Ichihara, 1998; Bhatia et al, 1999).

The **aims** of this study were to investigate the interactions between Kupffer cells and hepatocytes in paracetamol toxicity *in vitro*.

MATERIALS AND METHODS

Murine Macrophages

Murine macrophages were isolated using a method established in this laboratory using a modification of the method for primary culture of murine hepatocytes as described in chapter 4, in which hepatic and splenic macrophages were also isolated from the same mouse (M Dollinger, personal communication). Briefly, the discarded supernatant was retained after the first spin from the hepatocyte perfusion and the mouse spleen was taken, gently injected with digestion medium (appendix 1) and cut into small pieces using sterile scissors. The spleen was then gently passed through a cell strainer and centrifuged at 300g for 10 minutes and the pellet was resuspended in plating medium (Appendix 1). The retained hepatic and splenic perfusion supernatants were gently mixed and layered carefully over lymphoprep (Nycomed) then centrifuged at 300g for 30 minutes. The interface cell population was retained and cells were plated at 5×10^5 cells per culture insert. After 2 hours the supernatant was removed and the plates were washed three times with sterile PBS to remove all cells that were not firmly adherent; the adherent cells represent a population comprising mainly macrophages (M. Dollinger, personal communication). Plating

media supplemented with 10% fetal calf serum was added and cells were washed three times with PBS after 24 hours and cells were then ready for use in experiments.

Human Macrophages

Human macrophages were isolated from buffy coats from healthy blood donors (courtesy of Dr M Moore). The buffy coat (100-130ml) was diluted with sterile PBS to a final volume of 200ml, layered over lymphoprep and centrifuged at 1800rpm for 20 minutes at 4°C. The peripheral blood mononuclear cell (PBMC) layer (interface of plasma and lymphoprep) was carefully removed by pipette, diluted with sterile PBS and centrifuged at 1000rpm for 15 minutes. The supernatant was discarded. The cell pellet was resuspended in PBS, layered over lymphoprep and centrifuged at 1800rpm for 20 minutes. The PBMC were removed, resuspended in PBS and centrifuged at 300g for 10 minutes. The pellet was resuspended in Iscove's medium (Gibco) supplemented with 5% human serum (courtesy of Dr M Moore) and the cells were plated in culture inserts (5×10^5 cells per insert). After two hours the supernatant was removed, the cells were washed twice with PBS and the media replaced. The cells were washed twice more, and were left in media with 5% human serum for 72-96 hours prior to beginning each experiment.

Experimental Design

Cell culture experiments with hepatocytes were carried out as previously described, with either primary murine hepatocytes or a hepatocyte cell line (WRL 68 cells) grown on 12 well tissue culture plates in serum-free conditions (see chapter 4). For co-culture, culture inserts containing macrophages were placed in wells containing hepatocytes so that the cells shared the same culture media but had no direct contact. Macrophage and hepatocyte numbers were estimated using the MTT assay: apoptosis and BrdU incorporation were quantitated by direct counting. Immunohistochemistry was carried out on culture slides or inserts fixed in acetone / methanol (1:1) for 10 minutes and stored in TBS at 4°C for up to 1 week. The antibody used against CD68 (clone PGM-1, Dako; 1:50 dilution) was incubated for 1 hour at room temperature and no pretreatment was required. Statistical analysis was carried out using ANOVA.

RESULTS

Murine Co-Culture

Primary murine hepatocytes showed no change in cell number over a 72 hour culture period both when grown alone and in co-culture with macrophages (Figure 6.1). On the other hand, the macrophages decreased in number over the duration of the experiments, with a significant reduction at 72 hours ($p < 0.05$). When 10mM paracetamol was added, there was a significant reduction in hepatocyte number at 72 hours ($p < 0.05$) but no difference in paracetamol toxicity was noted comparing hepatocytes alone and in co-culture with macrophages (Figure 6.2). No differences in apoptosis, mitosis or BrdU incorporation were identified (not shown).

Figure 6.1

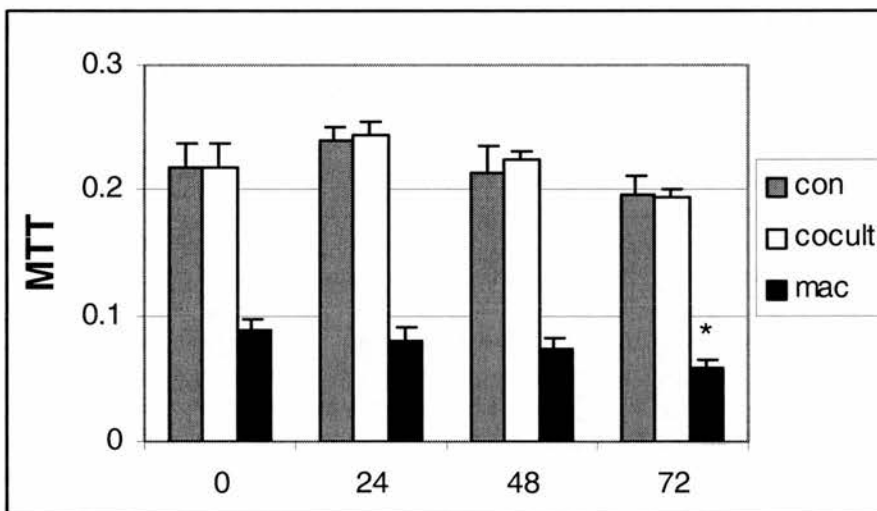


Figure 6.1

Cell number measured by MTT for hepatocytes alone (con), hepatocytes in co-culture with macrophages (cocult) and macrophages alone (mo). The data represent the mean (\pm standard deviation) of four repeat experiments. * = significant reduction compared with the cell number at time 0 ($p < 0.05$).

Figure 6.2

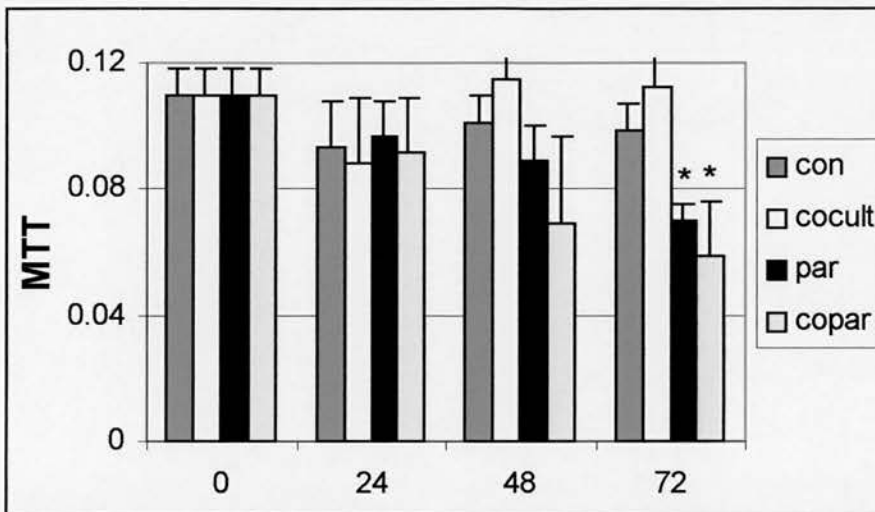


Figure 6.2

Cell number measured by MTT for murine hepatocytes alone (con), hepatocytes in coculture with macrophages (cocult), hepatocytes with 10mM paracetamol (par) and hepatocytes in coculture with paracetamol (copar). The data represent the mean of 3 repeat experiments. * = significant reduction compared with controls ($p < 0.05$).

Isolation of a pure population of human macrophages

The adherent cells isolated from the PBMC showed strong expression of CD68 (clone PGM-1, Dako) in all cells (Figure 6.3). This CD68 clone is specific for the monocyte / macrophage lineage, suggesting that this is a highly efficient method to isolate a pure population of macrophages.

Figure 6.3

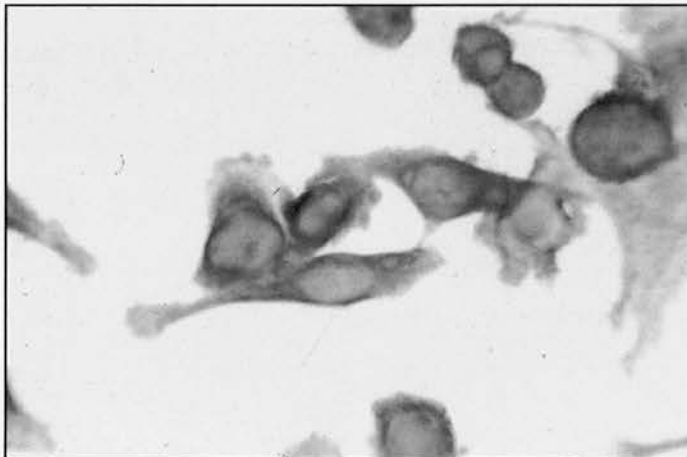


Figure 6.3 Photomicrograph showing CD68 expression by human macrophages grown on culture inserts. All adherent cells showed cytoplasmic staining.

Paracetamol Toxicity in Co-Culture

No differences were noted in cell number comparing hepatocytes alone and hepatocytes in coculture with macrophages when paracetamol was absent or present (Figure 6.4). Similarly, no differences were noted in the rate of apoptosis, mitosis or BrdU positivity, comparing hepatocytes alone or in coculture (not shown). The same results were found when HepG2 cells were substituted for WRL 68 cells, using 10mM paracetamol (not shown). Macrophages showed no change in cell number during the time-course of control experiments but showed a small reduction in cell number after incubation with 5mM paracetamol for 72 hours ($p < 0.05$; Figure 6.5).

Figure 6.4

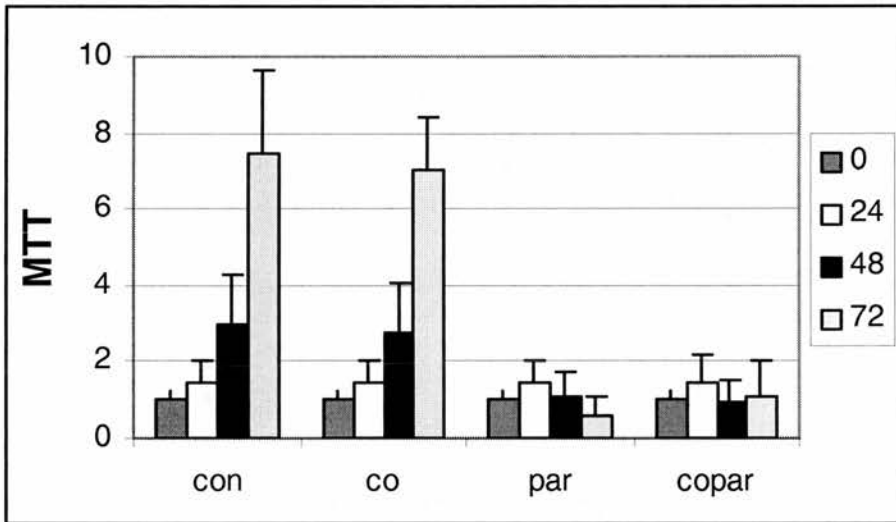


Figure 6.4

Cell number measured by MTT for hepatocytes (WRL 68 cells) alone (con), in co-culture with macrophages (co), hepatocytes with 5mM paracetamol (par) and co-culture with 5mM paracetamol (copar). The data are the mean \pm standard deviation of 4 experiments. There are no differences comparing con and co and comparing par and copar.

Figure 6.5

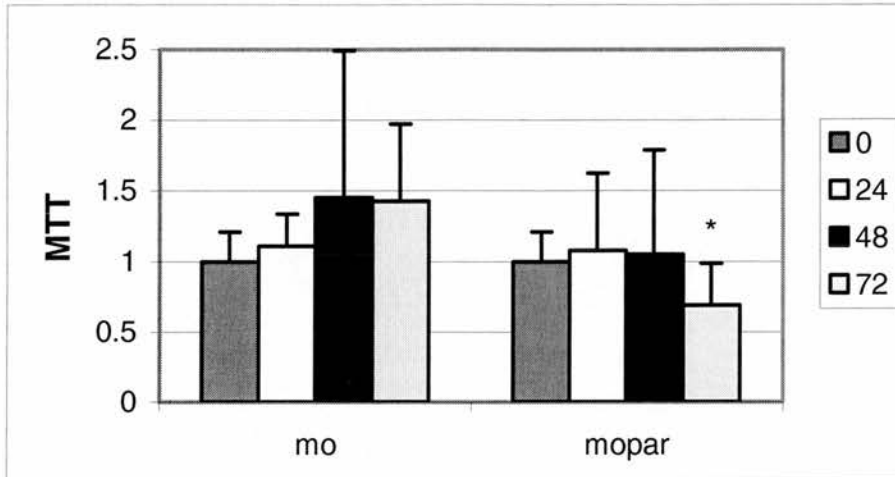


Figure 6.5

Macrophage cell number alone (mo) and with 5mM paracetamol (mopar) up to 72 hours. The data are mean \pm standard deviation for 3 experiments. * = significant reduction compared with controls ($p < 0.05$).

TNF- α production is augmented in co-culture and with paracetamol

Secreted TNF- α was detected in culture supernatants by ELISA. Hepatocytes alone showed no production of TNF- α with or without paracetamol (Table 6.2). Macrophages produced low levels of TNF- α in culture (mean 47pg/ml); on addition of paracetamol, the production of TNF- α was increased threefold by 72 hours (154pg/ml; $p < 0.05$). Co-culture of macrophages and hepatocytes resulted in an increase in TNF- α production at 24, 48 and 72 hours (mean 205pg/ml). When paracetamol was added to the co-cultures, a further substantial increase in TNF- α was noted at 48 and 72 hours (mean 609pg/ml; $p < 0.05$, Table 6.2).

Table 6.2

Group	Time	TNF-α	Range
Hepatocytes	24	0	0
	48	0	0
	72	0	0
Hepatocytes + Paracetamol	24	0	0
	48	0	0
	72	0	0
Macrophages	24	38	± 24
	48	64	± 22
	72	38	± 33
Macrophages + Paracetamol	24	42	± 36
	48	51	± 38
	72	154*	± 59
Co-Culture	24	152**	± 106
	48	229**	± 118
	72	235**	± 124
Co-Culture + Paracetamol	24	297	± 129
	48	654***	± 158
	72	564***	± 61

Table 6.2 TNF- α concentrations in co-culture

TNF- α concentration is in pg/ml (calculated compared with recombinant TNF- α as a standard up to 1000pg/ml) and is based on 3 or 4 observations. The range represents the confidence interval of the mean.

* Significant increase compared with macrophages alone at all time-points and macrophages + paracetamol at 24 and 48 hours ($p < 0.05$).

** Significant increase compared with macrophages alone at all time-points ($p < 0.05$).

*** Significant increase compared with co-culture alone at all time-points and co-culture + paracetamol at 24 hours ($p < 0.05$).

DISCUSSION

TNF- α production by Kupffer cells in co-culture

Co-culture of hepatocytes with macrophages resulted in a striking up-regulation of TNF- α production, which was further increased by addition of a toxic dose of paracetamol. The level of TNF- α produced was comparable with a concentration of recombinant TNF- α known to increase paracetamol hepatotoxicity (≥ 600 pg/ml; see chapter 5) but in the co-culture model no augmentation of toxicity was seen. This indicates that hepatocytes in co-culture behave differently to those grown alone and supports the hypothesis that macrophages (Kupffer cells) can condition the way in which hepatocytes respond to exogenous stimuli.

In this model, any interaction between macrophages and hepatocytes occurs by production of soluble factors as direct cellular contact cannot occur. Detection of TNF- α released by macrophages treated with paracetamol shows levels similar to those reported elsewhere (Goldin et al, 1995) although another study has reported inhibition of TNF- α release from Kupffer cells by paracetamol (Horbach et al, 1997). The paradoxical difference between TNF- α produced in co-culture and addition of recombinant TNF- α is unexplained, but presumably other cytokines including one or more survival factors are being produced in co-culture to antagonise the toxic effects of TNF- α . This has not been further studied but it is possible that the lack of TNF- α effect in co-culture may relate to production of soluble TNF receptors. Proteolytic cleavage of the external domain of TNFR1 and TNFR2 produces soluble TNFR which increase the half-life of TNF- α but blocks interaction with membrane TNFR and therefore act as a TNF antagonist (Van Zee et al, 1992).

The data from this study support the suggestion that macrophages produce TNF- α during paracetamol toxicity but do not rule out the possibility that other cell types produce TNF- α . Furthermore, the suggestion that N-acetyl cysteine suppresses TNF- α production from Kupffer cells may be of relevance in studying the role of TNF- α in after paracetamol overdose in humans (Fox et al, 1997). Macrophages and monocytes are thought to be the most important producers of TNF- α but

hepatocytes, biliary epithelial cells and sinusoidal endothelial cells can also produce TNF- α (Gonzalez-Amaro et al, 1994; Hoffmann et al, 1994; Nagano et al, 1992). After partial hepatectomy in rats the major sites of TNF- α production are the biliary epithelium and endothelial cells of hepatic and portal veins and not Kupffer cells (Loffreda et al, 1997). However, the cellular origins of TNF- α , IL-6 and IL-1 in regeneration and after liver injury are unknown (Taub, 1998). While this study suggests that macrophages produce TNF- α during paracetamol toxicity, it does not rule out the possibility that other cell types produce TNF- α . Furthermore, this data is in direct contrast to that described in an in vivo murine model of paracetamol toxicity (chapter 5), where TNF- α was elevated in serum but there was no elevation of TNF- α RNA or protein in liver.

Kupffer cells and paracetamol toxicity

The findings of this study, that no difference in toxicity is noted between hepatocytes alone or in co-culture with Kupffer cells, is an agreement with observations noted in other studies (Raiford and Thigpen, 1994; Milosevic et al, 1999). However, both these studies primarily addressed changes in CYP450 enzymes in paracetamol toxicity and the data on toxicity is not presented in detail so accurate comparison of extent of hepatocyte loss is not possible.

Three studies have identified a reduction in paracetamol toxicity after blockade of Kupffer cells (Table 6.3). However, the available data show significant variation, with one group showing protection up to 2 hours and no protection after that, one group showing protection at 8 hours and the third protection at 24 hours. Several potential problems in interpretation exist for these studies and comparability is difficult given the disparate experimental protocols (differences in species, sex, dose of paracetamol, method of macrophage depletion, end-points). No study has studied injury at multiple time-points related to outcome (regeneration, maximum severity of liver injury, survival). Gadolinium chloride is more efficient at protecting against toxicity than dextran sulphate, correlated with a greater reduction in macrophage number (Michael et al, 1996) but DMDP which is also efficient at reducing macrophage number (Goldin et al, 1996) appears to offer much shorter protection.

However, macrophage-depleting agents may affect hepatocytes and other non-parenchymal cell types within the liver (Laskin et al, 1995) and so the differences may not be solely dependent on Kupffer cells. Gadolinium chloride is known to cause minor changes in hepatic function (e.g. reduction in bile flow) and may result in activation of Kupffer cells with increased cytokine production (Ruttinger et al, 1996), the significance of which is unknown.

Table 6.3

Reference	Species	Paracetamol dose	Blocking agent	Time-course
Laskin et al, 1995	Rat LEH (m)	800mg/kg	GadCl DexS	24hr*
Goldin et al, 1996	Mouse C57BL/10 (f)	250µg per mouse	DMDP	2hr**
Michael et al, 1999	Mouse B6C3F1 (m)	300mg/kg	GadCl DexS	8hr*

Table 7.4: LEH = Long Evans Hooded rat; m = male, f = female; GadCl = gadolinium chloride; DexS = dextran sulphate; DMDP = dichloromethylene diphosphonate. * protection from toxicity but only one time point studied. ** protection from toxicity up to 2 hours but no protection at 4 hours.

Endotoxin and macrophage activation in hepatotoxicity

Activation of Kupffer cells, leading to increased functional capability including increased phagocytic capacity, increased cytotoxic ability and increased production of bioactive substances, is a fundamental component of Kupffer cell involvement in toxic liver injury (reviewed in Decker, 1990). Several soluble substances can activate Kupffer cells including interferon- γ , endotoxin and TNF- α (Decker, 1990). Endotoxin (lipopolysaccharide) is absorbed from the gut into the portal circulation and is normally detoxified by Kupffer cells blood (Mathison and Ulevitch, 1979; Fox et al, 1989). Endotoxin has an important immunomodulatory role for Kupffer cells (reviewed in Nolan, 1989; Decker, 1990; Laskin 1996; Roth et al, 1997) as increased endotoxin in portal blood leads to recruitment and activation of macrophages in the liver (Pilaro and Laskin, 1986) associated with increased cytokine production

(Chensue et al, 1991; Steinhorn and Cerra, 1997). Elevation of portal endotoxin probably occurs because of increased intestinal permeability to it (Iimuro et al, 1997). Endotoxin is established as an important element in hepatotoxicity induced by various agents including ethanol, carbon tetrachloride and galactosamine (Nolan and Liebowitz, 1978; Bjarnason et al, 1984; Chojkier and Fierer, 1985; Iimuro et al, 1997). In spite of this, no study has investigated the role of endotoxin in paracetamol toxicity.

Methodological aspects and usefulness of co-culture

This study raises intriguing questions regarding the role of Kupffer cells and TNF- α in paracetamol toxicity. A paradox exists where TNF- α alone augments paracetamol toxicity in vitro but in co-culture similar levels of TNF- α have no effect on toxicity. This co-culture system may resemble more closely the situation in vivo, where the balance of evidence suggests that TNF- α does not have a direct role in hepatotoxicity induced by paracetamol in vivo (chapter 5).

This study represents a simple method to study toxic liver injury using a co-culture of hepatocytes and macrophages, and is similar to the approach used to study interactions between Kupffer cells and hepatocytes by several other groups (Goldin et al, 1995; Steinhorn and Cerra, 1997). A critical feature of any model of hepatotoxicity is the ability to maintain differentiated phenotype in culture (Selden et al, 1999). A number of different studies have described to improve hepatocyte survival and phenotype. Methodologies have included use of complex media enriched for hepatotrophic nutrients (Block et al, 1996; Hino et al, 1999), manipulation of extracellular matrix (Bhatia et al, 1999), admixture of different cell types with hepatocytes (Corlu et al, 1997) and two- and three-dimensional culture systems (Bhatia et al, 1999; Michalopoulos et al, 1999). Many issues are unresolved regarding cellular interactions and whether direct contact or paracrine secretion of proteins is more important in determining hepatocyte responses (Shimaoka et al, 1987).

One problem is the difficulty in isolating and growing Kupffer cells *in vitro*. Kupffer cell isolation relies on proteolytic digestion of the liver followed by centrifugal elutriation and result in relatively low yields of cells (Knook et al, 1976; Hespeling et al, 1995; Namieno et al, 1996). Other authors, and this study, have used macrophages derived from peripheral blood (Goldin et al, 1995) as a surrogate for Kupffer cells. It is not clear how well such a system compares with co-culture using Kupffer cells and further studies are required to address this issue.

Cellular interactions in paracetamol toxicity

Other cell types have also been implicated in paracetamol toxicity, mainly relating to microvascular changes in zone 3 in the early stages of liver injury (Lim et al, 1995). Polymorph accumulation and microvascular plugging have been shown to precede development of necrosis in some studies but not others (Jaeschke and Mitchell, 1989; Welty et al, 1993), and the pathogenetic significance of this is not known. However, other studies have shown that inhibition of neutrophils may reduce paracetamol toxicity (Smith et al, 1998). Endothelial cells are more susceptible to paracetamol toxicity than hepatocytes in mice; in one strain paracetamol is directly toxic to endothelial cells, but in another strain it requires activation by hepatocytes, presumably dependent on CYP450 isoforms (DeLeve et al, 1997). A possible role for endothelial cells in paracetamol toxicity has therefore been proposed, where paracetamol-induced injury to endothelial cells may contribute to zone 3 necrosis in the early stages of injury.

CHAPTER 7

GENETIC POLYMORPHISMS IN TUMOUR NECROSIS FACTOR

CHAPTER 7**GENETIC POLYMORPHISMS IN TUMOUR NECROSIS FACTOR****SUMMARY**

Several polymorphisms have been identified in the TNF- α and lymphotoxin- α (TNF- β) genes, where the polymorphic alleles are associated with the level of TNF- α production and are associated with important disease. Polymorphisms in TNF- α and TNF- β characterised by *NcoI* restriction sites in one allele were identified by PCR, *NcoI* digestion and gel electrophoresis. The TNF- α and TNF- β genotypes had no association with severity of encephalopathy. However, the TNF β (B1B1) phenotype showed a strong association with a high rate of hepatocyte apoptosis ($p=0.011$). The B1B1 genotype is associated with lower levels of circulating TNF- α , and also has been linked with less frequent progression to severe encephalopathy after paracetamol overdose. TNF- α may play an important role in paracetamol toxicity but may not simply act as a mediator of hepatocyte death.

INTRODUCTION

Evidence that TNF- α may play a role in paracetamol toxicity has been discussed in previous chapters. In summary, TNF- α may influence hepatocyte death by apoptosis and hepatocyte proliferation, although the precise role of TNF- α in paracetamol toxicity is not known. However, TNF- α has a role in the pathogenesis of many infectious and inflammatory diseases in which an association between circulating TNF- α and disease activity has been suggested. For example TNF- α is involved in the pathogenesis of several autoimmune diseases (reviewed in Wilson et al, 1995) and elevated TNF- α correlates with poor outcome in sepsis of various causes (Waage et al, 1989; Dofferhoff et al, 1992; Stuber et al, 1998). Significant inter-individual variation exists in TNF- α levels and polymorphisms in the TNF- α and lymphotoxin- α (TNF- β) genes may be an important factor in determining this variability (Pociot et al, 1993; Louis et al, 1998; Stuber et al, 1998). Genetic polymorphisms in TNF genes are associated with leishmaniasis, leprosy and a poor prognosis in cerebral

malaria (Cabrera et al, 1995; Roy et al, 1997; McGuire et al, 1994) and have also been associated with Crohn's disease (Louis et al, 1996).

The TNF- α and TNF- β genes are located on chromosome 6 in the class 3 region of the major histocompatibility complex (MHC; Bodmer et al, 1990). Two important features of the MHC are that several of the MHC genes are in linkage disequilibrium and that many polymorphic sites exist (Wilson et al, 1995). As a result, high levels of TNF- α are frequently noted in individuals with HLA-DR3 or -DR4 and low levels of TNF- α noted with HLA-DR2, suggesting polymorphism in genes regulating TNF- α expression (Pociot et al, 1993). Several polymorphic sites have been identified on both the TNF- α and TNF- β genes (reviewed in Wilson et al, 1995). Common polymorphisms include the -308 position in the TNF- α gene, where adenosine is substituted for guanine in the uncommon allele (TNF2; Wilson et al, 1992), and the *Nco*I restriction fragment length polymorphism (RFLP) in TNF- β where the uncommon allele has a single base substitution in intron 1 (Messer et al, 1991). TNF2 (the uncommon allele) is a stronger transcriptional activator than TNF1 and TNF2 is associated with an increase in serum TNF- α (Wilson et al, 1997). Likewise the *Nco*I polymorphic site in the first intron of TNF- β (uncommon allele B2) is associated with increased levels of TNF- α after stimulation by endotoxin (Louis et al, 1998) although the mechanism whereby TNF- α is increased is not known. The TNFB1B1 phenotype has been associated with elevated TNF- β and may have no effect on or reduce the level of TNF- α (Messer et al, 1991; Pociot et al, 1993). In summary, the association between TNF genotype and TNF- α level in serum complex and has not been fully elucidated.

The aims of this study were to investigate TNF polymorphisms after paracetamol overdose and determine whether the TNF genotype was associated with various pathological parameters in the liver.

MATERIALS AND METHODS

Patients: This study was carried out with the approval of the local ethical committee and used the same patient group as described in chapter 2. Briefly, all patients presenting to the Scottish Liver Transplant Unit (SLTU) with fulminant hepatic failure (FHF) from 1991 – 1997 were identified. Patients were included in the study when tissue blocks were available. Cases secondary to paracetamol overdose (POD) were identified as the study group. Controls were a group of healthy adult blood donors with no history of paracetamol ingestion.

Clinical Parameters: A detailed database is maintained of all patients presenting to the SLTU in fulminant liver failure so extensive clinical information was available (see below). Pathological information was available as detailed in chapter 2 and below.

DNA extraction and PCR: Briefly TNF- α has two alleles, a common allele (1; G at position -308) and an uncommon allele (2; A at position -308). The common allele (1) lacks a *NcoI* cleavage site present in the uncommon allele (2) and the different genotypes can be easily identified by gel electrophoresis. Likewise the polymorphism in the first intron of TNF- β can be identified by the presence of an *NcoI* restriction site in the common allele (B1) but not the uncommon allele (B2; see Table 7.3). The PCR strategy was to amplify polymorphic regions in TNF- α (position -308 in the promoter region) and TNF- β (a base-pair polymorphism in intron 1).

DNA was extracted from 15 μ m sections using proteinase K digestion (Appendix 7). The PCR mix comprised 1.25 units red hot DNA polymerase (AbGene) in reaction buffer, 200 μ M of each dNTP, 50ng of each primer (Table 7.1) and 1.5mM MgCl₂. The PCR reaction was carried out in a Hybaid thermal cycler as detailed in Table 7.2. 10 μ l of each PCR product was digested with *NcoI* restriction enzyme, and the products and restriction digests were separated on a 12% polyacrylamide gel and visualised after ethidium bromide staining to allow determination of different

genotypes (Table 7.3; Figure 7.1). The allele nomenclature has been well-described (Wilson et al, 1992; Louis et al, 1998; Park et al, 1998; Bernal et al, 1998).

Table 7.1

Primers		Notes
TNF- α	TCC TCC CTG CTC CGA TTC CG	Primers supplied by Cruachem (Bearsden) based on sequences originally described in Wilson et al, 1992
	AGG CAA TAG GTT TTG AGG GCC AT	
TNF- β	TGT CTC TGA CTC TCC ATC TGT C	Primers supplied by Gibco-BRL
	AGA AAC CCC AAG GTG AGC AGA G	

Table 7.1 Primers for TNF- α and TNF- β

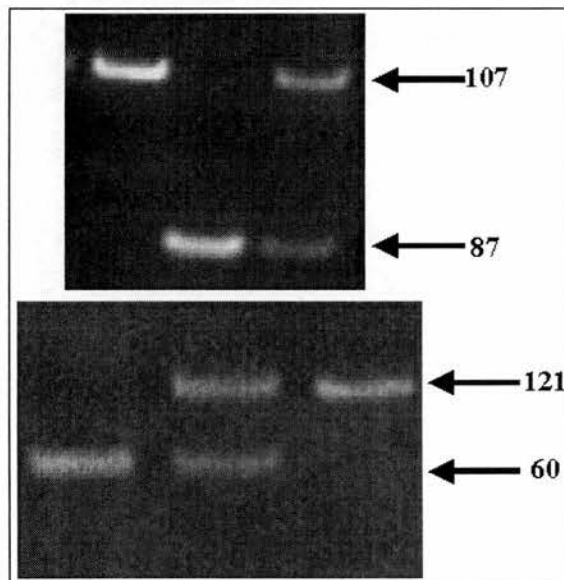
Table 7.2

PCR profile	
TNF- α	denaturation at 94°C for 3 min 38 cycles of 60°C for 1 min, 72°C for 1 min, 94°C for 1 min final extension at 72°C for 10 min
TNF- β	denaturation at 94°C for 3 min 38 cycles of 63°C for 1 min, 72°C for 1 min, 94°C for 1 min final extension at 72°C for 10 min

Table 7.2 PCR profile for TNF- α and TNF- β

Table 7.3

TNF- α	Genotype	11	12	22
	PCR product (bp)	107	107	107
	<i>NcoI</i> digest (bp)	107	107 87 20	87 20
TNF- β	Genotype	B1B1	B1B2	B2B2
	PCR product (bp)	121	121	121
	<i>NcoI</i> digest (bp)	61 60	121 61 60	121

Table 7.3 Nomenclature for different for TNF- α and TNF- β genotypes after *NcoI* digestion**Figure 7.1****Figure 7.1** Gel photograph for TNF- α (top) and TNF- β (bottom) demonstrating the typical bands identified.

Statistical Analysis

After advice on statistical advice from Mandy Lee, Department of Medical Statistics, University of Edinburgh, data were analysed using Kruskal-Wallis test (MedCalc, Belgium) with correction for multiple testing.

RESULTS

TNF Genotypes

Genotyping for TNF- α was successful in 47 cases (69%) and for TNF- β in 38 cases (56%) of paracetamol overdose. All control cases were successfully genotyped. The genotypes are given in Table 7.4.

Table 7.4

	TNF- α Genotype			TNF- β Genotype		
	11	12	22	B1B1	B1B2	B2B2
POD	55	36	9	29	32	39
control	66	29	5	10	44	46

Table 7.4 TNF- α and TNF- β genotypes

% of cases of each genotype - the nomenclature is described in the methods and is summarised in table 7.3. TNF- α n=47; TNF- β n=38.

TNF Genotype and Paracetamol Overdose

Detailed analysis revealed no association between any of the TNF- α and TNF- β genotypes and the various clinical parameters (Table 7.5). Analysis of the pathological factors revealed a significant association between the TNF- β genotype with apoptotic rate ($p=0.011$). Plotting the apoptotic rate for B1B1, B1B2 and B2B2 genotypes revealed a significantly higher rate of apoptosis in B1B1 than B1B2 and B2B2 (Figure 7.2).

Table 7.5

Parameter	TNF- α	TNF β
encephalopathy ¹	0.332	0.083
necrosis ²	0.094	0.209
apoptosis ³	0.280	0.011**
ki-67 positivity ⁴	0.432	0.745
death ⁵	0.192	0.985
liver transplantation	0.410	0.245

Table 7.5 TNF- α and TNF β genotype and paracetamol overdose

The data presented are the p values using the Kruskal-Wallis test – significant values in bold ($p < 0.05$). ¹ encephalopathy: grade of encephalopathy. ² necrosis: panacinar vs. severe necrosis (see chapter 2). ³ apoptosis: % of apoptotic hepatocytes – **association of B1B1 genotype and a higher apoptotic rate. ⁴ Ki-67 positivity: % of Ki-67 positive hepatocytes. ⁵ death without liver transplant being carried out.

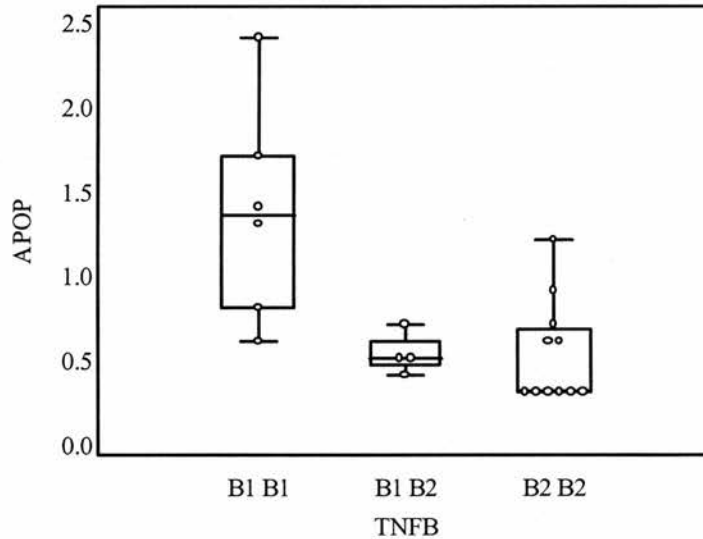
Figure 7.2

Figure 7.2 Apoptotic index plotted against TNF β genotype. The bars represent median, upper and lower quartiles and maximum / minimum values. B1B1 n= 6 ; B1B2 n= 5; B2B2 n= 11.

DISCUSSION

Association of TNF genotype and paracetamol overdose

The genotypes from this study show some differences from those reported elsewhere (Table 7.6) with an increased proportion of TNF- α 22 genotype in both paracetamol overdose cases and controls and an increase in TNF- β B1B1 genotype in cases of

paracetamol overdose. When looking simply at the allele frequency (Table 7.6), there is a very slight increase in the TNF- α 2 allele in controls compared with other studies, with a much higher frequency of the 2 allele in patients who have taken a paracetamol overdose. For TNF- β , the controls showed a similar allele frequency to that described elsewhere, but the TNFB1 allele was much more commonly identified after paracetamol overdose. The reasons for the difference in genotype distribution and allele frequency for TNF- α 2 and TNF- β 1 are not known. Possible explanations include the different populations studied; this study was in a Scottish population where other studies were carried out in London, Belgium, Germany and Korea. A further possible explanation for the differences in the POD cases is that in this study is the use of formalin-fixed blocks as a source of DNA for POD cases where the controls and all the other studies have used whole blood, a more reliable source of DNA; DNA extraction and PCR was unsuccessful in a significant proportion of POD cases (31% for TNF- α , 44% for TNF- β) which may have skewed the results.

Table 7.6a

Reference	Patient group	TNF- α genotype (%)			Allele frequency	
		11	12	22	1	2
Bernal et al, 1998a	Normal	67	32	1	0.83	0.17
	paracetamol overdose	65	33	2	0.92	0.18
Park et al, 1998	Normal	77	22	1	0.88	0.12
	colorectal carcinoma	82	17	1	0.91	0.09
Louis et al, 1998	Normal	72	28	0	0.86	0.14
This study	Normal	66	29	5	0.80	0.20
	Paracetamol overdose	55	36	9	0.74	0.36

Reference	Patient group	TNF- β genotype (%)			Allele frequency	
		B1B1	B1B2	B2B2	B1	B2
Stuber et al, 1996 ¹	Sepsis	10	48	42	0.35	0.65
Bernal et al. 1998a ²	Normal	11	47	42	0.34	0.66
	paracetamol overdose	10	46	43	0.34	0.66
Park et al, 1998 ³	Normal	10	45	45	0.32	0.68
	colorectal carcinoma*	18	52	29	0.44	0.56
This study	Normal	10	44	46	0.32	0.68
	Paracetamol overdose	29	32	39	0.45	0.55

Table 7.6 Studies of genetic polymorphisms in TNF- α and TNF- β .

¹ The study group was patients suffering severe sepsis of culture-proven infectious aetiology

² The study compared normal controls with patients who had taken a paracetamol overdose

³ The study compared normal controls with patients with colorectal carcinoma.

* A significant increase in allele B1 was found in this study

TNF genotype and hepatocyte apoptosis

While there are undoubted problems in the interpretation of studies associating genetic polymorphisms with complex disease (see next section) this study has identified an interesting association between the TNF B1B1 genotype and a higher apoptotic rate after paracetamol overdose. The explanation for this association is not known. One possibility is the known association of TNF genotype and circulating TNF- α . The TNFB1B1 genotype may be associated with normal or low levels of circulating TNF- α (Messer et al, 1991; Pociot et al, 1993), and may suggest that lower levels of circulating TNF- α are associated with higher rates of apoptosis. Conversely the higher levels of TNF- α in heterozygotes or B2B2 homozygotes may protect against apoptosis. The role of TNF- α in paracetamol toxicity remains controversial, as TNF- α augments or protects against hepatotoxicity in vitro depending when it is administered and in vivo studies suggest that TNF- α is not directly involved in hepatocyte death. This study once again suggests that TNF- α may be protective or pro-regenerative rather than pro-injurious after paracetamol overdose. The TNF β B1B1 genotype has been previously shown to be associated with less frequent progression to severe encephalopathy although that was not reproduced in this study.

Problems in interpreting genetic polymorphism studies

Recent conflicting studies of genetic polymorphisms and complex diseases have led to the criticism that this approach to study disease is flawed (Wilhelmsen, 1997; Nature Genetics, 1999; Todd, 1999; Gambaro et al, 2000). Many of the criticisms are valid, and general principles have been proposed that such studies require large sample sizes (>1000 cases, >1000 controls), small p values on statistical testing, biologically sensible associations between genotype and disease and a physiological understanding of how polymorphic alleles function. This study fulfils some, but not all of these criteria. The genetics of the TNF loci are well-characterised, with well-defined physiological effects on the serum concentration of TNF- α , the p values are small, and the biological logic that TNF- α may alter hepatocyte apoptosis is irrefutable. However, the sample size is much smaller than the suggested one, but

the number of cases where tissue is available limits the size. Furthermore, PCR was not successful in every case. The controls group is also imperfect, being normal blood donors rather than a more relevant group such as patients who did not die after paracetamol overdose. Nevertheless, despite some shortcomings this study has identified a very interesting association between the TNFB1B1 genotype and a lower rate of hepatocyte apoptosis after paracetamol overdose. While the significance of this is at present unclear, this study represents a potentially important contribution to the study of TNF- α and liver injury and regeneration.

CHAPTER EIGHT

SUMMARY AND CONCLUSIONS

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SUMMARY AND CONCLUSIONS

The problems of paracetamol overdose

Paracetamol overdose remains a pressing clinical problem as the commonest cause of fulminant liver failure in the UK. Drugs that replenish glutathione, such as N-acetyl cysteine or methionine, are effective in preventing severe liver injury and liver failure if given within 16 hours of overdose and have proved the value of understanding the pathogenesis of an injury to develop an effective cure.

Necrosis, apoptosis and regeneration in paracetamol overdose

The major pathological change after paracetamol overdose is severe hepatic necrosis. However, the extent of necrosis is not the only factor determining outcome and increasing evidence suggests that hepatocyte apoptosis may also occur in murine (Ray et al, 1996) and human liver (chapters 2 and 3). Two groups of patients were studied. Patients who die following coproxamol overdose usually do so because of opiate poisoning and do not develop features of paracetamol hepatotoxicity. However, in this study, hepatic necrosis was seen in 9% of cases and hepatocyte apoptosis was seen in 26% of cases of coproxamol overdose but were not seen in patients dying because of methadone (opiate only) overdose. These findings suggest that apoptosis may occur as an early event, possibly preceding necrosis as has been suggested in mice (Ray et al, 1996). Further studies in patients dying after paracetamol overdose revealed ongoing hepatocyte apoptosis at time of death or liver transplantation, and in the presence of striking regeneration. The apoptosis in this situation is important for three reasons. First, at this time paracetamol is undetectable in blood, suggesting that apoptosis may occur as a direct effect of paracetamol. Second, apoptosis occurs despite striking regenerative activity, raising the possibility that these two conflicting processes may be associated. Third, apoptosis occurs at low rates but may nevertheless indicate a significant attritional loss of hepatocytes at a critical time i.e. when necrosis is maximal. The mechanistic association between apoptosis and proliferation is not known, but the possibility that they relate to

Kupffer cell activation and the cytokine milieu in the injured and regenerating liver has been raised.

Models of paracetamol toxicity

An *in vitro* model of paracetamol hepatotoxicity was developed to further study apoptosis and regeneration. In this model, using murine or human hepatocytes, paracetamol caused dose-dependent hepatocyte death by necrosis and apoptosis (chapter 4). Human cell lines represented a much more reproducible model of toxic than primary murine cells. Apoptosis occurred at significant rates, suggesting that apoptotic cell loss is an important contributor to hepatic injury. However, the intrinsically high rate of proliferation in the cell lines made analysis of regeneration problematic.

The role of TNF- α in paracetamol toxicity

TNF- α has been implicated in hepatotoxicity caused by many different toxins, in most causes potentiating hepatocyte death by apoptosis. However, in paracetamol toxicity, conflicting data exist regarding the role of TNF- α . An *in vivo* study in mice (chapter 4) showed that TNF- α is elevated in serum after paracetamol overdose but that inhibition of TNF- α does not reduce injury or improve the outcome. Using an *in vitro* model with human hepatocytes (chapter 6), TNF- α augmented paracetamol toxicity, increasing rates of both necrosis and apoptosis. However, pretreatment with TNF- α protected against paracetamol toxicity, reducing subsequent paracetamol-induced apoptosis. Studies genetic polymorphisms in TNF genes (chapter 8) were problematic, given small numbers of patients but an interesting observation was an association of the B1B1 phenotype (associated with lower levels of TNF- α) with high rates of hepatocytes apoptosis.

The findings regarding TNF- α are conflicting and complex. The balance of evidence suggests that despite augmenting paracetamol toxicity *in vitro*, TNF- α is not a critical mediator of toxicity *in vivo*. However, TNF- α may have a different role in paracetamol toxicity, acting to signal regeneration or protect against injury. The mechanisms by which TNF- α acts are not clearly defined, and further study is

required. Many questions remain regarding the role of TNF- α in toxic liver injury, and specifically after paracetamol overdose but studies to address late time-points after overdose and hepatic regeneration and activation of hepatic survival signals such as NF- κ B activation in particular may help ascertain the role of TNF- α in toxic liver injury. Furthermore, the source of TNF- α has not been identified in most studies of liver injury and regeneration. While it is generally presumed that Kupffer cells are the source of most cytokines in liver injury, every cell-type within the liver is capable of producing TNF- α . Furthermore, macrophages in the spleen or intestine may produce high levels of TNF- α (Suzuki et al, 1996; Bathe et al, 1997). The particular difficulties of human studies must also be addressed, such as the effects of N-acetylcysteine therapy (Fox et al, 1997). Studies in other cell types have shown that paracetamol protects against cytotoxicity and apoptosis induced by non-steroidal drugs (Romano et al, 1988; Schonberg and Skorpen, 1997), suggesting that the effect of paracetamol may be cell type and context dependent.

Cellular interactions in paracetamol toxicity

Co-culture of hepatocytes with Kupffer cells allowed investigation of the cellular interactions in paracetamol toxicity. Compelling evidence exists for an active role for Kupffer cells in toxic liver injury, including after paracetamol overdose. In chapter 6, an in vitro model of co-culture of a human hepatocyte cell line with primary human macrophages revealed no apparent effect on hepatocyte number, apoptosis or proliferation, with or without paracetamol. However, TNF- α production by macrophages was augmented by paracetamol and was further augmented by co-culture with hepatocytes. The concentration of TNF- α produced was comparable with that of recombinant TNF- α known to augment paracetamol toxicity from previous studies. This suggests that hepatocytes behave differently in co-culture than when grown alone, and that macrophages may condition how hepatocytes respond to exogenous stimuli by production of soluble factors and suggests that Kupffer cells are important regulators of toxic injury to hepatocytes.

Macrophages and monocytes (but mainly Kupffer cells) are thought to be the most important producers of TNF- α but TNF- α can also be produced by hepatocytes,

biliary epithelial cells and sinusoidal endothelial cells (Hunt et al, 1992; Gonzalez-Amaro et al, 1994; Hoffmann et al, 1994; Nagano et al, 1992; Loffreda et al, 1997). However, the cellular origins of TNF- α , IL-6 and IL-1 in regeneration and after liver injury are unknown (Taub, 1998). These studies support the suggestion that macrophages, within or outwith the liver, produce TNF- α during paracetamol toxicity but do not rule out the possibility that TNF- α is not produced by other cell types. Further studies of Kupffer cell activation and patterns of cytokine and chemokines production during liver injury and subsequent regeneration would be of great interest to further delineate the role of Kupffer cells in these processes.

Conclusions

This work challenges the conventional approach to paracetamol toxicity as a simple case of boring liver necrosis. Several apparent paradoxes have been identified:

1. Ongoing hepatocyte apoptosis occurs during regeneration after paracetamol overdose
2. TNF- α has complex effects and can augment or protect against hepatocyte apoptosis depending on the timing of administration
3. Kupffer cells produce TNF- α at levels that should cause hepatocyte apoptosis but condition the hepatocyte response so that apoptosis is not noted.

The situation after paracetamol overdose is extremely complex and requires further study in a variety of areas. In terms of developing new therapies to improve the outcome, the data regarding cytokines and chemokines in controlling liver regeneration is intriguing. Utilisation and augmentation of the liver's regenerative capacity is an exciting prospect in therapy of acute liver failure (Kay and Fausto, 1997). The protective effects and pro-regenerative capacity in ELR-CXC chemokines is of interest as a potential therapeutic tool in toxic liver injury although further studies are required (Hogaboam et al, 1999).

Paracetamol toxicity – the future

While improved drug safety information and advances in packaging to limit availability of paracetamol may help in reducing overdoses, the effects have been

limited (Fagan and Wannan, 1996; Hawton et al, 1996) although recent data suggest that this approach has been increasingly successful (BMJ, 2001). Combination of methionine with paracetamol to completely prevent liver injury is possible but is relatively expensive, requires all persons taking paracetamol to take for most an unnecessary additional compound and may introduce side-effects where paracetamol alone has none at recommended dosage (Bray et al, 1993). A better understanding of paracetamol toxicity may lead to new therapies including additional methods to replenish glutathione, and administration of other potentially protective agents such as prostaglandin E2 or antioxidants (Farrell, 1994; Fry and Seeff, 1995). Most studies concentrate on prevention of significant liver injury and tend to ignore the group of patients who develop liver injury, often presenting after the time at which current antidotes work. This important group of patients has a high mortality and few therapeutic options apart from liver transplantation are limited (Peleman et al, 1988; O'Grady et al, 1989). Indirect approaches such as hepatocyte transplantation after genetic manipulation of donor hepatocytes to increase hepatocyte numbers may be useful (Kobayashi et al, 2000; Hagmann, 2000), but this does not specifically address the pathogenesis of liver injury after paracetamol overdose.

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APPENDIX

CONTENTS

1. Murine liver perfusion and culture
 2. Serum-free and supplemented culture of murine hepatocytes
 3. Culture conditions for HepG2 and WRL 68 cells
 4. Validation of MTT as a measure of cell number in hepatocyte cell lines
 5. Feulgen's method
 6. Running mean for scoring cell counts
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-

APPENDIX 1

MURINE LIVER PERFUSION AND CULTURE

Perfusion Medium (calcium-free, containing EDTA) was purchased from Gibco-BRL (Paisley) and supplemented with gentamicin (1 μ g/ml). The medium was pre-warmed to 37°C and oxygenated immediately prior to infusion.

Digestion Medium was made up fresh each fortnight.

Stock:	L15 medium (Sigma)	
	Gentamicin	1 μ g/ml
	L-glutamine	200mM
	Dexamethasone	18ng/ml
	Insulin	1 μ g/ml
	HEPES	20ml
	NaOH	3ml

Before perfusion 75mg collagenase (Sigma) and 5mg DNase I (Sigma) were added to 20ml of the stock and filter sterilised. The solution was made up to 100ml and pre-warmed to 37°C prior to infusion.

Plating Medium

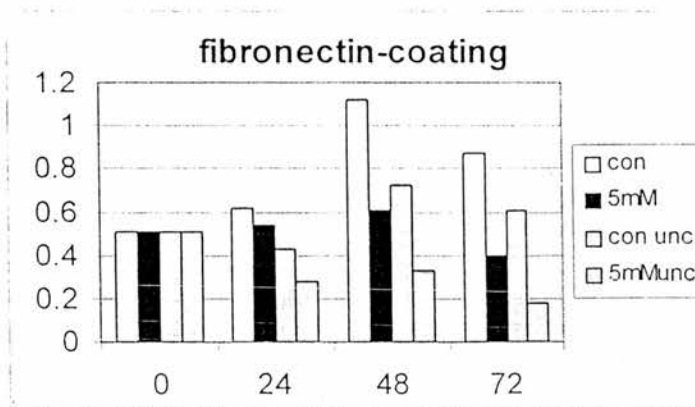
DMEM F12 (Sigma)	
Gentamicin	1 μ g/ml
L-glutamine	200mM
Dexamethasone	18ng/ml
Insulin	1 μ g/ml
pH 7.4	

Culture Medium

Modified Chee's (Gibco)	
Gentamicin	1 μ g/ml
L-glutamine	200mM
Dexamethasone	18ng/ml
Insulin	1 μ g/ml
EGF	12.5 μ g/ml

Fibronectin-coating of cultureware

Fibronectin (Sigma; 1mg/ml) was diluted 1:100 in sterile PBS, added in sufficient volume to cover the bottom of the plate / slide and left at 4°C overnight. Any excess fibronectin solution was aspirated and the coated plates were stored at 4°C for up to one week. Pilot experiments were carried out to assess whether fibronectin-coating made any difference to cell number, with and without paracetamol (Figure 1).

Figure 1

Comparison of cells grown on slides coated with fibronectin in media only (con) or with 5mM paracetamol. Comparison with same groups grown on uncoated slides (unc).

Cell numbers are lower on uncoated slides than on those coated with fibronectin. For all subsequent experiments, all groups utilised fibronectin-coated slides.

APPENDIX 2A SERUM-FREE CULTURE OF MURINE HEPATOCYTES

A variety of different culture media have been used for primary rodent hepatocyte culture. Appropriate supplements added (see appendix 1 – culture media) as previously developed in this laboratory, and five different media were compared (Figure 1).

Figure 1

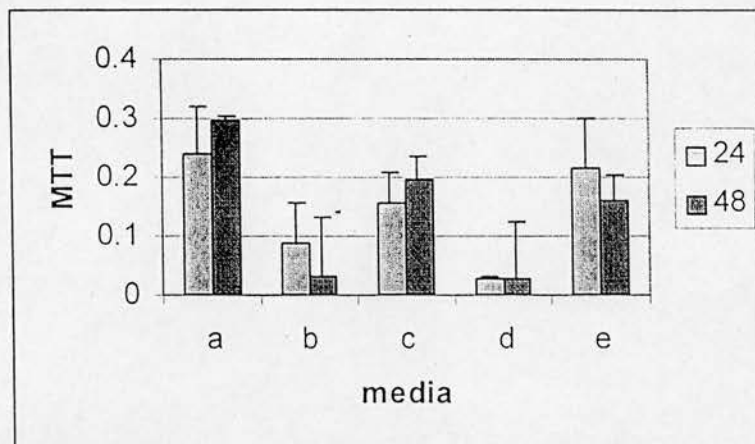


Figure 1

Primary murine hepatocytes plated and grown for 48 hours. a = modified Chee's. b = RPMI (Gibco). c = William's E (Gibco). d = DMEM F12. e = DMEM. Each bar represents four experiments; the error bars are 2 standard deviations.

Conclusions

After 48 hours, significantly more hepatocytes were present in Chee's media than in any other medium ($p < 0.05$). Chee's was therefore used for all subsequent experiments with murine hepatocytes.

APPENDIX 2B SUPPLEMENTED CULTURE OF PRIMARY MURINE HEPATOCYTES

Primary murine hepatocyte culture has been developed in this department over several years (C. Bellamy, personal communication) in a wide range of studies with certain supplements added to allow hepatocyte growth and survival in serum-free conditions. Several experiments were carried out to assess the value of these different supplements for use in the study of paracetamol toxicity in this study, illustrated in Figure 2A and B.

Figure 2A

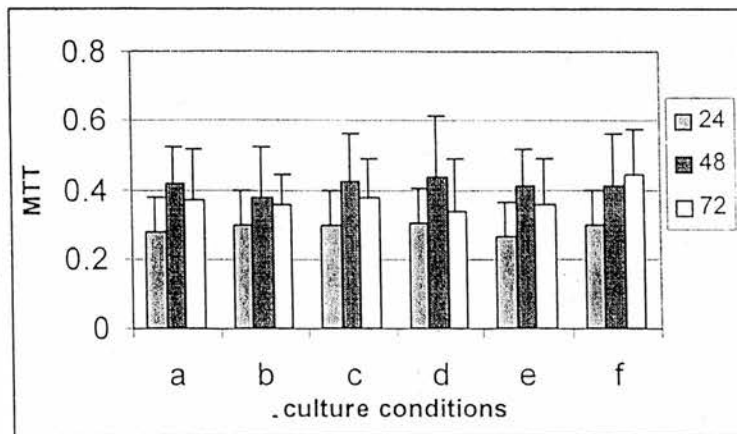


Figure 2B

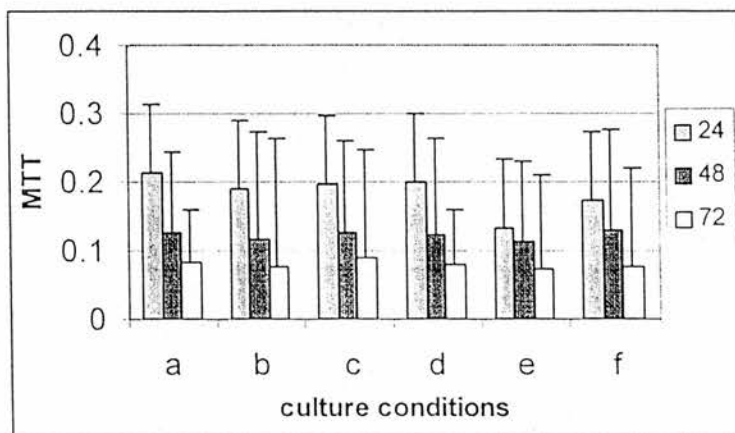


Figure 2

2A = primary hepatocytes. 2B = primary hepatocyte with 10mM paracetamol
 a = media only (modified Chee's; supplemented with L-glutamine and antibiotics). b = media plus ITS (Sigma: insulin, transferrin, selenium). c = media plus dexamethasone (18ng/ml). d = media plus ITS and dexamethasone. e = media plus ITS, dexamethasone and EGF (12.5 μ g/ml). f = media plus EGF. Each experiment represents four repeats; error bars represent 2 standard deviations.

Conclusions

No differences were noted comparing the different culture conditions, both with hepatocytes alone and in the presence of paracetamol. Experiments were therefore carried out using Chee's media only.

APPENDIX 3 CULTURE CONDITIONS FOR HEPG2 AND WRL 68 CELLS

Various culture supplements, used in a variety of studies of hepatocyte growth in the literature were tested as shown below for WRL 68 and HepG2 cells.

Figure 3.1 (WRL 68)

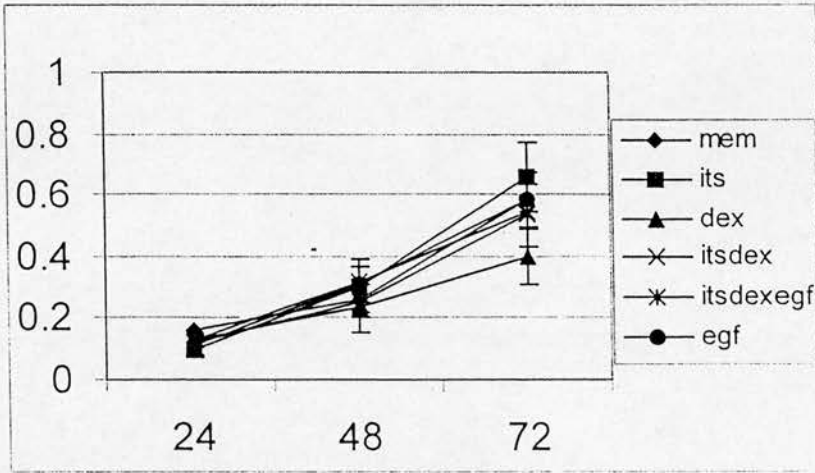
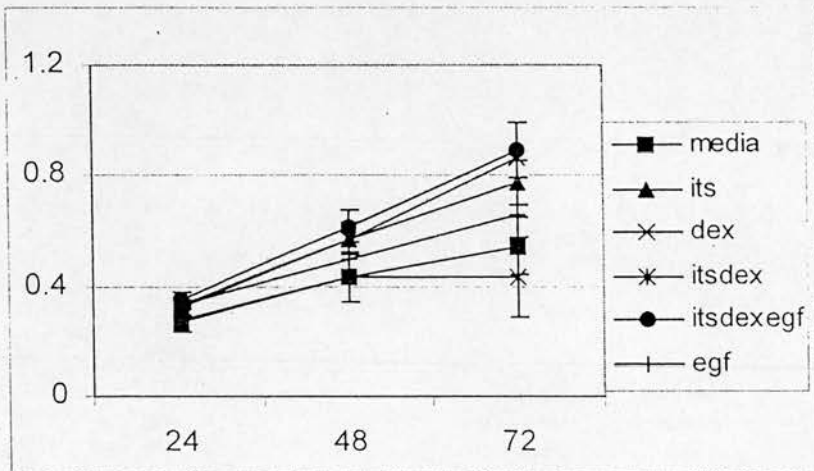


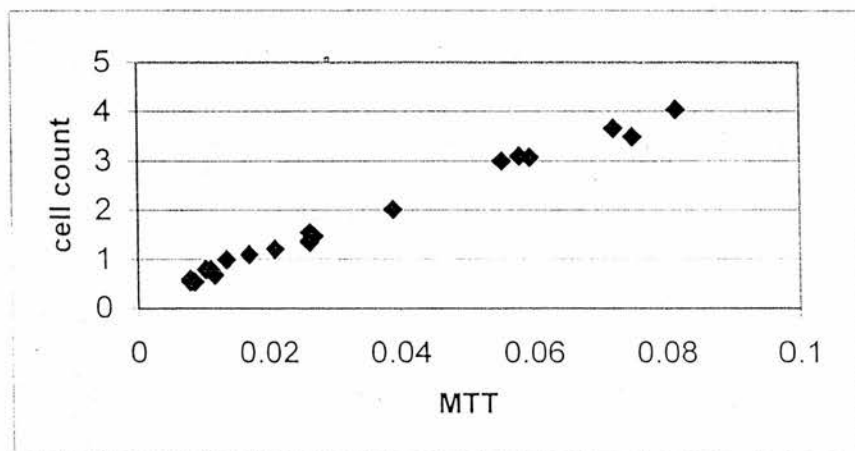
Figure 3.1 (HepG2)



Addition of various culture supplements to the hepatocyte cell lines. mem or media = media only; its = insulin-transferrin-selenium; dex = dexamethasone; egf = epidermal growth factor. Results are repeats of three experiments, error bars represent 2 x SD. None of the differences are significant at each time-point.

Both cell lines showed similar growth in media only, compared with a variety of putative growth supplements. All subsequent experiments were therefore carried out in unsupplemented media.

The MTT assay is a well-established method to measure cell number, based on detection of metabolic activity of cells. However, it is theoretically possible that paracetamol may reduce metabolic activity of cells, in addition to or instead of killing the cells, thereby reducing the MTT result. Pilot studies were carried out on WRL 68 cells to compare direct counting of cell number with MTT assay. Cell number was calculated by using a haemocytometer to count cells harvested from culture dishes with EDTA. MTT was carried out as detailed in the text of chapter 4.



The figure shows cell number by cell counting (y axis) plotted against cell number by MTT (x axis). The lower MTTs and cell numbers (left part of graph) are paracetamol-treated samples (5mM paracetamol) and the upper right MTTs are controls (media only).

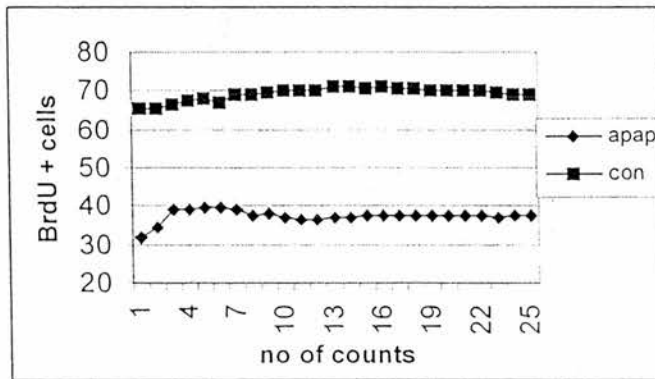
The graph shows a linear association between cell number measured by MTT and measured by direct counting. This suggests that MTT is a valid method to assess cell number in toxicity studies. HepG2 cells show a similar graph (not shown).

Culture slides were fixed in glacial acetic acid / methanol / formalin as detailed in chapter 4. Slides were immersed in 5M HCl for 45 minutes at room temperature then washed in tap water. Slides were then immersed in Schiff's reagent (Sigma) for 45 minutes and washed in tap water. Slides were counterstained with 0.3% light green for 1 minute and air dried. Slides were mounted in pertex and stored in the dark.

APPENDIX 6

RUNNING MEANS FOR CELL COUNTING

Running means were calculated for each morphological count in order to determine how many cells had to be counted to identify a significant difference. Each mean count was the total number of cells counted in a high power field (for most examples 150 – 200 cells). A cut-off of 2% around the derived mean was used (i.e. if that many cells was counted then a difference of greater than 2% was significant) and in practice most counts required counting of between 500 and 1000 cells.



Running mean for counting BrdU positive WRL 68 cells with (apap) and without (con) a toxic dose of paracetamol (5mM). Other counts had similar graphs and are not shown.

APPENDIX 7

DNA EXTRACTION FROM PARAFFIN SECTIONS

15µm sections were cut for extraction of DNA and placed in a sterile microfuge tube. The sections were washed twice in xylene and twice in absolute ethanol, centrifuging at 13000rpm after each wash. After the final wash and spin the pellet was dried for 10 minutes at room temperature. 200µl of lysis buffer (50mM KCl, 10mM Tris, 2.5mM MgCl₂, 0.1mg/ml gelatin, 0.45% NP40, 0.45% Tween 20, distilled water; PH 8.3) containing 200µg proteinase K was added and the sample incubated at 50°C for 2 hours, vortexing the tube after 1 hour. The tube was placed in boiling water for 20 minutes to inactivate the proteinase K. The sample was cooled on ice and stored at 4°C until use.

Original Paper

Inhibition of tumour necrosis factor alpha does not prevent experimental paracetamol-induced hepatic necrosis

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Abstract

Paracetamol-induced hepatic necrosis is the most common form of toxic liver injury experienced in clinical practice in the UK and USA. Recently, reports have described prevention of hepatic necrosis, induced by other hepato-toxins, by inhibiting tumour necrosis factor alpha (TNF α). The aim of the present study was to determine the role of TNF α in paracetamol-induced hepatic necrosis. Six-week-old CBA/J female mice were given 300 mg/kg paracetamol by intraperitoneal (IP) injection after an 8-h fast. Hepatic expression of TNF α was measured by enzyme-linked immunoassay (ELISA) and reverse transcriptase-polymerase chain reaction (RT-PCR). Serum TNF α was measured by ELISA. One hour prior to paracetamol injection, mice were also given blocking anti-TNF α antibodies, soluble TNF α receptor, interleukin 10 (IL-10), and dexamethasone. Hepatic injury was measured by serum aspartate aminotransferase and histological assessment on haematoxylin and eosin (H&E)-stained liver sections. There was a significant increase in serum TNF α at 6 h (control 0.002 ± 0.002 ng/ml, $n=7$; paracetamol-treated 0.022 ± 0.007 ng/ml, $n=5$, $p < 0.05$), but hepatic TNF α expression did not change up to 24 h following paracetamol injection. Histologically severe centrilobular hepatic necrosis was noted at 3 h and progressed for 24 h after paracetamol poisoning. Death rate, serum aspartate aminotransferase, and hepatic histology were not significantly different between the groups treated with blocking anti-TNF α antibodies, soluble TNF α receptor, IL-10, and dexamethasone, compared with controls. In conclusion, there is no evidence to suggest that modulation of TNF α expression affects hepatic injury following experimental paracetamol poisoning; anti-TNF α therapies are therefore unlikely to be effective in the corresponding clinical situation. Copyright © 2000 John Wiley & Sons, Ltd.

Keywords: paracetamol; acetaminophen; toxic liver injury; tumour necrosis factor α

Received: 9 January 1999
Revised: 31 May 1999
Accepted: 4 October 1999

Introduction

Paracetamol (acetaminophen) poisoning is the most common cause of fulminant hepatic failure and emergency liver transplantation in the United Kingdom [1]. Despite the availability of a safe and effective antidote (*N*-acetylcysteine), patients continue to die following paracetamol poisoning; there are around 100 deaths per year in Scotland (population 5 million) alone.

Historically, studies of paracetamol toxicity have concentrated on the metabolite *N*-acetyl-*p*-benzoquinomone-imine (NAPQI), which depletes glutathione and accumulates, causing injury with resulting zone 3 hepatic necrosis [2]. However, recent studies have shown that other factors are important in determining the severity of injury, including species and strain studied [3], sex, non-parenchymal liver cells, and local cytokine production. Kupffer cells have a central role in causing hepatic injury in experimental models; Kupffer cell blockade can abrogate hepatic injury

induced by paracetamol [4,5], galactosamine [6], and carbon tetrachloride [7].

Tumour necrosis factor alpha (TNF α), produced by activated Kupffer cells, is an important mediator of liver injury and regeneration in a variety of diseases and experimental models. TNF α can induce hepatocyte apoptosis and necrosis after hepatotoxin exposure [8,9]. In addition, toxic liver injury following administration of carbon tetrachloride [9], cadmium [10], galactosamine/lipopolysaccharide [11], and concanavalin A [12] can be inhibited by pretreatments that block hepatic TNF α expression. Evidence regarding the role of TNF α in the pathogenesis of paracetamol-induced liver injury, including use of transgenic technology, is contradictory. The aim of the present study was to determine the role of TNF α in the pathogenesis of hepatic necrosis following paracetamol poisoning and in particular, to ascertain whether TNF α inhibition could prevent hepatic necrosis in this experimental model.

Materials and methods

Experimental model

Six-week-old female CBA/J mice were fasted, but with free access to water, for 8 h prior to intraperitoneal (IP) injection of 300 mg/kg paracetamol dissolved in normal saline. After injection, the animals were allowed free access to laboratory chow. Rabbit anti-murine TNF α antibodies were prepared by multiple site immunization of New Zealand White rabbits with recombinant murine TNF α ; serum IgG was purified using a protein A column and 0.5 ml of immune serum injected intraperitoneally, 1 h prior to paracetamol administration. The resultant anti-murine TNF α antibodies are specific for TNF α , with no demonstrable cross-reactivity to interleukin (IL) 1 α , IL-1 β , IL-6, IL-10 or IL-8. The anti-TNF α antibody neutralizes TNF α in a WEHI killing assay, thus demonstrating its effectiveness. Non-immune serum was used as a control. Many previous studies have shown the efficacy of this immune serum in inhibiting the *in vivo* effects of both circulating and tissue-expressed TNF α when administered by IP injection 1 h prior to treatment with various noxious agents [13–16]. An Fc antibody linked to a soluble TNF α receptor (200 μ g; a kind gift from Immunex Corp., Seattle, WA, USA), which is more stable and 1000-fold more effective than monomeric naturally occurring soluble TNF α receptor, was administered 1 h prior to paracetamol injection [17]. Mice were also injected intraperitoneally with recombinant IL-10 (5 μ g; purchased from R & D Systems Inc., Minneapolis, MN, USA) and dexamethasone (4 μ g; purchased from Sigma-Aldrich, St Louis, MO, USA) or saline as control, 1 h prior to paracetamol administration. Such doses and treatments have also been shown to inhibit the *in vivo* effects of TNF α in experimental animals [18–20] in this laboratory.

TNF α expression

Samples of liver were weighed and homogenized in 1 ml of phosphate-buffered saline containing 0.5% Nonidet P40 and the supernatant was recovered following centrifugation. Measurements of antigenic TNF α from serum and liver homogenate were made using a double ligand ELISA specific for murine TNF α as previously described [17]. Liver was also 'snap-frozen' in liquid nitrogen and homogenized in 1 ml of 4.2 M guanidine isothiocyanate, 25 mM Tris (pH 8.0), 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. An equal volume of 100 mM Tris (pH 8.0), 10 mM EDTA, and 1.0% SDS was added and total RNA prepared following phenol-chloroform and chloroform-isoamyl alcohol extraction. The isolated RNA was quantitated by spectrophotometric analysis at 260 nm and 5 μ g was reverse-transcribed into cDNA using oligo (dT)12–18 primers and AMV reverse transcriptase. The cDNA was amplified using specific primers for TNF α and cyclophilin (TNF α sense CCTGTAGCCACGTCGTAGC, TNF α anti-sense TTGACCTCAGCGCTGA-

GTTG; cyclophilin sense CATCTGCACTGCCAA-GAC, cyclophilin antisense CTGCAATCCAGCTAG-GCATG). The amplification reaction was incubated initially at 94°C for 5 min, followed by 35 cycles at 93°C for 45 s, 52°C for 45 s, and 72°C for 90 s. The amplification products were visualized under UV light following separation in a 2% agarose gel containing 0.3 mg/ml ethidium bromide [18].

Assessment of hepatic injury

Hepatic injury was measured by serum aspartate aminotransferase activity determined using a standard colorimetric assay (Sigma Chemical Co., St Louis, MO, USA) and by histological assessment of H&E-stained sections of liver tissue. The histological assessment was conducted by two independent investigators (KJS and DJH) without prior knowledge of what, if any, anti-TNF α treatment the animal had received. Hepatic necrosis was scored as follows: +, less than 30% of the total area necrotic; ++, 30–60% of the total area necrotic; +++, greater than 60% of the total area necrotic.

Statistical analysis

A two-way analysis of variance was used to test for differences in serum and hepatic TNF α concentrations and serum aspartate aminotransferase activities. A chi-square test was used to test for significant differences in mortality. Both analysis of variance and chi-square tests were used to test for differences in hepatic histology. A result of $p < 0.05$ was chosen as significant. All data are presented as mean \pm standard error.

Results

To determine the time course of hepatic injury, we measured the serum concentration of the enzyme, aspartate aminotransferase, and scored hepatic necrosis on H&E-stained histological sections. Three hours following paracetamol administration, a significant increase in serum aspartate aminotransferase (control 22.5 ± 2.6 IU/l, $n = 6$; paracetamol-treated 2726 ± 629 IU/l, $n = 6$, $p < 0.05$) and the appearance of severe hepatic necrosis were observed. These changes were completely prevented by the prior injection of *N*-acetylcysteine (300 mg/kg). Thus, the murine model used in these experiments is similar to the liver damage induced in humans.

Induction of TNF α by paracetamol *in vivo* was determined by measuring serum and hepatic TNF α protein and gene expression. Serum TNF α (Figure 1) increased 3 h following paracetamol injection (20.4 ± 0.6 pg/ml, mean \pm SEM, $n = 5$, $p < 0.05$) and remained elevated for 24 h compared with controls (2.4 ± 2.4 pg/ml, $n = 7$). Hepatic TNF α protein concentration (Figure 2) was similar at all time points following paracetamol administration (range 0.498–4.818 ng/g wet weight) and was not significantly

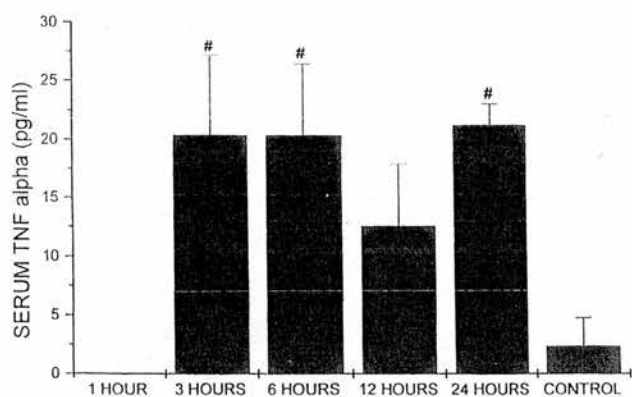


Figure 1. Serum TNF α following paracetamol poisoning. Paracetamol (300 mg/kg) was injected intraperitoneally and serum TNF α measured by ELISA at the given time points following administration. # = significant difference ($p < 0.05$) compared with saline-injected controls; $n = 5$ in each group except the control group, in which $n = 7$

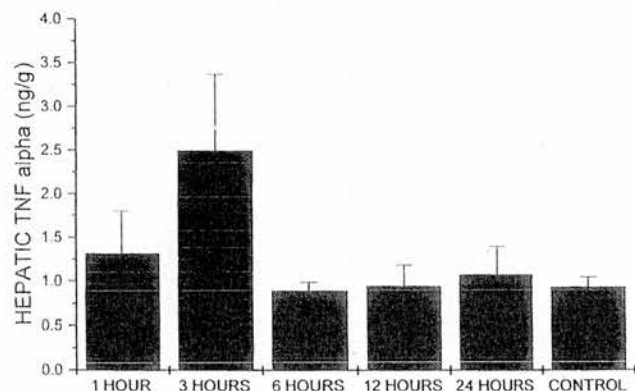


Figure 2. Hepatic TNF α following paracetamol poisoning. Paracetamol (300 mg/kg) was injected intraperitoneally and hepatic TNF α measured by ELISA at the given time points following administration. No significant differences ($p < 0.05$) were noted compared with saline-injected controls; $n = 5$ in each group except the control group, in which $n = 7$

different compared with controls (0.941 ± 0.114 ng/g wet weight, $n = 7$). Hepatic TNF α gene expression was not induced following paracetamol (Figure 3). Although we found a small, but significant increase in serum TNF α protein, there was therefore no induction of hepatic TNF α protein or gene expression following paracetamol poisoning.

To determine further the role of TNF α in the pathogenesis of hepatic injury induced by paracetamol, we inhibited the biological effects of TNF α *in vivo*, using anti-TNF α antibodies, soluble TNF receptors, IL-10, and dexamethasone. Mortality (Table 1) follow-

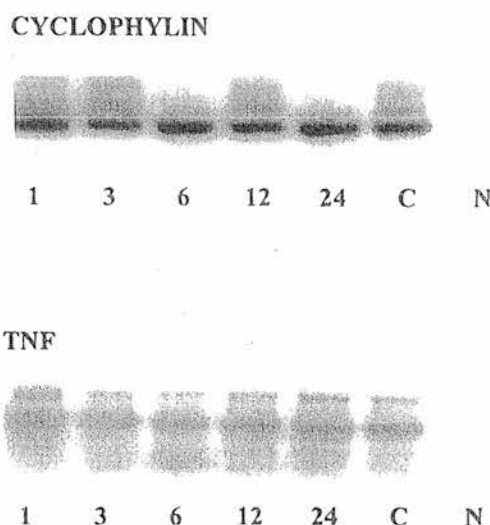


Figure 3. Hepatic TNF α mRNA expression following paracetamol poisoning. Total RNA was extracted from liver tissue after intraperitoneal paracetamol injection (300 mg/kg), cDNA synthesized, and TNF α mRNA expression determined using PCR amplification as described previously [17]. Cyclophillin was used as a control. 1, 3, 6, 12, and 24 correspond to the numbers of hours post-paracetamol injection; C = saline-injected control and N = negative PCR control. No significant difference in hepatic TNF mRNA expression was noted following paracetamol poisoning compared with saline-injected controls

ing paracetamol poisoning was similar in mice pretreated with blocking anti-TNF α antibodies (12.5%), soluble TNF receptor (8.3%), IL-10 (12.5%) and dexamethasone (0%) compared with controls (0%). Serum aspartate aminotransferase (Table 1) was also similar in all groups. Histological assessment of the extent of hepatic necrosis (Figure 4 and Table 2), determined 20 h post-paracetamol injection, was also similar in controls (2.0 ± 0.2 , mean score \pm SEM, $n = 16$) and mice pretreated with blocking anti-TNF α antibodies (2.5 ± 0.3 , $n = 8$), soluble TNF α receptor (2.3 ± 0.3 , $n = 10$), IL-10 (2.43 ± 0.2 , $n = 7$), and dexamethasone (2.1 ± 0.2 , $n = 8$).

Discussion

In this study, we found an increase in serum TNF α at 3 h post-paracetamol injection, but there was no increase in hepatic TNF α protein or gene expression. In addition, treatment with anti-TNF α antibodies, soluble TNF receptor, IL-10, and dexamethasone did not protect against paracetamol-induced hepatic necrosis, as determined 20 h post-paracetamol poisoning.

Table 1. Mortality and serum aspartate aminotransferase following paracetamol poisoning: effect of TNF α inhibition

	Control	Anti-TNF antibody	Soluble TNF receptor	IL-10	Dexamethasone
Mortality	0/18 (0)	1/8 (12.5)	1/12 (8.3)	1/8 (12.5)	0/10 (0)
Serum AST (IU/l)	6611 \pm 858	9684 \pm 762	5770 \pm 772	6137 \pm 884	5532 \pm 772

Results are mean \pm SEM. No statistical differences were detected.

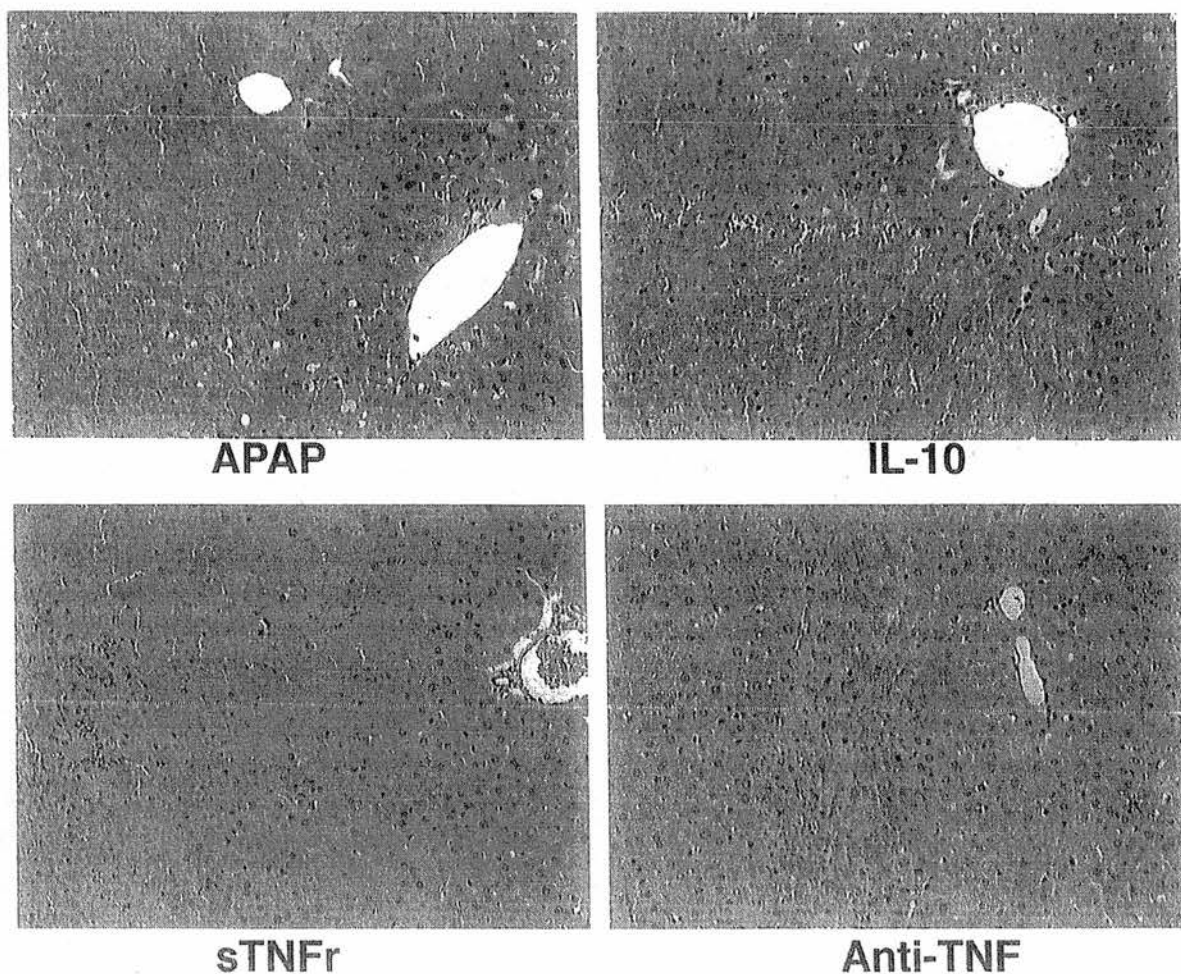


Figure 4. Hepatic histology following paracetamol poisoning; the effect of anti-TNF α therapies. Representative sections from control animals (APAP) and mice preinjected with anti-TNF α antibodies (Anti-TNF), soluble TNF receptor (sTNFr), and interleukin 10 (IL-10) prior to paracetamol injection are presented. No difference in hepatic histology was observed. The data from dexamethasone-treated animals are now shown, but no improvement in hepatic histology was observed

Table 2. Histological assessment of hepatic necrosis following paracetamol poisoning: effect of TNF α inhibition

	Control	Anti-TNF antibody	Soluble TNF receptor	IL-10	Dexamethasone
+	4	0	1	0	1
++	8	2	3	4	5
+++	4	5	6	3	3

Results are numbers of animals with hepatic necrosis assessed as +, ++ or +++; hepatic necrosis as defined in methods. No statistical differences were detected.

Although not measured in this study, the anti-TNF α therapies used in the paracetamol model have previously been shown to be highly effective in inhibiting TNF α in other animal models used in our laboratory. For example, the anti-TNF α antibodies administered by IP injection, 1 h prior to lipopolysaccharide (LPS) IP injection, reduce peak serum TNF α concentrations of 80 000 pg/ml at 1.5 h to undetectable levels [15]. Similarly, the anti-TNF α antibodies used in this study can significantly reduce circulating TNF α concentrations for at least 16 h when administered intraperitoneally at the time of caecal ligation and puncture [15]. These antibodies also significantly reduce TNF α -induced hepatic injury induced by endotoxin injection

[16]. The soluble TNF α receptors used in these studies are a chimera of an Fc antibody linked to soluble TNF α receptors. This chimera is more effective than the naturally occurring monomeric soluble TNF α receptor and inhibits 125 pg/ml TNF α in an L929 bioassay at concentrations of 10^{-9} – 10^{-10} M. The Fc–TNF α receptor chimera administered by IP injection 1 h prior to LPS reduces circulating concentrations of TNF α of approximately 1 ng/ml to undetectable concentrations. In addition, the Fc–TNF α receptor chimera, administered by IP injection, inhibits TNF α -dependent tissue infiltration with neutrophils and eosinophils for up to 24 h [17]. The dose and mode of administration of IL-10 significantly

inhibit TNF α release and improves murine survival following caecal ligation and puncture [18]. Similarly, the dose and mode of administration of dexamethasone significantly reduce circulating TNF α and hepatic expression of TNF α following endotoxin injection [20]. Therefore, the anti-TNF α therapies employed in this paper would be more than adequate to inhibit completely the circulating concentrations of TNF α observed in this study following paracetamol poisoning (maximum 30 pg/ml).

Other studies of paracetamol-induced injury have shown no increase in serum TNF α in Swiss mice [21] and no increase in serum or hepatic TNF α in male C57BL/6 mice up to 8 h after injection [22]. The latter study also showed no difference in hepatic injury between TNF α /LT double knockout mice and controls at up to 8 h, consistent with our observations that exogenous inhibitors of TNF α do not prevent paracetamol-induced liver injury in mice. The TNF α knockout study suggests that TNF α is not involved in paracetamol-induced toxicity. However, compensation of a deleted gene by altered expression of other genes is well recognized in transgenic knockout models. This may be particularly relevant in a complex area such as cytokine production. TNF α is a multifunctional cytokine produced in many pathological situations and TNF α synthesis and release are related to, and regulated by, other cytokines, chemokines, and other factors. TNF α production is related to that of IL-1 [23,24], which shares some of its biological effects, and many of the cytotoxic effects of TNF α may be accounted for by IL-18 [25], which could be up-regulated by other pathways. Conversely, a variety of factors may down-regulate TNF α synthesis and release, such as IL-10 [11,26] or chemical inhibitors [27], or neutralize 'active' TNF α (e.g. soluble TNF receptor) and inhibit TNF α effects *in vivo* [28]. Our data confirm the results of the earlier study using TNF α knockout mice, but we have used animals that have not had the opportunity to compensate for the lack of TNF α .

Others have also found increases in serum TNF α , but these workers showed that anti-TNF α antibodies limited paracetamol-induced liver damage [24,29]. We did not find any elevation of hepatic TNF α protein or RNA, despite the serum elevation. Whilst we have not identified the source of this TNF α , many cells produce this early response cytokine following appropriate stimulation, including circulating monocytes and splenic and enteric macrophages [30,31]. The role of TNF α in the pathogenesis of liver injury by another toxic agent, carbon tetrachloride (CCl₄), has been studied with similarly contradictory results [9,32]. Elevation of serum and hepatic TNF α has been described, with increases in the same range as our findings in serum in paracetamol-induced toxicity, with conflicting studies showing inhibition of injury by soluble TNF receptors but no prevention of injury by anti-TNF α antibodies.

In summary, the data presented in this paper do not implicate TNF α in the pathogenesis of hepatocyte

necrosis following paracetamol poisoning. Therapies directed against TNF α are therefore unlikely to be clinically effective in preventing or limiting hepatic injury.

Acknowledgements

This research was supported by the Scottish Hospital Endowments Research Trust, the Royal College of Pathologists, and by National Institute of Health Grants (numbers HL02401, HL31693, HL35276 and HL50057). K. J. Simpson was an MRC (UK) Travelling Fellow.

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