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

Kenneth J. Simpson[1,2]*, Nicholas W. Lukacs[2], Angus H. McGregor[3], David J. Harrison[3], Robert M. Strieter[4] and Steven L. Kunkel[2]

[1] *Scottish Liver Transplant Unit, Royal Infirmary of Edinburgh, Lauriston Place, Edinburgh, UK*
[2] *Department of Pathology, University of Michigan, Ann Arbor, Michigan, USA*
[3] *Department of Pathology, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, UK*
[4] *Division of Pulmonary and Critical Care Medicine, University of Michigan, Ann Arbor, Michigan, USA*

*Correspondence to:
Dr Kenneth J. Simpson,
Scottish Liver Transplant Unit,
Royal Infirmary of Edinburgh,
Lauriston Place,
Edinburgh EH3 9YW, UK.
E-mail: ksimp@srvl.med.ed.ac.uk

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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 (TNFα). The aim of the present study was to determine the role of TNFα 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 TNFα was measured by enzyme-linked immunoassay (ELISA) and reverse transcriptase-polymerase chain reaction (RT-PCR). Serum TNFα was measured by ELISA. One hour prior to paracetamol injection, mice were also given blocking anti-TNFα antibodies, soluble TNFα 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 TNFα 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 TNFα 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-TNFα antibodies, soluble TNFα receptor, IL-10, and dexamethasone, compared with controls. In conclusion, there is no evidence to suggest that modulation of TNFα expression affects hepatic injury following experimental paracetamol poisoning; anti-TNFα therapies are therefore unlikely to be effective in the corresponding clinical situation.

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

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 (TNFα), produced by activated Kupffer cells, is an important mediator of liver injury and regeneration in a variety of diseases and experimental models. TNFα 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 TNFα expression. Evidence regarding the role of TNFα 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 TNFα in the pathogenesis of hepatic necrosis following paracetamol poisoning and in particular, to ascertain whether TNFα 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 TNFα antibodies were prepared by multiple site immunization of New Zealand White rabbits with recombinant murine TNFα; 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 TNFα antibodies are specific for TNFα, with no demonstrable cross-reactivity to interleukin (IL) 1α, IL-1β, IL-6, IL-10 or IL-8. The anti-TNFα antibody neutralizes TNFα 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 TNFα when administered by IP injection 1 h prior to treatment with various noxious agents [13–16]. An Fc antibody linked to a soluble TNFα receptor (200 μg; a kind gift from Immunex Corp., Seattle, WA, USA), which is more stable and 1000-fold more effective than monomeric naturally occurring soluble TNFα receptor, was administered 1 h prior to paracetamol injection [17]. Mice were also injected intraperitoneally with recombinant IL-10 (5 μg; purchased from R & D Systems Inc., Minneapolis, MN, USA) and dexamethasone (4 μg; 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 TNFα in experimental animals [18–20] in this laboratory.

TNFα expression

Samples of liver were weighed and homogenized in 1 ml of phosphate-buffered saline containing 0.5% Nonidit P40 and the supernatant was recovered following centrifugation. Measurements of antigenic TNFα from serum and liver homogenate were made using a double ligand ELISA specific for murine TNFα 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 μg was reverse-transcribed into cDNA using oligo (dT)12–18 primers and AMV reverse transcriptase. The cDNA was amplified using specific primers for TNFα and cyclophylin (TNFα sense CCTGTAGCCCACGTCGTAGC, TNFα anti-sense TTGACCTCAGCGCTGAGTTG; cyclophylin sense CATCTGCACTGCCAAGAC, cyclophylin antisense CTGCAATCCAGCTAGGCATG). 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-TNFα 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 TNFα 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 ± 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 TNFα by paracetamol *in vivo* was determined by measuring serum and hepatic TNFα protein and gene expression. Serum TNFα (Figure 1) increased 3 h following paracetamol injection (20.4 ± 0.6 pg/ml, mean ± SEM, $n=5$, $p<0.05$) and remained elevated for 24 h compared with controls (2.4 ± 2.4 pg/ml, $n=7$). Hepatic TNFα 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

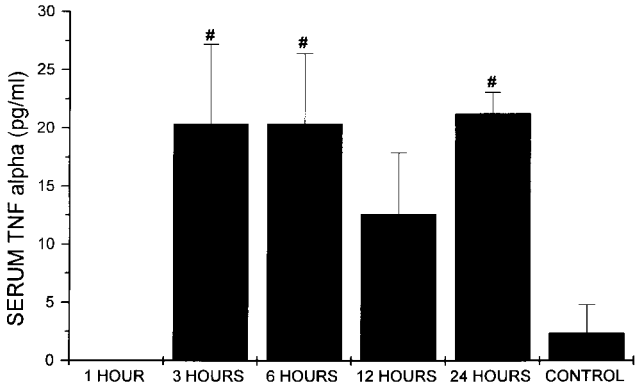

Figure 1. Serum TNFα following paracetamol poisoning. Paracetamol (300 mg/kg) was injected intraperitoneally and serum TNFα measured by ELISA at the given time points following administration. #=significant difference ($p<0.05$) compared with saline-injected controls; $n=5$ in each group except the control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

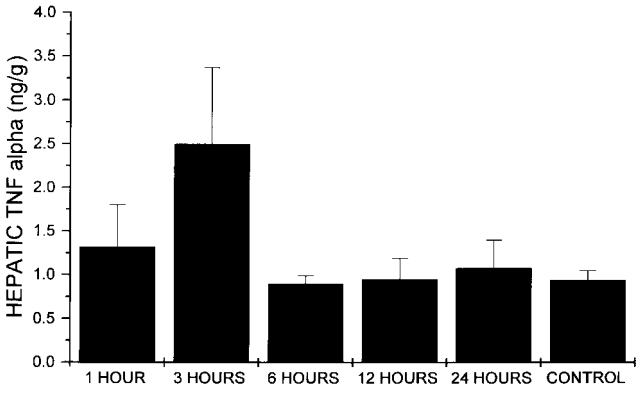

Figure 2. Hepatic TNFα following paracetamol poisoning. Paracetamol (300 mg/kg) was injected intraperitoneally and hepatic TNFα measured by ELISA at the given time points following administration. No significant differences ($p<0.05$) were noted compared with saline-injected controls; $n=5$ in each group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

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

To determine further the role of TNFα in the pathogenesis of hepatic injury induced by paracetamol, we inhibited the biological effects of TNFα *in vivo*, using anti-TNFα antibodies, soluble TNF receptors, IL-10, and dexamethasone. Mortality (Table 1) following paracetamol poisoning was similar in mice pretreated with blocking anti-TNFα 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±SEM, $n=16$) and mice pretreated with blocking anti-TNFα antibodies (2.5 ± 0.3, $n=8$), soluble TNFα receptor (2.3 ± 0.3, $n=10$), IL-10 (2.43 ± 0.2, $n=7$), and dexamethasone (2.1 ± 0.2, $n=8$).

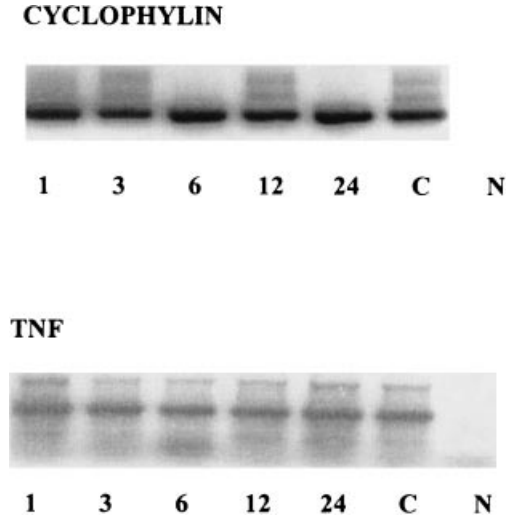

Figure 3. Hepatic TNFα mRNA expression following paracetamol poisoning. Total RNA was extracted from liver tissue after intraperitoneal paracetamol injection (300 mg/kg), cDNA synthesized, and TNFα mRNA expression determined using PCR amplification as described previously [17]. Cyclophylin was used as 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

Discussion

In this study, we found an increase in serum TNFα at 3 h post-paracetamol injection, but there was no increase in hepatic TNFα protein or gene expression. In addition, treatment with anti-TNFα 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 TNFα 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±858	9684±762	5770±772	6137±884	5532±772

Results are mean±SEM. No statistical differences were detected.

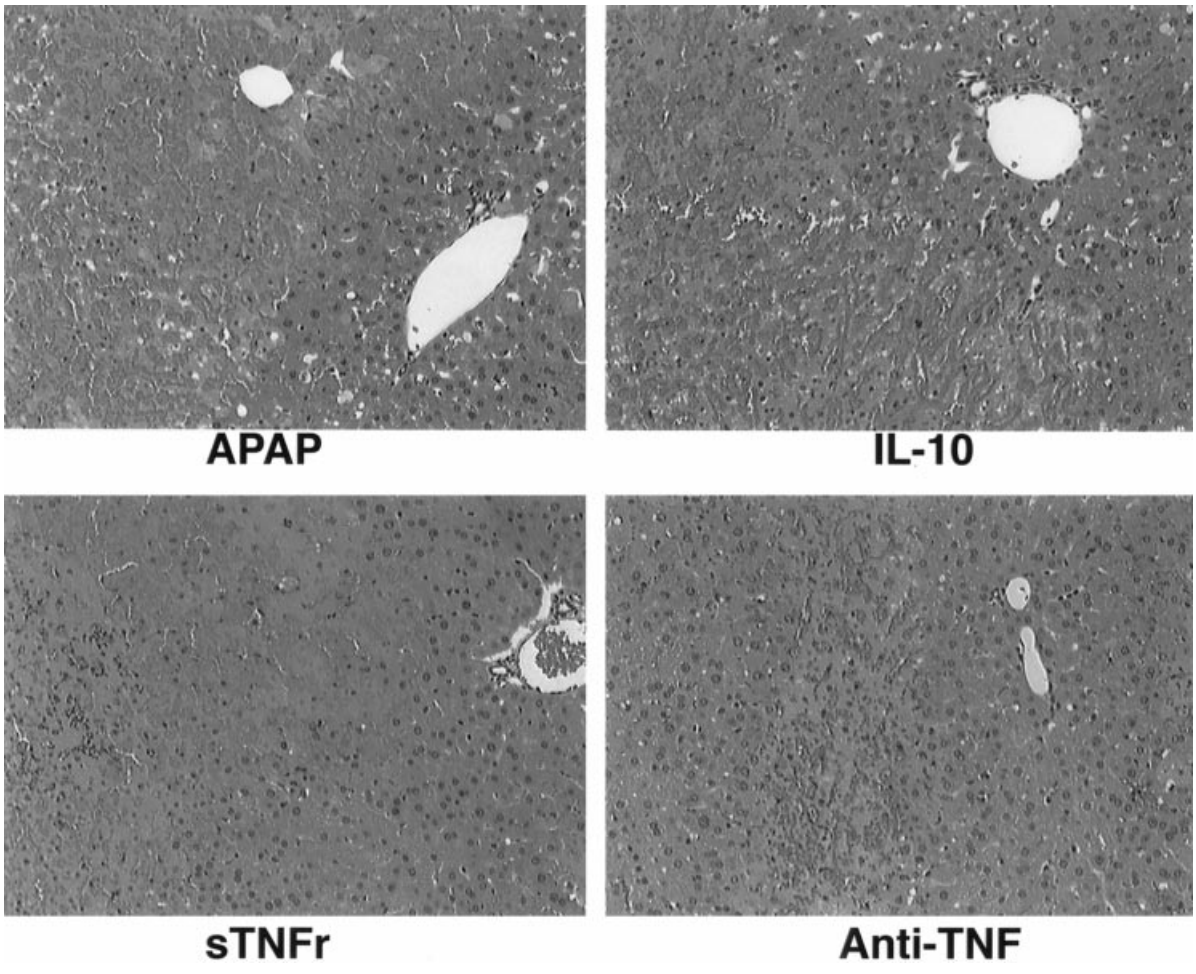

Figure 4. Hepatic histology following paracetamol poisoning; the effect of anti-TNFα therapies. Representative sections from control animals (APAP) and mice preinjected with anti-TNFα antibodies (Anti-TNF), soluble TNF receptor (sTNFr), and interleukin 10 (IL-10) prior to paracetamol injection are presented. 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 TNFα 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-TNFα therapies used in the paracetamol model have previously been shown to be highly effective in inhibiting TNFα in other animal models used in our laboratory. For example, the anti-TNFα antibodies administered by IP injection, 1 h prior to lipopolysaccharide (LPS) IP injection, reduce peak serum TNFα concentrations of 80 000 pg/ml at 1.5 h to undetectable levels [15]. Similarly, the anti-TNFα antibodies used in this study can significantly reduce circulating TNFα concentrations for at least 16 h when administered intraperitoneally at the time of caecal ligation and puncture [15]. These antibodies also significantly reduce TNFα-induced hepatic injury induced by endotoxin injection [16]. The soluble TNFα receptors used in these studies are a chimera of an Fc antibody linked to soluble TNFα receptors. This chimera is more effective than the naturally occurring monomeric soluble TNFα receptor and inhibits 125 pg/ml TNFα in an L929 bioassay at concentrations of 10^{-9}–10^{-10} M. The Fc–TNFα receptor chimera administered by IP injection 1 h prior to LPS reduces circulating concentrations of TNFα of approximately 1 ng/ml to undetectable concentrations. In addition, the Fc–TNFα receptor chimera, administered by IP injection, inhibits TNFα-dependent tissue infiltration with neutrophils and eosinophils for up to 24 h [17]. The dose and mode of administration of IL-10 significantly

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

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

Others have also found increases in serum TNFα, but these workers showed that anti-TNFα antibodies limited paracetamol-induced liver damage [24,29]. We did not find any elevation of hepatic TNFα protein or RNA, despite the serum elevation. Whilst we have not identified the source of this TNFα, many cells produce this early response cytokine following appropriate stimulation, including circulating monocytes and splenic and enteric macrophages [30,31]. The role of TNFα in the pathogenesis of liver injury by another toxic agent, carbon tetrachloride (CCl_4), has been studied with similarly contradictory results [9,32]. Elevation of serum and hepatic TNFα 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-TNFα antibodies.

In summary, the data presented in this paper do not implicate TNFα in the pathogenesis of hepatocyte necrosis following paracetamol poisoning. Therapies directed against TNFα are therefore unlikely to be clinically effective in preventing or limiting hepatic injury.

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