STUDIES ON CYTOKINES IN LIVER PATHOPHYSIOLOGY

BY

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FOR MY WIFE,

RONA,

AND CHILDREN,

FRASER, EILIDH and NIALL

DECLARATION

I hereby declare that this thesis is based on the results of my own experiments and that the thesis is exclusively of my own composition. The data presented in this thesis has not been submitted previously for a higher degree.

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TABLE OF CONTENTS

CHAPTER 3. ADHESION MEDIATED CHEMOKINE PRODUCTION. 79

CHAPTER 4. ROLE OF TNFα IN HEPATIC INJURY POST PARACETAMOL POISONING. 101

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Background: Hepatic inflammation has a variety of causes and is a common health care problem world-wide. Although much work has been undertaken on the pathogenesis of hepatitis, the underlying cytokine networks involved have remained elusive. In addition, the recently described family of small molecular weight chemoattractant cytokines, chemokines, have not been studied in relation to their potential role in the pathogenesis of liver disease.

Aims of the thesis: To study the mechanisms of chemokine production that may occur during hepatic disease, the role of chemokines in hepatic injury and repair following paracetamol poisoning and hepatic expression of chemokines in patients with liver disease. In addition, the role of tumour necrosis factor alpha (TNF) and stem cell factor (SCF) were also studied following paracetamol poisoning.

Materials and Methods: Human hepatoma cell line chemokine production was studied during co-culture with human monocytes and following tumour necrosis factor stimulation using enzyme linked immunoassay and reverse transcriptase PCR (RT-PCR). RT-PCR was used to determine the expression of CXC and CC chemokines in liver biopsy tissue from patients with hepatitis of various aetiologies. The role of chemokines in hepatic repair following paracetamol poisoning was studied in a murine model, using 300mg/kg paracetamol administered by intraperitoneal injection (IP). Repair was quantitated histologically using the AXIO-HOME microscope. Hepatic

chemokine expression was inhibited by IP injection of blocking anti-chemokine antibodies and augmented with an adenovirus MIP2 expression vector. Hepatic TNF expression was measured following paracetamol poisoning and inhibited with anti-TNF antibodies, soluble TNF receptor, interleukin (IL) 10 or dexamethasone. Hepatic injury was measured by serum transaminase concentrations and histologically using H&E sections. Hepatic SCF expression and role in hepatic regeneration was also determined following paracetamol poisoning using methods described above.

Results: Both CXC and CC chemokines were produced during monocyte adhesion with human hepatoma cell lines. IL-8 production was dependent on proinflammatory cytokine production. In contrast, CC chemokine production appeared to be dependent on free radical activation of NF-κB. Direct TNF stimulated IL-8 production was mediated by TNF RI receptors via a protein kinase C pathway and was inhibited by dexamethasone. Both CXC and CC chemokines were detectable in human liver biopsies in patients with hepatitis C, hepatic allograft rejection and alcoholic hepatitis. Hepatic CXC and CC chemokines were also induced following paracetamol poisoning, inhibition of MIP2 and MIP1 alpha increased 3 day mortality and augmenting MIP2 expression was associated with accelerated hepatic regeneration. Hepatic TNF expression was not induced following paracetamol poisoning and inhibiting TNF was not associated with protection from hepatic injury. SCF was present within the liver at high concentration and located in both hepatocytes and bile ducts. Paracetamol poisoning was associated with reduced hepatic SCF concentrations and inhibiting SCF was associated with delayed hepatic regeneration.

Conclusions: Both adhesion mediated and direct proinflammatory cytokine stimulation induce hepatic chemokine production. Chemokines are expressed in liver tissue from patients with hepatitis and therefore may be implicated in the pathogenesis of hepatic inflammation. The data regarding the role of chemokines in hepatic regeneration suggest that these cytokines have much wider biological functions than just chemoattraction. SCF may also play a role in hepatic regeneration, but TNF is not implicated in post paracetamol liver injury.

CHAPTER 1

INTRODUCTION.

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1.1. CYTOKINES: A DEFINITION.

A basic definition for most cytokines would classify these mediators as soluble hormone-like proteins produced by a variety of cells following stimulation with a variety of inducers, therefore lipid based inflammatory mediators such as platelet activating factor (PAF) and leukotrienes, which are not the focus of this thesis, will not be discussed further (*reviewed by Imaizumi et al 1995, Goetzl et al 1995*). In contrast to hormones which are synthesised by specific endocrine tissues, cytokines are produced by a variety of organs or cells. Historically, the term interleukin (IL) was proposed to describe proteins produced by lymphocytes acting on lymphocytes and further classified as monokines or lymphokines, depending upon whether their source of synthesis was a monocyte/macrophages or lymphocytes, respectively. However, the cellular source and biological targets of these proteins are not restricted to cells of the immune system, as endothelial cells, stellate (Ito cells, fat storing cells, myofibroblasts) cells and hepatocytes are all capable of producing and responding to a number of different cytokines. Cytokines can broadly be grouped according to their structure or function into interkeukins, growth factors, interferons, tumour necrosis factors and chemokines (Table 1.1). However, inclusion in one group does not exclude a cytokine from membership of another, for example IL-8 is both an interleukin and a chemokine. In addition, many of the interleukins are also growth and differentiation factors for both immune and haematopoietic cells.

Table 1.1. Cytokine families.

INTERLEUKINS	GROWTH FACTORS	INTERFERONS	TUMOUR NECROSIS FACTORS	CHEMOKINES
Interleukin 1 - Interleukin 18	Hepatocyte growth factor Insulin like growth factor I & II Platelet derived growth factor Fibroblast growth factor 1 - 9 Transforming growth factor α & β 1 - 5 Colony stimulating factors, M-CSF, G-CSF, GM-CSF, erythropoietin, thrombospondin Epidermal growth factors, EGF, TGF-α, heparin binding EGF like growth factor, amphiregulin Nerve growth factor	Interferon α, β & γ	Tumour necrosis factor α (cachectin) Tumour necrosis factor β (lymphotoxin)	CXC (alpha) ELR positive: Interleukin 8, Gro α, β & γ, ENA-78, NAP-2 ELR negative: IP10, MIG, SDF CC (beta) MIP1α & β, MCP 1-4, RANTES, eotaxin C (gamma) Lymphotactin

Cytokines are most distinguished for their activities associated with inflammation, immune reactivity, tissue injury or repair and organ dysfunction. Evidence for the crucial role of cytokines in the evolution of the inflammatory response comes from the demonstration that certain viruses encode the genetic information for soluble receptors for some cytokines, which when expressed by an infected cell inactivates a particular cytokine and allows the virus to evade the immune system. In addition, certain viruses may utilise cytokines or cytokine receptors to gain entry into cells, eg. human immunodeficiency virus (HIV) 1 and chemokine receptors, CCR-5 and CXCR-4 (*Kozak et al 1997*), or interfere with cellular functions, eg. hepatitis B and IL-6 (*Neurath et al 1992*). However, many cytokines also play important roles in the modulation of normal physiological and biochemical functions of cells.

1.2. INTERLEUKINS

As mentioned above, it was originally considered that interleukins were produced by leukocytes and acted on leukocytes. However, it has become increasingly clear that these cytokines are produced by cells other than leukocytes and they act on a wide variety of cells. At present there are 18 different interleukins, as this thesis concentrates on the roles of tumour necrosis factor α (TNFα) and chemokines in the pathobiology of liver disorders, the cell source, targets, types of cell receptor and actions of the interleukins have been summarised in table 1.2.

1.3. INTERFERONS

The interferons (IFN) were first identified by their anti-viral properties, but they have many other effects, serving as potent immunoregulatory and immunostimulatory factors. This group of cytokines are divided structurally into three, IFNα, IFNβ and

IFNγ. The cellular source, targets, types of cell receptor and actions of the interferons have been summarised in table 1.3.

1.4. GROWTH FACTORS

Growth factors are proteins that act on other cells to induce proliferation. Often this function and the target cell can be concluded from the cytokines name, such as granulocyte-monocyte colony stimulating factor. The cell source, targets, types of cell receptor and actions of the growth factors have been summarised in table 1.4. However, many other cytokines possess growth enhancing activity and these will be discussed under the appropriate sub-headings.

1.5. TUMOUR NECROSIS FACTORS

TNFα (cachectin) and TNFβ (lymphotoxin) have similar amino acid sequences (30% homology), bind to the same receptors and produce a wide range of similar effects. Not least of these is their ability to induce the necrosis of certain tumour cells (Carswell et al 1975), an effect from which they derive their name.

TNFα (*reviewed by Vilcek & Lee 1991*) is produced mainly from monocytes and tissue resident macrophages, but can be synthesised by other cells including neutrophils, activated lymphocytes, natural killer (NK) and lymphokine activated killer (LAK) cells, endothelial cells smooth muscle cells and hepatocytes. In contrast, TNFβ is produced by lymphocytes. The primary sequence of TNFα contains both hydrophobic and hydrophilic areas, thus TNFα is initially expressed as a biologically active 26-kDa transmembrane protein which is then cleaved to form the 17-kDa, 157 amino acid mature secreted protein. TNFβ can also be expressed in a biologically active

Table 1.2. INTERLEUKINS.

CYTOKINE	SOURCE	TARGETS	RECEPTORS	ACTIONS
IL 1α/β	Monocytes, macrophages, epithelial cells, fibroblasts, glial cells	Multiple	IL1R type 1, IL1R type 2	Multiple, acts as an early response cytokine inducing the production of other cytokines
IL 2	T cells	T, B, NK cells, neutrophils, macrophages, glial cells	IL2 receptor complex (triamer of α, β,γ chains)	T cell growth factor, activates B, NK and other cell types
IL 3	T cells, granulocytes, mast cells, epithelial cells, keratinocytes, neural cells	Haematopoietic stem cells, epithelial cells	IL3Rα/IL3Rβ diamer	Growth factor for haematopoietic stem cells, proliferation of epithelial, T, mast cells and basophils, immunactivator
IL 4	T cells	Multiple	IL4R (ligand binding)/IL2Rγ diamer	Anti-inflammatory, immunomodulatory, augments TH2 type cytokine response, growth factor
IL 5	T cells, eosinophils, mast cells	Eosinophils	IL5Rα/IL3Rβ diamer	Eosinophil activation, growth, differentiation and anti-apoptotic factor, stimulates Ig synthesis from B cells
IL 6#	T cells monocytes many other cells	Multiple	IL6R/gp130	Immunomodulatory, induces acute phase response, cellular growth factor
IL 7	Bone marrow stromal cells, thymic cortical epithelial cells	B and T cells	IL7R/IL2Rγ diamer	Proliferation and differentiation of preB cells and T cells
IL 8	Multiple cell types	Granulocytes, T cells, endothelial cells, keratinocytes and melanoma cells	CXCR1 and CXCR2	Cellular chemotactic and activation factor, growth factor
IL 9	T cells, peripheral blood mononuclear cells	T and B cells, bone marrow progenitors	IL9R/IL2Rγ diamer	T cell growth factor, immunomodulatory, haematopoietic growth factor
IL 10	T and B cells, monocytes/macro phages	Multiple	IL10R	Anti-inflammatory, immunomodulatory, drives Th2 and inhibits Th1 response
IL 11	Fibroblasts, articular cells, trophoblasts	Multiple	IL11R/gp130	Haematopoietic growth factor, cellular growth and differentiation factor , induces acute phase response
IL 12	Monocytes/macro phages, B cells, mast cells	T, NK cells	IL12R	Multiple effects on T and NK cells, immunomodulatory, erythropoietic growth factor

IL 6 family includes, Leukaemia inhibitory factor (LIF), oncostatin M and ciliary neurotrophic factor (CNTF).

INTERLEUKINS, continued.

CYTOKINE	SOURCE	TARGETS	RECEPTORS	ACTIONS
IL 13	T cells	Monocytes/macrophages, B cells, NK cells, endothelial cells, bone marrow progenitors	IL13R/IL2Rγ diamer	Similar effects to IL4
IL 14	B cells	B cells	IL14R	B cell proliferation
IL 15	T cells, epithelial cells, fibroblasts, skeletal muscle	NK cells, activated T and B cells	IL15Rα/IL2Rβ /IL2Rγ	Proliferation of NK, T and B cells, chemoattractant for T cells, induces LAK
IL 16		T cells		CD4 recruitment, T cell activation, inhibits HIV replication
IL 17	T cells	Fibroblasts, transformed cells, foetal hepatocytes	IL 17R	Haematopoiesis
IL 18	Osteoblastic cells, Kupffer cells, activated macrophages.	T & activated B cells, haemopoietic cells	?	Induces IFNγ, IL-2 & GM-CSF expression, augments NK activity

Table 1.3. INTERFERONS.

CYTOKINE	SOURCE	TARGETS	RECEPTORS	ACTIONS
Interferon α	T cells B cells Monocytes Fibroblasts	Multiple	Type I	Antiviral Anti-protozoal
Interferon β	Fibroblasts	Multiple	Type I	Antiviral Anti-protozoal
Interferon γ	T cells Natural killer cells	Multiple	Type II	Antiviral Anti-protozoal Immunomodulatory

Table 1.4. GROWTH FACTORS.

CYTOKINE	SOURCE	TARGETS	RECEPTORS	ACTIONS
Hepatocyte growth factor	Platelets, leucocytes, fibroblasts, hepatocytes	Multiple	c-MET	Multiple
Insulin like growth factor				
I	Hepatocytes, neurones, granulocytes, monocytes, fibroblasts, muscle cells	Multiple	IGF-I (type 1)receptor, insulin receptor	Skeletal growth and body protein metabolism
II	Hepatocytes, Kupffer cells, endothelial, epithelial cells and osteoblasts	Multiple	IGF-II (type 2) receptor, insulin receptor	
Platelet derived growth factors	Multiple	Multiple	PDGFα/PDGFRβ homo or heterodiamers	Cellular proliferation and activation, immunomodulatory
Fibroblast growth factors 1-9	Multiple, including immune and stromal cells	Multiple cell types	FGFR1-5	Proliferation of cells of mesodermal origin and most ecto and endodermal derived cells
olony stimulating factors.				Colony stimulating factors are best known for their mitogenic effect on monocytes and granulocytes. However these growth factors also affect the proliferation , differentiation and function of a wide variety of cells. They also have immunomodulatory and regulatory functions.
M-CSF	Monocytes/macro phages, fibroblasts, T and B cells, endothelial cells.	Multiple	c-*fms* gene product	
G-CSF	Monocytes/macro phages, fibroblasts, endothelial cells.	Multiple	GCSFR	
GM-CSF	T and B cells, macrophages, endothelial cells and fibroblasts	Multiple	GM-CSFRα/IL3R β diamer	

GROWTH FACTORS, continued.

CYTOKINE	SOURCE	TARGETS	RECEPTORS	ACTIONS
Transforming growth factor alpha	Macrophages, Keratinocytes	Multiple	TGFβR I, TGFβR II	Cellular proliferation and differentiation, activation and immunomodulatory role
beta	Platelets and many other cell types			

transmembrane form and is cleaved to produce the 171 amino acid, 25-kDa mature protein. The cleavage of membrane bound TNF can be inhibited by metalloproteinase inhibitors. Secreted TNFα and TNFβ combine to form active non-covalently linked homotriamers. Single TNFβ molecules can also triamerise with two membrane bound TNFβ molecules. This triamer of membrane bound and soluble TNFβ binds a specific TNF receptor, TNF-Rrp (LT-βR).

The two TNFs are induced following endotoxin (lipopolysaccaride, LPS) stimulation and are responsible for almost all of the clinical symptoms and signs of septic shock. These cytokines have wide ranging (pleiotropic) effects on multiple cell types, through their ability to induce the expression of other cytokines, such as growth factors, interleukins and chemokines. TNFs can also regulate the expression of cell surface receptors, transcription factors and acute phase proteins. In addition to inducing tumour necrosis, TNFs can also induce apoptosis of certain cell types.

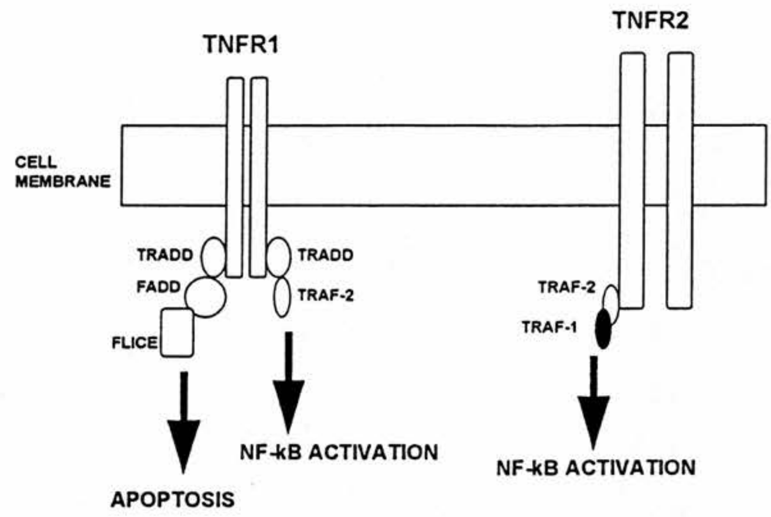
There are two TNF receptors, TNF-R55 (also referred to as TNFR1, TNFRβ, p55 or CD120a) and TNF-R75 (TNFR2, TNFRα, p75 or CD120b), which are expressed on all cell types (*reviewed by Bazzoni & Beutler 1996*). The TNF receptors are encoded by two different genes, which are differentially regulated. TNFR1 appears to be constitutively expressed, while TNFR2 can be induced by a variety of external stimuli, including mitogens, cAMP, lipopolysaccaride and interleukins. Structure/functional studies have shown that the TNF receptors are only able to bind triamers of TNFα or TNFβ. The intracellular domains of TNFR1 and TNFR2 are different suggesting that they induce different intracellular signalling pathways. There is also evidence that both receptors have different biological functions; depending on the cell type studied, TNFR1 translates the cytotoxic signals of the TNFs and TNFR2 the proliferative and regulatory signals. Although TNFR2 is constitutively phosphorylated, neither TNFR has intrinsic kinase activity. Recently several elegant studies have elucidated the novel family of TNFR associated proteins that are essential for signal transduction (Figure 1.1.). Binding of TNFs with their receptors can subsequently induce a wide variety of intracellular signalling pathways which are listed in Table 1.5.

1.6. CHEMOKINES

The chemokines are a recently characterised family of chemoattractant cytokines that are structurally divided into at least three different groups (Table 1.6) depending on the arrangement of the first two cysteine residues in the amino acid sequence (*reviewed by Adams & Lloyd 1997, Baggiolini et al 1997*). The CXC chemokines, for example IL-8 or Growth related gene (Gro), have a non-conserved amino acid between the cysteine residues. In contrast, the CC chemokines, for example monocyte chemoattractant protein (MCP) 1 or macrophage inflammatory protein 1 alpha (MIP1α), have no amino acid between the cysteine residues. Two recently characterised chemokine molecules have a different primary structure, lymphotactin has only a single cysteine at the N-terminus and neurotactin has 4 amino acids between the first two cysteine residues. The genes for the CXC chemokines are clustered on chromosome 4, and the genes for the CC chemokines are found on chromosome 17. Stromal cell derived factor (SDF) a recently characterised CXC chemokine is encoded on the long arm of chromosome 10. Most cell types can synthesise the CXC chemokines, while the cells able to express the CC chemokines are more limited. MIP1α, for example, is only produced by immune cells. The nomenclature of the chemokine family is some what confusing, however a comparison of human, murien and rat chemokines is shown in table 1.6.

The early response cytokines, TNFα and IL-1β, are primary stimuli to the production of chemokines. Many other stimuli exist, including other cytokines such as IFN-γ, oxidant stress, bacterial or viral infection. Chemokines were originally described

Figure 1.1. Tumour necrosis factor associated proteins.



TNFR = tumour necrosis factor receptors, TRADD = TNFR associated proteins with death domains, FADD = Fas assocaited proteins with death domains, TRAF = TNF associated factors.

Table 1.5. TNFα intracellular signalling pathways.

Activation of phospholipases;

- Neutral and acidic sphingomyelinase,
- Phospholipase C (phosphatidylcholine and phosphatidylinositol specific),
- Phospholipase A,
- Phospholipase D.

Activation of kinases and phosphatases;

- MAP kinase,
- Ceramide activated kinase,
- Protein kinase C,
- c-jun N-terminal protein kinase,
- c-raf-1 serine/threonine kinase.

Induction of free radical generation.

Increased intracellular calcium concentration.

Induction of nitric oxide production.

Activation of pertussis-toxin sensitive G proteins.

Activation of NF-κB.

Table 1.6. Human and rodent chemokine nomenclature.

HUMAN	MURINE	ALTERNATIVE NAMES*
CC CHEMOKINES		
Macrophage inflammatory protein (MIP)		
MIP1 alpha	MIP1 alpha	LD78, pAT464, GOS 19
MIP1 beta	MIP1 beta	Act-2, pAT744, G-26.
No human homologue	MIP1 gamma	CCF 18
MIP3 alpha	No murine homologue	Liver and Activation Regulated Chemokine (LARC), Exodus 1
MIP3 beta	No murine homologue	Epstein Barr virus induced gene 1 Ligand Chemokine (ELC)
MIP4	No murine homologue	Pulmonary and Activation Regulated Chemokine (PARC)
MIP5	No murine homologue	Hemofiltrate CC chemokine 2 (HCC 2)
Monocyte chemotactic protein (MCP)		
MCP-1	JE	Monocyte Chemotactic and Activating Factor (MCAF)
MCP-2	No murine homologue	
MCP-3	MARC	FIC
MCP-4	No murine homologue	NCC-1
No human homologue	MCP-5	
Regulated upon Activation, Normal T cell Expressed and Secreted (RANTES)	RANTES	SIS-delta
Eotaxin	Eotaxin	Eosinophil chemotactic protein
Eotaxin 2	No murine homologue	MPIF-2, CK-beta-6
Thymus and Activation Regulated Chemokine (TARC)	No murine homologue	
I 309	TCA3	
Hemofiltrate CC chemokine (HCC)		
HCC 1	No murine homologue	NCC-2
HCC 3	No murine homologue	
Thymus expressed chemokine (TECK)	TECK	
C6Kine	C6Kine	Exodus 2, Secondary Lymphoid tissue Chemokine (SLC)
Macrophage Derived Chemokine (MDC)	No murine homologue	Stimulated T cell Chemotactic Protein 1 (STCP-1)
No human homologue	C10	

* Includes some of the more common examples of alternative chemokine nomenclature and is not an exhaustive list.

Table 1.6. Human and rodent chemokine nomenclature (continued).

HUMAN	MURINE	ALTERNATIVE NAMES*
CXC CHEMOKINES		
ELR POSTIVE		
Interleukin 8	No murine homologue	NAP-1
Growth Related Oncogene (Gro)#		
Gro alpha	KC	Melanoma Growth Stimulating Activity (MGSA)
Gro beta	MIP2 alpha	
Gro gamma	MIP2 beta	
Epithelial Neutrophil Activating peptide (ENA) 78	ENA 78	
Platelet Basic Protein (PBP)@	No murine homologue	
Granulocyte Chemotactic Protein (GCP) 2	GCP 2	LPS Induced CXC chemokine (LIX)
ELR NEGATIVE		
Interferon gamma inducible Protein (IP) 10	CRG 2	
Monokine Induced by interferon Gamma (MIG)	MIG	
Stromal cell Derived Factor (SDF) 1	SDF	
Platelet Factor 4 (PF4)	No murine homologue	
CX3C CHEMOKINES		
Fractalkine	Fractalkine	Neurotactin
C CHEMOKINES		
Lymphotactin	Lymphotactin	

* Includes some of the more common examples of alternative chemokine nomenclature and is not an exhaustive list. # Human Gro alpha and murine KC have similar protein sequence to rat cytokine induced neutrophil chemoattractant (CINC) 1. CINC 2 and CINC 3 have similar protein sequences to human Gro beta and Gro gamma respectively. CINC 2 and CINC 3 are also similar to murine MIP2. @ Platelet basic protein is proteolytically cleaved to produce CTAP-III, beta-thromboglobulin and NAP-2).

regarding their chemoattractant properties. In general, CXC chemokines are neutrophil chemoattractants and CC chemokines are monocyte chemoattractants. However, some recently described members of the CXC chemokines, interferon inducible protein 10 (IP-10) and monokine induced by interferon gamma (MIG), are not biologically active on neutrophils. Both IP-10 and MIG do not have the amino acid sequence Glu-Leu-Arg (ELR) at the N-terminal side of the CXC motif, which is essential for binding and activation of the neutrophil IL-8 receptors that induce chemotaxis. Both CXC and CC chemokines act as lymphocyte chemoattractants with some selectivity on certain lymphocyte subsets. Chemokines are also growth factors for haemopoietic cells, activating factors for a variety of cell types, are able to induce angiogenesis and inhibit entry of human immunodeficency virus into permissive cells.

There are several chemokine receptors characterised, including several orphan receptors which have no characterised ligand at present. The chemokine receptors and their ligands are detailed in Table 1.7. Chemokine receptors are members of the seven transmembrane domain G-protein signalling family of cull surface receptors, and as such are sensitive to inhibition by pertussis toxin. There is evidence that some of the specificity of chemokine action is dependent on the interaction with different G-protein isoforms, the IL-8 receptors can bind Gαi2, Gαi3, Gα14, Gα15 and Gα16 while CCR1 can couple Gα14 but not Gα16. Coupling of the receptor and ligand induces phospholipase C hydrolysis of phosphatidylinositol 4,5-bisphosphate into two potentially active products, inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 mobilises intracellular calcium and activates calmodulin dependent protein kinases. Both DAG and the elevated intercellular calcium can also activate the serine/threonine protein kinase C. Protein kinase C comprises a family of at least 7 different but structurally related enzymes which have different substrate specificity. G proteins can also associate with adenylate cyclase, cGMP phosphodiesterase and Ras, with the activation of other intracellular signalling pathways. For example, the IL-8 receptor,

Table 1.7. Chemokine receptors and ligands.

CHEMOKINE RECEPTOR	LIGANDS
CXC chemokine receptors	
CXC R1	IL-8
CXC R2	IL-8, Gro, ENA-78, NAP-2
CXC R3	IP-10, MIG
CXC R4	SDF-1
CC chemokine receptors	
CC R1	MIP1α, MIP1β, RANTES, MCP-2, MCP-3, MCP-4
CC R2a/b	MCP-1, MCP-2, MCP-3, MCP-4
CC R3	Eotaxin, RANTES, MCP-3, MCP-4, MIP1α, MIP1β
CC R4	RANTES, MIP1α, MCP-1
CC R5	RANTES, MIP1α, MIP1β
CC R6	Liver and activation regulated chemokine (LARC)
CC R7	EBI1-ligand chemokine
CC R 8	I-309
Duffy Antigen	CXC chemokines (IL-8, Gro, ENA-78) and CC chemokines (RANTES, MIP1α, MIP1β, MCP proteins)
Orphan chemokine receptors	Unknown
Viral chemokine receptors	Various

CXCR1, is able to increase intracellular calcium, activate phospholipase D and induce free radical generation. Both IL-8 receptors can phosphorylate the p42/p44 mitogen activated protein kinase (MAP-kinase) and phosphatidylinositol 3-kinase. The former kinase can also be induced by MCP-1 stimulation of the CCR2 chemokine receptor.

1.7. CYTOKINE PRODUCTION: The importance of intercellular networking.

The liver is an important organ in the metabolism of cytokines, with the capacity both to produce and remove cytokines. Hepatic uptake of circulating cytokines is inhibited by alcohol (*Deaciuc et al 1996*) and this may contribute to the elevated levels of TNFα and IL-6 that are observed in such patients. All cells normally resident in the liver have the capacity to produce cytokines which by stimulating surrounding cells (paracrine effect) or themselves (autocrine effect) lead to further cytokine production and amplification of an inflammatory response. While some cytokines are released by resting cells, the concentrations and variety of cytokines released is considerably increased following stimulation by a variety of inducers.

1.7.1. Hepatic injury during sepsis.

The production of cytokines within the liver often depends upon the initial induction of early response cytokines released from tissue resident macrophages (Kupffer cells). A good example of this complex network of cytokine signals, leading to the amplification of an immune response, follows the injection of lipopolysaccaride/endotoxin. In the liver, lipopolysaccaride binds to CD14 and other cell surface molecules on the Kupffer cell and stimulates the production of both TNFα and IL-1β. These early response proinflammatory cytokines activate macrophages via an autocrine effect and recruit the stromal cells of the liver (endothelial cells, stellate cells and hepatocytes) to participate in the inflammatory response (paracrine effect) by inducing expression of cytokines and chemokines by these cells (*Thornton et al 1990, 1992*). TNFα and IL-1β can also induce the expression of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), on the endothelial surface and the corresponding ligand Mac-1 on circulating inflammatory cells. Adhesion between circulating mononuclear cells and activated endothelium via ICAM-1 induces both CXC and CC chemokine expression (*Lukacs et al 1994, 1995a*), which attract and activate circulating inflammatory cells and further amplifies an inflammatory response. In addition to inducing and amplifying a local inflammatory response, TNFα may induce both necrosis and apoptosis of hepatocytes via a direct mechanism and through the induction of nitric oxide expression by the hepatocytes themselves (*Kurose et al 1996*). Cell damage may also be mediated via free radical and elastase production induced by TNFα.

1.7.2. Ischaemia reperfusion injury

Another example of the importance of cytokine networks in the development of hepatic injury occurs consequent to ischaemia/reperfusion (I/R). This important clinical problem can occur following hepatic resection or surgery and after hypotension and resuscitation. There are two phases of hepatic injury following I/R; an initial phase (1-3 hours post reperfusion), which is associated with free radical generation and Kupffer cell activation and a later phase (6-24 hours post reperfusion) associated with neutrophil influx into the liver. The accumulation of neutrophils following I/R occurs in both experimental animal models and patients with cirrhosis and are important effectors of hepatic damage. The cascade of events leading to neutrophil accumulation is now better understood following the publication of several recent studies. Free radicals released following reperfusion stimulate the production of PAF and TNFα (*Suzuki et al 1994, Serizawa et al 1996*) from Kupffer and endothelial cells. Hepatic expression of IL-1α, another early response cytokine, is also elevated following I/R (*Shito et al 1997*). Both PAF and TNFα can stimulate the production of each other and therefore further amplify the inflammatory response. Neither TNFα, PAF or IL-1α are chemoattractant for neutrophils, but these cytokines can stimulate the release of neutrophilic chemoattractants, such as epithelial neutrophil activating peptide (ENA-78) and cytokine induced neutrophil chemoattractants (CINC), from parenchyma and non-parenchyma cells of the liver (*Colletti et al 1996a, Deutschman et al 1996*). Inhibition of early response or chemoattractant cytokines and increasing circulating IL-1 receptor antagonist attenuates hepatic neutrophil accumulation and damage in animal models of I/R. Hepatic neutrophil infiltration and damage also occurs following 70% hepatectomy and similar cytokine/chemokine networks have been reported with increased TNFα expression inducing local production of the neutrophil chemoattractant, ENA-78 in the liver (*Colletti et al 1996b*). LPS stimulated TNFα release from monocytes is also increased in patients after partial hepatectomy and therefore similar mechanisms of hepatic damage may occur in humans (*Sato et al 1996*).

1.7.3. Other stimuli to cytokine production

Reactive oxygen intermediates are produced by hepatocytes during the metabolism of ethanol, paracetamol and a variety of other drugs. Free radicals can activate the transcription factor, NF-κB, which can induce the transcription of a variety of cytokines and chemokines (*Sen & Packer 1996*). Hepatitis B viral infection stimulates the production of TNFα (*Larapezzi et al 1996*) and human herpesvirus 6 may induce IL-8 expression (*Inagi et al 1996*) in Hep G2 hepatoma cells. Ethanol induces IL-8 (*Shiratori et al 1993*) and Gro (*Shiratori et al 1994*) production in rodent primary hepatocyte cultures and supernatants from ethanol treated hepatocytes can induce the production of CINC from rat Kupffer cells (*Mawet et al 1996*). This latter effect is dependent on hepatocyte ethanol metabolism and is inhibited by 4-methylpyrazole. Cytokine production in the liver is also affected by hormones (*Van Gool et al 1990*), adrenaline increases LPS induced IL-6 production, but reduces TNF

α synthesis. Low concentrations of corticosterone, similar to those found in vivo, enhance the production of IL-6 and TNFα by LPS, but higher concentrations of corticosterone inhibit cytokine production.

Unopposed amplification of the immune response could lead to the development of autoimmune disease or massive release of proinflammatory mediators with multiorgan failure and death, as is seen during sepsis. Several negative immune modulators exist including anti-inflammatory cytokines (eg. IL-10 and IL-4), soluble cytokine receptors (soluble TNF receptors) and cytokine antagonists (IL-1 receptor antagonist). IL-10 for example down regulates endotoxin mediated IL6 release from Kupffer and sinusoidal endothelial cells (*Knolle et al 1997*). However, not all soluble cytokine receptors are inhibitory, soluble IL-6R (p80) can induce IL-6 effects on cells not normally expressing this component of the IL-6 receptor by binding to the ubiquitously expressed gp130 signal transduction polypeptide (*Fernandezbotran et al 1996*).

1.8. CYTOKINE RECEPTORS.

The functions of cytokines are mediated by binding to specific receptors expressed on the surface of target cells (*Foxwell et al 1992*). Cytokine receptors can be classified structurally into 5 different groups (Table 1.8). The immunoglobulin superfamily contain several extracellular immunoglobulin like domains and include other cell surface molecules such as MHC, the T cell receptor and ICAM-1. The haematopoietic growth factor family have a conserved amino acid sequence (tryptophan-serine-X-tryptophan-serine or WSXWS) usually located on the extracellular domain just proximal to the transmembrane region of the receptor. The TNF receptor family also includes the CD40 antigen on B cells and Fas (*Van den Abeele et al 1995*). There are 2 TNF receptors, p55 (TNFRI) and p75 (TNFRII), which have been discussed above.

Table 1.8. Cytokine receptors.

IMMUNOGLOBULIN	HAEMATOPOIETIC	INTERFERON	TUMOUR NECROSIS FACTOR
Interleukin 1	Interleukin 2 (β & γ)	Type I interferon (α & β)	Tumour necrosis factor
Interleukin 6	Interleukin 3	Type II interferon (γ)	Type I/α/55 kDa
	Interleukin 4		Type II/β/75 kDa
	Interleukin 5		Nerve growth factor
	Interelukin 7		Fas
	Interleukin 9		CD 40
	Leukaemia inhibitory factor		
	gp 130		

Chemokine receptors are detailed in Table 1.6

The interferon receptor family comprises of the receptors for IFN-α/β and IFN-γ. Lastly is the chemokine receptor family, characterised by 7 transmembrane alpha-helical structures, which are discussed in detail above. (*Horuk 1994*). Some cytokine receptors are hybrids, for example the IL6R contains both immunoglobulin and WSXWS domains and most cytokine receptors are not single polypeptide chains, but complexes of two or more. One polypeptide chain binds the cytokine ligand which then diamerises with a signal transducing polypeptide generating a receptor with greater ligand affinity. Signal transducing polypeptides may bind to a number of different cytokine binding chains; for example, gp130, the signal transducing portion of the IL-6 receptor can also interact with the receptors binding leukaemia inhibitory factor (LIF), oncostatin M and ciliary neurotrophic factor (CNTF).

1.8.1. Intracellular signalling.

Binding of cytokines to their receptors, stimulates a complex system of intracellular signals ultimately leading to the biological effect of the cytokine on its target cell (*Foxwell et al 1992, Horuk 1994, Schindeler & Darnell 1995, Van den Abeele et al 1995, Diehl & Rai 1996, Mufson 1997, Darnay & Aggarwal 1997*). Some of the growth factor receptors, eg. monocyte colony stimulating factor (M-CSF), platelet derived growth factor (PDGF) and epidermal growth factor (EGF), have intrinsic tyrosine kinase activity. Ligand binding induces receptor diamerisation, autophosphorylation and attracts cytosolic proteins with Src homology which subsequently activate the MAP-kinase pathway. Other intracellular signalling pathways may also be activated by receptors with intrinsic tyrosine kinase activity, including phosphoinositol, protein kinase C and Ras-GTPase. Most cytokine receptors do not posses intrinsic tyrosine kinase activity but associate with soluble cytoplasmic protein tyrosine kinases such as those of the Janus (Jak) or Tyk tyrosine kinase families. Jak phosphorylates members of the STAT (signal transduction and activator of transcription) protein family, which associate with other cytoplasmic phosphorylated

STAT proteins, translocate to the nucleus and activate gene transcription by binding to specific sequences in target genes. STAT proteins can also be phosphorylated directly by the membrane bound receptor tyrosine kinases. The specificity of the intracellular signals activated by different cytokines is dependent on the activation of specific STAT proteins (Table 1.9) and the different target gene sequences recognised by the different STAT protein diamers. Phosphorylation dependant activation of cytosolic protein phosphatases by tyrosine kinases also affects the degree of protein tyrosine phosphorylation and hence activation of these enzyme systems. Signal transduction of the interferon receptors is also mediated via the Jak/Tyk STAT pathway. TNFα induces a wide variety of intracellular second messengers including NF-kB, serine/threonine (stress activated protein kinase, SAPK) and tyrosine kinases, ceramide/sphingomyelin, reactive oxygen species and phosphoinositols. Intracellular signalling induced by chemokine receptor binding has been discussed above.

1.8.2. Intracellular signalling in liver cells

Most studies regarding cytokine intracellular signalling have been conducted in cells of the immune system rather than hepatocytes or the nonparenchymal cells of the liver. Increased intracellular cGMP is involved in the production of TNFα by Kupffer cells during sepsis (*Harbrecht et al 1995*). Phosphorylation of Jak1 and to a lesser extent Jak2 with STAT phosphorylation, Ras and MAPK activation has been recently reported following IL-4 stimulation of hepatocytes (*Chuang et al 1996*). IFN-γ and IL-6, but not IL-1β, TNFα and EGF, activate STAT3 in rat hepatocytes and human hepatoma cells (*Kordula et al 1995*). In contrast with IL-6, which produces rapid and transient activation of STAT1 and STAT3 in HepG2 cells, hepatocyte growth factor (HGF) induces delayed and sustained activation of STAT3 only (*Schaper et al 1997*). Insulin inhibits hepatocyte IL-6 induced acute phase response by transcriptional inhibition of STAT3 expression (*Campos et al 1996*). IL-1β induces the SAPK pathway in HepG2 cells, but does not activate Ras or MAP-kinase (*Bird et al 1994*).

Table 1.9. Signal transducer and activator of transcription.

STAT	CYTOKINE STIMULUS	GENE SEQUENCE RECOGNISED
1	Interferon γ, Interleukin 5, 7, CNTF, PDGF, GM-CSF	GAS
2	Interferon α & β	ISRE
3	Interleukin 6 (and related cytokines), 2, 15, G-CSF, HGF, PDGF	GAS
4	Interleukin 12	GAS
5	Interleukin 2, 3, 5, 7, 15, GM-CSF, SCF, Epo.	GAS
6	Interleukin 3, 4, 13, PDGF	GAS

ISRE = interferon stimulated response element, GAS = gamma activated sequence. These are palindromic DNA sequences (GAS = TT N(i) AA, binding of STAT 1 and STAT 4 is best when N(i) = 5, binding of STAT 3 is best when N(i) = 4 and binding of STAT 6 is best when N(i) = 6).

Oxidative stress can also induce the MAP-kinase which via phosphorylation of protein phosphatases can dephosphorylate and inactivate hepatic p38, a phosphoprotein constitutively active in the liver (*Mendelson et al 1996*). In addition TNFα can induce specific hepatocyte phosphatase expression and hence modulate signalling via cytoplasmic or membrane bound protein kinases (*Ahmad et al 1997*).

1.9. CYTOKINES AND HEPATIC METABOLISM.

The functions of cytokines are diverse and clearly include a role in normal physiologic functions and homeostasis. Body temperature fluctuation during the day, appetite regulation and lethargy/sleep patterns are all controlled at least in part by cytokines. The normal metabolic functions of the liver, including gluconeogenesis, lipid and protein metabolism are affected by cytokines. TNFα increases hepatic HMG CoA reductase activity, lipid synthesis and secretion and expression of LDL receptors (*Laio & Floren 1994*) but reduces both hepatic and plasma LCAT activity. Transforming growth factor β (TGFβ) inhibits LCAT activity in Hep G2 cells (*Skettering et al 1995*). IL-4 induces the expression of cytochrome P450 2E1 and glutathione S-transferases. In contrast, IL-2, TNFα, IL-1β and IL-6 can inhibit the induction of certain cytochrome P450 isoforms. Endocytosis of hepatocytic mannose and scavenger receptors is enhanced by TNFα and IL-1β (*Asumendi et al 1996*), but these cytokines inhibit the activity of bile acid transporters on the biliary canalicular membrane (*Moseley et al 1996, Green et al 1996*). The biological effect of cytokines, such as TNFα is dependent on the effective concentration; low concentrations are involved in homeostasis, increasing local concentration of TNFα is associated with local inflammatory response and focal hepatic necrosis and massive release of TNFα into the circulation results in systemic activation with adult respiratory distress syndrome and multiple organ failure.

1.10. CYTOKINES AND THE ACUTE PHASE RESPONSE.

The acute phase response is characterised by increased circulating concentrations of several different plasma proteins, including C-reactive protein (CRP), serum amyloid A (SAA) protein, haptoglobin, fibrinogen and $\alpha 2$ macroglobulin (positive acute phase proteins), and the reduction in others, such as albumin, alpha fetoprotein, fibronectin and transferrin (negative acute phase proteins). The altered serum concentrations of these proteins is the direct result of changes in protein synthesis and release from hepatocytes under the influence of cytokines. The effects of cytokines on the hepatic acute phase response has been the subject of several recent reviews (*Baumann & Gauldie 1994, Koj 1996, Moshage 1997*).

IL-6 affects the synthesis and secretion of a wide range of acute phase proteins from cultured rodent and human primary hepatocytes, hepatoma cell lines and *in vivo* in animal models and man (*Banks et al 1995*). The synthesis of positive acute phase proteins is induced by IL-6, while the production of negative acute phase proteins is inhibited. Other cytokines utilising the common gp130 signal transducing receptor (LIF, oncostatin M, CNTF and cardiotropin-1) can also induce similar changes in acute phase protein production by cultured hepatocytes. The importance of gp130 in signal transduction during the acute phase response in vivo is supported by the inhibition of IL-6 stimulated acute phase protein production by monoclonal anti-gp130 antibodies (*Harrison et al 1996*). Many other cytokines, eg. TNFα, IL-1β, IL-4, IL-8, IL-10, IL-13 and IFN-γ, and growth factors, such as TGFβ and HGF can induce an acute phase response or modulate the effects of IL-6. Hep G2 cells also express G-protein receptors for the chemoattractants, C5a and FMLP, which are also able to induce production of the positive acute phase proteins and inhibit negative acute phase protein production (*Buchner et al 1995, McCoy et al 1995*). Classic hormones, eg. corticosteroids, can also modulate the cytokine stimulated acute phase response.

1.11. CYTOKINES, HEPATIC INJURY AND INFLAMMATION.

Many animal models of hepatic inflammation exist, two well studied examples are lipopolysaccaride administration to rodents sensitised with galactosamine (LPS/GalN) and Concanavallin A injection. Serum cytokines are not elevated following LPS/GalN, however hepatocyte apoptosis is followed by inflammatory cell infiltration, hepatic necrosis, and elevated serum transaminases. The histological changes in the liver and elevated serum transaminases occurring following LPS/GalN administration are prevented by prior administration of anti-TNF antibodies, soluble TNF receptors, exogenous recombinant IL-10, cyclosporin or dexamethasone (*Santucci et al 1996, Louis et al 1997*). Phosphodiesterase inhibitors can also inhibit hepatic injury in this model by altering the cytokine balance in favour of anti-inflammatory cytokines, such as IL-10 (*Gantner et al 1997*). Recently, transcriptional blockers other than GalN, such as actinomycin D and alpha amanitin were also shown to induce apoptosis, hepatic inflammation and necrosis which could be prevented by anti-TNF antibodies (*Leist et al 1997*). TNFα dependant hepatocyte apoptosis appears to signal via the IL-1 converting enzyme (ICE) like protease pathway and can also be prevented in vitro and vivo by specific ICE-protease inhibitors (*Knustle et al 1997*). Pretreatment with IL-1β, by inducing tolerance, can also inhibit hepatocyte apoptosis induced by TNFα in the LPS/GalN model (*Bohlinger et al 1995*).

Concanavallin A (Con-A) injection induces massive T cell activation, infiltration of the liver with neutrophils, CD4+ and CD8+ lymphocytes and focal hepatic necrosis. Serum elevation of IL-2, TNFα and IFN-γ have been reported. In vitro studies have implicated perforin mediated hepatocyte injury, observing that TNFα is not directly cytotoxic to Con-A treated hepatocytes (*Watanabe et al 1996*). In contrast, others have shown ConA is directly cytotoxic to cultured hepatocytes (*Leist & Wendel 1996*).

However, in vivo administration of anti-TNF antibody, anti-IFN-γ antibody or recombinant IL-6 prevents hepatic damage without affecting IL-2 release or hepatic infiltration of inflammatory cells (*Mizuhara et al 1996*). Soluble TNF receptor is also effective in preventing liver damage in this model (*Bruck et al 1997*)

The advent of transgenic technology has lead to its use in the study of hepatic inflammation. IFN-γ transgenic mice develop a chronic hepatitis, which appears mediated by TNFα (*Okamoto et al 1996*). The HBV transgenic mouse has improved greatly the understanding of the pathogenesis of hepatic damage in HBV infection (*Chisari & Ferrari 1995*). The observed reduction in HBV transcription following infection with other hepatotropic viruses or bacteria in HBV transgenic mice appears secondary to the local production of IFN-γ and TNFα by macrophages and cytotoxic lymphocytes (*Guidotti et al 1996*). An inhibitory effect on HBV replication in these mice can also be induced by exogenous recombinant IL-12 administration via the induction of IFN-γ (*Cavanaugh et al 1997*). IFN-γ can induce apoptotic cell death in primary hepatocyte culture (*Morita et al 1995*) and this mechanism may be important in clearance of viral infected cells in vivo. Adenovirus transfection or liposomal encapsulation, the latter with or without retrovirus infection (*Wu & Zern 1996*), have been used to study the transient expression of cytokines in the liver. For example, adenovirus mediated expression of CINC, a CXC chemokine, in the liver is associated with the development of a neutrophilic hepatitis (*Maher et al 1997*).

1.11.1. Clinical studies of cytokines and hepatic inflammation

The serum or plasma concentrations of a variety of cytokines and cytokine antagonists are elevated in patients with liver disease. However, the potential pathogenic role of elevated circulating cytokines to the development of hepatic inflammation is not clear. Increased circulating proinflammatory cytokines can contribute to the multiorgan failure seen in some patients with liver disease. Elevated circulating TNFα or IL-1β have been observed in patients with alcoholic liver disease, especially those who are malnourished, and have been correlated with survival (*Means et al 1996*). Increased circulating TNFα has been reported in patients with the HELLP syndrome (*Haeger et al 1996*) and others with acute liver failure (*Keane et al 1996*). IFN-γ is found in the serum of patients with viral hepatitis and acute cellular rejection (*Cacciarelli et al 1996*). Increased circulating TNFα and M-CSF have been reported in patients with viral hepatitis (*Spengler et al 1996*). Increased circulating IL-6 occurs in patients with HCV infection and is correlated with hepatic inflammatory activity and serum HCV-RNA levels (*Malaguarnera et al 1997*). In addition to increased TNFα, elevated IL-8 has been reported in patients with alcoholic liver disease (*Sheron et al 1993*). Increased circulating IL-8 may have a suppressive effect on neutrophil function as has been reported in IL-8 transgenic mice; these mice are unable to mount a neutrophil response to intraperitoneal injection of bacteria (*Simonet et al 1994*).

Cytokine production from peripheral blood mononuclear cells have also been studied in a variety of liver conditions, but again their importance in understanding the pathogenic mechanisms within the liver are unclear. LPS induced TNFα expression is increased in blood monocytes from patients with alcoholic liver disease due to a decreased responsiveness to the anti-inflammatory effects of IL-10 (*Lemoine et al 1995*). Monocyte production of proinflammatory cytokines is increased in patients with hepatitis C virus (HCV) infection, although others have shown impaired phorbol-12-myristate-13-acetate (PMA) stimulated TNFα and IL-1β release (*Mendoza et al 1996*). Mitogen and HCV peptide stimulated T cells, derived from peripheral blood, produce TNFα and IFN-γ but little IL-4. In contrast, spontaneous IL-10 production and HCV peptide stimulated IL-12 production was similar in patients with HCV infection compared with patients with liver cirrhosis and controls (*Kakumu et al 1997*).

Many studies using reverse transcriptase, polymerase chain reaction (RT-PCR), in-situ hybridisation or immunohistochemistry have demonstrated hepatic expression of a variety of different cytokines in patients with liver disease. Hepatic expression of TNF α has been reported in hepatitis B, hepatitis C, autoimmune liver disease and alcoholic hepatitis (*Llorente et al 1996, Fang et al 1996*). Recent interest has focused on differentiating the T helper (Th) cell responses in various inflammatory conditions into Th1, characterised by IFNγ and IL-2 expression and a cell-mediated response, or Th2, characterised by IL-4 and IL-5 expression and an antibody mediated immune response. Th0 denotes a mixed response. A Th2 response may be associated with fibrosis and viral persistence, in contrast a vigorous Th1 response may damage surrounding hepatocytes (a bystander effect). Hepatic expression of Th1 cytokines has been reported in patients with hepatitis C infection, in addition, T cell clones prepared from liver tissue produce Th1 cytokines following stimulation with recombinant HCV proteins (*Napoli et al 1996, Lohr et al 1996*). Chronic cellular rejection following hepatic transplantation is also associated with the expression of Th1 cytokines in the liver (*Gorczynski et al 1996*). In contrast with HCV infection the situation in patients with hepatitis B infection is more complicated. Patients infected with HBV express both IL-4 and IFN-γ (Th0) when the liver is severely inflamed (*Bertoletti et al 1997*). Others have shown hepatic expression of Th1 cytokines in patients with acute HBV and active inflammation and Th2 cytokines in patients with chronic infection and low levels of hepatic inflammation (*Fukuda et al 1995*). However, it is not clear if this latter finding is related to switching of the immune response from Th1 to Th2, as has been reported in the natural evolution of immune responses in mice. The T helper response in hepatitis B may be dependent on the antigen expressed at a particular time. Hepatitis B core antigen can induce a Th1 response and hepatitis B e antigen induces Th2 or Th0 helper T cells in vivo, this effect can also be modulated by exogenous IFN α (*Milich et al 1997*). Differentiation of the immune response into Th1 or Th2 like responses in patients with primary biliary cirrhosis (PBC) is more confused. Mitogenic stimulation of liver infiltrating T cells from patients with PBC and some with autoimmune hepatitis produce Th2 cytokines (*Lohr et al 1994*). However others, studying the intrahepatic expression of IFN-γ and IL-4, have suggested a predominance of a Th1 phenotype in PBC (*Harada et al 1997*). Defective purified protein derivative (PPD) stimulated Th1 and Th2 cytokine production by peripheral blood cells has also been reported in patients with PBC (*Jones et al 1997*)

In some conditions the cellular infiltrate in the liver has been correlated with hepatic expression of chemoattractant cytokines. Hepatic neutrophil infiltrates are prominent in alcoholic hepatitis and have been correlated with the local expression of the CXC chemokines, IL-8 (*Sheron et al 1993*) and Gro (*Maltby et al 1996*). In addition, a hepatotoxic effect of the murine Gro homologue, macrophage inflammatory protein 2 (MIP2), has also been implicated in the pathogenesis of alcoholic liver disease (*Bautista 1997*). Hepatic expression of IL-5, a potent eosinophil chemoattractant, has been reported in conditions such as PBC (*Martinez et al 1995*) and acute cellular rejection following liver transplantation (*Martinez et al 1993*), which can both be associated with hepatic eosinophilia. Acute cellular rejection is also characterised by lymphocytic inflammation and hepatic expression of the CC chemokines, MIP-1α and MIP-1β (*Adams et al 1996*).

1.12. CYTOKINES AND HEPATIC FIBROSIS.

The stellate cell plays a central role in the development of hepatic fibrosis and cirrhosis. The effect of cytokines on stellate cell biology have been reviewed recently by several authors (*Davis & Kresina 1996, Alcolando et al 1997*). In summary, stellate cell proliferation and collagen synthesis can be influenced by Kupffer cell (eg. TGFβ and TNFα), endothelial cell (eg. PDGF) and hepatocyte (eg. insulin like growth factor and IGF-binding protein) derived factors (*Gressner et al 1995*). Stellate cell derived PDGF also has an autocrine effect on the producing cells. IL-1β, IL-4 and IL-6 can also modulate stellate cell collagen and cytokine synthesis. Another example of cytokine networking involving hepatic stellate cells (*Benyon et al 1997*) is their recently reported ability to secrete stem cell factor (SCF). SCF activates mast cells and prolongs their survival by inhibiting apoptosis. Recent reports have correlated the degree of mast cell infiltration with the severity of hepatic fibrosis (*Farrell et al 1995*). Hepatic SCF mRNA and protein concentration is increased in fibrotic liver diseases in patients and following bile duct ligation in experimental animals (*Omori et al 1997a*). Interestingly, human hepatic stellate cells proliferate when exposed to human mast cell tryptase (*Benyon et al 1997*). Therefore, both mast cells and stellate cells may interact via SCF and tryptase to promote fibrogenesis within the liver.

In addition to its importance in the pathophysiology of hepatic fibrosis, the stellate cell is also the major source of the CC chemokine, MCP-1, in patients with acute liver disease (*Czaja et al 1994*). The expression of stellate cell MCP-1 is induced by reactive oxygen species and the early response cytokines, TNFα and IL-1β (*Xu et al 1996*). Interestingly, MCP-1 is also chemoattractant for mast cells and basophils.

1.13. CYTOKINES AND HEPATIC REGENERATION.

The role of cytokines in hepatic regeneration has also been the subject of several recent reviews (*Hoffman et al 1994, Fausto & Webber 1994, Diehl & Rai 1996b*). Many cytokines are hepatocyte mitogens in vitro, but their relative importance in vivo remains unclear. EGF, TGFα and HGF are complete hepatocyte mitogens, but recent studies have suggested a central role for TNFα, in hepatic regeneration following both toxic and partial, 70%, hepatectomy (PH). Exogenous TNFα stimulates hepatic DNA synthesis in rodents and accelerates recovery of liver weight following PH. Although serum TNFα concentrations are not elevated following PH, anti-TNF antibodies inhibit regeneration in this model and following toxic liver injury (*Bruccoleri et al 1997*). Hepatic regeneration following PH is also impaired in TNFR1 knock-out mice (*Yamada et al 1997*). In addition, Kupffer cell blockade augments hepatic regeneration following PH secondary to impaired IL-10 release from these cells allowing sustained unopposed TNFα production from endothelial cells (*Rai et al 1997*). Anti-TNF antibodies have an even greater inhibitory effect on hepatic regeneration following PH in chronic ethanol fed rats. TNFα released following PH has also been implicated in the activation of several intracellular signalling pathways (e.g. STAT 3) and transcription regulators (e.g. AP1, C/ERB, NF-kB) which may control the expression of proto-oncogenes and the progression of hepatocytes through the cell cycle during regeneration (*Cressman et al 1995, Diehl et al 1995, Taub 1996*). Lead nitrate induces liver cell hyperplasia and increases hepatic TNFα concentrations but two other inducing agents, cyproterone acetate or nafenopin, are not associated with increased hepatic TNFα expression (*Menegazzi et al 1997*). Dexamethasone, a relatively non-specific anti-inflammatory agent, reduces TNFα concentrations, without affecting HGF expression, and inhibits hepatocyte proliferation induced by lead nitrate .

1.14. CYTOKINES AND HEPATIC CANCER.

Certain cytokines have been implicated in the development of hepatic cancer. TGFβ over expression in trangenic mice is associated with a 60% incidence of spontaneous hepatoma, however the incidence in HGF/TGFβ double transgenics is only 30%, suggesting coexpression of HGF may protect against hepatocarcinogenesis. Hepatoblastomas are often associated with extramedullary haematopoiesis within the liver and can express the haematopoietic cytokines, erythropoietin, stem cell factor, granulocyte colony stimulating factor (G-CSF) and granulocyte-monocyte colony stimulating factor (GM-CSF). Carcinoembryonic antigen can induce Kupffer cell production of TNFα and IL-1β (*Gangopadhyay et al 1996*), these proinflammatory cytokines may induce endothelial cell adhesion molecule expression and thus enhance the potential for metastasis. Elevated circulating concentrations of IL-8 have been reported in patients with hepatoma (*Sakamoto et al 1992*) and may contribute to the immunodeficiency of such patients. Transcatherter arterial embolisation is a commonly used therapy for hepatoma. Tumour embolisation is often followed by fever and an inflammatory response, this has been associated with increased circulating IL-6 concentrations, systemic TNFα or IL-1β are unaffected (*Matsuda et al 1994*).

TNFα, IL-1β, IL-6 and IFN-γ production have been implicated in the weight loss and cachexia induced by malignant tumours (*Argiles & Lopez Soriano 1997*). Implantation of Morris 7777 hepatoma cells in severe combined immunodeficiency (SCID) mice induces TNFα, IL-1β and IL-6 production from spleen cells and is associated with profound weight loss (*Murray et al 1997*). In contrast, transplantation of MCA sarcoma was not associated with loss or increased proinflammatory cytokine expression (*Murray et al 1997*). Rats bearing the Yoshida AH-130 ascites hepatoma have a hypercatabolic state with increased circulating TNFα concentrations. Anti-TNF antibodies normalise the muscle protein synthesis abnormalities and disturbances in hormone balance, but are unable to prevent weight loss in this model (*Costelli et al 1993, 1995*).

1.15. THERAPEUTIC POTENTIAL OF CYTOKINES.

Use of interferon alpha in the viral hepatitis is the most widely used cytokine in the treatment of liver disease (*Tilg 1997*). TNFα, GM-CSF and IL-12 have also been used to treat patients with viral hepatitis, IL-12 may be especially efficacious in chronic HBV infection which appears characterised by a Th2 type cytokine response. Prior inhibition of macrophage function with gadolinium chloride or liposome encapsulated diphosphonates has been shown to inhibit liver injury in a number of experimental models of toxic liver injury. As noted above inhibition of TNFα with a variety of agents inhibits liver injury in these models. Inhibition of cytokine production by steroids can explain their efficacy in treating hepatic allograft rejection, alcoholic hepatitis and autoimmune hepatitis.

Cytokine therapy has been used to prevent hepatic tumour metastasis or tumour growth in a variety of animal models, but the use of cytokines in the management of patients with hepatoma is much more limited and often disappointing. Conventional antitumour therapies, such as mitomycin C and adriamycin, are able to activate Kupffer cells and induce the antitumoral activity of these cells (*Adachi et al 1992*). IFN-γ administration activates hepatic Kupffer cells and protects rodents against hepatic metastasis from intrasplenic hepatoma cell injection (*Karpoff et al 1996*). Using a similar model, injection of irradiated hepatoma cells transfected with vectors expressing GM-CSF or IL-2 to induce T cell activation also prevented hepatic tumour metastasis. Combination treatment with GM-CSF or IL-2 expressing cells and IFN-γ was more effective in preventing tumour growth than either therapy alone. Reinjection of tumour cells incubated in vitro with cytokines that upregulate MHC and adhesion molecule expression is also effective in inducing cytotoxic T cell responses and protects against parenteral tumour cell injection (*Guo et al 1997*). In vivo transfection of primary and metastatic hepatoma with adenovirus-IL-2 expressing vectors can cure upto 75% of tumour bearing animals (*Huang et al 1996*). Direct injection of IL-2 into hepatomas can also induce an effective anti-tumoural systemic T cell response in animals with implanted tumours (*Balemans et al 1993*). An alternative strategy is to induce lymphokine activated killer cells from peripheral blood mononuclear cells and reinfuse these cells, this has been found safe and effective in a small pilot study of patients with advanced carcinoma (*Yeung et al 1993*). Ultrasound guided injection of LAK and IL-2, combined with subcutaneous IL-2 has also been used safely in patients with primary and secondary liver tumours (*Ferlazzo et al 1997*). Intratumour injection of TNFα is effective in animal hepatoma models. Development of closed perfusion techniques has allowed the use of local TNFα, in conjunction with conventional cytotoxic therapy, in patients with hepatic malignancy. Intra-arterial TNFα and IFN-γ can also significantly improve the response to hepatic artery ligation in a rodent model of hepatoma (*Yang et al 1995*). In a small pilot study, thymostimulin has been shown to be effective in treating patients with hepatoma. The antitumoral activity of thymostimulin appears secondary to Kupffer cell TNFα production (*Balach et al 1997*).

CHAPTER 2

TNFα STIMULATED CHEMOKINE PRODUCTION FROM HUMAN HEPATOMA CELLS

CHAPTER 2

TNFα STIMULATED CHEMOKINE PRODUCTION FROM HUMAN HEPATOMA CELLS

2.1. INTRODUCTION

The cellular composition of the liver includes tissue resident macrophages (Kupffer cells), endothelial cells, biliary epithelial cells and lipocytes. However, hepatocytes form the largest cell mass within the liver. Although stromal cells have not been classically considered as immune cells, there is increasing evidence that these cells can play an active role in the development and maintenance of an inflammatory response through their ability to produce cytokines, including proinflammatory cytokines and chemokines. Relatively little is known regarding the production of chemokines by hepatocytes. Studies have shown that cultured rodent liver cells can produce IL-8 (*Shiratori et al 1993*) and Gro (*Shiratori et al 1994*) when exposed to ethanol and more recently human hepatoma cell lines (*Thornton et al 1990*) and primary human hepatocytes (*Rowell et al 1997*) have been shown to produce both CXC and CC chemokines following proinflammatory cytokine stimulation. However, contamination of the primary cultures with Kupffer cells, which are potent sources of chemokines, may produce confounding results in studies using primary cell cultures. In addition, no data exists on the potential intracellular pathways induced by proinflammatory cytokines resulting in hepatocyte chemokine production. Knowledge of signalling mechanisms may direct development of specific therapy to modulate chemokine production from this large mass of cells, preventing their recruitment into the inflammatory response.

In view of the problems of supply and purity of preparation of primary human hepatocytes, the studies in this chapter focused on chemokine production from human hepatoma cells (Hep G2 and SK-Heps lines) induced by TNFα (in view of the potential importance of this early response cytokine in the pathogenesis of hepatic disease), although preliminary data is presented on the few experiments undertaken using normal human hepatocytes (Clonetics Inc). The aims were as follows;

- to determine which chemokines were produced by cultured human hepatoma cells.
- to assess the effect of soluble TNF receptors and other cytokines on TNFα induced chemokine production.
- Using specific inhibitors and inducers to investigated the intracellular pathways involved in hepatoma cell chemokines production.
- To measure the effect of drugs previously used in the treatment of liver disease, on hepatoma cell chemokine production.

2.2. METHODS

2.2.1. Cell culture and stimulation.

Human hepatoma cells (SK-Heps and Hep G2) from the American Type Culture Collection, were cultured in Dulbecco's modified Eagles medium (DMEM) in 6 or 24 well plates. Normal human hepatocytes (a kind gift from Dr S Kamil, Clonetics Inc, CA) were cultured in 6 well matrigel coated plates in Hepato-STIM (Becton Dickinson, Bedford, MA). In the majority of experiments, TNFα was added at 5ug/ml and the cells cultured for a further 20 hours. The supernatants were recovered and stored at -20^{o}C for ELISA and guanidine isothiocyanate added to the cells before freezing at -20^{o}C and subsequent RNA extraction. The inhibitors (purchased from Sigma and Fugisawa), dexamethasone (10^{-4} mol/L), cyclosporin (5 ug/ml), FK 506

(100 ug/ml), staurosporine (100 ng/ml) genistein (10 umol/L), calphostin C (1 umol/L), and anti-TNF receptor antibodies (from R&D Systems Inc, 20 ng/ml) were preincubated with the culture human hepatoma cells at 37°C for 30 minutes before addition of TNFα. The antioxidant, N-acetylcysteine (Sigma, 10 mmol/L), and the NF-κB inhibitor, pyrrolidine dithiocarbamate (Sigma, PDTC, 1 mmol/L), were preincubated with the hepatoma cells for 6 hours prior to addition of TNFα. Soluble TNF receptors (R&D Systems Inc) were added at the time of TNFα stimulation. Phorbol-12-myristate-13-acetate, (PMA, 2.5 umol/L) stimulation of Hep G2 cells and SK-Heps was continued for 4 hours before the media was replace and the hepatoma cells cultured for a further 20 hours.

2.2.2. Enzyme linked immunoassay (ELISA).

Flat bottomed 96-well microtitre plates (Nunc Immunoplate I 96-F, Copenhagen, Denmark) were coated with 50 ul/well of anti-chemokine antibody for 16 hours at 4°C and then washed with phosphate buffered saline (PBS), pH 7.5 and 0.05% Tween-20 (wash buffer). Nonspecific binding sites were blocked with 2% bovine serum albumin in PBS and incubated for 90 minutes at 37°C. Plates were rinsed four times with wash buffer, and 50 ul of supernatant (neat and at 1/10 dilution) added in duplicate, followed by incubation for 1 hour at 37°C. Plates were washed four times, followed by addition of 50 ul/well of biotinylated anti-murine antibody (3.5 ug/ml in PBS, pH 7.5, 0.05% Tween-20, and 2% fetal calf serum), and then incubated at 37°C for 30 minutes. Plates were washed as before, streptavidin-peroxidase conjugate (Bio-Rad Labs) was added, and the plates incubated for 30 minutes at 37°C. Plates were then washed four times and chromogen substrate (Bio-Rad Labs) added, the reaction was terminated with 50 ul/well of 3 M H_2SO_4 and the plates read at 490 nm in an ELISA reader. Standards were $^1/_2$ log dilutions of human chemokines from 1 pg/ml to 100 ng/ml. The ELISA methods used consistently detected chemokine concentrations > 30 pg/ml and were specific without detectable cross-reaction with other CXC or CC chemokines, IL-6, IL-1 or TNFα.

2.2.3. RNA extraction, cDNA synthesis and PCR.

Cultured cells were scraped from the culture plates and homogenised in 1 ml of 4.2 mol/L guanidine isothiocyanate, 25 mmol/L Tris (pH 8.0), 0.5% Sarkosyl and 0.1 mol/L 2-mercaptoethanol. An equal volume of 100 mmol/L Tris (pH 8.0), 10 mmol/L ethylenediamine tetraacetic acid (EDTA) and 1.0% sodium dodecyl sulphate (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 5ug reverse transcribed into cDNA using oligo (dT)12-18 primers and AMV reverse transcriptase. The cDNA was amplified using specific primers for the chemokines detected by ELISA using cyclophylin as control (cyclophylin sense CATCTGCACTGCCAAGAC, cyclophylin antisense CTGCAATCCAGCTAGGCATG). The amplification reaction was incubated initially at 94^{o}C for 5 minutes followed by 35 cycles at 93^{o}C for 45 seconds, 52^{o}C for 45 seconds and 72^{o}C for 90 seconds. The amplification products were visualised under UV light following separation in a 2% agarose gel containing 0.3 mg/ml ethidium bromide.

2.2.3. Statistical analysis

Results are presented as mean $\pm$ standard error of at least 3 separate experiments. Statistical significance was determined by analysis of variance and students t-test as appropriate, with $p < 0.05$ considered significant.

<u>2.3. RESULTS</u>

2.3.1. Hepatoma cells produce CXC and CC chemokines following proinflammatory cytokine stimulation.

Both Hep G2 cells (Figure 2.1) and SK-Heps produced IL-8 immunoreactive protein following stimulation with TNFα, in a concentration and time dependent fashion and this was associated with induction of mRNA expression. Hep G2 cells produced none of the other CXC chemokines measured, ie. Gro, ENA-78, IP 10 or MIG. In contrast, SK-Heps constitutively produced low concentrations of the CXC chemokine, Gro (0.120 $\pm$ 0.023 ng/ml, mean $\pm$ SEM, n = 12), which were not induced further by TNFα (0.176 $\pm$ 0.022, n = 16). SK-Heps produced the CC chemokines MCP-1 and RANTES during stimulation with TNFα, and the production of these chemokines was induced in a time and concentration dependent manner. Induction of MCP-1 and RANTES immunoreactive protein was associated with increased expression of the mRNA.

2.3.2. Cultured human hepatocytes produce CXC and CC chemokines.

Normal human hepatocytes (Figure 2.2) stimulated with TNFα produced significantly ($P < 0.05$) increased immunoreactive IL-8 (control 1.692 $\pm$ 0.252 ng/ml, mean $\pm$ SEM, n = 6, TNFα stimulated 7.773 $\pm$ 0.230, n = 4), Gro (control 0.190 $\pm$ 0.033 ng/ml, n = 6, TNFα stimulated 0.771 $\pm$ 0.099, n = 4), ENA-78 (control 0.694 $\pm$ 0.157 ng/ml, n = 6, TNFα stimulated 1.655 $\pm$ 0.136, n = 4) and IP-10 (control 0.134 $\pm$ 0.060 ng/ml, n = 6, TNFα stimulated 0.933 $\pm$ 0.239, n = 4). Low concentrations of MIG were also detectable (0.089 $\pm$ 0.021 ng/ml, n = 6), but TNFα stimulation had no significant effect on MIG expression (0.045 $\pm$ 0.028, n = 4). No immunoreactive MIP1 alpha or beta were detectable following TNFα stimulation, however TNFα significantly increased MCP-1 production (control 0.229 $\pm$ 0.058 ng/ml, n = 6, TNFα 0.461 $\pm$ 0.038, n = 4). Unfortunately RANTES production was not studied in these experiments.

In preliminary experiments (n = 2), a similar pattern of chemokine production was induced in human primary hepatocytes by IL-1β, with the suggestion that this early response cytokine was more potent in inducing chemokine expression. In addition, these preliminary experiments revealed that IFNγ (5 ng/ml) was generally inhibitory in regard to human hepatocyte chemokine production, reducing chemokine concentration below basal expression. However significant production of both IP-10 (12.996 ng/ml, n = 2) and MIG (1.196 ng/ml, n = 2) was noted following IFNγ stimulation.

2.3.3. TNFα signals via TNFR1 to induce chemokine production.

Preincubation of hepatoma cells with anti-TNFR1 antibody resulted in significant reduction ($p < 0.05$) in the production of IL-8 (4.12 $\pm$ 0.43 ng/ml, n = 10), MCP-1 (0.17 $\pm$ 0.04 ng/ml, n = 10) and RANTES (0.05 $\pm$ 0.03 ng/ml, n = 8) from SK-Heps following TNFα stimulation compared with controls (IL-8, 22.28 $\pm$ 2.46, n = 10, MCP-1, 0.63 $\pm$ 0.10, n = 10, RANTES, 0.85 $\pm$ 0.21, n = 10). The concentration of IL-8 induced by TNFα stimulation of Hep G2 cells (2.58 $\pm$ 0.42, n = 16) was also significantly reduced by anti-TNFR1 antibodies (0.58 $\pm$ 0.08, n = 13). Anti-TNFR2 antibodies had no effect on TNFα stimulated IL-8 production from Hep G2 cells (control 2.58 $\pm$ 0.42, n = 16, anti-TNFR2 antibody 2.54 $\pm$ 0.39, n = 13) and MCP-1 (control 0.63 $\pm$ 0.10, n = 10, anti-TNFR2 antibody 0.66 $\pm$ 0.13, n = 10) or RANTES (control 0.85 $\pm$ 0.21, n = 10, anti-TNFR2 antibody 0.62 $\pm$ 0.05, n = 8) released by SK-Heps. Interestingly, IL-8 production by SK-Heps was significantly enhanced by anti-TNFR2 antibodies (40.11 $\pm$ 5.24, n = 10), compared with controls (IL-8, 22.28 $\pm$ 2.46, n = 10). These data suggest that TNFα stimulated chemokine production from human hepatoma cells, signals via TNFR1, rather than TNFR2.

2.3.4. Soluble TNFR inhibits chemokine production.

Inclusion of either soluble TNFR1 or TNFR2 in the culture media did not affect chemokine synthesis induced by TNFα. However, when both soluble TNFRs were combined in the culture media, there was a dose dependent inhibition of chemokine production by both the SK-Heps and Hep G2 cells (figure 2.3). Soluble TNFR1 and TNFR2 (1000 ng/ml) also completely inhibited RANTES production (TNFα stimulated 0.85 ± 0.21 ng/ml, mean $\pm$ SEM, n = 8, TNFα and soluble TNFR 0, n = 4) and significantly inhibited MCP-1 expression (TNFα stimulated 0.63 ± 0.10 ng/ml, mean $\pm$ SEM, n = 10, TNFα and soluble TNFR 0.14 ± 0.06, n = 6).

2.3.5. Other cytokines modulate TNFα stimulated chemokine production.

IFN-γ significantly inhibited TNFα stimulated, IL-8 production from Hep G2 cells (TNFα alone 2.94 ± 0.33 ng/ml, mean $\pm$ SEM, n = 12, TNFα + IFN-γ 1.84 ± 0.25, n = 8, figure 2.4). IL-10 and IL-4 had no effect on either basal or TNFα stimulated IL-8 production from Hep G2 cells (figure 2.4). In contrast, these cytokines had no effect on IL-8 expression in SK-Heps (figure 2.5), but IFN-γ significantly increased the TNFα stimulated RANTES (TNFα alone 0.90 ± 0.17 ng/ml, n = 12, TNFα + IFN-γ 1.85 ± 0.48, n = 8) and TNFα stimulated MCP-1 (TNFα alone 0.51 ± 0.05 ng/ml, n = 12, TNFα + IFN-γ 1.09 ± 0.17, n = 8) production in these cells.

2.3.6. Dexamethasone inhibits TNFα stimulated, IL-8 expression.

High dose dexamethasone significantly reduced the production of IL-8 by Hep G2 (TNFα stimulated IL-8 production 2.94 ± 0.33 ng/ml, mean $\pm$ SEM, n = 10, TNFα + dexamethasone 1.45 ± 0.08, n = 8) and SK-Heps (TNFα stimulated IL-8 production 19.61 ± 2.74, n = 10, TNFα + dexamethasone 10.11 ± 1.16, n = 8) following TNFα stimulation (figure 2.6 & 2.7). Cyclosporin and FK 506 had no effect on IL-8 production by either cell line, and the synthesis of RANTES and MCP-1 by SK-Heps was unaffected by dexamethasone, cyclosporin and FK 506.

2.3.7. TNFα induced IL-8 production is mediated via protein kinase C.

The phorbol ester, PMA, significantly induced the expression of IL-8 (figure 2.8 & 2.9) by SK-Heps (control 2.928 $\pm$ 0.195 ng/ml, mean $\pm$ SEM, n = 12, PMA 15.489 $\pm$ 1.477, n = 6) and Hep G2 cells (control 0.733 $\pm$ 0.083, n = 12, PMA 6.874 $\pm$ 0.579, n = 6). Staurosporine (0.1ug/ml), a non-specific protein kinase inhibitor, and calphostin C (1 umol/L), a specific protein kinase C inhibitor, significantly inhibited TNFα stimulated IL-8 production (figure 2.8 & 2.9) from both Hep G2 (TNFα stimulated IL-8 production 2.689 $\pm$ 0.346, n = 12, TNFα + staurosporine 0.556 $\pm$ 0.427, n = 6, TNFα + calphostin C 1.307 $\pm$ 0.186, n = 8) and SK-Heps (TNFα 19.479 $\pm$ 2.680, n = 12, TNFα + staurosporine 1.672 $\pm$ 0.481, n = 6, TNFα + calphostin C 5.837 $\pm$ 0.820, n = 8). Staurosporine at lower concentrations (10ng/ml and 1ng/ml) also significantly reduced IL-8 production in both SK-Heps and Hep G2 cells compared with controls. Genistein (10umol/L), a protein tyrosine kinase inhibitor, had no effect on IL-8 production (figure 2.8 & 2.9) by Hep G2 cells, but significantly reduced IL-8 expression in SK-Heps (TNFα + genistein 8.138 $\pm$ 1.438, n = 8). Therefore, IL-8 production in human hepatoma cell lines is mediated via protein kinase C, however, in SK-Heps other protein tyrosine kinases are also implicated.

2.3.8. TNFα induced CC chemokine production is mediated via protein kinases.

PMA significantly induced the expression of RANTES (0.393 $\pm$ 0.159 ng/ml, mean $\pm$ SEM, n = 6, figure 2.10) compared with controls (0.135 $\pm$ 0.057, n = 12). Staurosporine completely abolished the production of RANTES induced by TNFα (TNFα stimulated RANTES production, 0.899 $\pm$ 0.174, n = 12, TNFα + staurosporine 0, n = 6), but calphostin C and genistein had no effect on RANTES expression in SK-Heps (figure 2.10). PMA did not significantly induce the expression of MCP-1 in SK-Heps, compared with controls (figure 2.11). Staurosporine and calphostin C significantly inhibited the production of MCP-1 (TNFα alone 0.509 $\pm$ 0.051 ng/ml, n = 12, TNFα + staurosporine 0.077 $\pm$ 0.030, n = 6, TNFα + calphostin C 0.104 $\pm$ 0.027, n = 8) induced by TNFα (figure 2.11). In addition, MCP-1 expression in SK-Heps was inhibited by genistein (0.171 $\pm$ 0.047, n = 8, figure 2.14).

2.3.9 TNFα induced chemokine production is unaffected by antioxidants and pyrrolidine dithiocarbamate.

In contrast with data presented in chapter 3, regarding adhesion mediated chemokine production, N-acetylcysteine (NAC) did not alter TNFα stimulated chemokine production by Hep G2 or SK-Heps. Pyrrolidine dithiocarbamate (PDTC) significantly enhanced IL-8 expression induced by TNFα in SK-Heps and Hep G2 cells (table 2.1).

2.3.10. Figures for Chapter 2.

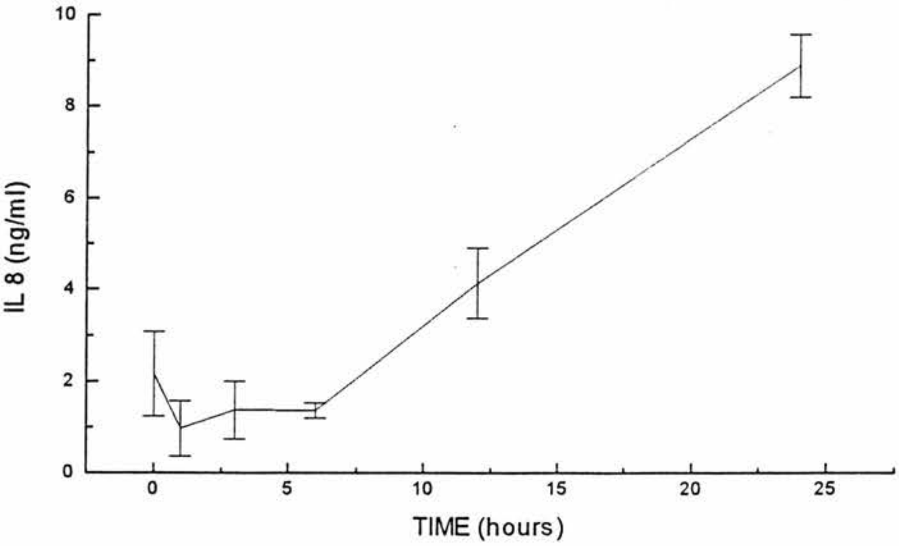


Figure 2.1a. Time course of TNFα induced IL-8 protein production from Hep G2 cells.

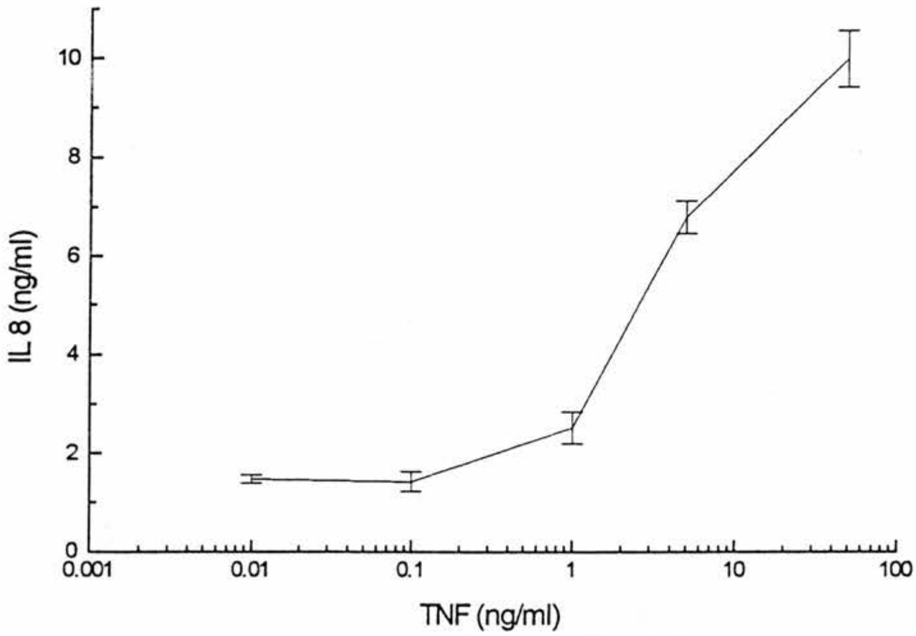


Figure 2.1b. Concentration curve of TNFα induced IL-8 protein production from Hep G2 cells.

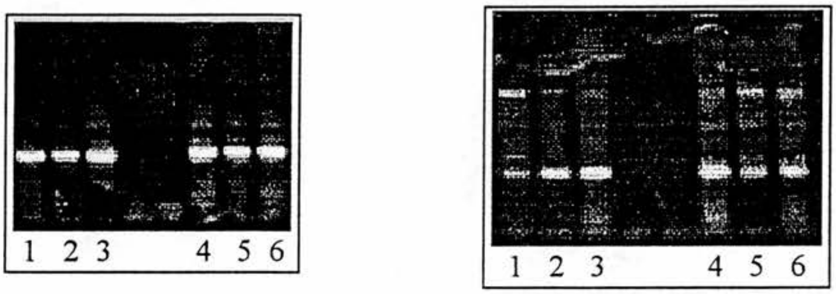


Figure 2.1c. Induction of Hep G2 IL-8 gene expression following TNFα stimulation. 1 = 3 hours, 2 = 12 hours, 3 = 24 hours, 4 = 5 ng/ml TNF alpha, 5 = 0.1 ng/ml TNF alpha, 6 = 0.01 ng/ml TNF alpha.

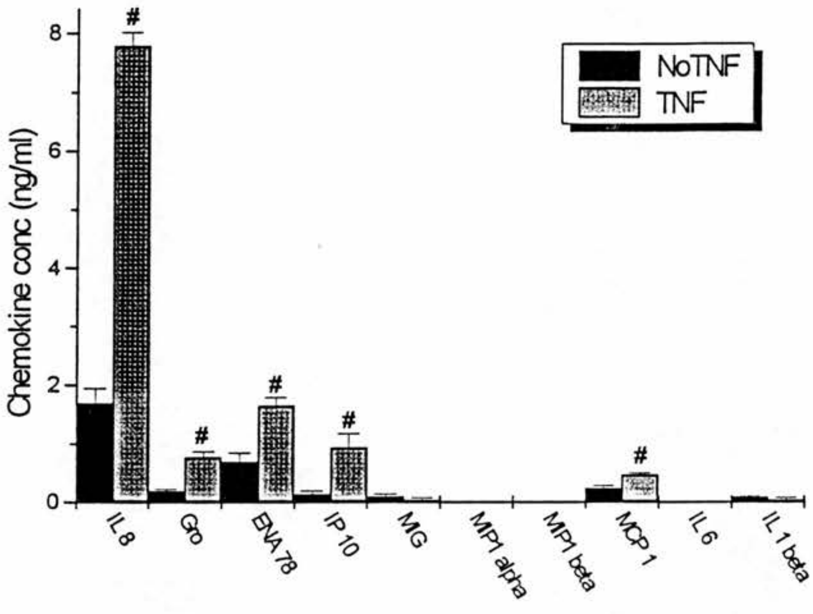


Figure 2.2. TNFα (5 ng/ml) induced chemokine production by normal human hepatocytes. # = significantly different (P< 0.05, n = 4) from unstimulated controls (n= 6).

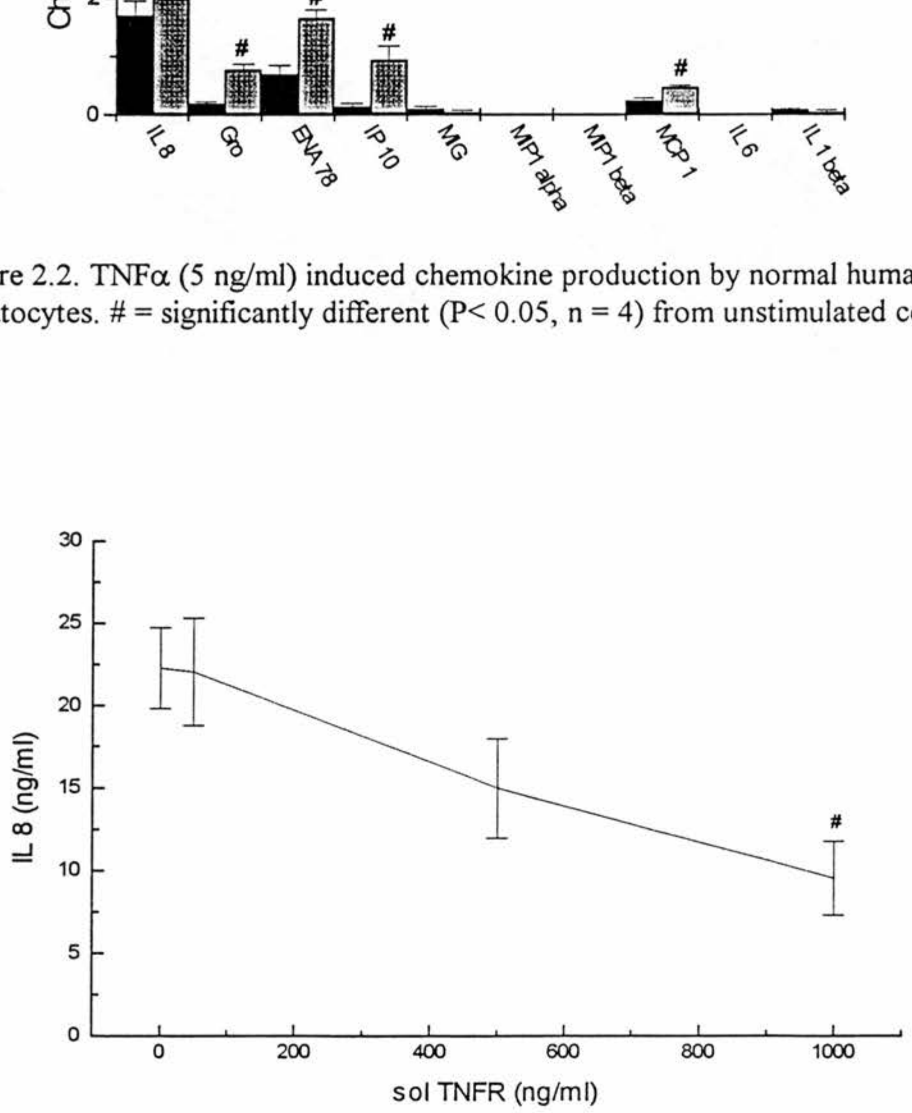


Figure 2.3a. Effect of soluble TNF receptors (sTNFR1 and sTNFR2) on IL-8 production by SK-Heps, # = significantly different (P < 0.05) v control.

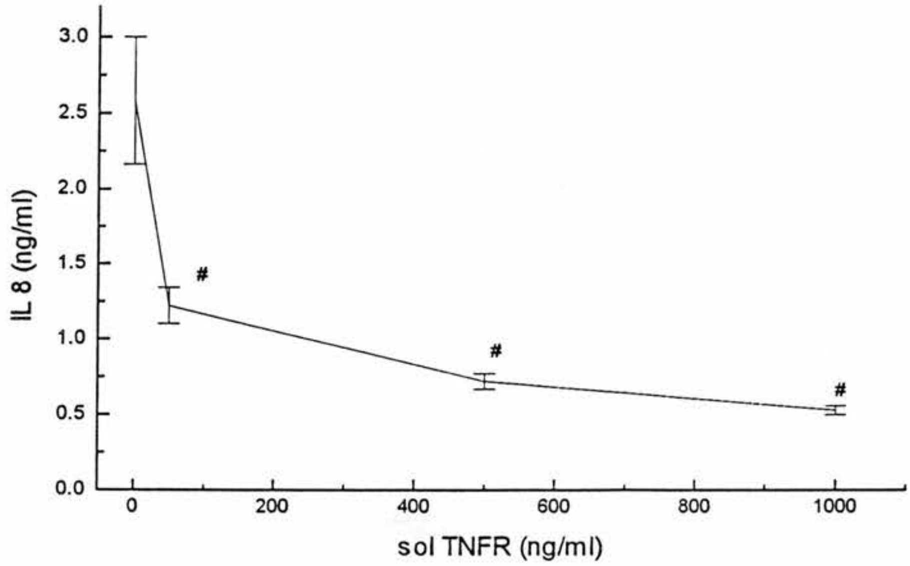


Figure 2.3b. Effect of soluble TNF receptors (sTNFR1 and sTNFR2) on IL-8 production by Hep G2 cells. # = significantly different (P < 0.05) v control.

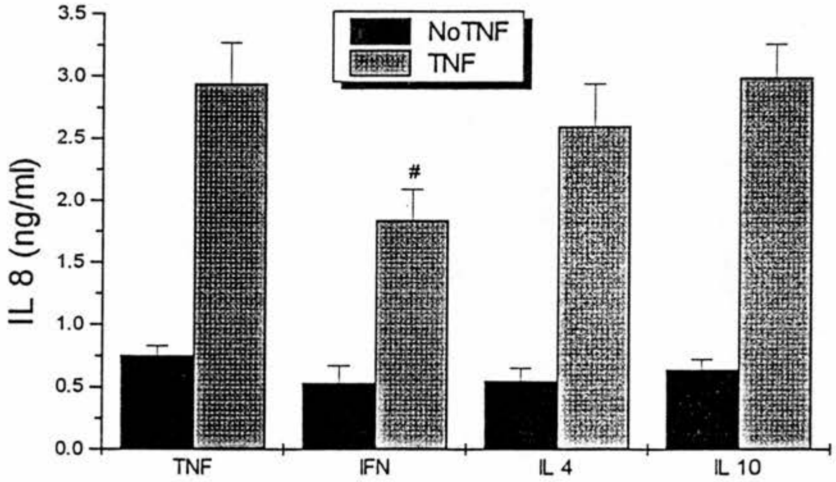


Figure 2.4. Effect of other cytokines on TNFα induced IL-8 expression in Hep G2 cells, # = significantly different (P < 0.05) from controls.

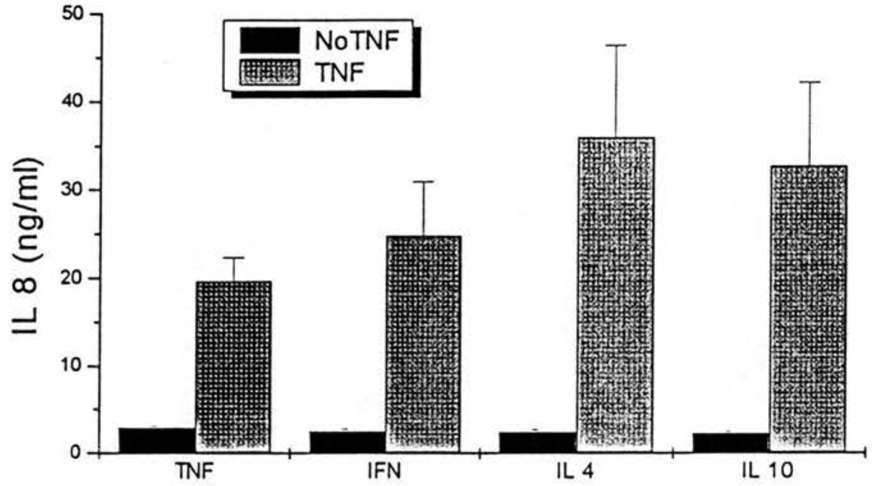


Figure 2.5. Effect of other cytokines on TNFα induced IL-8 expression in SK-Heps, no significant differences were noted.

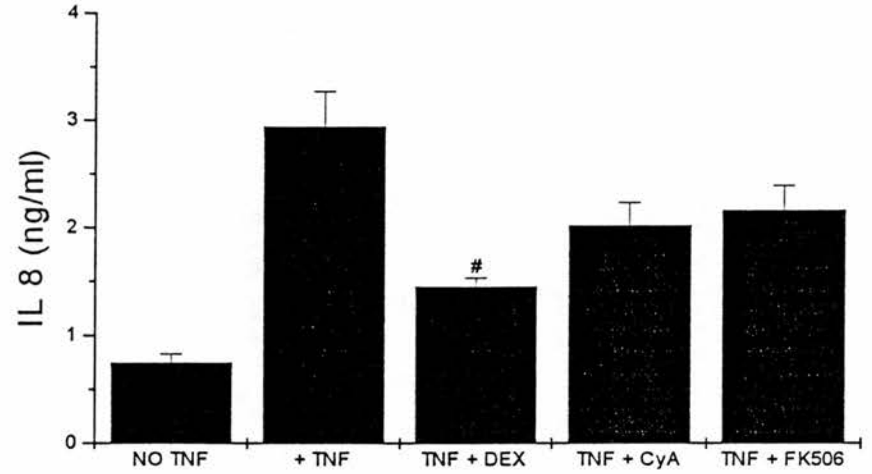


Figure 2.6. Effect of dexamethasone, cyclosporin and FK 506 on TNFα induced IL-8 production by Hep G2 cells, # = significantly different ($P < 0.05$) from control.

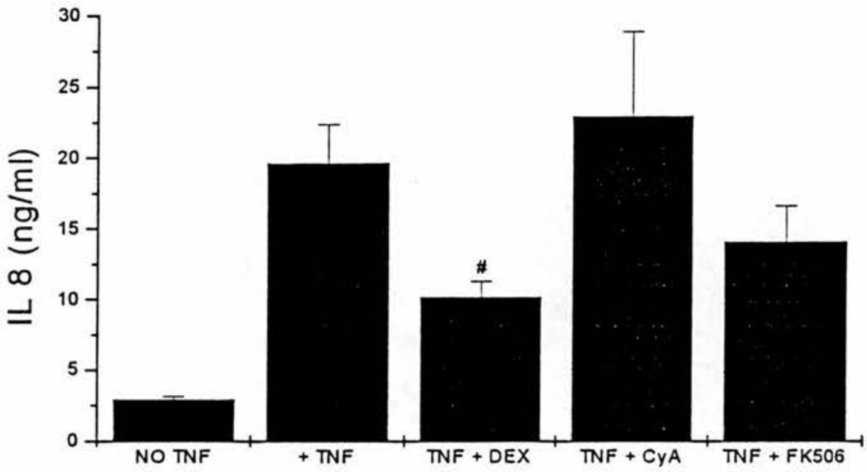


Figure 2.7. Effect of dexamethasone, cyclosporin and FK 506 on TNFα induced IL-8 production by SK-Heps, # = significantly different ($P < 0.05$) from control.

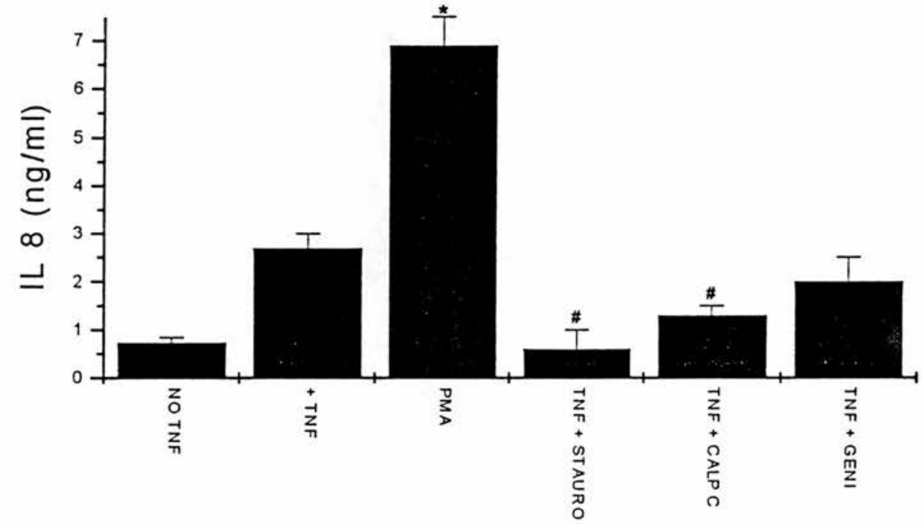


Figure 2.8. Effect of protein kinase inhibitors on IL-8 production by Hep G2 cells following TNFα stimulation. Stauro = staurosporine, calp C = calphostin C and geni = genistein. * = significantly different ($P < 0.05$) compared with no TNF, # = significantly different compared with TNF stimulated (+ TNF).

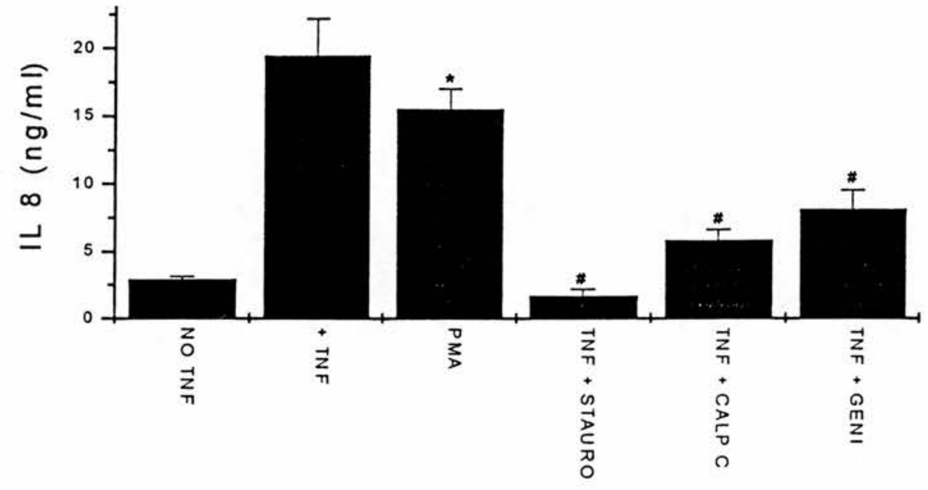


Figure 2.9. Effect of protein kinase inhibitors on IL-8 production by SK-Heps following TNFα stimulation. Stauro = staurosporine, calp C = calphostin C and geni = genistein. * = significantly different (P < 0.05) compared with no TNF, # = significantly different compared with TNF stimulated (+ TNF).

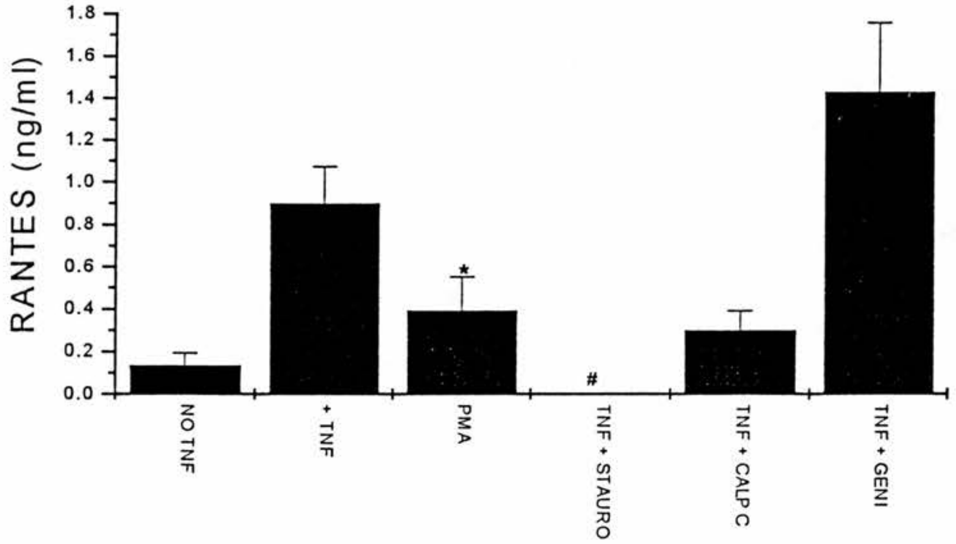


Figure 2.10. Effect of protein kinase inhibitors on RANTES production by SK-Heps following TNFα stimulation. Stauro = staurosporine, calp C = calphostin C and geni = genistein. * = significantly different (P < 0.05) compared with no TNF, # = significantly different compared with TNF stimulated (+ TNF).

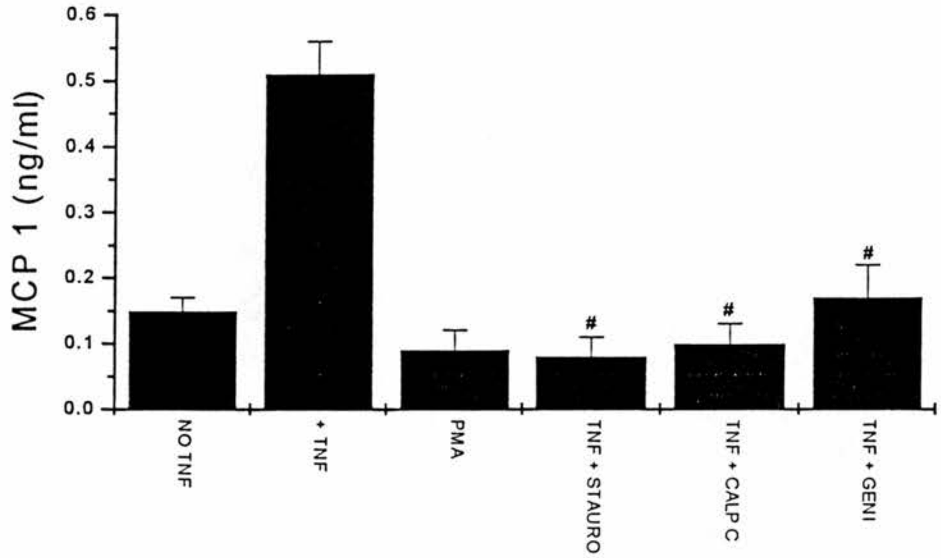


Figure 2.11. Effect of protein kinase inhibitors on MCP-1 production by SK-Heps following TNFα stimulation. Stauro = staurosporine, calp C = calphostin C and geni = genistein. # = significantly different ($P < 0.05$) compared with TNF stimulated (+ TNF).

2.3.11. Table 2.1. Effect of N-acetylcysteine and pyrrolidine dithiocarbamate on TNFα stimulated chemokine production in human hepatoma cell lines.

	TNFα alone	TNFα + NAC (10 mmol/L)	TNFα + PDTC (1 mmol/L)
Hep G2 IL-8	2.722 ± 0.681	1.561 ± 0.322	12.051 ± 0.795#
SK-Hep IL-8	9.731 ± 0.955	9.417 ± 0.811	21.486 ± 3.886#
SK-Hep MCP-1	1.453 ± 0.394	1.069 ± 0.396	0.945 ± 0.413
SK-Hep RANTES	0.455 ± 0.094	0.184 ± 0.074	0.584 ± 0.155

N = 6 in all groups. # = significantly different ($P < 0.05$) compared with unstimulated and TNFα stimulated hepatoma cells.

2.4. DISCUSSION

In this chapter the production of CXC and CC chemokines following TNFα stimulation was studied. Both SK-Heps and Hep G2 cell lines produced IL-8 constitutively and the expression of IL-8 was induced by TNFα. SK-Heps also expressed Gro, RANTES and MCP-1, although TNFα stimulation only induced the expression of the latter two CC chemokines and not Gro. Chemokine expression induced by TNFα was mediated via TNFR1 and inhibited by soluble TNF receptors. Although dexamethasone inhibited IL-8 expression in both cell lines other drugs used in the treatment of hepatic disorders, FK 506 and cyclosporin, had no effect on chemokine production by Hep G2 cells or SK-Heps. The effect of other cytokines had a different effect on IL-8 production in Hep G2 cells compared with SK-Heps. IFN-γ inhibited Hep G2, TNFα stimulated IL-8 expression, but had no effect on IL-8 expression induced in SK-Heps. Induction of IL-8 expression by PMA and inhibition of IL-8 expression induced by TNFα after treatment with the protein kinases C inhibitors, staurosporine and calphostin C, implicate this intracellular pathway in TNFα stimulated IL-8 production in this cell line. Similar results were observed regarding TNFα stimulated IL-8 production in SK-Heps. However, the tyrosine kinase inhibitor, genistein, also inhibited TNFα stimulated IL-8 production in this cell line, thus implicating protein tyrosine kinases in IL-8 production by this cell line. These data regarding the intracellular pathways involved in CC chemokine production by SK-Heps is less clear. MCP-1 production induced by TNFα, like IL-8, is inhibited by staurosporine, calphostin C and genistein, but PMA has no effect. In contrast, PMA stimulated RANTES expression in SK-Heps, but TNFα induced RANTES production was only inhibited by staurosporine. Calphostin C and genistein had no effect on TNFα induced RANTES production. These data implicate intracellular pathways utilising protein kinases in TNFα induced CC chemokine production by SK-Heps, but do not clarify further which pathways may be involved. Thus, although TNFα signals via TNFR1 to induce chemokine production by human hepatoma cells, divergent intracellular pathways lead to the expression of CXC and CC chemokines.

In these studies human hepatocytes and hepatoma cell lines were found to secrete a variety of CXC and CC chemokines. The pattern of chemokine production in normal human hepatocytes was most similar to the SK-Hep cell line and is similar to other epithelial cells. Hep G2 cells in contrast produced only IL-8 following TNFα stimulation. It is not surprising that the human hepatoma cell lines secrete different chemokines in view of their different origins. Hep G2 cell were derived from a hepatoblastoma and therefore may be more "immature" compared with SK-Heps, which were derived from ascitic fluid cells in a patient with a hepatoma. These data contrast with the study of Rowell and colleagues (*1997*), who found expression of a variety of CXC chemokines by Hep G2 cells and absence of MCP-1 expression in any of the hepatoma cell lines or human hepatocyte cultures tested. In addition, they found an inverse relationship in the concentration of chemokines produced and the age of the donor; foetal liver cells were more potent producers of IL-8 and Gro than normal human hepatocytes from an elderly donor.

Previous studies have shown that the majority of the neutrophilic chemoattractant activity in hepatocyte conditioned media is dependent on IL-8 (*Thornton et al 1990, Rowell et al 1997*). The production of other CXC chemokines, such as Gro and ENA-78, may contribute the residual neutrophil chemoattractant activity observed in hepatocyte conditioned media after neutralisation of IL-8 with blocking antibodies. The role of CXC chemokine production in the pathogenesis of liver disorders is becoming clearer. Both IL-8 and Gro expression in hepatocytes can be induced by ethanol (*Shiratori et al 1993, 1994*). These CXC chemokines are expressed in liver tissue from patients with alcoholic liver disease and are correlated with the degree of neutrophilic infiltrate (*Sheron et al 1993, Maltby et al 1996*). Recently, hepatic over expression of Gro/CINC, using adenovirus expression vectors, was found to induce a neutrophilic hepatitis (*Maher et al 1997*). However, CXC chemokines can also function as lymphocyte chemoattractants, with some subset specificity (*Baggiolini et al 1997*). Presumably whether a certain proinflammatory stimulus induces a neutrophilic or lymphocytic infiltrate may depend on expression of other immunomodulatory cytokines, adhesion molecules or the activation state of the circulating inflammatory cells. ELR negative, CXC chemokines (eg. IP-10 and MIG) can inhibit the biological functions of the ELR positive CXC chemokines (eg. IL-8 and ENA-78). Thus the cellular composition of an inflammatory infiltrate may also be influenced by the relative local concentrations of ELR negative and ELR positive CXC chemokines.

The data presented in this chapter clearly show that TNFα stimulation of hepatocyte chemokine production is mediated via TNFR1, rather than TNFR2. This confirms previous data showing that IL-8 expression in HeLa cells was mediated via TNFR1 (*Boldin et al 1995*). The inhibitory effect of soluble TNF receptors, at physiological concentrations, on cellular chemokine production, suggests that these soluble receptors may have an immunomodulatory role *in vivo*. These data presented in this chapter also suggest that TNFα signals via intracellular protein kinase C, at least in regard to IL-8 production. Staurosporine is a broad spectrum inhibitor of protein kinases but has other effects such as the induction of apoptosis in some cell lines. In view of the general inhibitory nature of staurosporine on chemokine production and because cell death was not directly studied, a direct toxic effect of staurosporine on the hepatoma cells cannot be excluded. However, the concentrations of staurosporine necessary to induce apoptosis in sensitive cell lines were in excess of those used in this study and no detachment of adherent hepatoma cells into the culture media were observed after incubation with staurosporine. Calphostin C and genistein can also induce apoptosis in certain cells, but their differential effect on TNFα stimulated chemokine production is evidence against a direct toxic effect on the hepatoma cells used in these studies. In addition, no detachment of the hepatoma cells was observed after incubation with these kinase inhibitors. Genistein is an inhibitor of protein kinases and calphostin C can also inhibit protein tyrosine kinases and other protein kinases such as PKA and PKG. However the latter effect of calphostin C occurs at concentrations 5-50 fold those used in the experiments reported. In addition, studies in other cell types have implicated several intracellular cellular signalling pathways, such as protein tyrosine kinases, protein kinase C and phosphatases, in the modulation of proinflammatory cytokine stimulation of chemokine production (*Jordan et al 1995, Jordan et al 1996, Alonso et al 1996, Beales & Calam 1997*).

In summary, the stimulatory effect of TNFα on hepatoma cell lines has been studied. These data show that hepatocytes can produce a variety of CXC and CC chemokines following proinflammatory cytokine stimulation. Chemokine expression induced by TNFα is modulated by soluble TNF receptors, IFNγ and dexamethasone and involves the activation of intracellular protein kinases.

CHAPTER 3

ADHESION MEDIATED CHEMOKINE PRODUCTION

CHAPTER 3
ADHESION MEDIATED CHEMOKINE PRODUCTION

3.1. INTRODUCTION

Recent studies have shown an intimate association between adhesion molecules and chemokines, with MIP1β, a member of the C-C chemokine family, binding to CD44 and thereby activating β1 integrin binding to vascular cell adhesion molecule 1 (VCAM-1) adhesion molecules, resulting in the attachment and chemotaxis of CD8+ T cells (*Tanaka et al 1993*). In addition, monocyte adhesion to plastic and tissue matrix is sufficient for the expression of various cytokine genes (*Haskill et al 1988, Eierman et al 1989, Kasahara et al 1991, 1993*). Lymphocytes activated via CD3 and ICAM-1 or VCAM-1 receptor (CD11a and VLA 4, respectively) have shown induced proliferative and cytokine responses (*Van Seventer et al 1990, Damle et al 1992*). Adhesion between monocytes and human umbilical endothelial cells (HUVEC) via ICAM-1, but not VCAM-1, can induce the production of MIP1α (Lukacs et al 1994). MIP1α, a member of the C-C chemokine family, is chemoattractant for monocytes, T lymphocytes and natural killer cells (*Davatelis et al 1988, Wolpe et al 1988, Taub et al 1993, Maghazachi et al 1994*). Thus, MIP1α may be important in attracting these cells to sites of malignant disease. Both MCP-1 and IL-8 are produced during monocyte adhesion with IFN-γ treated HUVEC (*Lukacs et al 1995*). In addition to its chemoattractant properties, IL-8 is a potent angiogenic factor which may promote tumour growth through its angiogenic properties (*Strieter et al 1995*) and inhibition *in vivo* of IL-8 in SCID mice results in reduced non-small cell lung cancer tumour size and tendency to metastasis (*Arenberg et al 1996*).

The aims of the studies presented in this chapter study were to determine if monocyte interaction with the human hepatoma cell lines, SK-Heps and Hep-G2, induced the production of chemokines in an adhesion dependent manner, to determine the cellular origin of the chemokines and the specific adhesion molecules important in the monocyte/hepatoma cell interaction, and to assess the potential role of reactive oxygen intermediates as a mechanism for chemokine expression in this model.

3.2. MATERIALS AND METHODS

3.2.1. Mononuclear cell isolation

Peripheral blood was drawn into a heparinised syringe from healthy volunteers, diluted 1:1 with normal saline and mononuclear cells separated by density gradient centrifugation. The recovered cells were washed three times with normal saline and layered onto a density gradient (1.068 g/ml) for the enrichment of monocytes (Ficol-lite, Atlanta Biologics, Atlanta, GA). The isolated cells were then washed, cytospun onto a glass slide, stained with Diff-Quik and differentially counted. The purity of the monocytes from the gradient was consistently above 80% monocytes, with the remainder lymphocytes.

3.2.2. Hepatoma:monocyte co-culture.

SK-Heps and Hep-G2 were grown in DMEM to confluence in 6 or 24 well plates and the enriched monocyte population (1×10^6 cell/ml) layered on top. Culture supernatants were collected at 24 hours of co-culture, peak chemokine production was detected at this time point in previous studies (*Lukacs et al 1994*). To determine if cell contact was obligatory for chemokine production, experiments were conducted in which the monocyte population was separated from the hepatoma cells using a transwell system (Costar, Kennebunkport, ME). Hepatoma cells and monocytes were fixed by addition of 1% paraformaldehyde as previously described (*Takafuji et al 1996*). Normal human hepatocytes (a kind gift from Dr S Kamil, Clonetics Inc, CA) were cultured in 6 well matrigel coated plates in Hepato-STIM (Becton Dickinson, Bedford, MA).

Monoclonal antibodies (5ng/ml) to TNF, IL1 beta and adhesion molecules (R & D Systems, Minneapolis, MN) were preincubated with the hepatocytes for 15 minutes before addition of the monocytes and the supernatants recovered at 24 hours. N-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC) and 3'-aminobenzamine (ABZ) were added to the co-cultures with the monocytes, NAC was preincubated with the hepatocytes for 6 hours prior to the addition of the monocytes.

3.2.3. Chemokine ELISA.

Extracellular immunoreactive chemokines were measured as described previously in chapter 2, using human anti-chemokine antibodies raised in New Zealand white rabbits by multiple site injection. Chemokine gene expression was determined by RT-PCR as previously described in chapter 2, using human primers for the genes of interest.

3.2.4. Statistics.

Data are presented as mean $\pm$ standard error. If the data was noted to be skewed then non-parametric statistical tests were used (Wilcoxons rank sum test or Kruskal-Wallis test). Statistical significance between groups was determined using the unpaired Students t-test and comparison between multiple groups was performed by one-way ANOVA. $P < 0.05$ was considered significant.

3.3. RESULTS

3.3.1. Monocyte interaction with human hepatoma cell lines induces chemokine production.

Unstimulated cultures of SK-Heps or monocytes produce minimal amounts of MIP1α (SK-Heps, 0.030 ± 0.01 ng/ml, mean ± SEM, n = 8; monocytes, 0.010 ± 0.010, n = 8), IL-8 (SK-Heps, 0.030 ± 0.01, n = 8, monocytes 0.420 ± 0.020, n = 8) or MCP-1 (SK-Heps, 0.528 ± 0.034 ng/ml, n = 8, monocytes 0.228 ± 0.068, n = 8). In addition, Hep G2 cells cultured alone in 6 well plates produced some IL-8 (0.030 ± 0.01, n = 8) but no background MIP1α and MCP-1.

Enriched monocytes co-cultured with the hepatoma cells (Figure 3.1) produced significant concentrations of MIP1α (SK-Heps & monocytes 3.480 ± 0.580, Hep G2 & monocytes 3.638 ± 0.160, n = 8), IL-8 (SK-Hep & monocytes, 362.0 ± 80.8, n = 8, Hep G2 & monocytes, 19.58 ± 2.50, n = 8) and MCP-1 (SK-Hep & monocytes, 2.334 ± 0.207, n = 8, Hep G2 & monocytes, 0.968 ± 0.142, n = 8). Increased chemokine protein concentration was associated with increased expression of mRNA (Figure 3.2). Separation of the monocytes and hepatocytes using transwell plates showed significant reduction in the production of both MIP1α (SK-Heps & monocytes 0.303 ± 0.060, Hep G2 & monocytes 0.395 ± 0.029, n = 8) and IL-8 (SK-Hep & monocytes 47.9 ± 7.6, n = 8; Hep G2 & monocytes 5.66 ± 0.43, n = 8), indicating a requirement for cell-to-cell contact (Figure 3.1). In contrast, MCP-1 production was significantly reduced when the SK-Heps were separated from the mononuclear cells by an insert (0.968 ± 0.163, n = 8), but not when the Hep G2 hepatoma cells were cultured with the monocytes in transwell plates (0.813 ± 0.183, n = 8). These data implicate adhesion as essential in the production of MCP-1 during monocyte adhesion with SK-Heps, but not in MCP-1 production during monocyte interaction with Hep G2 cells. In contrast with previous studies (*Lukacs et al 1994,1995*), adhesion mediated chemokine production was not dependent on the preactivation of the hepatoma cells with the proinflammatory cytokines, TNFα, IL-1β, IFN-γ or LPS (Table 3.1).

Unstimulated normal human hepatocytes produced no MIP1α either spontaneously or when co-cultured with monocytes. In contrast, small amounts of IL-8 were produced by normal human hepatocytes (1.692 $\pm$ 0.252, n = 6), with increased IL-8 concentration (4.026 $\pm$ 0.544, n = 6) during monocyte:hepatocyte co-culture. MCP-1 production was similar in cultures of hepatocytes alone (0.229 $\pm$ 0.058, n = 6) compared with cultures of hepatocytes and monocytes (0.359 $\pm$ 0.037, n = 6).

In summary, both CXC and CC chemokines are produced during monocyte:hepatoma cell co-culture by an adhesion dependent mechanism and does not require preactivation of the hepatoma cells. In contrast, normal human hepatocytes and mononuclear cells only produce the CXC chemokine, IL-8, during co-culture.

3.3.2. Both monocytes and hepatoma cells produce chemokines during adhesion.

To examine which cell populations were producing the chemokines during the co-cultures individual cell populations were fixed with paraformaldehyde before culture. No MIP1α was produced (Figure 3.3) when paraformaldehyde fixed monocytes were co-cultured with Hep G2 hepatoma cells and low concentrations were observed when paraformaldehyde fixed monocytes were cultured with SK-Heps (0.144 $\pm$ 0.049 ng/ml, n = 6). In contrast, culture of normal monocytes with paraformaldehyde fixed hepatoma cells resulted in significant production of MIP1α (fixed SK-Heps & normal monocytes 1.348 $\pm$ 0.273, fixed Hep G2 & normal monocytes 1.276 $\pm$ 0.125, n = 6), indicating that the monocytes and not the hepatoma cells were the source of MIP1α.

Paraformaldehyde fixed monocytes co-cultured with paraformaldehyde fixed hepatocytes (either SK-Hep or Hep G2) produced no IL-8. Normal monocytes co-cultured with fixed SK-Heps (31.42 $\pm$ 4.44, n = 6) and paraformaldehyde fixed monocytes co-cultured with normal SK-Heps (24.08 $\pm$ 6.47, n = 6) synthesised IL-8, suggesting that the IL-8 produced during monocyte-SK Hep interaction is derived from both cell populations. IL-8 was also produced in the Hep G2 cultures (fixed Hep G2 & normal monocytes 5.87 ± 0.93, n = 6; Hep G2 & fixed monocytes 0.56 ± 0.06, n = 6). However, the significantly higher production of IL-8 during monocyte interaction with fixed Hep G2 cells, implies that the monocytes are the major source of IL-8 in this culture system.

MCP-1 production during adhesion was significantly reduced when either fixed monocytes (0.105 ± 0.017, n = 4) or fixed hepatocytes (0.056 ± 0.019, n = 4) were co-cultured with the respective unfixed cell populations, compared with the adhesion between unfixed monocytes and unfixed SK-Heps (0.260 ± 0.030, n = 4). Although MCP-1 production during monocyte interaction with Hep G2 cells did not appear to be adhesion mediated, co-culturing normal monocytes with fixed Hep G2 cells did not affect MCP-1 production and MCP-1 production was completely abolished when fixed mononuclear cells were used. This suggests the monocytes produced the MCP-1 in the Hep G2:monocyte co-cultures, but that both the monocytes and SK-Heps were the source of MCP-1 in this co-culture system.

In summary, MIP1α production during monocyte adhesion with human hepatoma cells is from the former cell type. In contrast, the production of IL-8 and MCP-1 during monocyte:hepatoma cell co-culture is from both cell types, suggesting that adhesion can activate both cell populations.

3.3.3. Inhibition of IL-8 production by neutralising antibodies to TNFα or IL1 β.

The proinflammatory cytokines, TNFα and IL-1β, are potent stimuli for the production of chemokines in many different cell types. In co-culture supernatants, both TNFα (SK-Heps & monocytes 0.128 ± 0.018 ng/ml, Hep G2 & monocytes 0.207 ± 0.015, n = 8) and IL-1β (SK-Heps & monocytes 0.781 ± 0.050, Hep G2 & monocytes

0.253 ± 0.014, n = 8) were detected. The concentrations of IL-1β (0.192 ± 0.056, n = 8) were lower in supernatants from monocytes cultured alone, in contrast, TNFα (0.276 ± 0.094, n = 8) concentrations were similar in supernatants from monocytes cultured alone compared with the co-cultures of monocytes with hepatoma cells. No IL-6 was produced during monocyte:hepatoma cell co-culture.

To further study the potential role of proinflammatory cytokine stimulation in the production of chemokines, neutralising antibodies to either IL-1β or TNFα were included in the cultures (Figure 3.4). Production of IL-8 was significantly reduced when anti-TNFα (87.98 ± 16.79, n = 12) or anti-IL-1β (80.74 ± 11.62, n = 12) antibody were included in monocyte:SK Hep co-cultures. However, IL-8 production was not significantly reduced when anti-TNFα (10.96 ± 1.63, n = 8) or anti-IL-1β (7.63 ± 1.58, n = 8) antibody were included in monocyte:Hep G2 co-cultures. MIP1α and MCP-1 production were also unaffected by anti-TNFα or anti-IL-1β antibodies.

In summary, IL-8 production during adhesion appears dependent on proinflammatory cytokine release during monocyte adhesion with SK Hep cells, but not Hep G2 cells. MIP1α and MCP-1 production was not dependent on proinflammatory cytokine production with either cell population.

3.3.4. Inhibition of MIP1α and MCP-1 production by neutralising antibodies to beta integrins.

To determine which adhesion molecules are involved in the monocyte adhesion with hepatoma cells leading to chemokine production, neutralising monoclonal antibodies for specific adhesion molecules were included in the co-cultures. Inclusion of ICAM-1 blocking antibodies in the co-cultures did not affect the production of MIP1α, MCP-1 or IL-8 (Figure 3.5, 3.6). In contrast, monoclonal antibodies directed against VCAM-1 or its β1 integrin ligand, resulted in significant reduction in the production of MIP1α during monocyte adhesion with either hepatoma cell line (Figure

3.5). Inclusion of monoclonal antibodies directed against E-selectin and β3 integrin also resulted in significant reduction in the production of MIP1α (Figure 3.5). Similar data were observed regarding the effect of anti-adhesion molecule antibodies on the production of MCP-1, during monocyte adhesion with SK-Heps (Figure 3.6). As the production of MCP-1 in the Hep G2 cultures with monocytes was not adhesion dependent, this interaction was not studied with blocking adhesion molecules. The production of IL-8 was unaffected by the inclusion of any of these neutralising antibodies in cultures of monocytes and SK-Heps or Hep G2 cells. Therefore, in contrast with the CXC chemokine IL-8, adhesion dependent production of the CC chemokines, MIP1α and MCP-1, occurs via selectin and β1 and β3 integrin mediated adhesive interactions.

3.3.5. Effect of antioxidants on MIP1α production.

Cross linking of beta integrins has previously been shown to lead to an intracellular oxygen burst and free radical formation. Many chemokine genes, including MIP1α and IL-8, contain a NF-κB binding site in their 5' noncoding region which may be activated by reactive oxygen species via NF-κB. To study the potential role of this pathway, N-acetylcysteine (NAC) and the specific inhibitor of oxidant mediated NF-κB activation, PDTC, or an AP-1 inhibitor, ABZ, were included in the co-cultures. Inclusion of either NAC (Figure 3.7) or PDTC (Figure 3.8) resulted in a dose dependent inhibition of MIP1α production in cultures of monocytes with SK-Heps or Hep G2 cells. In contrast, the AP-1 inhibitor, ABZ had no effect on MIP1α production. Similar results were obtained for MCP-1 production during monocyte adhesion with SK-Hep cells (SK-Heps + monocytes, 2.40 $\pm$ 0.30 ng/ml, mean $\pm$ SEM, n = 9, SK-Heps + monocytes + NAC 10 mmol/L, 0.67 $\pm$ 0.11, n = 8, SK-Heps + monocytes + PDTC 1 mmol/L, 0.60 $\pm$ 0.19, n = 8, SK-Heps + monocytes + ABZ 1 mmol/L, 1.95 $\pm$ 0.12, n = 6). The production of IL-8 during monocyte adhesion with

SK-Hep or Hep G2 hepatoma cells was unaffected by the inclusion of NAC, PDTC and ABZ in the culture media.

This specific effect of NAC and PDTC on adhesion mediated CC chemokine production implicates the intracellular production of reactive oxygen intermediates and subsequent activation of the nuclear transcription factor, NF-κB in the synthesis of MCP-1 and MIP1α during monocyte adhesion with hepatoma cell lines and contrasts with the expression of IL-8, a CXC chemokine.

3.3.6. Figures for Chapter 3.

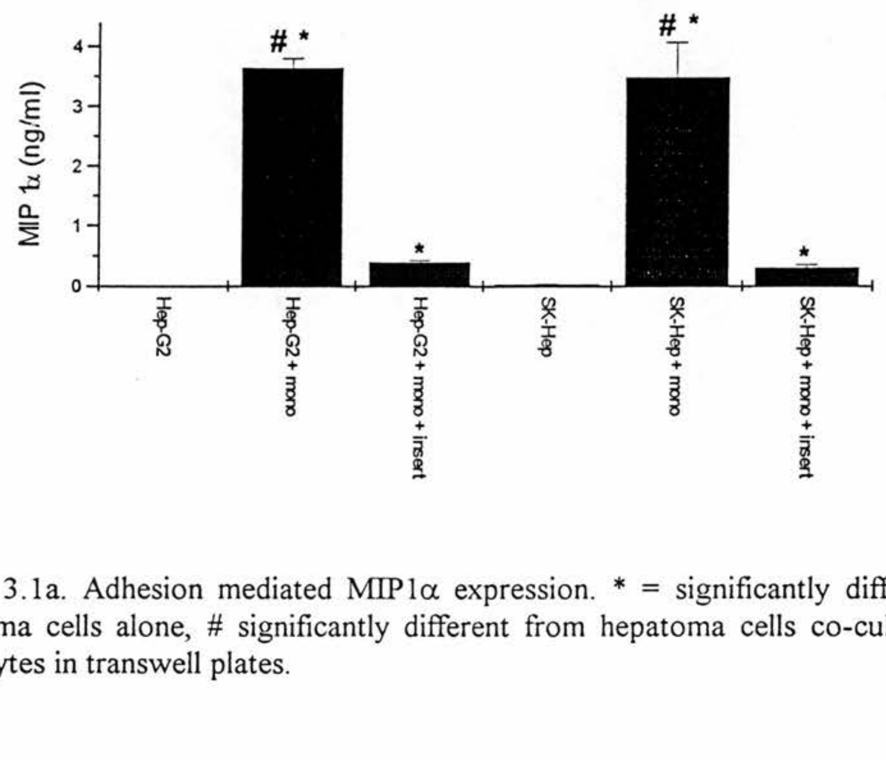


Figure 3.1a. Adhesion mediated MIP1α expression. * = significantly different from hepatoma cells alone, # significantly different from hepatoma cells co-cultured with monocytes in transwell plates.

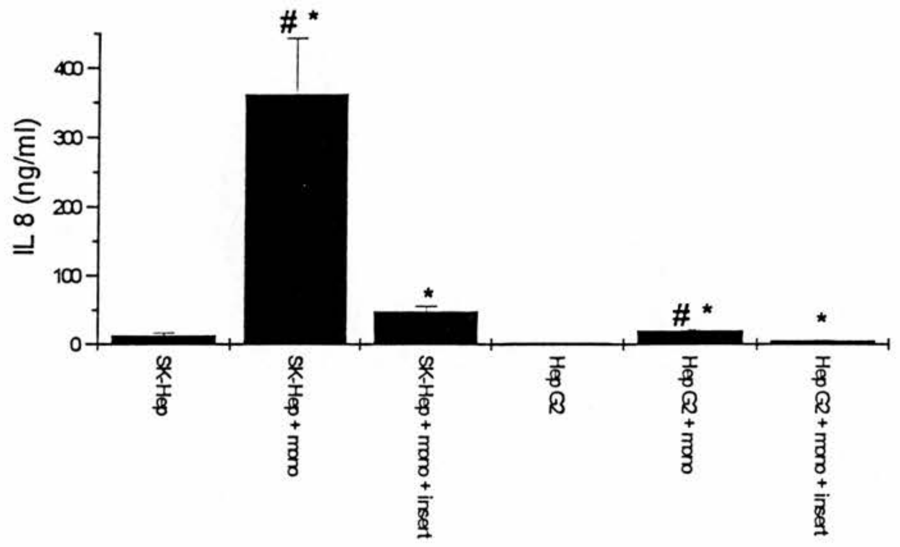


Figure 3.1b. Adhesion mediated IL-8 expression. * = significantly different from hepatoma cells alone, # significantly different from hepatoma cells co-cultured with monocytes in transwell plates.

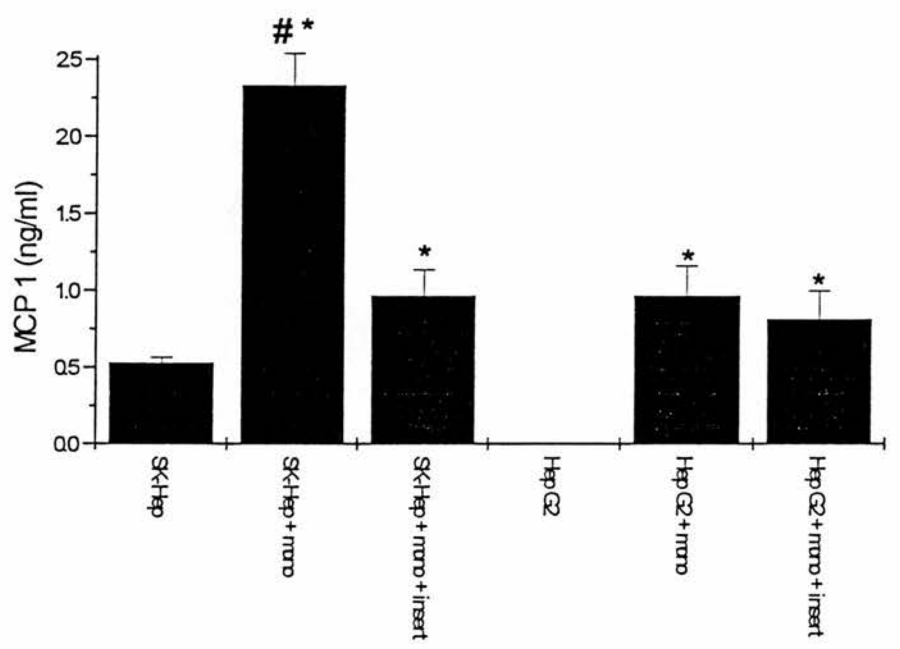


Figure 3.1c. Adhesion mediated MCP-1 expression. * = significantly different from hepatoma cells alone, # significantly different from hepatoma cells co-cultured with monocytes in transwell plates

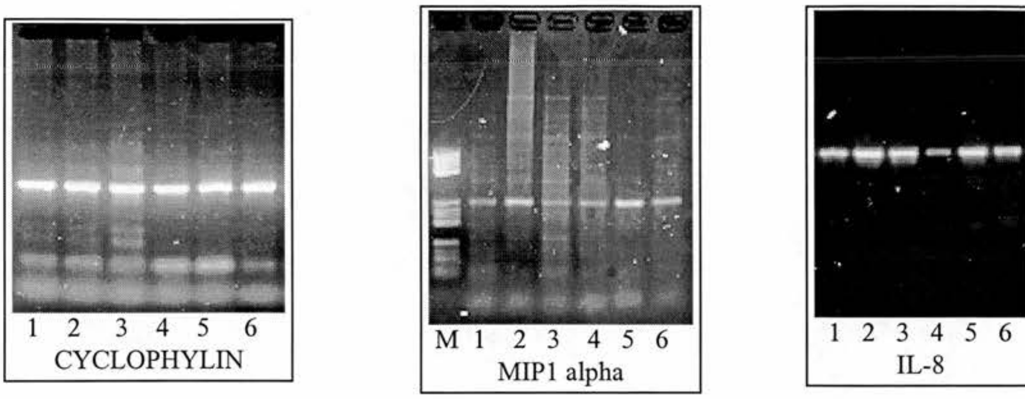


Figure 3.2. Adhesion mediated chemokine production is associated with enhanced gene expression. 1 = SK-Heps alone, 2 = SK-Heps + monocytes, 3 = SK-Heps + monocytes + insert, 4 = Hep G2 alone, 5 = Hep G2 + monocytes, 6 = Hep G2 + monocytes + insert. M = molecular weight markers. Data for MCP-1 is not shown but similar results were obtained.

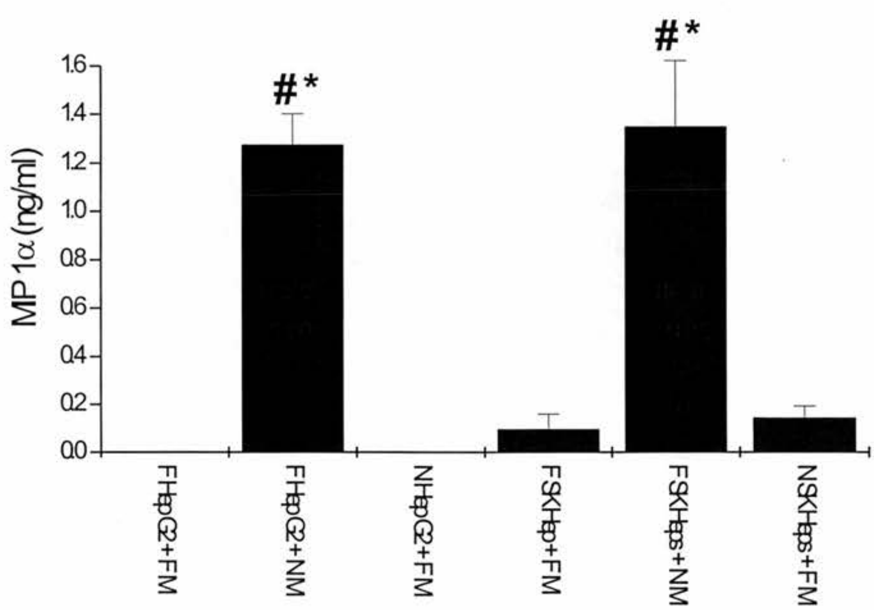


Figure 3.3a Effect of paraformaldehyde fixation on MIP1α production during adhesion. N = unfixed cells, F = paraformaldehyde fixed cells, M = monocytes. # = significantly different (P < 0.05) compared with controls.

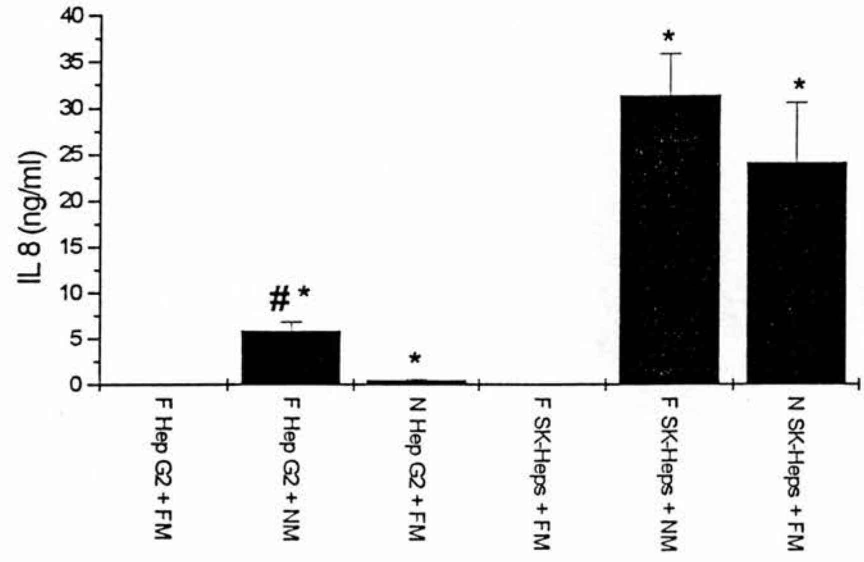


Figure 3.3b Effect of formaldehyde fixation on IL-8 production during adhesion. N = unfixed cells, F = fixed cells, M = monocytes. # = significantly different ($P < 0.05$) compared with controls.

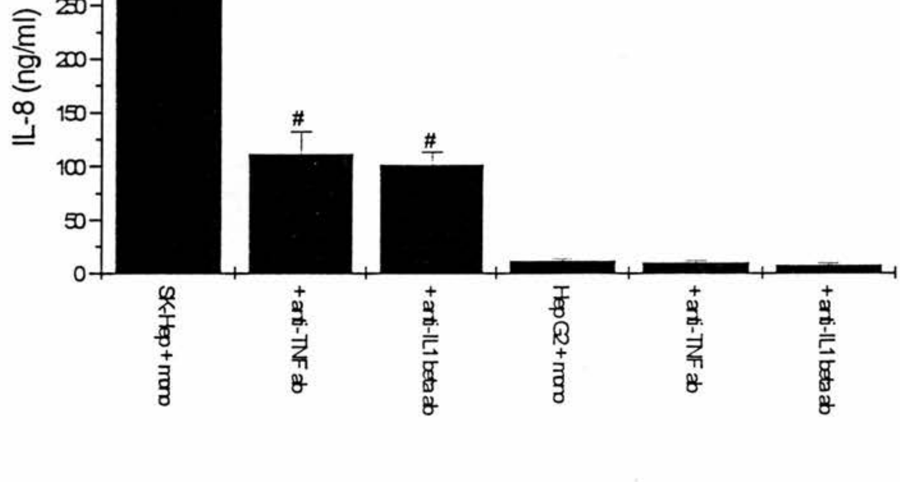


Figure 3.4. Effect of blocking TNFα or IL-1β on IL-8 production during monocyte adhesion with hepatoma cells. # = significantly different ($P < 0.05$) compared with controls.

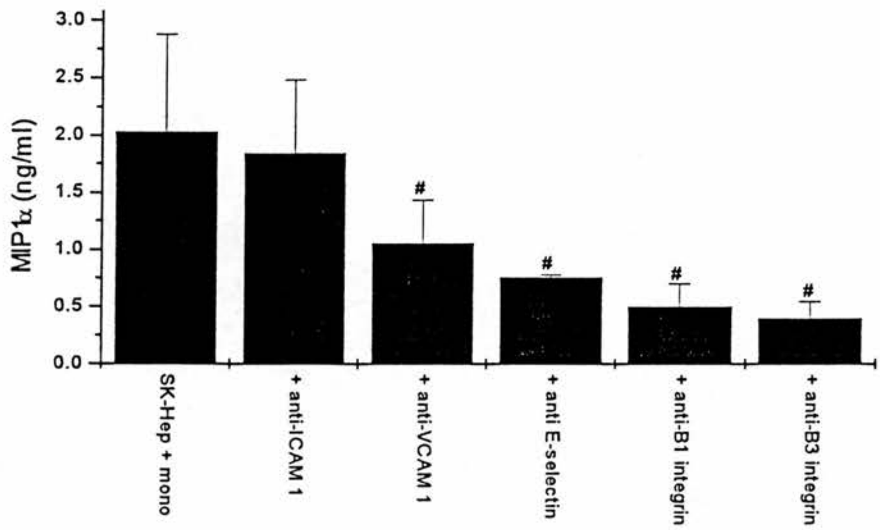


Figure 3.5a. Effect of blocking anti-adhesion molecule antibodies on MIP1α production during adhesion using SK-Heps. # = significantly different (P < 0.05) compared with controls.

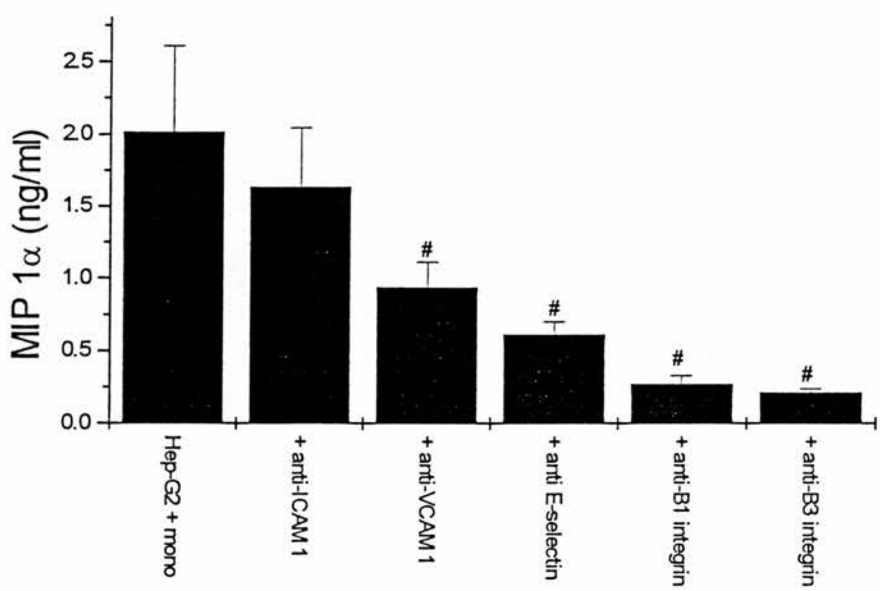


Figure 3.5b. Effect of blocking anti-adhesion molecule antibodies on MIP1α production during adhesion using Hep G2 cells. # = significantly different (P < 0.05) compared with controls.

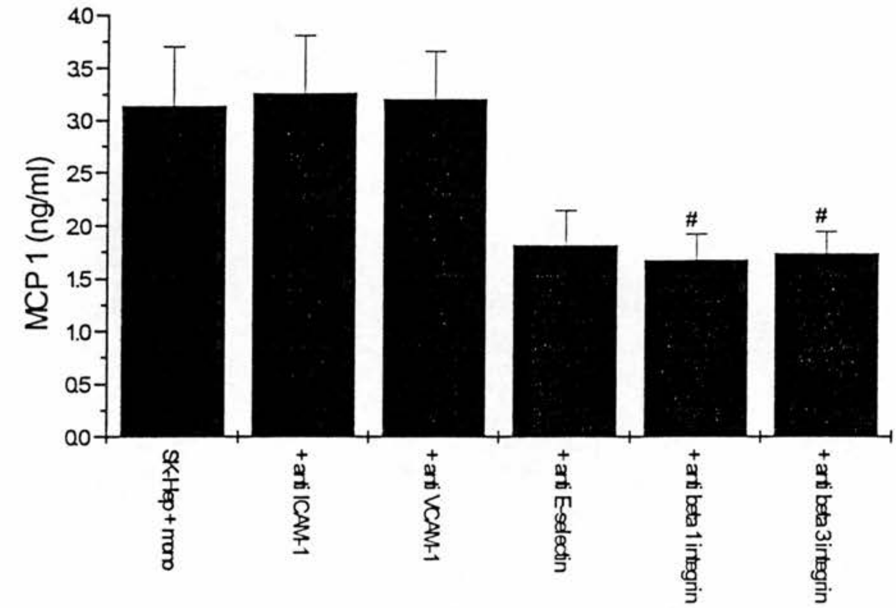


Figure 3.6. Effect of blocking anti-adhesion molecule antibodies on MCP-1 production during adhesion using SK-Heps. # = significantly different (P < 0.05) compared with control.

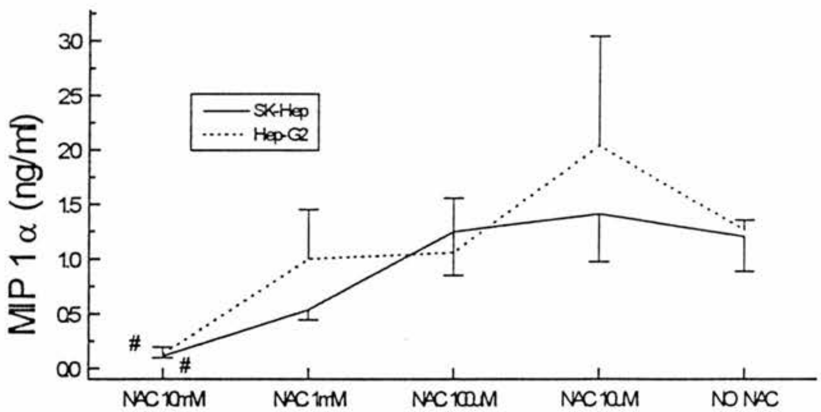


Figure 3.7. Effect of N-acetylcysteine on MIP1α production during adhesion. # = significantly different (P < 0.05) compared with controls.

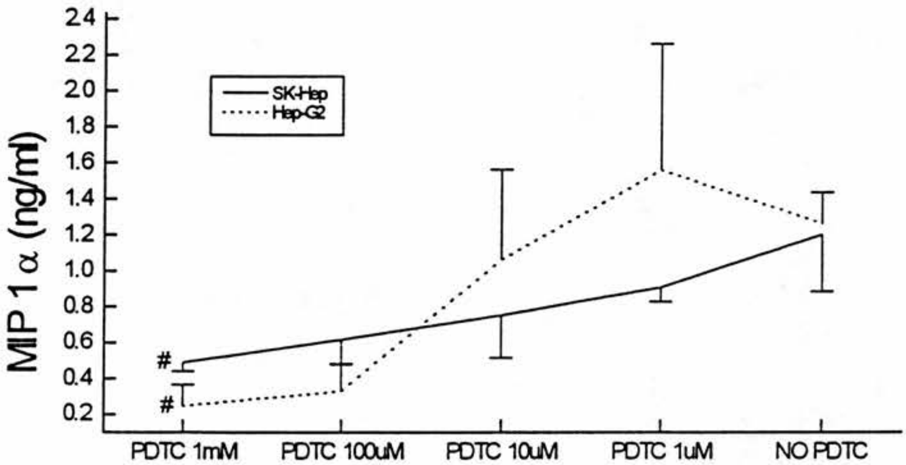


Figure 3.8. Effect of pyrrolidine dithiocarbamate on MIP1α production during adhesion. # = significantly different (P < 0.05) compared with controls.

3.3.7. Table 3.1. Effect of cytokine prestimulation on chemokine production during monocyte adhesion with hepatoma cells.

	MCP-1		MIP1α	
	NO MONO	**+ MONO**	**NO MONO**	**+ MONO**
SK-Hep				
unstimulated	0.676 ± 0.122	1.993 ± 0.073	0.040 ± 0.013	2.699 ± 0.365
TNFα	1.32 ± 0.044	1.588 ± 0.110	0.130 ± 0.012	3.078 ± 0.373
IL-1β	0.784 ± 0.092	1.476 ± 0.076	0.065 ± 0.009	2.757 ± 0.500
IFN-γ	0.540 ± 0.006	1.478 ± 0.118	0.150 ± 0.063	4.697 ± 0.606
LPS	0.390 ± 0.006	1.240 ± 0.082	0.025 ± 0.003	2.061 ± 0.316
Hep G2				
unstimulated	0	1.052 ± 0.092	0	5.363 ± 0.592
TNFα	0	1.002 ± 0.111	0	4.540 ± 0.430
IL-1β	0	1.158 ± 0.088	0	4.506 ± 0.178
IFN-γ	0	1.596 ± 0.322	0	5.778 ± 0.372
LPS	ND	ND	ND	ND

IL-8 was not measured in these studies. ND = not determined. No significant differences were noted between unstimulated and cytokine stimulated hepatoma cells in the chemokine concentration produced following adhesion.

3.4. DISCUSSION.

These data presented in this chapter show that monocyte adhesion to hepatoma cells induces the production of the potent inflammatory chemokines, MIP1α, MCP-1 and IL-8. The mononuclear cells, rather than the hepatocytes, appeared to be the source of the MIP1α. In contrast, both cell populations synthesised IL-8 and MCP-1. Chemokine production was dependent on adhesion between the two cell populations, except in the case of MCP-1 production induced by monocyte interaction with Hep G2 cells, because the addition of monocytes to hepatoma cells in transwell separated culture dishes significantly reduced the IL-8 and MIP1α concentrations. IL-8 production during monocyte adhesion with the SK-Hep hepatoma cell line was dependent on release of TNFα and IL-1β, but inhibition of these proinflammatory cytokines had no effect on MIP1α or MCP-1 production nor production of IL-8 during monocyte adhesion with Hep G2 cells. MIP1α and MCP-1 production, but not IL-8, was inhibited by neutralising monoclonal antibodies directed against VCAM-1 and the β1 integrin component of its ligand (VLA 4, α4β1) and also antibodies directed to β3 integrins and E-selectin. Adhesion mediated IL-8 production during the Hep G2:monocyte co-culture could not be inhibited by the anti-adhesion molecule antibodies used. Studies of the potential intercellular pathways implicated release of intracellular reactive oxygen intermediates with the subsequent induction of NF-κB in the production of the CC chemokines studied, but not in CXC chemokine (IL-8) expression.

The results of this study support previous reports of adhesion mediated chemokine production (*Lukacs et al 1994, 1995*) during monocyte adhesion with human umbilical endothelial cells (HUVEC). However there are several important differences. Pretreatment of either Hep G2 or SK-Hep cells with IFN-γ was unnecessary to induce chemokine production during adhesion, preincubation of the HUVEC cultures with IFN-γ was essential to induce expression of MIP1α, MCP-1 and IL-8. TNFα and IL-1 β were not produced during monocyte adhesion with HUVEC and chemokine production was unaffected by anti-TNFα and anti-IL-1β antibodies, in contrast with the IL-8 produced during monocyte:SK-Hep co-culture. In addition, during the monocyte:HUVEC interaction, MIP1α production was inhibited by monoclonal anti-ICAM-1 antibodies (*Lukacs et al 1994*). This may be explained by investigations in other laboratories which found no expression of ICAM-1 on human hepatoma cell lines (*Volpes et al 1992, Kvale et al 1993*). However, MIP1α production was not inhibited by anti-ICAM-1 antibodies but by neutralisation of interaction with E-selectin, VCAM-1 and the β1 component of its integrin ligand VLA-4 (α4β1), and β3 integrin. Thus, following transendothelial migration, non-ICAM-1 dependent adhesive interactions, namely with E-selectin and β1 and β3 integrins, may be important in stimulating chemokine production from the mononuclear cells. A recent study has reported induction of hepatoma cell β1 and β4 integrin expression following adhesion with endothelial cells and HGF treatment. E-selectin expression occurs on inflammed hepatic endothelium, but not normal endothelium, but the expressionof selectins on human hepatoma cells has not been reported. However, in a small study of 10 hepatomas, none expressed VCAM-1 or E-selectin.

Immunohistochemical studies have not demonstrated E-selectin, ICAM-1 or VCAM-1 expression on normal human hepatocytes. In addition, the lack of MCP-1 and MIP1α production during monocyte adhesion with normal human hepatocytes suggests that CC chemokine production from non-proinflammatory cytokine stimulated cells is specific for transformed cells. Interestingly, it has previously been shown that IL-8 and MCP-1 production by monocyte:HUVEC adhesion was inhibited by soluble collagen and fibronectin (*Lukacs et al 1995*), both of these extracellular matrix proteins are potential ligands for β1 and β3 integrins.

It has been proposed that reactive oxygen intermediates are the common pathway through which differing stimuli induce NF-κB activation and gene expression (*Schreck et al 1991*), although this may be dependent on the cell type studied (*Brennan et al 1995*). Intracellular signalling via β1, β2 or β3 integrins may trigger a respiratory burst and the formation of reactive oxygen species in a variety of inflammatory cells, including mononuclear cells (*Nagata et al 1995, Berton et al 1992, Zhou et al 1993*). In addition, selectin cross-linking significantly increases the oxidative burst induced in neutrophils by either formyl-Met-Leu-Phe or TNFα (*Waddell et al 1994*). A NF-κB site is located in the 5' region of several chemokine genes and is important in the transcriptional regulation of these genes. Therefore the effects of antioxidants on chemokine production during monocyte hepatoma cell interaction were studied. In this study, NAC induced a dose dependent inhibition of MIP1α production in monocytes co-cultured with either Hep G2 or SK-Heps cells and inhibited MCP-1 production during monocyte adhesion with SK-Heps. In contrast, IL-8 production was unaffected, thus excluding a toxic or anti-adhesive effect of NAC on the cells in the co-culture. NAC increases intracellular thiols and interacts directly with oxygen free radicals (*Aruoma et al 1989*) and hence can inhibit NF-κB activation and gene transcription (*Staal et al 1990*). PDTC is a specific inhibitor of oxidant mediated NF-κB activation, while other transcription factors, such as SP1, AP-1 and C/ERP are unaffected (*Schreck et al 1992, Ziegler-Heitbrock et al 1993*). PDTC also inhibited adhesion mediated MIP1α and MCP-1 production, but not IL-8 synthesis, in a dose-dependent fashion. Although both NAC and PDTC may affect AP-1 binding in certain cells (*Meyer et al 1993, Bergelson et al 1994*), another AP-1 inhibitor, 3'-aminobenzamine, had no effect on either IL-8, MCP-1 or MIP1α production in the studies presented here. Although the activation of NF-κB was not directly demonstrated, inhibitors were used to assess the differential effect of inhibiting this transcription factor on adhesion mediated chemokine expression. Therefore, these data indirectly implicate intercellular free radial mediated NF-κB activation as the mechanism inducing CC chemokine production during adhesion.

Previous studies have shown a relationship between monocyte adhesion and chemokine production (*Lukacs et al 1994, 1995*). Such studies have focused on adhesion mediated chemokine expression in the setting of inflammation. This is the first study to report monocyte adhesion to transformed human hepatoma cell lines also induces the production of chemokines. In the context of malignant disease this finding may have several potential biological effects. The activation of mononuclear cells during contact with tumour cells would allow the local deposition of MIP1α, intensifying the chemotactic gradient, and increasing both the numbers and activational status of the infiltrating inflammatory cells. IL-8 is a potent angiogenic factor in vivo (*Strieter et al 1995*) and IL-8 inhibition reduces tumour size and frequency of metastasis in SCID mice (*Arenberg et al 1996*). Interferon inducible protein 10 (IP-10), a non-ELR CXC chemokine is an angiostatic factor both in vitro and in vivo (*Strieter et al 1995*). However negligible production of IP-10, in comparison with the concentrations of IL-8, were noted during monocyte adhesion with either SK-Heps or Hep G2 cells (data not shown). Therefore, IL-8 production during adhesion, through its angiogenic properties, could enhance tumour cell growth and metastatic potential. This effect would be further amplified by the inflammatory cells recruited to the site of a tumour via adhesion mediated CC chemokine production. Thus, tumours may not just evade the body's immune response, but may potentially turn the host defence systems to its own advantage. Adhesion of monocytes with normal human hepatocytes did not induce MIP1α or MCP-1 expression and therefore adhesion mediated chemokine production and subsequent immune amplification is unlikely to occur in normal uninflammed non-transformed liver. These data suggest that CXC chemokine production is mediated via proinflammatory cytokine production and CC chemokine expression via integrin mediated free radical production and subsequent NF-κB activation. Therefore, targeted anti-cancer therapies may enhance local inflammatory chemokine production, but inhibit angiogenic chemokine expression. However, with the observed minor differences in adhesion mediated chemokine production between SK-Heps and Hep G2, suggest that individual tumours may respond in slightly differing ways.

In summary, monocyte adhesion with hepatoma cells induces the production of MIP1α, MCP-1 and IL-8. IL-8 production during monocyte adhesion with SK-Hep cells was dependent on proinflammatory cytokine induction. Integrins, β1 and β3, VCAM-1 and E-selectin are important adhesion molecules involved in CC chemokine, MCP-1 and MIP1α, production, which was not dependent on classical proinflammatory cytokine stimulation, but appears to follow upon free radical mediated activation of NF-κB. Thus, adhesion between monocytes and hepatoma cells may induce the production of potent chemoattractant and angiogenic chemokines resulting in enhanced tumour cell growth and potential for metastasis and may act as novel targets for anti-tumour therapy.

CHAPTER 4

ROLE OF TNFα IN HEPATIC INJURY POST PARACETAMOL POISONING

CHAPTER 4

ROLE OF TNFα IN HEPATIC INJURY POST PARACETAMOL POISONING

4.1 INTRODUCTION.

Paracetamol is metabolised to the highly reactive compound N-acetyl-p-benzoquinonone-imine (NAPQI), which is normally inactivated by intracellular glutathione. Intracellular NAPQI accumulates following overdose and binds to intracellular enzymes ultimately leading to hepatocyte cell death (*Mitchell et al 1973*). This understanding of the metabolic fate of paracetamol does not explain the observations that inhibition of macrophage function will prevent paracetamol induced hepatic necrosis in experimental models. A recent report has shown that pretreatment with gadolinium chloride, to block Kupffer cell function, does not alter the metabolic fate of paracetamol in vivo and has implicated a direct contribution of Kupffer cells in the pathogenesis of hepatic necrosis following paracetamol overdose (*Laskin et al 1995*). Kupffer cells are primary sources of tumour necrosis factor alpha (TNFα) which has been shown to induce hepatocyte apoptosis and necrosis after hepatotoxin exposure (*Leist et al 1995, Kimura et al 1997*). In addition, toxic liver injury following administration of carbon tetrachloride (*Czaja et al 1995*), cadmium (*Kayama et al 1995*), galactosamine/lipopolysaccaride (*Louis et al 1997*) and Concanavallin A (*Gantner et al 1995*) can be inhibited by blocking hepatic TNFα expression or Kupffer cell function (*Edwards et al 1993*).

The aims of these studies were to;

- determine if TNFα expression was induced by paracetamol poisoning
- measure the effect of inhibiting TNFα on hepatic necrosis in this experimental model.

4.2 MATERIALS AND METHODS.

4.1. Experimental model.

Six week old female CBA/J mice were fasted, but with free access to water, for 8 hours prior to intraperitoneal (I.P.) injection of paracetamol 300 mg/kg, 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 immunisation 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 IP, 1 hour prior to paracetamol administration. Non-immune serum was used as control. Many previous studies have shown the efficacy of the immune serum in inhibiting the in vivo effects of TNFα (*Remick et al 1990, Eskandari et al 1992, Hewett et al 1993, Colletti et al 1995*). Mice were also injected IP. with interleukin 10 (5 ug) soluble TNF receptor (200ug) and dexamethasone (4 ug) or saline as control, 1 hour prior to paracetamol administration, which have also been shown to inhibit the in vivo effects of TNFα in experimental animals (*Standiford et al 1995, Walley et al 1996, Lukacs et al 1995, Chensue et al 1990*). In addition, soluble interleukin 1 receptors (200ug/animal) were injected I.P. 1 hour prior to paracetamol administration.

4.2. TNFα expression.

Samples of liver were weighed and homogenised in 1 ml of phosphate buffered saline containing 0.5% Nonidit P40 and the supernatant 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 (*Walley et al 1996*). Liver was also "snap frozen" in liquid nitrogen and homogenised in 1 ml of 4.2 mol/L guanidine isothiocyanate, 25 mmol/L Tris (pH 8.0), 0.5% Sarkosyl and 0.1 mol/L 2 mercaptethanol. An equal volume of 100 mmol/L Tris (pH 8.0), 10 mmol/L 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 5ug 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 minutes followed by 35 cycles at 93°C for 45 seconds, 52°C for 45 seconds and 72°C for 90 seconds. The amplification products were visualised under UV light following separation in a 2% agarose gel containing 0.3 mg/ml ethidium bromide.

4.3. Assessment of hepatic injury.

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

4.4. 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 $\pm$ standard error.

4.3 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 $\pm$ 2.6 IU/L, n = 6, paracetamol treated 2726 $\pm$ 629 IU/L, n = 6, $p < 0.05$) and the appearance of severe hepatic necrosis was observed (Figure 4.1 & 4.2). These changes were completely prevented by the prior injection of 300 mg/kg, N-acetylcysteine (Figure 4.3). 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 4.4) increased 3 hours following paracetamol injection (20.4 $\pm$ 0.6 pg/ml, mean $\pm$ SEM, n = 5, $p < 0.05$) and remained elevated for 24 hours compared with control (2.4 $\pm$ 2.4 pg/ml, n = 7). Hepatic TNFα protein concentration (figure 4.5) was similar at all time points following paracetamol administration (range 0.498-4.818 ng/gm wet weight) and was not significantly different compared with controls (0.941 $\pm$ 0.114 ng/gm wet weight, n = 7). Hepatic TNFα gene expression was not induced following paracetamol (Figure 4.6). Therefore, although a small, but significant increase in serum TNFα protein was observed, there was no induction of hepatic TNFα protein or gene expression following paracetamol poisoning.

To further determine the role of TNFα in the pathogenesis of hepatic injury induced by paracetamol, we inhibited the biological effects of TNFα *in vivo* using anti-TNF antibodies, soluble TNF receptors, interleukin 10 and dexamethasone. Mortality (Table 4.1) following paracetamol poisoning was minimally increased in mice pre-treated with blocking anti-TNFα antibodies (12.5%), soluble TNF receptor (8.3%), interleukin 10 (12.5%) and dexamethasone (0%) compared with controls (0%). Serum aspartate (Table 4.1) was also similar in all groups. Histological assessment of the extent of hepatic necrosis (Figure 4.7, Table 4.2), determined 20 hours post paracetamol injection, was also similar in controls (2.0 $\pm$ 0.2, mean score $\pm$ SEM, n = 16) and mice pretreated with blocking anti-TNFα antibodies (2.5 $\pm$ 0.3, n = 8), soluble TNFα receptor (2.3 $\pm$ 0.3, n = 10), interleukin 10 (2.43 $\pm$ 0.2, n = 7) and dexamethasone (2.1 $\pm$ 0.2, n = 8). Soluble interleukin 1 receptors had no effect on hepatic injury post-paracetamol as determined by serum transaminases (7378 $\pm$ 1092, n = 8), death rate (0%) or histological score (2.5 $\pm$ 0.2).

4.3.1. Figures for Chapter 4.

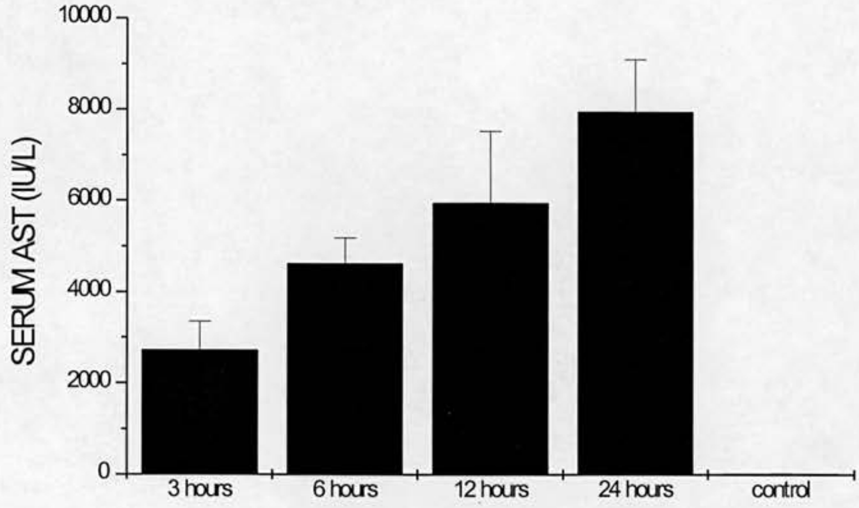


Figure 4.1. Serum aspartate aminotransferase post-paracetamol poisoning. N = 5 in each group, except control n = 7.

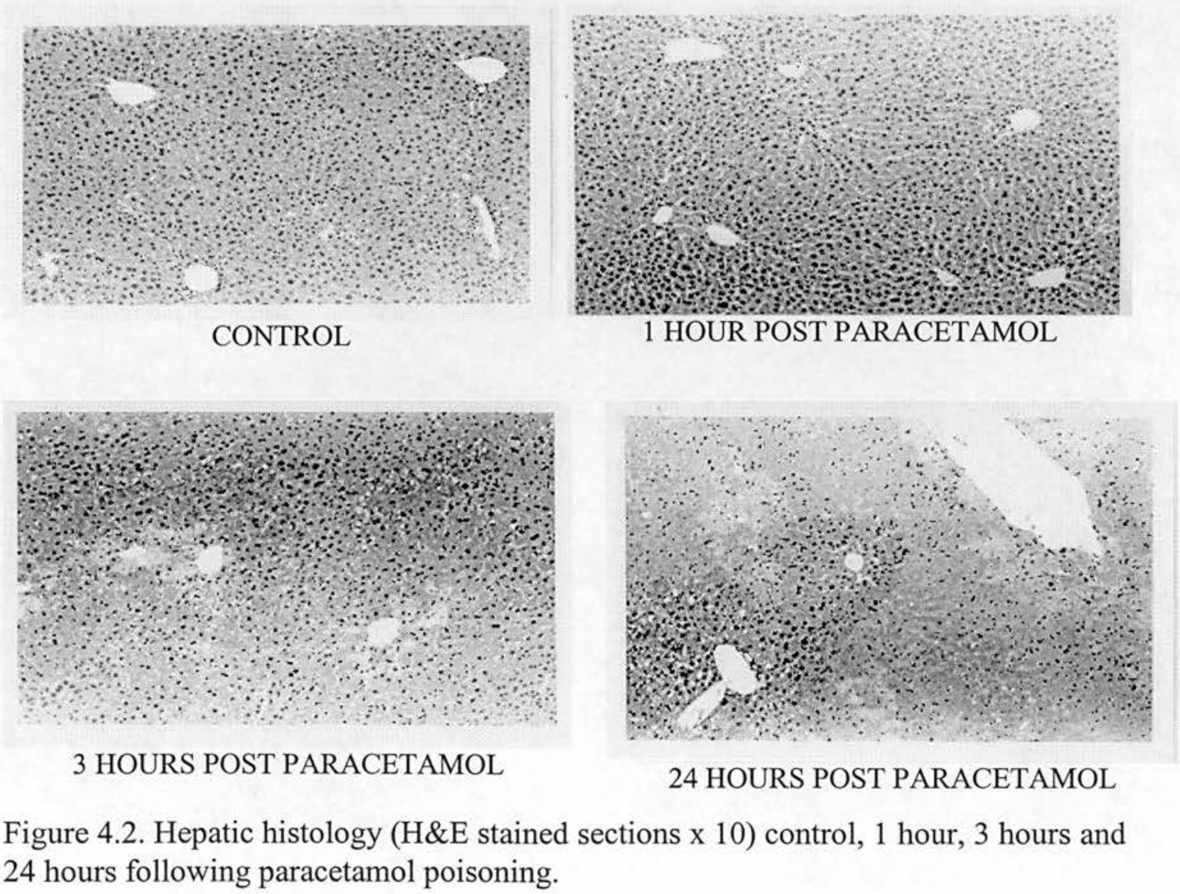


Figure 4.2. Hepatic histology (H&E stained sections x 10) control, 1 hour, 3 hours and 24 hours following paracetamol poisoning.

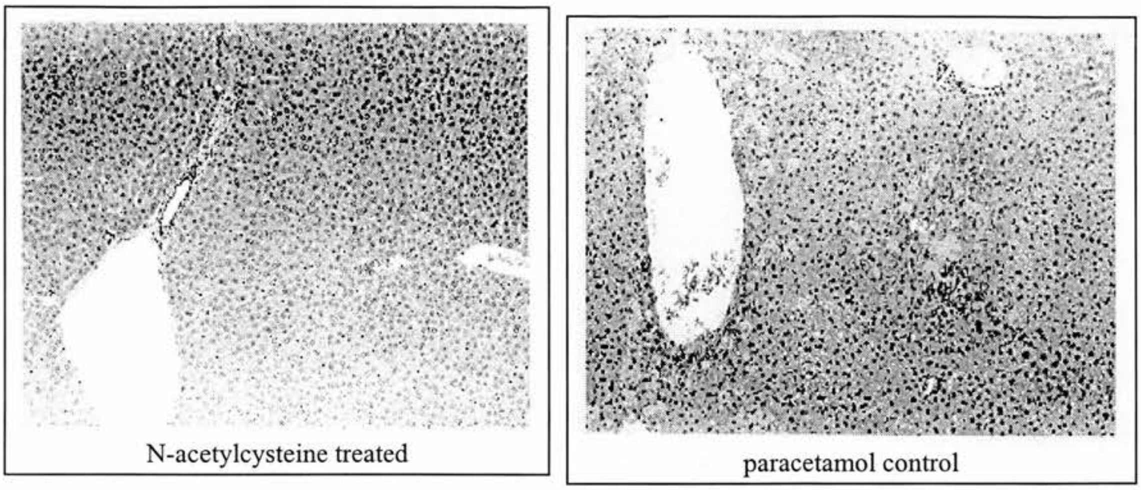


Figure 4.3. Hepatic histology (H&E stained sections x 10) 24 hours post-paracetamol poisoning, with and without pretreatment with N-acetylcysteine (300 mg/kg).

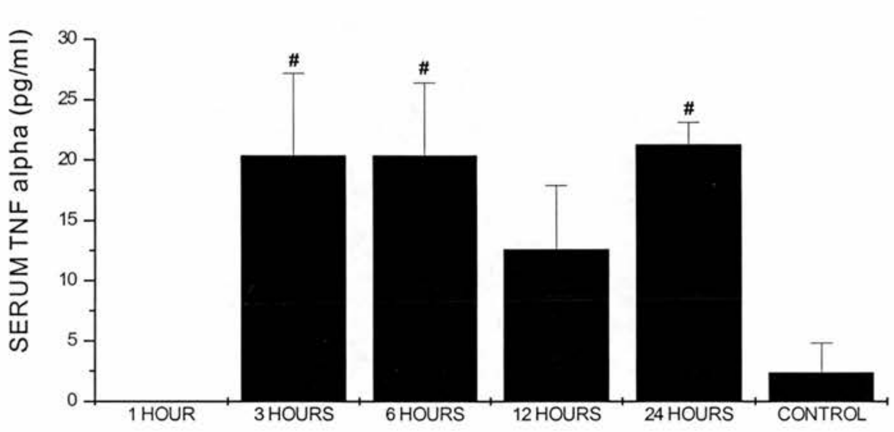


Figure 4.4. Serum TNFα following paracetamol poisoning. # = significant difference ($p < 0.05$) compared with saline injected controls, n = 5 in each group except the control group in which n = 7.

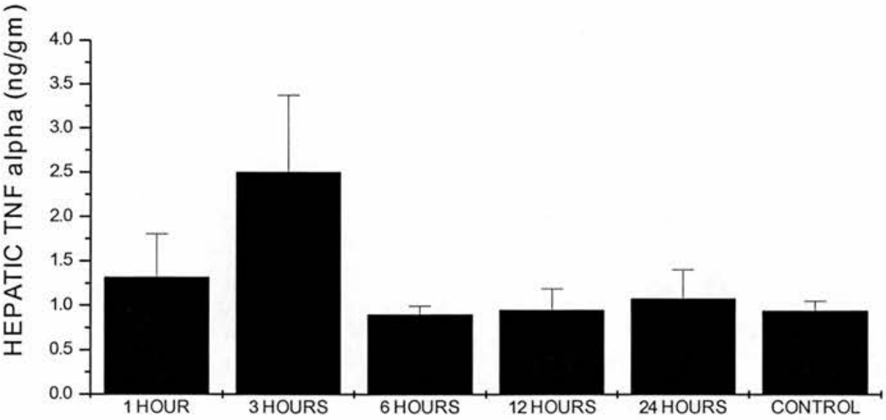


Figure 4.5. Hepatic TNFα following paracetamol poisoning. No significant differences ($p < 0.05$) were noted compared with saline injected controls, n = 5 in each group except the control group in which n = 7.

CYCLOPHYLIN

1 3 6 12 24 C N

TNF

1 3 6 12 24 C N

Figure 4.6. Hepatic TNFα mRNA expression following paracetamol poisoning. Cyclophylin was used as control. 1, 3, 6, 12, 24 = hours post-paracetamol, C = control, N = negative control. Images are negatives of polaroid photographs.

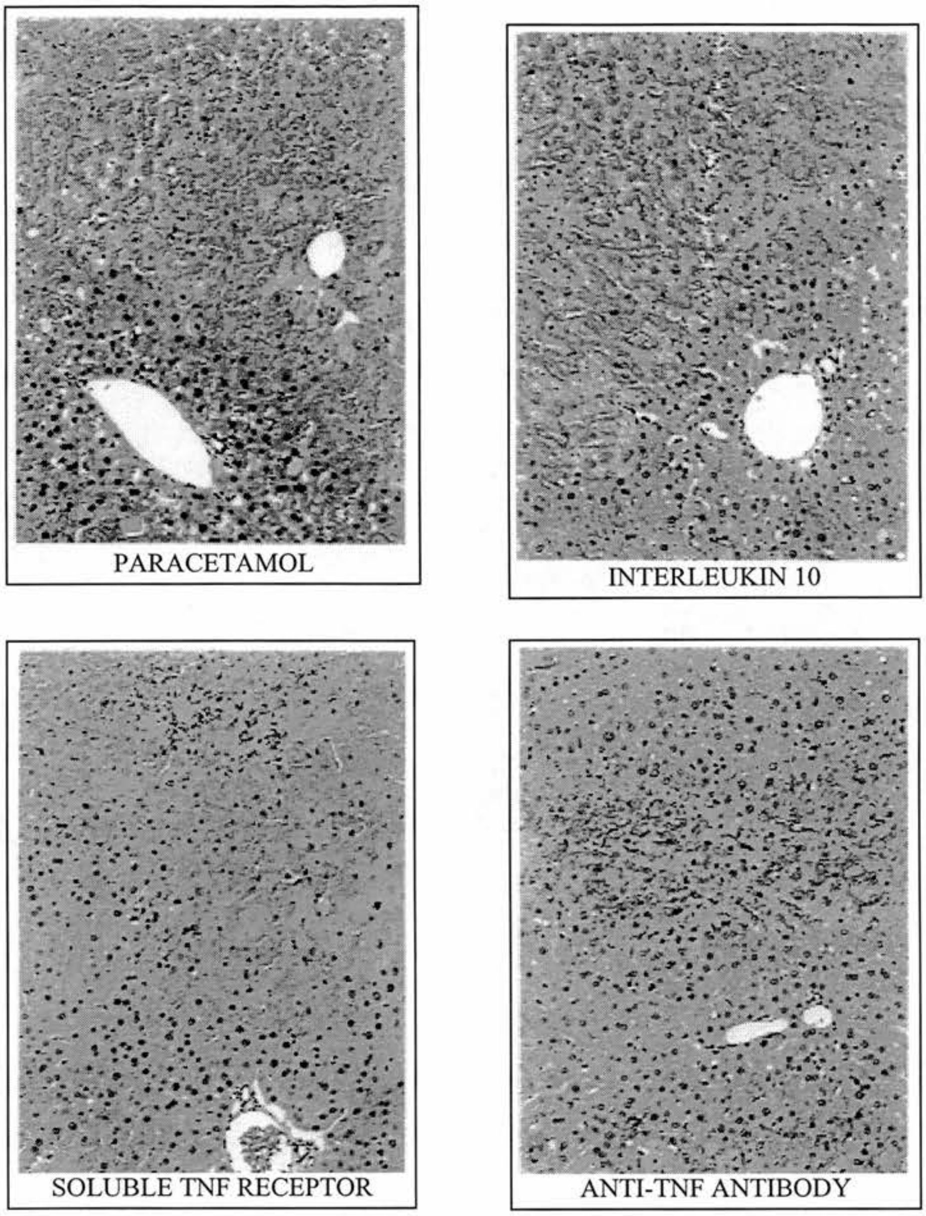


Figure 4.7. Hepatic histology following paracetamol poisoning, the effect of anti-TNF α therapies. Representative sections from control animals and mice preinjected with anti-TNFα antibodies, soluble TNF receptor and interleukin 10 prior to paracetamol injection are presented. No difference in hepatic histology was observed. The data from dexamethasone treated animals is not shown, but no improvement in hepatic histology was observed.

4.3.2. Tables for Chapter 4.

TABLE 4.1. Mortality and serum aspartate aminotransferase following paracetamol poisoning: effect of TNFα inhibition.

	CONTROL	ANTI-TNF ANTIBODY	SOLUBLE TNF RECEPTOR	INTERLEUKIN 10	DEXAMETH-ASONE
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.

TABLE 4.2. Histological assesment of hepatic necrosis following paracetamol poisoning; effect of TNFα inhibtion.

	CONTROL	ANTI-TNF ANTIBODY	SOLUBLE TNF RECEPTOR	INTERLEUKIN 10	DEXAMETH-ASONE
+	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.

4.4 DISCUSSION.

In this study, an increase in serum TNFα at 3 hours post paracetamol was observed, but there was no increase in hepatic TNFα protein or gene expression. In addition, treatment with anti-TNFα antibodies, soluble TNF receptor, interleukin 10 and dexamethasone did not protect against paracetamol induced hepatic necrosis as determined 20 hours post paracetamol poisoning. The concentrations of anti-TNFα therapies used were sufficient to inhibit the high serum concentrations observed during sepsis, for example the anti-TNFα antibodies reduce TNFα serum concentrations of 80,000 pg/ml observed following caecal ligation and puncture, to undetectable levels (*Eskandari et al 1992*). Therefore, the anti-TNFα therapies employed in this paper should have been more than adequate to completely inhibit circulating concentrations of TNFα observed following paracetamol poisoning (maximum 30 pg/ml).

Kupffer cells have been implicated in the development of hepatic necrosis following paracetamol poisoning. Cultured hepatocytes exposed to paracetamol release unidentified mediators that activate Kupffer cells in vitro (*Laskin et al 1986a*). Activated Kupffer cells have been identified in liver tissue following paracetamol poisoning in experimental animals (*Laskin et al 1986b*). Gadolinium chloride pretreatment blocks Kupffer cell function and inhibits hepatic necrosis in a rat model of paracetamol poisoning, without affecting the metabolism of paracetamol (*Laskin et al 1995*). Interestingly, pretreatment with lipopolysaccaride, a macrophage activator, also prevented hepatic necrosis in this model (*Laskin et al 1995*). Goldin and colleagues (*1996*), using dichloromethylene diphosphonate containing liposomes, found Kupffer cell blockade only delayed, but did not prevent, hepatic necrosis following paracetamol poisoning in a murine model. Kupffer cells are potent sources of TNFα, which may induce hepatocyte death *in vivo* and *in vitro* (*Hill et al 1995, Leist et al 1996*). Previous studies have shown anti-TNFα antibodies (*Hewett et al 1993*), soluble TNF receptors (*Czaja et al 1995*) or interleukin 10 (*Louis et al 1997*) can inhibit liver injury induced by other nonparacetamol hepatotoxic agents. Gadolinium chloride pretreatment is associated with hepatic release of TNFα (*Ruttinger et al 1996*). This may protect the liver from the subsequent toxic effects of TNFα through the induction of tolerance. In addition, elevated circulating TNFα has been reported in patients with fulminant liver failure from a variety of causes (*Muto et al 1988, Sekiyama et al 1994, Haeger et al 1996*).

In contrast with the results presented here, other workers have shown an increase in serum TNFα using a bioassay from 6 hours to 48 hours and increased hepatic TNFα expression at 8 hours following paracetamol administration (*Blazka et al 1995*). Serum transaminases and hepatic histological damage were also reduced but not inhibited by anti-TNFα antibodies (*Blazka et al 1996*). It is likely that the differences between the two studies are due to differences in the strain of mice used and the dosage of paracetamol (500 mg/kg). Interestingly, no effect of blocking TNFα on mortality was reported in these previous studies and the serum transaminases at 24 hours were returning to normal (*Blazka et al 1995, 1996*), in contrast with this study in which the aspartate aminotransferase was 2000-11,000 IU/L.

The role of TNFα in the pathogenesis of toxic liver injury has been studied in other models, including carbon tetrachloride (CCl4) or lipopolysaccaride/galactosamine (LPS/GalN) administration. Although serum or hepatic TNFα concentrations were not reported, administration of soluble TNF receptors inhibited liver injury in rats following CCl4 (5ml/kg) (*Czaja et al 1995*). However in another study, using a murine model, injection of anti-TNFα antibodies did not prevent CCl4 (0.1 ml/kg) induced hepatic injury, despite increased concentrations of serum and hepatic TNFα (*Bruccoleri et al 1997*). Interestingly, the reported concentrations of serum TNFα (30 pg/ml), were similar to those observed in this study. LPS/Galn administration induces hepatic necrosis in mice and elevated serum and hepatic TNFα concentrations. The increased TNFα expression and toxic liver injury in this model can be prevented by anti-TNFα therapies (*Louis et al 1997, Santucci et al 1996*).

TNFα can also play an important role in hepatic regeneration (*Diehl & Rai 1996*). Liver regeneration following partial hepatectomy is delayed by inhibiting the biological effects of TNFα *in vivo* and hepatic proliferation induced by lead nitrate is also prevented by anti-TNF antibodies. In addition, liver regeneration following partial hepatectomy is impaired in mice lacking the TNF p55 receptor (*Yamada et al 1997*). However, following toxic liver injury, TNFα may have two diametrically opposing pathogenic roles. One the one hand, TNFα can induce hepatic apoptosis and necrosis following toxin exposure, both *in vivo* and *in vitro* and on the other hand TNFα may also initiate and potentate hepatic regeneration. It is not clear from published data, whether this is a species difference (ie. rat v mouse), related to the route or dose of hepatotoxin administration or degree of hepatic injury induced. (ie. TNFα inhibition prevents injury following high dose hepatotoxin or induction of extensive hepatic necrosis; TNFα inhibition delays regeneration following lower dose hepatotoxin administration or induction of milder hepatic damage).

Kupffer cells are also potent sources of interleukin 1. Interleukin 1 can induce the production of TNFα from hepatocytes *in vitro* (*Frede et al 1996*) and pretreatment of animals with interleukin 1 does not induce hepatic damage, but prevents direct TNFα induced hepatic necrosis (*Bohlinger et al 1995*). However, no protective effect of administering soluble interleukin 1 receptors on hepatic necrosis induced by paracetamol poisoning was identified, suggesting that Kupffer cell derived interleukin 1 does not play a pathogenic role in this model. Another Kupffer cell derived cytokine, macrophage inflammatory protein 2 (MIP2), has recently been implicated in hepatic necrosis following chronic alcohol ingestion (*Bautista 1997*). Although hepatic expression of the CC chemokine monocyte chemoattractant protein 1 (MCP1) is increased in toxic liver injury (*Czaja et al 1994*), the production of other chemokines, such as MIP2, has not been studied.

In summary, the data presented in this chapter does not implicate TNFα in the pathogenesis of hepatocyte necrosis following paracetamol poisoning. Therefore therapies directed against TNFα are unlikely to be clinically effective. However, TNFα may play a central role in hepatic regeneration following toxic liver injury and partial (70%) hepatectomy. Therefore, although no role for TNFα in the acute necrosis following paracetamol poisoning was identified, this multifunctional cytokine may play a central role in initiating hepatic regeneration in this model.

CHAPTER 5

ROLE OF CHEMOKINES IN HEPATIC REGENERATION FOLLOWING PARACETAMOL POISONING

CHAPTER 5

ROLE OF CHEMOKINES IN HEPATIC REGENERATION FOLLOWING PARACETAMOL POISONING

5.1. INTRODUCTION AND AIMS

At present most of the published data regarding hepatic regeneration has been derived from studying the rat 70% hepatectomy model and much important data have been obtained from these studies regarding the role of cytokines in hepatic regeneration. Following 70% hepatectomy there is some infiltration of the parenchyma with neutrophils, which may induce some hepatocyte damage, but this animal model essentially investigates the regeneration of normal hepatocytes in an undamaged liver. This situation is rarely encountered in the clinical setting. Although other animal models of toxic liver injury exist and have been discussed above, the major cause of toxic liver injury in medical practice follows paracetamol poisoning. In this chapter the regeneration of the liver following paracetamol poisoning was investigated, particularly the potential role of chemokines.

The specific aims of this chapter were to

- Determine if chemokine production was induced in the liver after paracetamol poisoning.
- Subsequently, to examine the effect on liver regeneration of inhibiting or enhancing hepatic chemokine expression in this model

5.2. METHODS

5.2.1. Animal model.

The murine model of paracetamol poisoning was described previously in chapter 4.

5.2.2. Modulation of hepatic chemokine expression *in vivo*.

Rabbit anti-murine chemokine blocking antibodies were raised in New Zealand White rabbits with recombinant chemokines. Antiserum was administered by intraperitoneal injections, one hour prior to paracetamol poisoning, and every second day there after. Non-immune serum was used as control.

The adenovirus was a kind gift from Professor J Gauldie. Rat MIP-2 cDNA was inserted into the E3 region of the genome of recombinant human type 5 adenovirus, made replication deficient by deleting the E1 region. Rat MIP-2 is 90% homologous in base sequence with murine MIP-2. A human cytomegalovirus promoter and SV40 polyadenylation sequence inserted upstream and downstream of the MIP-2 cDNA respectively. Replication deficient type 5 adenovirus with *Escherichia coli* β-galactosidase (*lacZ*) inserted into the E3 region of the viral genome served as control. Adenovirus (2.5 x 10^8 plaque forming units) was injected via tail vein injection 24 hours before paracetamol administration.

5.2.3. Murine chemokine ELISA.

Hepatic chemokine expression was measured as described in chapter 4.

5.2.4. Assessment of hepatic regeneration.

Hepatic repair was measured using an AXIO-HOME microscope. Sections of liver tissue (2 from each animal) were stained with haematoxylin and eosin, the area of hepatic necrosis was quantitated and expressed as % total area.

5.2.5. Statistical analysis.

Results are presented as mean $\pm$ SEM. Statistical significance was determined by analysis of variance and students t-test as appropriate, with $p < 0.05$ considered significant.

5.3. RESULTS

5.3.1. Hepatic chemokine expression is induced following paracetamol poisoning.

To determine if chemokine expression was induced during hepatic injury and regeneration following paracetamol poisoning, the concentration of immunoreactive MIP-2, JE, MIP1α and MIP1β was measured in liver homogenate supernatants. Preliminary experiments identified 300 mg/kg as a dose which induced extensive hepatic necrosis, elevated serum transaminases and a mortality rate of upto 30%. Paracetamol administration at concentrations (ie. 200 mg/kg and less) were not associated with histological hepatic damage and only mild elevation of serum aspartate aminotransferase (ie. < 50 IU/L). Injection of paracetamol at these sub-necrotic concentrations did not alter hepatic chemokine expression. In contrast, protein concentrations of the CXC chemokine, MIP-2, were significantly increased compared with controls from 12 hours to 48 hours post-paracetamol poisoning using 300 mg/kg (Figure 5.1). Although hepatic congestion and necrosis are histologically evident from 3 hours post-paracetamol, the increased MIP-2 at 12 hours corresponds with the increasing serum aspartate aminotransferase concentrations over the first 24 hours. Hepatic MIP-2 concentrations are similar to controls by 3 days after paracetamol poisoning, at which time the liver histology has not returned to normal (Figure 5.2).

Despite the dense mononuclear inflammatory infiltrate surrounding the necrotic areas of liver tissue, although these was some increase in hepatic CC chemokines, JE, MIP1 α and MIP1β (Figure 5.3-5.5), post-paracetamol, these changes did not reach statistical significance.

5.3.2. Modulation of hepatic chemokine expression alters liver regeneration.

Intraperitoneal injection of neutralising anti-chemokine antibodies was associated with a significantly delayed liver regeneration (Figure 5.6) on day 6 in animals treated with neutralising antibodies directed against the CXC chemokine, ENA-78 (8.67 $\pm$ 2.50, mean $\pm$ SEM, n = 9, mean of 2 experiments) compared with controls (0.8 $\pm$ 0.67, n = 8, mean of 2 experiments). In contrast, neutralisation of MIP-2, JE (MCP-1), MIP1α or administration of neutralising anti-ICAM-1 antibodies, was not associated with delayed liver regeneration on day 6 (Figure 5.6). During these experiments the observed mortality rate in the animals treated with anti-ICAM-1 (35%), anti-MIP-2 (31.6%) and anti-MIP1α (25%) was noted to be increased compared with control animals (10.5%) and those treated with anti-JE (10%) and anti-ENA-78 (10%) antibodies (n = 19 or 20 in each group, mean of 2 experiments).

An adenovirus expression vector was used to study the effect of enhancing hepatic expression of the CXC chemokine, MIP-2, on liver regeneration following paracetamol poisoning. Both control and MIP-2 adenovirus treated animals had similar histological damage 24 hours post-paracetamol injection. In addition, a significant neutrophilic infiltrate was not observed in the MIP-2 adenovirus injected animals compared with controls (Figure 5.7). All animals treated with the MIP-2 expressing adenovirus had complete liver regeneration four days following paracetamol poisoning, compared with controls, where almost 50% of the liver remained necrotic (Figure 5.8, 5.9). Therefore, remained necrotic (Figure 5.8, 5.9). Therefore, increasing hepatic MIP-2 expression accelerates hepatic regeneration following paracetamol poisoning.

5.3.3. Figures for Chapter 5.

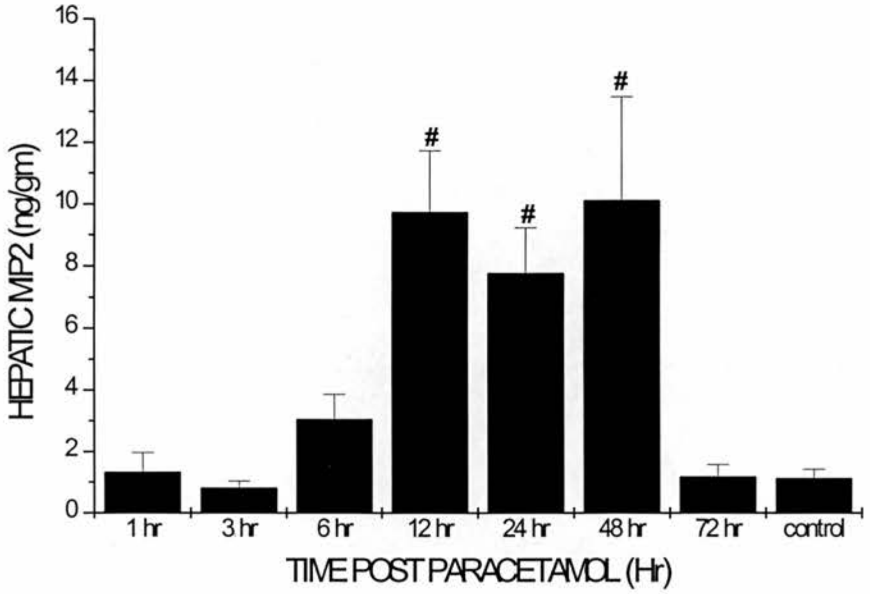


Figure 5.1. Hepatic expression of MIP 2 post-paracetamol poisoning. # = significantly different from controls, n = 5 in each group.

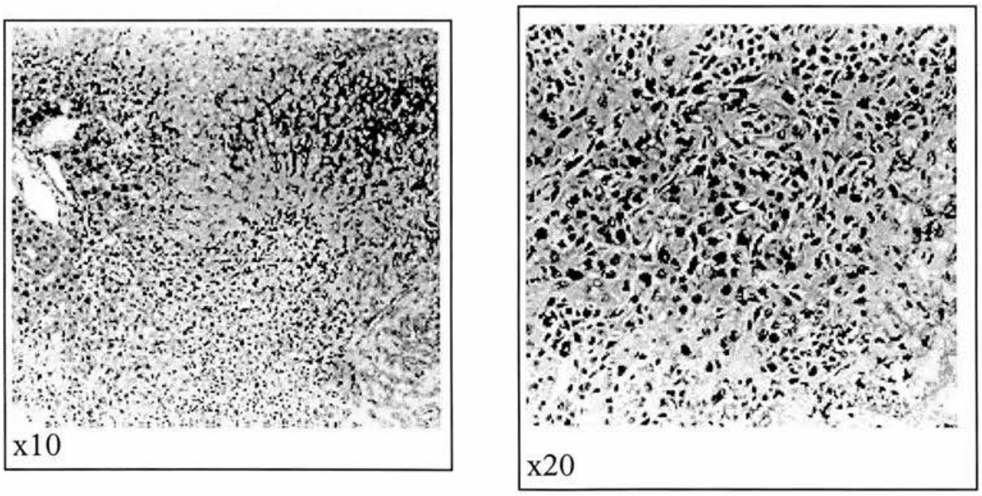


Figure 5.2. Hepatic histology (H&E stained sections) showing dense mononuclear inflammatory cell infiltrate 2 days post paracetamol poisoning

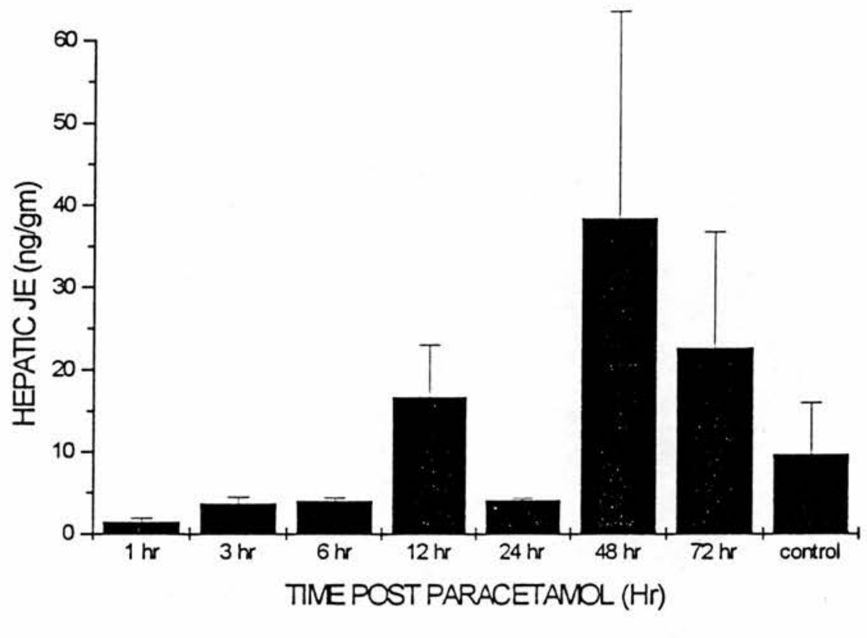


Figure 5.3. Hepatic JE (MCP-1) expression post-paracetamol.

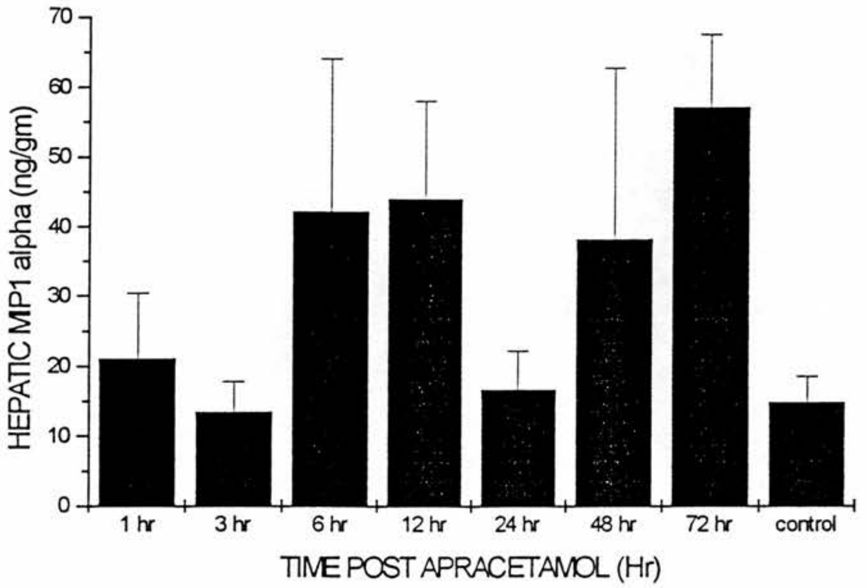


Figure 5.4. Hepatic MIP1α expression post-paracetamol.

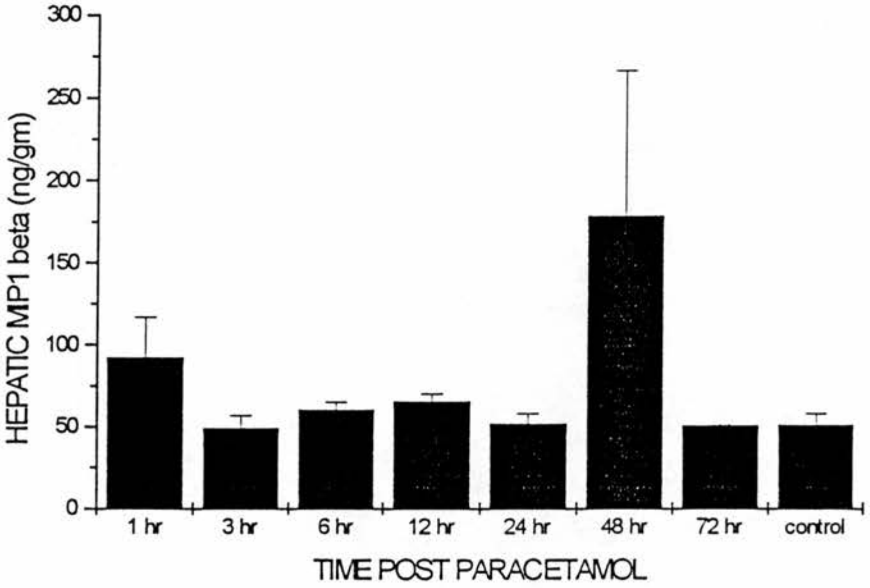


Figure 5.5. Hepatic MIP1β expression post-paracetamol.

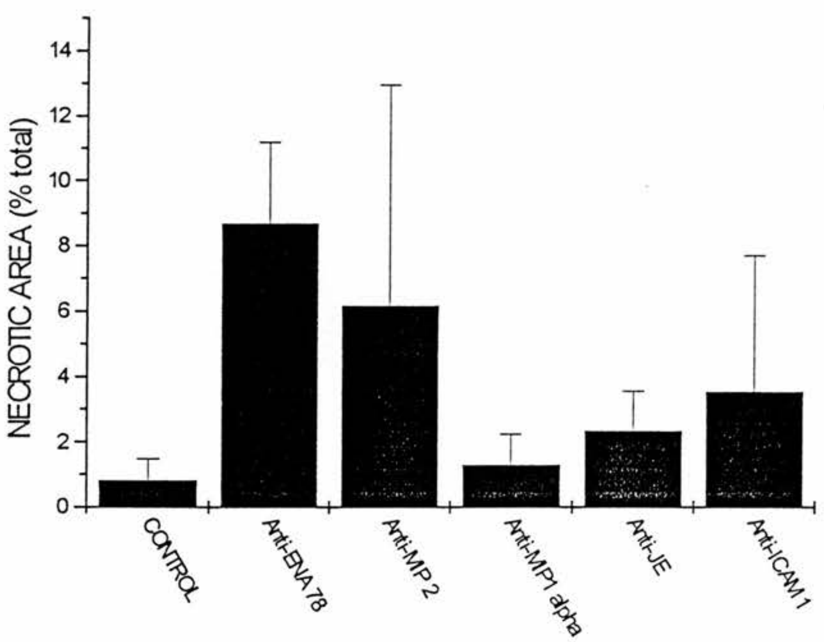


Figure 5.6. Effect of inhibiting hepatic chemokine expression on the resolution of the necrotic areas of liver, following paracetamol poisoning. # = significantly different from controls.

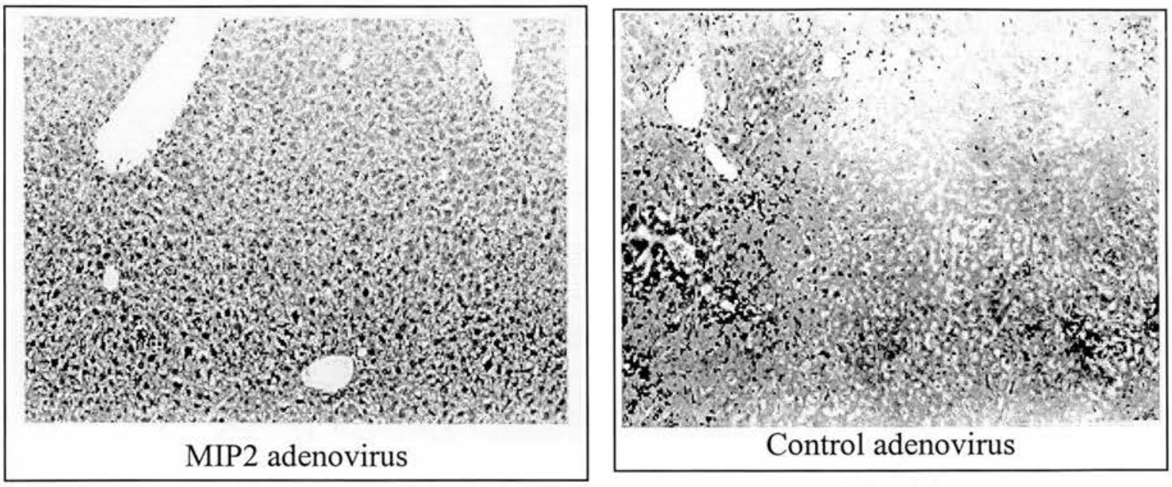


Figure 5.7. Effect of adenovirus MIP 2 on hepatic histology 4 days after paracetamol poisoning (H&E stained sections, x 10). Complete resolution of the hepatic damage in the MIP 2 treated animals was noted.

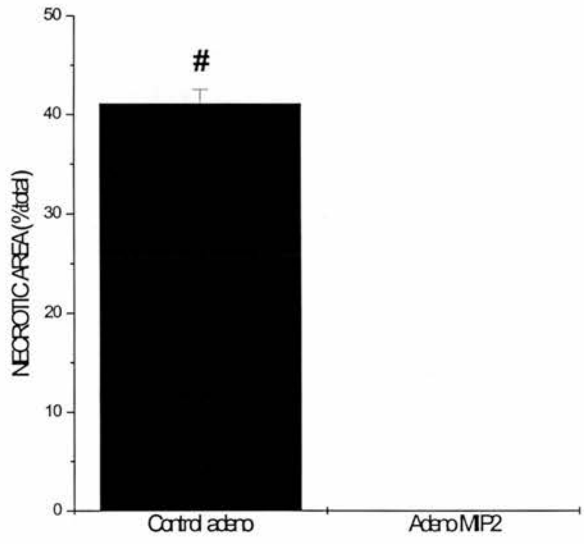
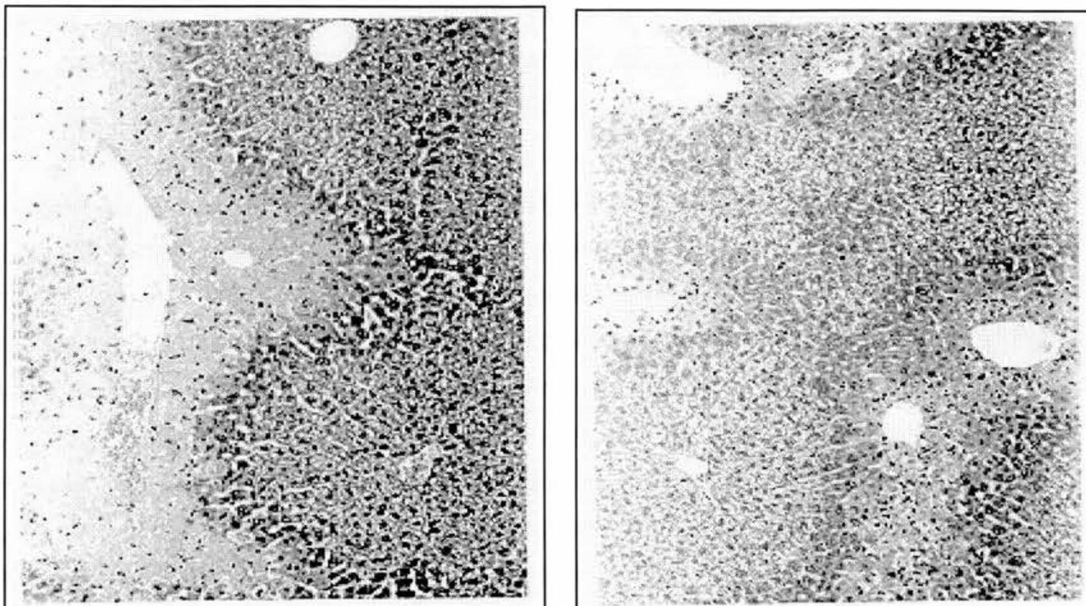


Figure 5.8. Quantitation of the area of hepatic necrosis at day 4 in animals treated with adenovirus MIP 2 compared with controls. # = significantly different from controls.



Control adenovirus | MIP2 adenovirus

Figure 5.9 Hepatic histology (H&E stained sections, x ?) 24 hours post paracetamol in control and adenovirus MIP 2 treated animals, no significant neutrophilic hepatitis was noted in the adenoMIP 2 treated animals.

5.4. DISCUSSION

These experiments have shown paracetamol poisoning in hepatotoxic doses induces the hepatic expression of the CXC chemokine, MIP-2, blocking the effects of MIP-2 increases post-poisoning mortality rates and augmenting hepatic MIP-2 expression enhances regeneration following paracetamol induced liver injury. Intravenous injection of adenovirus expression vectors is associated with a high level of gene expression in the liver compared with other tissues (*Jaffe et al 1992*). Although blocking MIP-2 antibodies did not delay liver regeneration, neutralisation of another CXC chemokine, ENA-78, significantly inhibited liver regeneration after paracetamol poisoning. These data suggest that CXC chemokines may play a permissive role *in vivo* in liver regeneration following toxic liver injury and, that augmenting the hepatic expression of these chemokines may accelerate regeneration. In contrast, although a dense mononuclear infiltrate was observed surrounding the necrotic areas of liver, hepatic expression of CC chemokines was not induced by lethal doses of paracetamol. However, the observation that mortality rates are increased in mice treated with anti-ICAM-1 and anti-MIP1α antibodies suggests that pathways utilising these molecules may also be important in the response to paracetamol poisoning.

Correlation of increased protein expression with increased mRNA, potential stimuli or cells expressing MIP-2 were not determined in this study. Kupffer cells are potent sources of MIP-2 following stimulation with proinflammatory cytokines, such as TNFα and IL1β. Both these stimuli and bacterial infection can induce the production of Gro, the human MIP-2 homologue, from primary human hepatocytes (*Rowell et al 1997*) and the production of Gro from human hepatoma cell lines was noted to be constitutive and was not induced by proinflammatory cytokine stimulation (presented in chapter 2). Biliary epithelial cells (*Morland et al 1997*), sinusoidal endothelial cells and fat-storing cells (*Xu et al 1996*) can all be induced to produce chemokines, including Gro or MIP-2 by proinflammatory stimuli. Studies reported in the previous chapter, using the murine model of paracetamol poisoning, did not find induction of TNFα expression. The effect of neutralising TNFα on hepatic expression of MIP-2 following paracetamol poisoning was not examined, but the lack of TNFα induction post-paracetamol poisoning would suggest that this cytokine is not the proximal stimulus to MIP-2 expression. Oxidative stress has also been implicated in the production of chemokines (*Xu et al 1996*). Hepatocyte metabolism of paracetamol metabolism results in free radical generation and oxidative stress (*Arnaiz et al 1995*), which may be the stimulus to MIP-2 production observed in this study. In a recent study, primary rat hepatocytes released Gro (a rat MIP-2 homologue) following exposure to paracetamol (10-50 mmol/L) or hydrogen peroxide (1-10 mmol/L) and was correlated with hepatocyte damage as determined by leakage of lactate dehydrogenase (*Horbach et al 1997*).

There are no studies published on chemokine expression following any form of toxic liver injury. A recent paper implicated MIP-2 in the hepatic injury following alcohol ingestion, but MIP-2 was not directly toxic to primary cultures of normal rat hepatocytes (*Bautista 1997*). These data presented in this chapter would suggest that the CXC chemokines have the opposite effect on the liver following paracetamol poisoning. The increased mortality rate in the anti-MIP-2 antibody treated group compared with controls suggests that MIP-2, expressed during paracetamol induced hepatic damage, may play a protective role. This clearly needs to be further studied both in vivo, assessing the effect of neutralising MIP-2 on hepatic injury, and in vitro using cultured primary murine hepatocytes.

The potential role of chemokines as mediators of hepatic regeneration have not previously been reported. In a rather unfocused study, 70% partial hepatectomy was noted to induce the hepatic expression of IP-10, KC and chemokine genes, as well as 67 other genes (*Haber et al 1993*). Partial hepatectomy also increases the hepatic expression of Gro/CINC in rats, and this has been implicated in the neutrophilic infiltrate that occurs in the liver remnant post-resection (*Ohtsuka et al 1997*). Although there is still some confusion in the literature, human Gro α is homologus with rat CINC1 and murine KC. Murine MIP-2 is most similar to rat CINC 2 and CINC 3 and human Gro β and Gro γ. In humans Gro α, β and γ bind to the same receptor, CXCR2 which can also bind all the other ELR positive CXC chemokines (*Ahuja & Murphy 1996*). In mice the Gro homologues, KC and MIP-2, also bind the same IL-8 like receptor. All members of the Gro family can act as neutrophil chemoattractants and can also induce a respiratory burst and Mac 1 expression in neutrophils (*Shibata et al 1995*). However, depending on the cell type studied Gro or its rodent homologues can induce cell proliferation. Gro is alternatively known as melanoma growth stimulatory activity because of its properties as an autocrine growth factor for human melanoma cells (*Norgauer et al 1996*), Gro induces DNA synthesis in human melanoma cells. Gro is expressed following injury to the skin (*Nanney et al 1995, Tsuruta et al 1996*) and plays a role in healing via induction of keratinocyte proliferation (*Rennekampff et al 1997*). MIP-2/CINC and KC/CINC1 induce proliferation of cultured rat alveolar epithelial cells and it has been suggested that the proliferative effects of IL1 are induced via Gro production (*Rangnekar et al 1991*). In contrast, MIP-2 may inhibit the proliferation of myeloid progenitors (*Broxmeyer et al 1996*) and Gro α and β can inhibit endothelial cell proliferation and angiogenesis (*Cao et al 1995*). Proliferation can be induced by MIP-2 and ENA-78 in rat primary hepatocyte cultures and this effect can be inhibited by the non-ELR CXC chemokines, IP-10 and MIG (L Colletti, personal communication). Therefore the enhanced regeneration induced by the MIP-2 expressing adenovirus may be mediated via the induction of hepatocyte cell division.

In summary, hepatotoxic doses of paracetamol were found to induce the production of the CXC chemokine, MIP-2. Inhibition of another CXC chemokine ENA-78 was associated with delay in liver regeneration following paracetamol induced hepatic necrosis and augmenting MIP-2 expression accelerated regeneration in this model. It is tempting to speculate therefore, that adenovirus transfection of chemokine genes may be a novel therapy to augment liver regeneration following toxic liver injury.

CHAPTER 6

STEM CELL FACTOR AND TOXIC LIVER INJURY.

CHAPTER 6

STEM CELL FACTOR AND TOXIC LIVER INJURY.

6.1. INTRODUCTION AND AIMS.

Hepatic regeneration following paracetamol poisoning is likely to involve a number of cytokines and growth factors. In the previous chapter MIP-2 was implicated as a cytokine with the potential to accelerate hepatic regeneration following paracetamol poisoning. Stem cell factor (SCF) is best known as a haemopoetic growth factor which induces leukocyte maturation and differentiation (*Zsebo et al 1990*). However, recent evidence would suggest that SCF may serve an important role in tissue repair and protection from injury in certain disease states. SCF is a growth factor for dermal melanocytes and is essential for mast cell survival, proliferation and maturation (*Tsai et al 1991, Mitsui et al 1993*). In addition, SCF and its receptor, c-kit, have been shown to be expressed on numerous tumour cells, including neuroblastomas, small cell lung adenocarcinoma, and hepatoblastomas (*Vonschweinitz et al 1995*). Finally, SCF has been shown to attenuate radiation-induced injury in several cell populations (*Leigh et al 1993*), indicating that it may have an protective role during cell injury. Previous studies have demonstrated SCF expression in the liver (*Rowell et al 1997, Omori et al 1997a*), the aims of these studies were therefore to

- Quantitate the expression on SCF in murine liver.
- Assess the effect of toxic liver injury on hepatic SCF expression.

- Determine the role of SCF in hepatic regeneration following paracetamol poisoning.

6.2. MATERIALS AND METHODS.

6.2.1. Animal model.

The model of paracetamol induced hepatic necrosis has been described previously in Chapter 4. To inhibit the effect of SCF *in vivo*, rabbit anti-murine SCF immune serum (0.5ml) was injected 1 hour prior to paracetamol injection and daily thereafter.

6.2.2. Stem cell factor ELISA.

Immunoreactive SCF was measured in serum and hepatic extracts using a specific murine SCF ELISA, which was similar to those described in previous chapters.

6.2.3. Immunohistochemistry.

Paraffin embedded sections were rehydrated in xylene and graded alcohol, treated with acetone for 2 minutes and 1:1 H_2O_2:methanol for 30 minutes at room temperature. After rinsing the slides were blocked with normal goats serum for 30 minutes at 37°C, then rabbit anti-murine SCF antibody or control antibody added (1:100 dilution) and the slides incubated for a further 30 minutes at 37°C. The slides were washed with PBS and overlaid for 30 minutes with biotinylated goat anti-rabbit IgG (Biogenex, CA, supersensitive reagent, 1:20). After further washing, the slides were incubated with streptavidin-peroxidase (Biogenex, 1:1000) in PBS for 30 minutes at 37°C and the colour developed with 3'-amino-9-ethylcarbazole (AEC) solution containing 0.3% hydrogen peroxide. The slides were counter-stained with Mayers haematoxylin and photographed.

6.2.4. Assessment of hepatic regeneration.

Hepatic repair was quantified as described in chapter 5.

6.2.5. Statistical analysis.

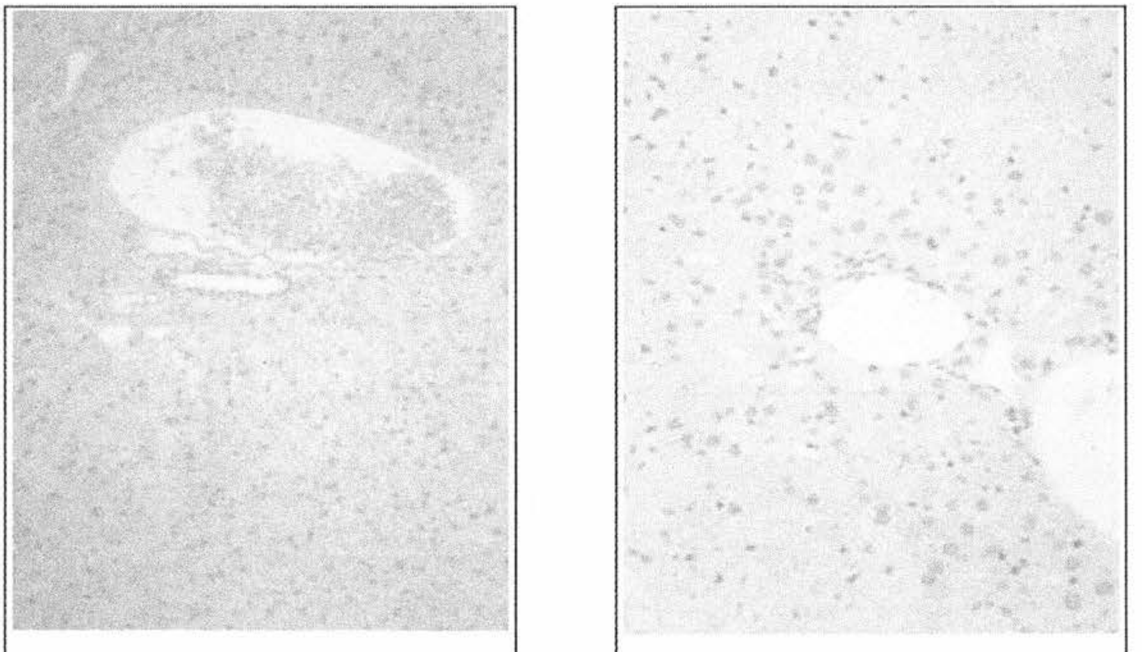
Results are presented as mean ± SEM. Statistical significance was determined by analysis of variance and students t-test as appropriate, with $p < 0.05$ considered significant.

6.3. RESULTS.

Very high concentrations of SCF were observed in control mouse liver (1000-2000 ng/gm wet weight). Immunohistochemical studies showed SCF staining in the biliary epithelial cells (Figure 6.1), although staining was also observed in hepatocyte cytoplasm and nuclei.

A significant reduction in hepatic immunoreactive SCF protein was noted at 12 and 24 hours following a non-necrotic dose (200 mg/kg) of paracetamol (Figure 6.2). This dose of paracetamol did not induce any histological damage, but serum AST was mildly elevated upto 2 times controls. This reduction in hepatic concentration of SCF was not associated with any alteration in serum SCF concentration (Figure 6.3). In contrast, hepatic SCF concentrations following 300 mg/kg paracetamol significantly increased at 1 hour following paracetamol injection. At this dose of paracetamol extensive hepatic damage is observed, but the increase in hepatic SCF concentrations in this model preceded any biochemical or histological evidence of liver necrosis (as presented in chapter 4). To further study the potential role of SCF in this model of paracetamol induced hepatic damage, anti-SCF antibodies were injected IP. before paracetamol poisoning and daily thereafter. A significant delay in hepatic repair was observed (Figure 6.5, 6.6), at 4 days post-paracetamol injection, in animals treated with anti-SCF antibodies (25.7 $\pm$ 8.5 % total area necrotic, mean $\pm$ SEM, n = 5) compared with controls (11.6 $\pm$ 5.3, n = 5).

6.3.1. Figures for Chapter 6.



Anti-SCF antibody

Control antibody

Figure 6.1. Immunohistochemistry of hepatic SCF expression (x 40). Cell were counterstained with haematoxylin.

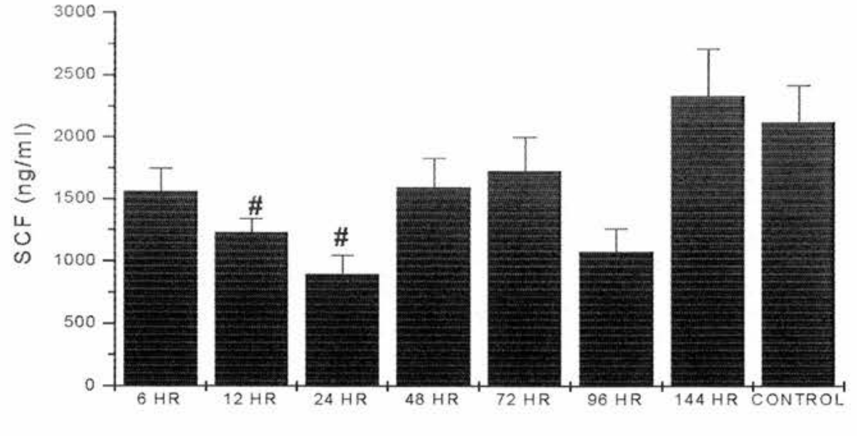


Figure 6.2. Hepatic SCF protein post-paracetamol 200 mg/kg. # = significantly different ($P < 0.05$) compared with controls.

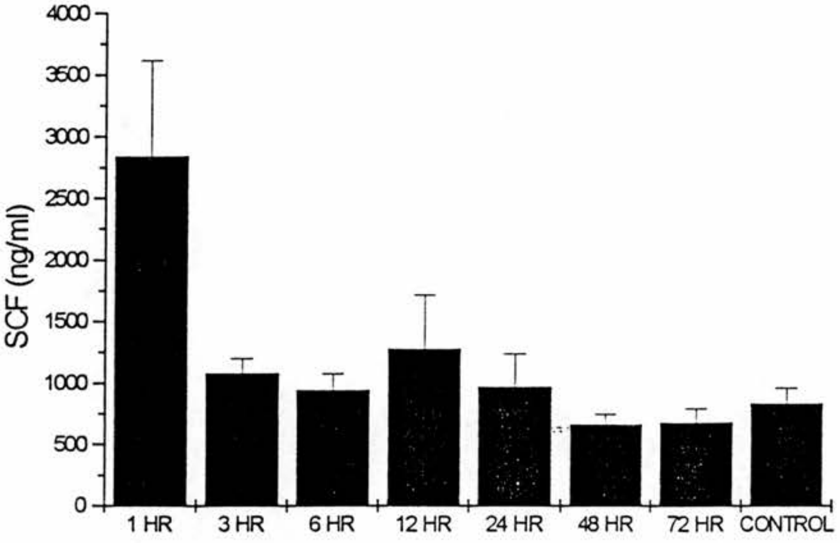


Figure 6.3. Hepatic SCF protein post-paracetamol 300 mg/kg. # = significantly different ($P < 0.05$) compared with controls.

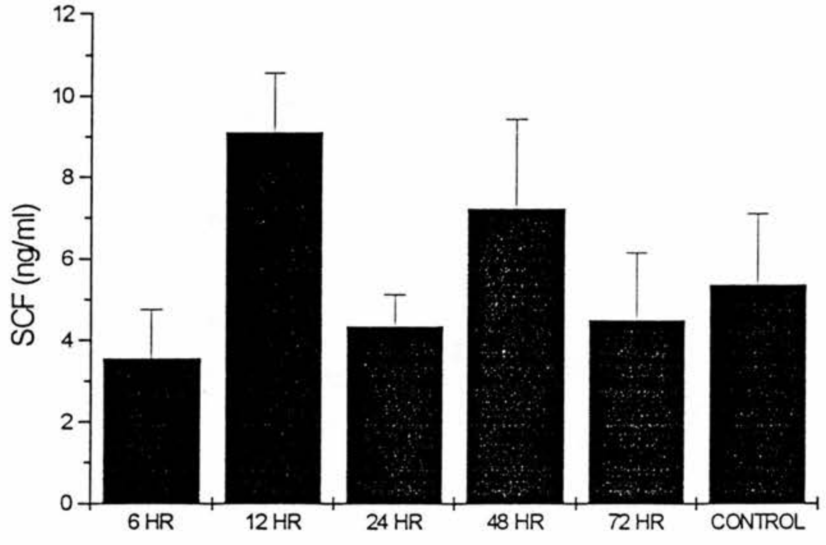


Figure 6.4. Serum SCF protein post-paracetamol 200 mg/kg. No significant differences were noted.

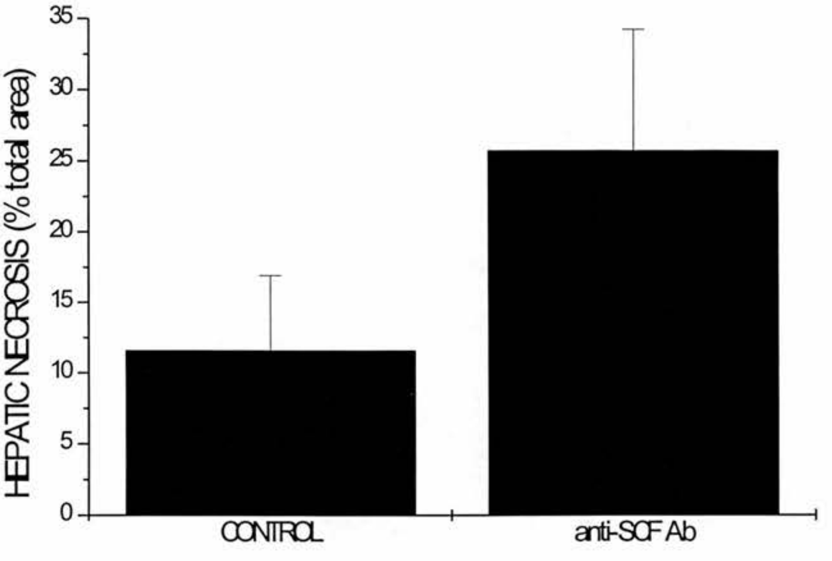


Figure 6.5. Hepatic necrotic area at 4 days post-paracetamol poisoning, effect of blocking SCF *in vivo*. # = significantly different ($P < 0.05$) compared with controls.

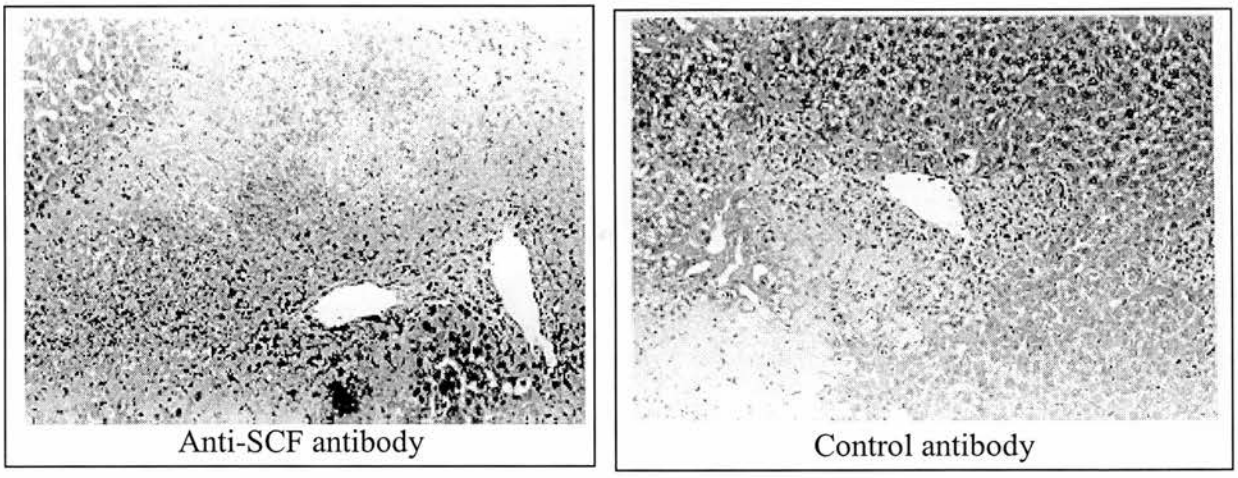


Figure 6.6. Hepatic histology, control antibody or anti-SCF antibody treated animals post-paracetamol (H&E staining, x 20).

6.4. DISCUSSION.

In this chapter high concentrations of hepatic SCF were detected, which increased early (1 hour) following hepatotoxic doses of paracetamol. In contrast, lower doses were associated with significant reduction in hepatic SCF concentrations 12 and 24 hours post-paracetamol poisoning. Hepatic SCF expression was noted in the biliary epithelial cells and hepatocytes, but SCF distribution was unaffected by paracetamol poisoning. Interestingly, *in vivo* inhibition of SCF was associated with significant delay in hepatic regeneration, 4 days following paracetamol injection.

These data regarding the role of SCF in the pathophysiology of liver damage following paracetamol poisoning may be interpreted in two ways. Firstly, SCF may protect the liver from paracetamol induced liver injury. SCF has been shown to attenuate radiation induced cellular damage in both hematopoietic and non-hematopoietic stem cell populations (*Leigh et al 1993, 1995*). Reactive oxygen intermediates have been implicated in cell injury induced by both radiation or paracetamol poisoning. In addition, SCF reduces apoptosis in mast cells and primordial germ cells from foetal gonads (*Pesce et al 1993*). Paracetamol can induce hepatocyte apoptosis both *in vivo* and *in vitro* (*DJ Harrison, personal communication*). However, the effect of SCF on paracetamol induced apoptosis or necrosis has not been studied to date .in this model.

SCF may have an alternative effect of augmenting hepatocyte proliferation and regeneration following toxic liver injury, but like the hypothesised effects on injury this was not studied directly in the experiments discussed in this chapter. SCF has a well recognised role in controlling cell proliferation and differentiation in hematopoietic cells, germ line cells and melanocytes. Observational studies have also implicated SCF in controlling hepatic regeneration from the oval cell compartment following partial hepatectomy and acetylaminofluorene administration (*Fujio et al 1994*). SCF has also been implicated in the development of differentiated hepatocytes in the pancreas of rats subjected to copper depletion and repletion and in the bile ductular proliferation induced in immature rats following bile duct ligation (*Rao et al 1996*). Therefore SCF may also play a permissive role in hepatocyte proliferation and differentiation following paracetamol poisoning. Hepatoblastomas are able to produce SCF (*Vonschweinitz et al 1995*). This may explain the extramedullary erythropoiesis that may occur within the liver in such patients, but the role of SCF in hepatoblastoma proliferation has not been studied. In addition, SCF can synergise with hepatocyte growth factor, a potent hepatocyte mitogen, in proliferation of bone marrow stem cells (*Galimi et al 1994*). SCF can also synergise with erythropoietin (*Jacobs Helber et al 1997*) and the flt-flt-3 tyrosine kinase signalling system, the latter has also been implicated in bile ductular proliferation following bile duct ligation (*Omori et al 1997b*). Therefore the interaction of SCF with these other cytokine systems in the paracetamol model would be worthy of further study.

In addition to the potential role of SCF in hepatic proliferation and repair, SCF may also be an important cytokine in the pathogenesis of liver fibrosis. Intrahepatic mast cells have been correlated with the degree of hepatic fibrosis in chronic liver disease (*Farrell et al 1995*). Systemic mastocytosis is associated with hepatic fibrosis, portal hypertension and death from bleeding oesophageal varices (*Mican et al 1995*). SCF is an essential factor for mast cell survival. Recent data has shown production of SCF from transformed stellate cells when exposed to mast cell conditioned media, possibly due to mast cell derived TNFα (*Benyon et al 1997, Brito & Borojevic 1997*)

These data presented in this chapter have implicated SCF in the pathogenesis of liver injury or repair following paracetamol poisoning. The mechanism underlying this effect is not clear at present, but it is tempting to speculate that this cytokine, in addition to MIP 2, may be clinically useful in treating toxic liver injury.

CHAPTER 7.

HEPATIC CHEMOKINE EXPRESSION IN PATHOLOGICAL STATES.

CHAPTER 7.

HEPATIC CHEMOKINE EXPRESSION IN PATHOLOGICAL STATES.

7.1. INTRODUCTION AND AIMS.

In previous chapters the role of chemokines and cytokines in the pathogenesis of liver disorders has been studied using *in vitro* cell culture and *in vivo* animal studies. When this work was initiated in 1995, there was little data regarding the hepatic expression of chemokines in the patients with liver disease, although this has changed more recently. Most inflammatory liver diseases are characterised by infiltration of the liver with lymphocytes, for example viral hepatitis and allograft rejection following liver transplantation. However, granulocytes, such as eosinophils in acute cellular rejection and neutrophils in alcoholic hepatitis, have also been implicated in the pathogenesis of hepatic disease.

The aim of these studies was to

- develop reproducible PCR assays for chemokine genes in human needle liver biopsies.
- to determine the expression of potential chemoattractant cytokines in patients with differing liver conditions.

7.2. MATERIALS AND METHODS.

7.2.1. Liver biopsy, RNA extraction and cDNA synthesis.

These studies were approved by the Royal Infirmary NHS Trust Medical Ethics Committee. After obtaining informed consent, a laparoscopic liver biopsy was performed using a Trucut needle or percutaneously using a Menghini needle. Unless the biopsy was being performed for suspected malignant disease (which was an exclusion criteria), the biopsy was halved, washed three times in ice-cold sterile saline, snap frozen in liquid nitrogen and stored at -70^{o}C.

Preliminary experiments lead to the following protocol for RNA isolation and cDNA synthesis. Liver tissue was thawed, weighed on a torsion balance and immersed in lysis buffer. The samples were homogenised and 1 x volume of 70% ethanol added. After mixing, the samples were applied to an RNeasy spin column (Quiagen), centrifuged at 10,000 rpm for 15 seconds. the columns were washed three times and the total RNA eluted from the column using DEPC treated water. Total RNA (1ug) was incubated with 1 unit DNase (GibcoBRL) in buffer (20 mmol/L Tris HCl, pH 8.3, 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$) for 15 minutes at room temperature. The RNA was then reverse transcribed into cDNA using AMV reverse transcriptase (15U) in buffer (10mmol/L Tris-HCl, pH 8.8, 50 mmol/L KCl, 5 mmol/L $MgCl_{2,}$ 1% Triton X-100, 20 units RNasin ribonuclease inhibitor, 1 mmol/L dTTP, 1 mmol/L dATP, 1 mmol/L dGTP, 1 mmol/L dCTP) using oligo$(dT)_{15}$ (0.5 ug) as a primer. The reverse transcriptase reaction was continued for 60 minutes at 42^{o}C and terminated by heating to 95^{o}C for 5 minutes.

7.2.2. Polymerase chain reaction.

Primers were designed for human MCP-1 (*Yoshimura et al 1989*), MCP-3 (*Minty et al 1993*), IL-8 (*Mukaida et al 1989*), MIP1α (*Nakao et al 1990*), MIP1β (*Napolitano et al 1991*), IP 10 (*Luster & Ravetch 1987*), RANTES (*Schall et al*

1988), ENA-78 (*Chang et al 1994*) and Gro α (*Haskill et al 1990*) using published sequence data. Where possible the primers were designed so that either the sense or anti-sense primer spanned an intron-exon boundary, thus avoiding amplification of genomic DNA. In addition, the primers were designed so that within the PCR product there was a restriction site, which would allow confirmation of the specificity of the product and allow insertion of further sequences for development of competitive templates and hence potential quantitation (Table 7.1).

The primers (0.5 umol/L) were added to buffer containing KCl (50 mmol/L), Tris HCl (10mmol/L), pH 9.0, 0.1% triton X-100, $MgCl_2$ (2 mmol/L), dNTP (0.2 mmol/L) and Taq DNA polymerase (2.5 units, Promega) was added after heating the reactions to 95^oC for 5 minutes ("hot-start" PCR). A Techne PHC-3 thermal cycler was used. The annealing, extension and heating temperatures and timings are shown in table 7.2. PCR reactions were performed in duplicate with 100% concordance. Preliminary experiments revealed that a positive PCR result was reproducible in over 90% of repeated cases.

7.2.3. Histological assessment.

The presence or absence of cirrhosis was determine by both macroscopic and microscopic assessment of liver tissue. The degree of lymphocytic, neutrophilic or eosinophilic infiltrate was assessed on H & E stained histological sections by two independent observers and graded on a three point scale as mild, moderate or severe.

7.2.4. Statistical analysis.

Data is presented as expression (% total number of samples in group). Chi squared tests were used to compare of chemokine gene expression between groups. A value of $p < 0.05$ was considered significant.

TABLE 7.1. Human chemokine PCR primers.

CHEMOKINE	SENSE PRIMER	ANTI-SENSE PRIMER	PRODUCT SIZE	SIZE OF GENOMIC SEQUENCE	RESTRICTION ENZYME
MCP-1	GAAGCTGTGATCTTCAAGAC	GTGGTTCAAGAGGAAAAGCA	361	0	Bgl II
MCP-3	GAAGCTGTGATCTTCAAGAC	GCACTGAGATGACATATAC	344	0	Bgl II
IL8	GACATACTCCAAACCTTTCC	TGCACCCAGTTTTCCT	108	410	Hind III
MIP1 beta	ACAGTGGTCAGTCCTTTC	ATTCTGTGGAATCTGCCG	200	888	Ava 1/Xho 1
MIP1 alpha	GTGACTGTCCTGTCTCTG	AAGAAAAGCAGCAGGCGGT	100	700	Eco 47 III
IP 10	GGCCATCAAGAATTTACTG	TCCATCCTTGGAAGCACT	100	620	Bgl II
RANTES	CAGCAGTCGTCTTTGTCACC	ATCTCGGCTCACTGCAAGCT	486	0	Kpn 1
ENA-78	TCTCTTGACCACTATGAGCC	TTCAGGGAGGCTACCACTT	264	0	Eco 47 III
Gro alpha	AACCGAAGTCATAGCCACAC	GGCATGTTGCAGGCTCCTCA	500	0	Hind III

Table 7.2. PCR conditions.

CHEMOKINE	MELTING	ANNEALING	EXTENSION
MCP-1	95°C for 45 seconds	57°C for 45 seconds	72°C for 60 seconds
MCP-3	95°C for 45 seconds	60°C for 45 seconds	72°C for 60 seconds
IL8	95°C for 45 seconds	57°C for 45 seconds	72°C for 60 seconds
MIP1 beta	95°C for 45 seconds	63°C for 45 seconds	72°C for 10 seconds
MIP1 alpha	95°C for 45 seconds	55°C for 45 seconds	72°C for 60 seconds
IP 10	95°C for 45 seconds	55°C for 45 seconds	72°C for 60 seconds
RANTES	95°C for 45 seconds	63°C for 30 seconds	72°C for 30 seconds
ENA-78	95°C for 60 seconds	55°C for 60 seconds	72°C for 120 seconds
Gro alpha	95°C for 60 seconds	55°C for 60 seconds	72°C for 120 seconds

7.3. RESULTS.

7.3.1. Chemokine gene expression in patients with Hepatitis C infection.

Expression of the lymphocytic chemokines, IL-8, IP 10, MCP-1, MCP-3, RANTES, MIP1α and MIP1β were determined in patients with chronic hepatitis C viral infection (clinical details are shown in table 7.3). In addition, the hepatic expression of these chemokines in 6 patients at the end of interferon therapy (3 x 10^6 units, three times per week for 6 months) were studied, all of these patients had responded to interferon, but relapsed following discontinuing interferon therapy. Representative pictures of PCR results are shown in Figure 7.1. Similar hepatic expression of chemokine genes was observed in patients before and following interferon therapy (Figure 7.2). Compared with chemokine gene expression in "cut-down" liver tissue (a kind gift from Dr C Benyon, University of Southampton, UK), IP 10 (39% v 100%) and RANTES (14.3% v 100%) genes were expressed at significantly lower frequency and IL-8 at significantly increased frequency (65% v 12.5%) in patients with hepatitis C virus infection (Figure 7.2). Compared with another control group, patients with non-alcoholic fatty liver, patients with hepatitis C virus infection had significantly increased frequency of IL-8 expression (65% v 0%). There was no correlation with hepatic chemokine expression and the degree of lymphocytic infiltrate or presence of cirrhosis.

7.3.2. Hepatic chemokine gene expression in patients with alcoholic liver disease.

In view of the characteristic neutrophilic infiltrate found in patients with alcoholic hepatitis, the expression of the CXC chemokines, IL-8, IP 10, Gro α and ENA-78 was studied. The patients clinical details are presented in table 7.3. Representative pictures of PCR results of Gro α and ENA-78 are shown in Figure 7.3. Hepatic CXC chemokine expression was similar in patients with alcoholic liver disease compared with gene expression found in "cut-down" liver tissue (Figure 7.4).

Figure 7.3. Clinical details of patients studied.

	ALD (N = 21)	HCV (N = 24)	POST-TP (N = 22)
Male/Female	9/12	14/10	7/15
Cirrhotic (n)	15	5	N/A
Bilirubin (umol/L)	50 $\pm$ 16	12 $\pm$ 4	201 $\pm$ 39
ALT (IU/L)	42 $\pm$ 8	88 $\pm$ 13	192 $\pm$ 35
Alkaline phosphatase (IU/L)	148 $\pm$ 27	88 $\pm$ 12	392 $\pm$ 66
GGTP (IU/L)	253 $\pm$ 71	66 $\pm$ 14	346 $\pm$ 46
Albumin (g/L)	37 $\pm$ 3	40 $\pm$ 1	31 $\pm$ 1

Results are mean $\pm$ SEM. ALD = alcoholic liver disease, HCV = Hepatitis C infection, Post-TP = post liver transplantation. ALT = alanine aminotransferase. GGTP = gamma glutamyl transferase.

However, IL-8 expression was significantly more common in patients with alcoholic liver disease (52.4%) compared with patients with non-alcoholic fatty liver (0%). There was no correlation with hepatic chemokine expression and the degree of neutrophilic infiltrate or presence of cirrhosis.

7.3.3. Chemokine gene expression in liver tissue from patients following liver transplantation.

Expression of the eosinophilic chemokines, MCP-3 and RANTES, was determined in patients routine day 7 liver biopsies. Although more than 50% of patients in this group expressed MCP-3, this difference was not significantly different compared with the two control groups studied (Figure 7.5). RANTES expression was significantly less frequent in the post-transplant patients (52.2%) compared with RANTES expression in "cut-down" liver tissue (100%). There was no correlation with hepatic chemokine expression and the degree of eosinophilic infiltrate or the histological degree of acute cellular rejection.

7.3.4. Comparison of hepatic chemokine expression in different disease states.

Hepatic chemokine expression was compared in the three disease groups studied. IP 10 expression was significantly reduced in patients with hepatitis C viral infection (39.1%) compared with patients with alcoholic liver disease (85.7%). In addition, the frequency of RANTES (14.3%) and MCP-3 (0%) expression in patients with hepatitis C viral infection was significantly reduced compared with patients following liver transplantation (RANTES, 52.5%, MCP-3, 56.5%).

7.3.6. Figures for Chapter 7.

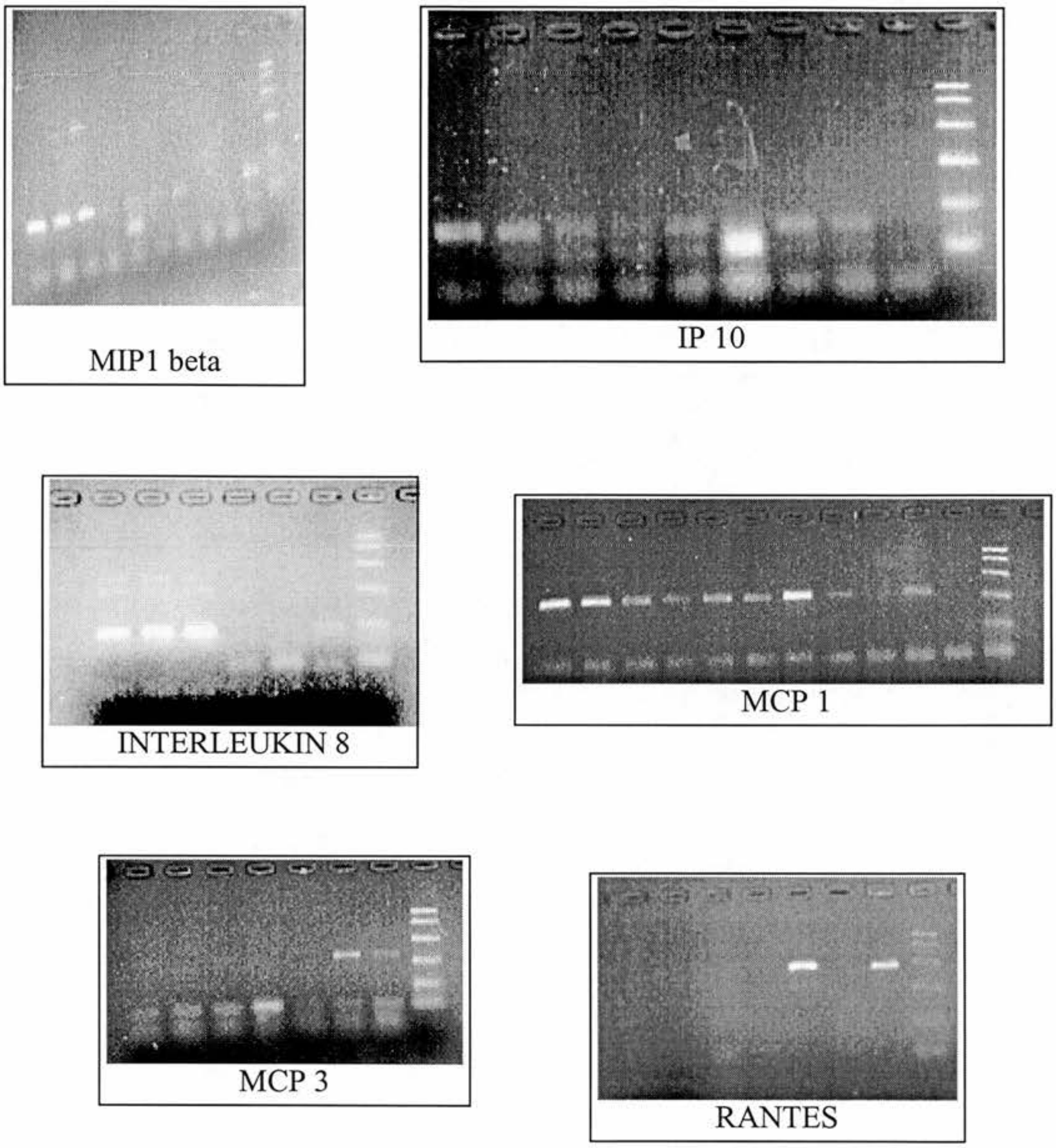


Figure 7.1. PCR results of chemokine genes in human liver tissue. Examples of results of MIP1 alpha PCR are not shown, but a single band was obtained.

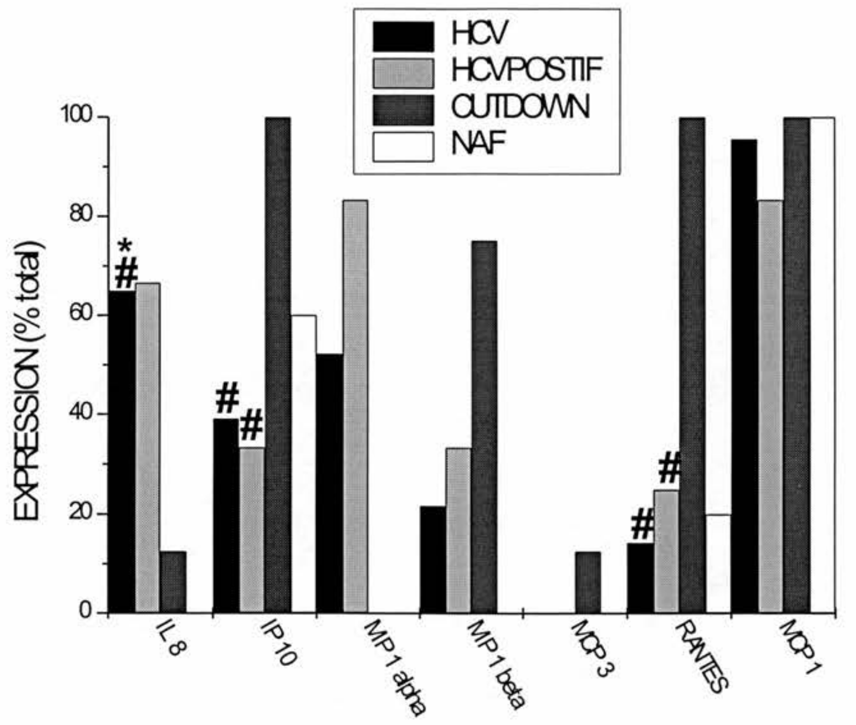


Figure 7.2. Chemokine gene expression in controls (cutdowns, NAF = non-alcoholic fatty liver) and patients with hepatitis C infection (HCV, HCVPOSTIF = following interferon alpha). # = significantly different from cutdown livers and * = significantly different from NAF.

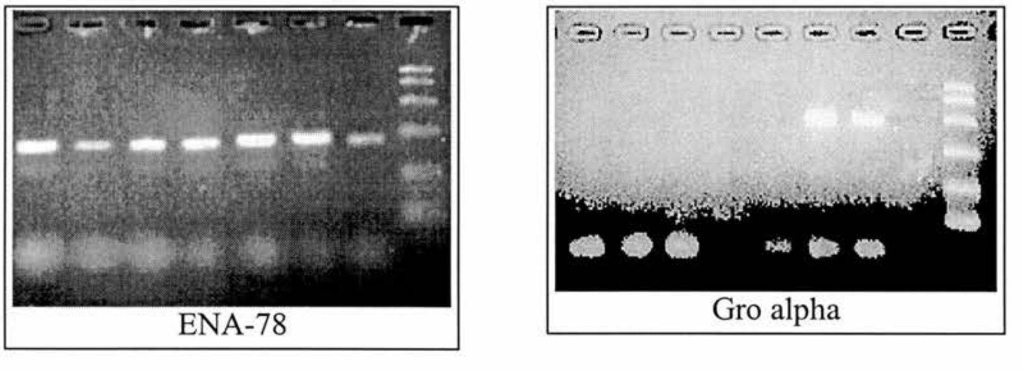


Figure 7.3. ENA-78 and Gro expression in human liver tissue

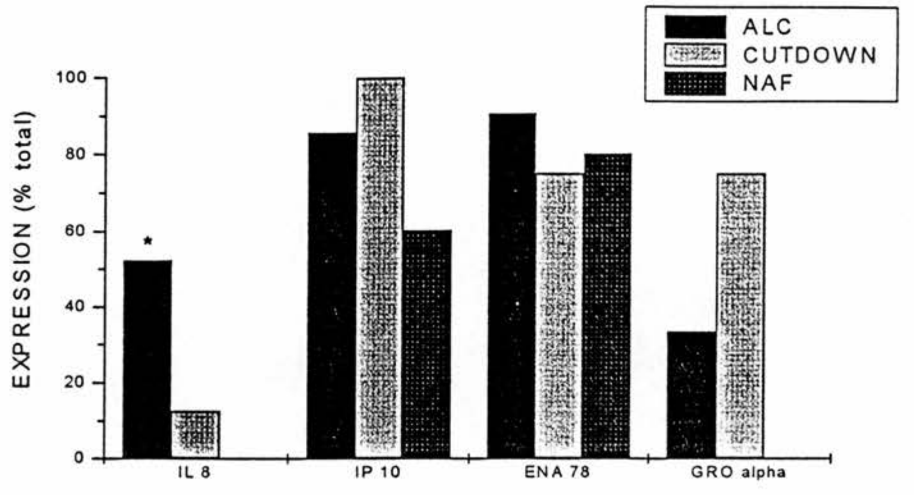


Figure 7.4. Chemokine gene expression in controls (cutdowns, NAF = non-alcoholic fatty liver) and patients with alcoholic liver disease. # = significantly different from cutdown livers and * = significantly different from NAF.

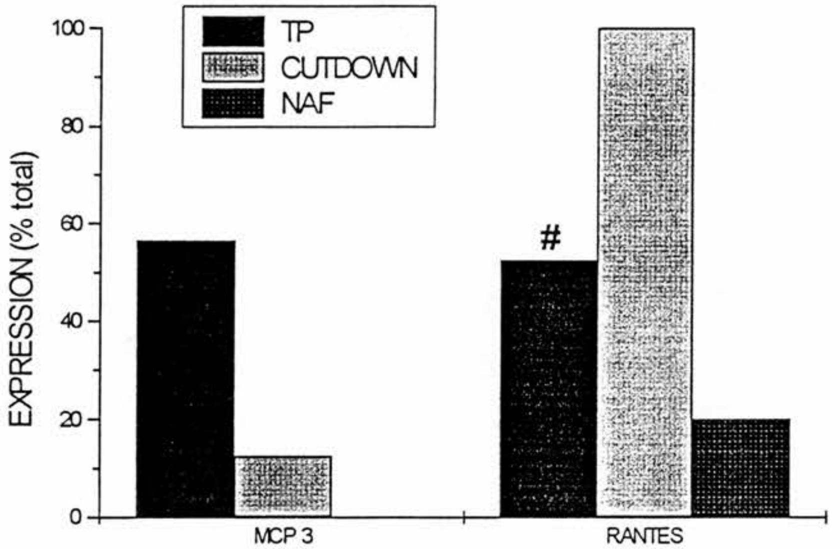


Figure 7.5. Chemokine gene expression in controls (cutdowns, NAF = non-alcoholic fatty liver) and patients after liver transplantation (TP). # = significantly different from cutdown livers and * = significantly different from NAF.

7.4. DISCUSSION.

In this chapter the expression of chemokine genes was studied in liver biopsy tissue from patients with a variety of diseases. A variety of both CXC and CC chemokines were found to be expressed in the disease groups studied, hepatitis C infection, post liver transplant and alcoholic liver disease, and also the controls, "cut-down" liver tissue and non-alcoholic fatty liver. Increased IL-8 expression was noted in both the hepatitis C virus infected patients and those with alcoholic liver disease compared with the control groups. The expression of chemokine genes in hepatitis C infected individuals was found to be unaffected by interferon alpha therapy. Although successful interferon therapy down regulates the hepatic expression of the adhesion molecule, ICAM-1, the continued expression of lymphocytic chemokines may explain the rapid relapse in many patients, once interferon therapy is withdrawn. A reduced frequency of IP 10 expression was also noted in patents with hepatitis C infection compared with controls and other disease groups, suggesting the intrahepatic production of interferon may be deficient in such patients, thus predisposing them to chronic viral infection. In addition, comparing hepatitis C viral infection, characterised by a lymphocytic infiltrate, and acute cellular rejection, in which a marked eosinophilia can be found, increased frequency of expression of the eosinophilic chemokines, MCP-3 and RANTES was noted in the latter. Others have noted increased expression of IL-5, MIP1α and MIP1β in liver tissue of patients with acute liver allograft rejection (*Martinez et al 1993, Adams et al 1996*).

Others have shown increased protein concentrations of the CXC chemokines, Gro and IL-8, in patients with alcoholic liver disease and have correlated the concentrations with the degree of neutrophilic hepatitis (*Sheron et al 1993, Maltby et al 1996*). These data presented in this chapter confirm the increased IL-8 expression and show that other CXC chemokines are also expressed in liver tissue from patients with alcoholic liver disease. IP 10 was frequently expressed in liver tissue in patients with alcoholic liver disease. This CXC chemokine lacks the ELR amino acid motif, essential for neutrophil attraction and activation, and can inhibit the effects on neutrophils of other ELR positive CXC chemokines (*Baggiolini et al 1997*). These data suggest that the balance between ELR positive and ELR negative CXC chemokines may be important in determining the extent of neutrophilic infiltrate in the liver in disease states.

Both MCP-3 and RANTES are potent eosinophilic chemoattractants and the lymphocytic chemokines studied in patients with hepatitis C virus infection have some specificity for different lymphocyte subsets (*Baggiolini et al 1997*). However no correlation was noted between chemokine gene expression and the degree of inflammatory cell infiltrate in any of the liver disease groups. These data do not exclude a role for these chemokines in the development of an inflammatory infiltrate in these conditions. The relative concentrations and site of production of the various chemokines and the expression of adhesion molecules on endothelial and other cell types are likely to be as important in the development of an inflammatory infiltrate. In addition, as discussed in previous chapters, chemokines have other biological roles, in cell proliferation, angiogenesis and tissue repair (*Baggiolini et al 1997*), and so their expression within the liver may have effects rather than chemoattraction.

In summary, these data presented in this chapter reveal diverse patterns of chemokine expression in the liver in various disease states. Further understanding of the pathological role of these chemokines in liver diseases will follow quantitation and localisation of their expression and the effect of therapy.

CHAPTER 8.

SUMMARY AND CONCLUSIONS.

Before starting the work on this thesis, little was known regarding the synthesis chemokines by liver cells and their potential role in the pathogenesis of liver diseases. Chapter 2 confirms and expands the published data (Thornton et al 1990, Rowell et al 1997). Human hepatoma cell lines, used in many studies to model the responses of hepatocytes, were found to express a number of CXC and CC chemokines following TNFα stimulation. Only protein concentrations in culture supernatant were measured, but most reports have shown increased protein expression is mediated via increased mRNA transcription. The stimulatory effects of TNFα on hepatoma cell chemokine expression were found to be mediated via TNFR1. There after the intracellular pathways inducing expression of the CXC chemokine, IL-8, diverged from the pathways inducing the expression of the CC chemokines, RANTES and MCP-1. Production of IL-8 involved activation of protein kinase C and was inhibited by staurosporine and calphostin C. In contrast, activation of protein kinase C and tyrosine kinase pathways were implicated in the expression of RANTES and MCP-1 induced by TNFα. These data suggest that if certain chemokines were found to play a primary role in the pathogenesis of a certain liver disorder, that the expression of this chemokine may be specifically inhibited by targeting the intracellular pathways involved in its expression.

Chapter 3 aimed to study adhesion mediated chemokine production in cells other than endothelial cells, such adhesion mediated chemokine production has not been reported previously. In contrast with endothelial cells, hepatoma cells do not require preactivation with proinflammatory cytokines for adhesion mediated chemokine production to occur. Chemokine production was induced in both the monocyte and hepatoma cell populations. IL-8 production was mediated via adhesion mediated production of TNFα and IL-1β, whereas the production of the CC chemokines MIP1β and MCP-1 were mediated via beta integrin mediated activation of intracellular reactive oxygen generation. Preliminary data in normal human hepatocytes suggests that small concentrations of IL-8 are produced during monocyte-hepatocyte adhesion, but no synthesis of CC chemokines occurs. Therefore during tumour development the hosts immune response may, via the synthesis of the angiogenic chemokine IL-8, augment tumour growth and metastatic potential. These data suggest that targeted inhibition of CXC chemokine production may be a novel therapeutic modality in anti-tumour therapies.

Chapters 4, 5 and 6 studied various aspects of a murine model of paracetamol poisoning. This model is analogous to the human situation, in that high serum concentrations of transaminases occur at doses of paracetamol that are hepatotoxic in humans. In addition, the liver damage is prevented by pretreatment with N-acetylcysteine, the antidote used in human poison cases. Despite the compelling data implicating Kupffer cells in the development of hepatocyte necrosis, inhibition of TNF α had no effect on the degree of liver damage. Similar data have been reported in models of carbon tetrachloride poisoning. Paracetamol poisoning induces the expression of CXC chemokines in the liver, inhibiting MIP-2 increased mortality and increasing hepatic MIP-2 expression with an adenovirus transfection vector accelerated hepatic regeneration. Although the effect of these treatments on hepatic inflammatory cell infiltrate were not studied, the published data suggests that the effects of the CXC chemokines are mediated via their action on cell proliferation and may allow development of novel therapies to augment hepatic regeneration following toxic liver injury. SCF was also induced and was implicated in either injury or repair following paracetamol poisoning. SCF plays a role in protecting cells against free radical mediated damage and if subsequent experiments demonstrate this effect also occurs in paracetamol poisoning, then SCF administration may also be helpful therapeutically.

The final experimental chapter described the expression of CXC and CC chemokines in liver tissue, as determined by PCR. Multiple chemokines were found to be expressed, although there was poor correlation with the inflammatory phenotype. It is likely that the localisation of expression is as important as the types of chemokines expressed in dictating the phenotype of an inflammatory infiltrate. In addition the expression and activation state of adhesion molecules is also important in movement of inflammatory cells into tissues. Perhaps the chemokines expressed have other actions such as a role in hepatocyte proliferation or death and is why there is poor correlation with the inflammatory phenotype.

In summary this thesis has reported data that expands the current knowledge regarding the production and role of chemokines in hepatic disease. In addition, potentially exciting therapeutic targets have been suggested that may revolutionise the treatment of liver disorders.

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