Increased tumour necrosis factor α production by neutrophils in patients with hepatitis B

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Abstract

Aims—To investigate the role of serum and neutrophil tumour necrosis factor α (TNF α) in patients with viral hepatitis

Methods—The activities of serum and neutrophil TNF α were measured using a bioassay of in vitro cytotoxicity against L929 cells in 57 patients with viral hepatitis and 20 healthy blood donors.

Results—Both serum and neutrophil TNF α in patients with chronic active hepatitis (CAH) and subacute fulminant hepatitis (SAFH) increased compared with those in normal controls ($p < 0.01$). No such differences were seen in patients with acute hepatitis. Serum and neutrophil TNF α were obviously reduced in patients with CAH and SAFH during convalescence compared with the active period ($p < 0.05$; $p < 0.01$). Furthermore, serum TNF α was significantly increased in patients with SAFH and complications compared with those without ($p < 0.01$), and in patients with SAFH who died compared with those who survived ($p < 0.01$). Neutrophil TNF α was significantly higher in patients with SAFH and secondary bacterial infections ($p < 0.05$).

Conclusions—Production of serum and neutrophil TNF α is increased in patients with CAH and SAFH, suggesting that neutrophil TNF α causes liver injury in these patients.

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Tumour necrosis factor α (TNF α) is a cytokine which mediates many important biological actions.^{1,2} Many experimental and clinical studies have shown that TNF α has a pivotal role in hepatocyte necrosis, including alcoholic hepatitis, viral hepatitis, and galactosamine liver damage.³⁻⁵ Dubravec *et al* recently found that peripheral blood neutrophils can also synthesise and secrete TNF α .⁶

Although there have been some reports on the importance of monocyte or macrophage derived TNF α in liver cell necrosis,^{3,7} the activity of neutrophil TNF α in patients with hepatitis and its role in the pathogenesis of liver injury in viral hepatitis are unknown. To evaluate the role of neutrophil TNF α in patients with viral hepatitis, we measured basal and lipopolysaccharide (LPS)-stimulated TNF α concentrations in peripheral blood neutrophils, a new source of TNF α production, using a bioassay.

Methods

Fifty seven inpatients (13 women, 44 men, 20-54 years old) with various clinical types of viral hepatitis were studied. All had hepatitis B (HBV) except nine subjects diagnosed with hepatitis A, all of whom had acute hepatitis. The serological profiles of patients with hepatitis B were HBsAg and HBeAg positive, or HBsAg and anti-HBc positive, or HBsAg, HBeAg, and anti-HBc positive. Serum HBV DNA was positive in 42 of 48 patients with HBV infection. The clinical types of patients were defined according to clinical signs and the results of liver function tests and liver histology.

Twenty healthy blood donors (11 women, nine men, 22-41 years old) with negative serum markers for hepatitis A, B, and C virus, and normal liver function tests were used as normal controls.

Peripheral blood neutrophils were isolated from anticoagulated venous blood by dextran sedimentation and Ficoll-Hypaque gradient and the residual erythrocytes were removed using hypotonic water lysis.⁶ Neutrophils (1×10^6 cells/ml) were suspended in RPMI-1640 medium containing 10% complement inactivated fetal calf serum, 20 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Neutrophil purity was 97% by Giemsa stain and the viability was 99% by trypan blue exclusion.

Neutrophils (1×10^6 /Well) were seeded into 24-well cell culture plates. Duplicate cultures were incubated either in the above medium or medium plus lipopolysaccharide (Sigma, final concentration 5 μ g/ml) at 37°C and a 5% CO₂ environment. After 24 hours culture supernatant fluids were collected and stored at -70°C for TNF α activity assay. Serum samples from patients and controls were obtained simultaneously and stored at -70°C.

Monocytes and macrophages were also isolated from normal controls by adherence of Ficoll-Hypaque-separated mononuclear cells to plastic Petri dishes for two hours at 37°C and stimulated in the same way except that they were cultured at a final concentration of

Table 1 Mean (SD) TNF α activities in normal controls (U/ml)

Age (years)	No of cases	Serum TNF α	Neutrophil TNF α
20	7	8.86 (3.98)	6.0 (3.06)
30	10	9.11 (3.02)	6.44 (2.19)
40	3	6.0 (2.0)	4.67 (1.15)
Total	20	8.50 (3.24)	5.90 (2.38)

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Table 2 Mean (SD) TNF α activities in patients

	No of cases	Mean age (years)	TNF α (U/ml)	
			Serum	Neutrophil
Acute hepatitis	15	33	8.27 (3.99)	5.60 (2.29)
CAH	20	38	17.20 (7.47)**	12.40 (6.82)**
SAFH	22	39	28.0 (18.02)**	17.36 (9.94)**

**p < 0.01 compared with controls.

Table 3 Correlation between serum and neutrophil TNF α

	No of cases	r =	p Value
Controls	20	0.5803	< 0.01
Acute hepatitis	15	0.7307	< 0.01
CAH	20	0.8826	< 0.01
SAFH	22	0.4468	< 0.05

2×10^5 cells per well. This population represents one fifth of the total number of neutrophils that were similarly stimulated.

The activities of serum and neutrophil TNF α were measured by bioassay of in vitro cytotoxicity against L929 cells.^{6,8} Briefly, L929 cells (5×10^5 /ml) were seeded into 55-well cell culture plates in 0.1 ml per well and incubated at 5% CO₂ at 37°C for 24 hours. Spent medium was removed and L929 cells were washed. Sera or supernatant fluids of serial two-fold dilutions were added to each well in 0.1 ml, and actinomycin D (final concentration 1 μ g/ml) was added to all wells at the same time. Control wells contained lipopolysaccharide and actinomycin D only. After 24 hours of incubation at 37°C in a 5% CO₂ atmosphere medium was removed and plates were stained with 0.2% crystal violet in 0.1 ml per well for 10 minutes. Excess stain was removed by washing in tap water. After drying, 1% sodium dodecyl sulphate was added to all wells, and absorbance determined at 570 nm using an enzyme linked immunosorbent assay (ELISA) reader (ME 891, China). Sample TNF α activities were calcu-

lated as the reciprocal of the dilution resulting in 50% cytotoxicity.

Data were expressed as mean (standard deviation) and analysed using Student's *t* test, the Mann-Whitney U test, and linear regression analysis.

Results

TNF α ACTIVITY OF NORMAL CONTROLS

The mean (SD) serum TNF α activities of normal men and women were 9.11 (4.14) U/ml and 8.0 (2.37) U/ml, respectively; the neutrophil TNF α was 6.67 (2.64) U/ml and 5.27 (2.05) U/ml, respectively. No significant differences were found between the two groups in serum or neutrophil TNF α (*p* > 0.05). There were also no differences between the different age groups for serum as well as neutrophil TNF α activities (table 1). Neutrophil TNF α concentrations were undetectable in culture supernatant fluids without lipopolysaccharide stimulation in 20 normal controls.

Secretion of TNF α by monocytes or macrophages was also detected. The results showed that a quantity of monocytes equal to a fifth of the neutrophils produced significantly less TNF α . It was confirmed that the TNF α determined in this study was produced by neutrophils.

PATIENTS' TNF α ACTIVITY

The activities of serum TNF α in patients with acute hepatitis A (*n* = 9) and with hepatitis B (*n* = 6) were 7.86 (3.62) U/ml and 8.63 (4.16) U/ml, respectively; neutrophil TNF α was 5.71 (2.78) U/ml and 5.59 (2.20) U/ml, respectively. There were no significant differences (*p* > 0.05) between the two groups of acute patients for serum and neutrophil TNF α activities. Both serum and neutrophil TNF α activities in patients with chronic active hepatitis and subacute fulminant hepatitis were increased compared with those found in normal controls. No such differences were found in patients with acute hepatitis (table 2). TNF α production of neutrophils without lipopolysaccharide stimulation was detectable in only three of 20 cases with CAH (4, 8, and 4 U/ml, respectively) and five of 22 cases with SAFH (6, 8, 8, 8, and 4 U/ml, respectively).

There was a significant correlation between serum and neutrophil TNF α activities (table 3).

Serum and neutrophil TNF α were obviously reduced in patients with CAH and SAFH during convalescence compared with the active period. However, serum TNF α in convalescing patients with SAFH was still much higher than that of normal controls (table 4).

Serum TNF α was significantly increased in patients with SAFH and complications, including secondary bacterial infections, upper gastrointestinal haemorrhage, hepatorenal syndrome and hepatic coma, compared with those free of these. This was also seen in patients with SAFH who died com-

Table 4 Serial observations of mean (SD) TNF α in patients

	TNF α (U/ml)			
	Serum	Neutrophil		
CAH <i>n</i> = 6				
At admission	20.0 (9.47)	16.67 (9.61)		
Convalescence	9.0 (4.69)	6.33 (3.32)	<i>p</i> < 0.01	<i>p</i> < 0.05
SAFH <i>n</i> = 8				
At admission	23.0 (11.66)	17.50 (13.34)		
Convalescence	13.50 (8.26)*	6.75 (3.37)	<i>p</i> < 0.05	<i>p</i> < 0.05

* *p* < 0.05 compared with controls.Table 5 Association between TNF α and clinical course in patients with SAFH

	TNF α (U/ml)			
	Serum	Neutrophil		
Secondary infections:				
Yes 11	39.27 (19.08)	22.36 (12.03)		
No 11	16.73 (6.15)	12.36 (2.80)	<i>p</i> < 0.01	<i>p</i> < 0.05
Complications*:				
Yes 8	43.0 (19.57)	20.75 (8.94)		
No 14	19.43 (10.03)	15.43 (10.27)	<i>p</i> < 0.01	<i>p</i> > 0.05
Prognosis:				
Died 8	43.0 (19.57)	20.75 (8.94)		
Survived 14	19.43 (10.03)	15.43 (10.27)	<i>p</i> < 0.01	<i>p</i> > 0.05

* Includes upper gastrointestinal haemorrhage, hepatorenal syndrome, and hepatic coma.

pared with those who survived. However, neutrophil TNF α was significantly higher in patients with SAFH and secondary bacterial infections as the only complication (table 5).

No correlations were found between the concentrations of serum or neutrophil TNF α and patients' weight, serum albumin, or numbers of peripheral blood neutrophils.

Discussion

The factors which cause liver cell necrosis in patients with hepatitis B are not fully understood, but clearly both the cellular and humoral immune responses of the hosts are important determinants.^{9,10} Some studies have shown that TNF α , as a factor mediating liver injury, has a pivotal role in the pathogenesis of hepatic necrosis in patients with hepatitis B.^{4,5,11} However, the importance of TNF α produced by neutrophils, and not monocytes or macrophages, has not been documented in such patients. We have shown that high activities of neutrophil TNF α as well as serum TNF α were present in patients with CAH or SAFH, and were closely associated with the severity of the patient's condition. Our results lend further support to the important role TNF α has in hepatocyte necrosis of hepatitis B. They also show that neutrophil TNF α is associated with liver injury. The concentrations of both serum and neutrophil TNF α were not increased in patients with acute hepatitis, including hepatitis A and B. This may be one of the explanations why severe hepatocyte necrosis occurs only in patients with CAH and SAFH and not in patients with acute hepatitis.

One of the most potent stimulators of TNF α release is endotoxin. Endotoxaemia occurs in patients with hepatitis, especially CAH and SAFH.¹² Furthermore, recent studies have shown that most of the effects of endotoxin are mediated through cytokines, such as interleukin 1 (IL-1) and TNF α .^{5,13} We propose, therefore, that endotoxaemia or secondary bacterial infections may be one of the causes for increased activity of neutrophil TNF α in patients with CAH and SAFH. Our results showed that both serum and neutrophil TNF α activities were much higher in patients with secondary bacterial infections than those without, further supporting the notion that increased TNF α activity was at least partly induced by endotoxin stimulation.

Neutrophils from three patients with CAH and five with SAFH produced some TNF α without lipopolysaccharide stimulation. No evidence of bacterial infections was apparent in three patients with CAH, but there was spontaneous bacterial peritonitis in five with SAFH. This might be explained by the possibility that the neutrophils were pre-activated in vivo, as a result of endotoxaemia. The bacterial contamination of samples should not be a factor because basal neutrophil TNF α was not found in normal controls or patients with concomitant acute hepatitis. The increase in serum TNF α activity differed from the level of

neutrophil TNF α activity in patients with complications and in those who subsequently died. One possible explanation is that serum TNF α activity was influenced by TNF α released from neutrophils in addition to that produced by monocytes or macrophages.

Despite numerous studies, the precise mechanisms for liver cell necrosis induced by TNF α remain obscure.¹⁴ Several potential factors could be implicated. (i) TNF α , as an antiviral agent, can quickly lyse and cause necrosis of hepatocytes infected with HBV. When HBV is eradicated, massive liver cell necrosis occurs.¹⁴ (ii) TNF α may promote the production of some oxygen free radicals,^{15,16} and it has been confirmed that oxygen free radicals can cause liver injury and necrosis.¹⁷ (iii) TNF α may stimulate release of other inflammatory cytokines, such as IL-1, IL-6, and IL-8, which can cause or aggravate liver damage.^{18,19}

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