Hepatic expression of tumour necrosis factor- α in chronic hepatitis B virus infection

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Abstract

Aim—To determine the hepatic expression of tumour necrosis factor- α (TNF α) in patients with chronic hepatitis B virus (HBV) infection.

Methods—Frozen liver biopsy sections from 19 patients with chronic HBV infection were studied, 12 of whom were HBeAg positive and 10 serum HBV DNA positive. Hepatic expression of TNF α was determined using immunohistochemistry.

Results—Only infiltrating mononuclear cells showed immunoreactive staining for TNF α (median 2, range 0–3; $n = 19$) which appeared as diffuse positive staining material in the cytoplasm. Patients with active liver disease, assessed histologically and biochemically, had a higher level of expression, both in the number of TNF α positive cells and the proportion of TNF α positive infiltrating mononuclear cells. There was no correlation between the expression of TNF α and serological parameters of viral infection (HBeAg and HBV DNA status and HBV DNA concentrations).

Conclusion—Hepatic expression of TNF α is increased in chronic HBV infection and is related to the activity of liver disease and not to the level of HBV replication.

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Immune recognition and clearance of hepatitis B virus (HBV) infected hepatocytes in chronic HBV infection is believed to result from the T lymphocyte response to viral antigens associated with human class I major histocompatibility complex molecules.¹⁻³ The observation that a successful response to interferon- α (IFN α) therapy is heralded by a hepatitis-like illness occurring eight to 12 weeks after the start of treatment further supports the role of the immune system in the virus–host interaction.^{2,4} HBV infection is also related to a variety of immunoregulatory defects including deficient IFN α and IFN γ production,^{5,6} resistance to IFNs,⁷ and dysfunction of peripheral blood monocytes associated with abnormal T cell activation.⁸

The production of tumour necrosis factor- α (TNF α) by peripheral blood mononuclear cells (PBMCs) has been shown to be increased in patients with chronic HBV infection.⁹ The expression of TNF α receptor on

PBMC was also increased despite raised circulating TNF α concentrations.¹⁰ In patients with chronic HBV infection who responded successfully to IFN α treatment there was a marked spontaneous production of TNF α by PBMCs during the e-seroconversion period which was not observed in non-responders.¹¹ Whether local production of TNF α in the liver tissue in chronic HBV infection is increased or not has yet to be established. The purpose of the present study was to determine the intrahepatic expression of TNF α in chronic HBV infection and correlate this with clinical parameters and HBV replication.

Methods

The study population comprised 19 patients seropositive for HBV surface antigen (HBsAg) for more than 12 months who presented to the Institute of Liver Studies, King's College Hospital, London. Histological diagnosis was based on internationally accepted criteria (table).¹² Of the patients, 10 were seropositive for HBV DNA and all were seronegative for antibody to hepatitis C virus (HCV), hepatitis D virus (HDV), and HIV. None of the patients had received antiviral or immunomodulatory treatment in the 12 months before entering the study. Blood was obtained on the biopsy day for serology.

Clinical, serological and histological data of the 19 patients studied

Characteristics	Data
Demographic data	
Number of patients	19
M:F ratio	18:1
Median age in years (range)	36 (19–61)
White:Asian:African	15:5:1
History of intravenous drug use	5
Homosexual practice	3
Biochemistry and serology	
Increased serum AST	12
HBsAg positive	19
Median HBsAg titre (range)	1:6400 (1:128–214,800)
HBeAg:anti-HBe positive	12:7
Serum HBV DNA positive	10
Median HBV DNA level (pg/40 μ l serum) (range)	180 (8–291)
HDV marker positive	0
HIV marker positive	0
HCV marker positive	0
Histology	
Non-specific reactive changes	2
Chronic persistent hepatitis	6
Chronic active hepatitis	11
Chronic active hepatitis (with cirrhosis)	2

AST = aspartate aminotransferase.

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Figure 1 Relation between intrahepatic expression of TNF α and liver disease activity. Active disease was defined as chronic active hepatitis (CAH) with or without cirrhosis. Inactive disease was defined as chronic persistent hepatitis (CPH) and non-specific reactive changes (NSRC).

Serum was tested for HBsAg, HBeAg, anti-HBe, and total antibody to HDV by radioimmunoassay (Abbott Diagnostics, Maidenhead, UK). The HBsAg titre was quantified by serial dilution using reverse passive haemagglutination (Wellcome Diagnostics, Dartford, UK). Antibodies to HCV and HIV were tested by enzyme immunoassay (Ortho Diagnostic Systems, Raritan, New Jersey, and Abbott Diagnostics, respectively) and serum HBV DNA was assayed using a quantitative dot blot technique.¹³

The specimens obtained by percutaneous liver biopsy were divided into two parts: one part was fixed in 10% formaldehyde and processed for routine histological studies, the other part was snap-frozen in liquid nitrogen cooled isopentane. Cryostat liver tissue sections (5 μ m) were then prepared on slides previously coated with 0.1% poly-L-lysine

(Sigma, Dorset, UK) to enhance attachment.¹⁴ The sections were dried overnight at room temperature, wrapped individually with aluminum foil, and stored at -20°C . After thawing on the day of the experiment, the sections were fixed in acetone for five minutes and extensively washed with phosphate buffered saline (PBS; pH 7.4).

After inactivating the endogenous peroxidase activity with methanol containing 0.3% hydrogen peroxide, the sections were covered with 5% normal rabbit serum (NRS; Dako, Bucks, UK) and 3% bovine serum albumin (BSA) in PBS for 15 minutes to block non-specific absorption. TNF α was then sought using mouse monoclonal antibodies to TNF α diluted 1 in 10 in PBS for one hour at room temperature (anti-TNF α , cell clone 357-27-2; kind gift of Dr A Meager, Mill Hill, UK). The concentration of monoclonal antibody used was 50 $\mu\text{g}/\text{ml}$. After washing with PBS, the sections were incubated with horseradish peroxidase conjugated rabbit anti-mouse antibody (Serotec, Oxford, UK), at a dilution of 1 in 50 in PBS, for one hour at room temperature. Diaminobenzidine (0.5 mg/ml) containing 0.01% hydrogen peroxide was used as the substrate (for 10 minutes) and the sections were counterstained with haematoxylin, dehydrated in graded alcohol solutions and xylene, and mounted in DPX. In all experiments normal mouse serum was used in the first antibody layer to serve as a control to verify the specificity of the reaction. Each slide was individually assessed by two authors (MJH and JYNL), and the average score was used for analysis.

Expression of TNF α in mononuclear cells was graded as follows: 0 = absent; 1 = occasional positive cells; 2 = positive cells in most portal tracts; and 3 = positive cells in nearly all portal tracts and in the lobular areas. The proportion of infiltrating mononuclear cells expressing TNF α was also counted in a blinded fashion for all biopsy specimens from all patients.

The Wilcoxon non-parametric rank sum test, the χ^2 test and linear regression analysis were used for data analysis.

Results

Hepatocytes, bile duct cells, sinusoidal cells, polymorphs, and fibroblasts did not show any immunoreactive staining for TNF α . Infiltrating mononuclear cells expressed TNF α most strongly (median 2, range 0–3; $n = 19$), with positively staining material present exclusively in the cytoplasm.

Patients with chronic active hepatitis had significantly higher number (median 3, range 2–3; $n = 11$) of TNF α expressing infiltrating mononuclear cells compared with patients with chronic persistent hepatitis and non-specific reactive changes ($n = 8$; median 1, range 0–1.2; $p < 0.01$; fig 1). The percentage of TNF α positive infiltrating mononuclear cells also increased significantly from a median of 0.02% (range 0–0.04%) in chronic persistent hepatitis and non-specific reactive changes to

Figure 2 Expression of TNF α in liver tissue. Only infiltrating mononuclear cells with large cytoplasm were positive for TNF α . Note that some of these TNF α positive cells are in the lobular area (magnification $\times 17$).

0.6% in chronic active liver disease (range 0.1–1.2%; $p < 0.01$). Positive cells were usually large with abundant cytoplasm and morphologically likely to be part of the monocyte/macrophage lineage (fig 2).

Patients with raised serum aspartate aminotransferase (AST) activities also showed increased expression of TNF α (median 2, range 1–3; $n = 12$) compared with patients with normal serum AST activities (median 1, range 0–1; $p < 0.01$). There was no correlation between the levels of TNF α expression and serological markers of viral infection.

Discussion

The present study shows that intrahepatic expression of TNF α is increased in patients with chronic HBV infection and active liver disease compared with those with inactive liver disease. Our data are consistent with a previous study which also showed an increase in the expression of TNF α within the infiltrating mononuclear cells of patients with chronic HBV infection.¹⁵ In contrast to that study we observed TNF α expression in infiltrating mononuclear cells and not in hepatocytes. The increase in TNF α expression was not simply related to a higher number of inflammatory cells in patients with chronic HBV infection as the proportion of mononuclear cells expressing TNF α was higher in patients with active than in those with inactive liver disease.

The production of TNF α by PBMCs as well as the expression of TNF α receptor on PBMCs are increased in patients with chronic HBV infection.^{9–10} The demonstration of increased intrahepatic expression of TNF α suggests that the TNF α system is also active locally in the liver in these patients. Recently, it has been shown that hepatocytic expression of TNF α receptor is also increased in patients with chronic active liver disease, irrespective of the aetiological trigger.¹⁶ Taken together, these data suggest that TNF α may play a role in host–HBV interaction.

There are at least three possible ways in which TNF α could affect host–HBV interaction. Firstly, TNF α may have a direct stimulatory or inhibitory effect on HBV. TNF α has been shown to have an antiviral effect on a wide range of DNA viruses,^{17–18} and to enhance HIV replication.^{19–20} In a pilot study on the use of TNF α to treat chronic HBV patients TNF α increased the serum levels of HBsAg and HBV DNA.²¹ Secondly, TNF α may affect immune recognition and clearance of HBV infected hepatocytes. TNF α has been shown to enhance HLA class I expression, which is likely to be important in the immune recognition of HBV infected cells.^{22–23} Interferon- α , a cytokine known to be effective in clearing HBV replication in a proportion of patients with chronic HBV infection, is also effective in enhancing hepatocytic expression of HLA class I antigens.¹⁴ TNF α may also stimulate cytotoxic T lymphocytes, which are believed to be essential for the elimination of HBV infected

hepatocytes.^{24–25} Thirdly, cytokines such as IFN γ and TNF α have been found to destroy hepatocytes containing HBsAg, thereby exhibiting another mechanism by which HBV infected hepatocytes may be eliminated.^{26–27}

The trigger for enhanced expression/production of TNF α in patients with chronic HBV infection and active liver disease remains unknown. It is possible that the induction of TNF α production is secondary to other events in the cytokine cascade. Interferon- γ , a cytokine with a central role in inflammatory response, has been shown to induce the production of TNF α .²² Recently, we have shown that hepatic expression of TNF α is also increased in patients with acute alcoholic hepatitis (Lau *et al*, 1994, unpublished data). Enhanced expression of TNF α in another liver disease further supports the hypothesis that the trigger for the local production of TNF α is secondary to other inflammatory events and is unlikely to be specific to HBV infection.

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