Serum concentrations and peripheral secretion of the beta chemokines monocyte chemoattractant protein 1 and macrophage inflammatory protein 1α in alcoholic liver disease

N C Fisher, D A H Neil, A Williams, D H Adams

Abstract

Background—Alcoholic liver disease is associated with increased hepatic expression of monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α).

Aims—To determine whether concentrations of chemokines in the peripheral circulation reflect disease activity, and whether chemokine secretion is restricted to the liver or is part of a systemic inflammatory response in alcoholic liver disease.

Patients—Fifty one patients with alcoholic liver disease and 12 healthy controls.

Methods—Peripheral vein (and hepatic vein in patients undergoing transjugular liver biopsy) chemokine concentrations were measured by ELISA. Chemokine secretion and transcription in isolated peripheral mononuclear cells were assessed using ELISA and in situ hybridisation in patients with severe alcoholic hepatitis.

Results—Serum MCP-1 concentrations were higher in alcoholic hepatitis compared with cirrhosis or healthy controls. MIP-1α concentrations were below the assay sensitivity in most patients. Serum MCP-1 concentrations correlated significantly with serum aspartate aminotransferase and creatinine. In severe alcoholic hepatitis, MCP-1 concentrations were higher in hepatic compared with peripheral veins; in mild alcoholic hepatitis there was no difference. Mononuclear cell secretion of both MCP-1 and MIP-1α was higher in severe alcoholic hepatitis compared with healthy controls, and chemokine mRNA was identified in monocytes.

Conclusions—Serum MCP-1 concentrations are raised in alcoholic liver disease and reflect severity of hepatic inflammation. Monocyte secretion of both MCP-1 and MIP-1α is increased in severe alcoholic hepatitis. Both intrahepatic sources and peripheral mononuclear cells contribute to the raised serum MCP-1 concentrations.

Keywords: alcoholic liver disease; chemokines; monocyte chemoattractant protein 1; macrophage inflammatory protein 1α

Alcoholic hepatitis is characterised by an intense leucocytic infiltration of the liver combined with hepatocyte damage and a variable degree of fibrosis. While the key hepatotoxin underlying alcoholic liver disease (ALD) is self evident, the cascade of immunological events leading to inflammatory damage is complex and the variation in inflammatory response between individuals consuming similar amounts of alcohol is not understood. We and others have previously shown that the expression of chemokines, a subgroup of cytokines with chemoattractant activity, correlates with clinical severity and inflammatory activity within the liver in alcoholic and other inflammatory liver diseases. The principal role of chemokines is in the recruitment of leucocytes to sites of inflammation and they play a vital role in a variety of infective and inflammatory diseases.

Chemokines can be subdivided according to their structure into subgroups, of which the largest are the CXC, or alpha, and CC, or beta groups defined by the presence or absence respectively of an additional amino acid (“X”) between the first two cysteine residues in a conserved four cysteine motif. The alpha chemokines are further subdivided according to the presence or absence of a glutamine-leucine-arginine (ELR) amino acid sequence near the active terminal; those possessing this sequence are potent chemoattractants for neutrophils while those that do not are chemotactic for lymphocytes. Interleukin 8 (IL-8) possesses an ELR amino acid sequence and is the prototypic alpha chemokine, being exclusively chemotactic for neutrophils. The beta chemokines are exclusively chemotactic for mononuclear cells; the prototypes of this group are monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α), both of which are monocyte and lymphocyte chemoattractants.

Although the effects of chemokines are predominantly localised to sites of inflammation by binding to proteoglycans in the glyocalyx, they can also be detected in the circulation, and serum concentrations of the alpha chemokine IL-8 have been shown to correlate closely with clinical, histological, and laboratory indicators of severity in ALD. However, alcoholic hepatitis and active cirrhosis are also typically associated with a mononuclear...
cell infiltrate including monocytes and lymphocytes, and the hepatic expression of the chemokines MCP-1 and MIP-1α correlates with intensity of infiltration with these mononuclear cells in ALD. Serum concentrations of beta chemokines have not been studied in inflammatory liver diseases, although MCP-1 concentrations are elevated in human sepsis. For these reasons we studied circulating concentrations and peripheral secretion of MCP-1 and MIP-1α in ALD in order to determine: (a) whether concentrations of chemokines in the peripheral circulation reflect hepatic disease activity; and (b) whether chemokine secretion is restricted to the liver or is part of a systemic inflammatory response.

Table 1  Summary characteristics of patients with severe alcoholic hepatitis (AH), mild to moderate AH, and quiescent cirrhosis, as defined histologically

<table>
<thead>
<tr>
<th></th>
<th>Severe AH (n=24)</th>
<th>Mild/moderate AH (n=15)</th>
<th>Cirrhosis (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/l)</td>
<td>64</td>
<td>50</td>
<td>36</td>
</tr>
<tr>
<td>Range</td>
<td>20-405</td>
<td>20-113</td>
<td>13-61</td>
</tr>
<tr>
<td>Bilirubin (µmol/l)</td>
<td>232</td>
<td>100</td>
<td>54</td>
</tr>
<tr>
<td>Median</td>
<td>45-1050</td>
<td>14-313</td>
<td>11-550</td>
</tr>
<tr>
<td>PT (s)</td>
<td>23</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>Discriminant function†</td>
<td>65.2</td>
<td>40.5</td>
<td>28.4</td>
</tr>
<tr>
<td>Median</td>
<td>29.6-151</td>
<td>5.6-138</td>
<td>9.8-111</td>
</tr>
<tr>
<td>PSE</td>
<td>13</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Transplanted‡</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Deaths‡</td>
<td>10</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

*After Maddrey,15 where discriminant function = [(prolongation in PT × 4.6) + (serum bilirubin/17)]. A discriminant function of >32 is indicative of a risk of mortality of at least 50%.
†Severe AH versus mild or moderate AH, p<0.05; severe AH versus quiescent cirrhosis, p<0.05; mild to moderate AH versus quiescent cirrhosis, p<0.05 (Mann-Whitney U test).
‡Occurring within three months after serum sampling. All deaths were directly related to liver disease.
AST, aspartate aminotransferase; PT, prothrombin time; PSE, portosystemic encephalopathy (defined where clinically obvious).

SERUM SAMPLING
Serum samples obtained from peripheral venous blood were analysed for all subjects. Some of the patients with coagulopathy underwent transjugular biopsy for clinical indications and in these patients we took the opportunity to sample blood from the hepatic vein. In a further five patients, transjugular intrahepatic portosystemic shunts (TIPSS) were inserted radiologically for control of ascites or because of previous problematic variceal haemorrhage; in these patients we took blood samples from peripheral, hepatic, and portal veins. Blood was collected into tubes containing clot activator; serum was separated within 30 minutes by centrifugation and stored at −70°C prior to analysis.

PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) CHEMOKINE SECRETION
MCP-1 and MIP-1α secretion from cultured PBMCs was measured in selected patients with severe alcoholic hepatitis (n=12) and healthy controls (n=12). PBMCs were isolated from citrated venous blood by Ficoll/Hypaque density gradient centrifugation for 30 minutes at 1600 rpm; cells were then washed twice in Hank's balanced salt solution, counted by haemocytometry, and cultured in 1 ml aliquots at a concentration of 10⁶ cells/ml. Cells were cultured for 24 hours at 37°C at 10⁶ cells/ml in RPMI containing 10% fetal bovine serum, glutamine (2 mM/l), and antibiotics (penicillin 100 U/ml and streptomycin 100 µg/ml) without additional stimulation. Supernatants were collected by centrifugation and stored at −70°C prior to analysis.

CHEMOKINE MEASUREMENT
MCP-1 and MIP-1α concentrations were measured using commercially available ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer’s instructions. The lower limits of sensitivity for these assays were 31 and 47 pg/ml respectively. For measurement of PBMC secretion of MCP-1 we developed a customised ELISA. Ninety six microwell plates were coated with mouse monoclonal antihuman MCP-1 (500 ng/ml; R&D Systems, Oxon, UK) overnight at 4°C. Plates were then washed and non-specific binding blocked with 0.1% bovine serum albumin before adding the test sample for two hours at room temperature. Rabbit antihuman MCP-1 (2.5 µg/ml; Peprotech EC Ltd, London, UK) was added for one hour, followed by swine antirabbit antibody conjugated with horseradish peroxidase (1.3 µg/ml, Dako Ltd, Cambridge, UK) for a further hour, with washing between each step. Bound antibody was then detected using tetramethylene blue liquid substrate and colorimetric analysis. Reference samples consisted of serial dilutions of recombinant human MCP-1 (Peprotech EC Ltd, London, UK). This ELISA gave reproducible results with values within 10% of the commercial ELISA, with both intra-assay and interassay variability.
of less than 5% and with a lower limit of sensitivity of 250 pg/ml.

**ANALYSIS FOR PBMC CHEMOKINE mRNA**
Chemokine mRNA in peripheral blood leukocytes was determined by in situ hybridisation of cytoplasm preparations of PBMCs isolated as described above. Freshly isolated PBMCs were resuspended and mounted onto coated slides, fixed in 1% paraformaldehyde, and stored at −70°C prior to analysis. Chemokine mRNA hybridisation was done with a probe for MCP-1 using techniques described elsewhere. Briefly, 35S uridine triphosphate labelled antisense or sense cRNA probes were synthesised from a cDNA sequence specific for MCP-1, which was ligated into an Eco R1/Bam H1 restriction site of the plasmid vector Bluescript KS+/-.

**STATISTICAL ANALYSIS**
Tests for non-parametric data (Mann-Whitney U test, Wilcoxon rank sum, and multivariate linear regression) were done using SPSS statistical software. A p value of less than 0.05 was considered to indicate statistical significance. This study was approved by the Research Ethics Committee of the University Hospital Birmingham NHS Trust and patients gave informed consent before participation in the study.

**Results**

**SERUM CHEMOKINE CONCENTRATIONS**
Peripheral vein MCP-1 concentrations were higher in alcoholic hepatitis (severe, 640 (SD 285) pg/ml; mild, 435 (110) pg/ml) compared with mild to moderate hepatitis or cirrhosis.

**Table 2** Correlation of laboratory indexes of disease severity with serum monocyte chemoattractant protein (MCP-1) concentrations in patients with alcoholic liver disease, using Spearman’s rank test

<table>
<thead>
<tr>
<th>Laboratory index</th>
<th>r Value</th>
<th>p Value</th>
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<tbody>
<tr>
<td>Serum aspartate aminotransferase</td>
<td>+0.47</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Creatinine*</td>
<td>+0.36</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Bilirubin*</td>
<td>+0.35</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Albumin</td>
<td>−0.33</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Prothrombin time increase</td>
<td>+0.15</td>
<td>NS</td>
</tr>
<tr>
<td>Peripheral leucocyte count</td>
<td>+0.18</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Values transformed logarithmically for statistical analysis. Linear regression analysis identified serum aspartate aminotransferase (p<0.01) and creatinine (p<0.05) as significant independent variables.
detection of the assay (as for peripheral vein concentrations) in all patients with ALD.

**PBMC CHEMOKINE SECRETION**
Spontaneous secretion of MCP-1 and MIP-1α from cultured PBMCs was higher in patients with severe alcoholic hepatitis compared with healthy controls (fig 3). In alcoholic hepatitis MCP-1 secretion was 13.13 (11.34) ng/ml and for controls 5.27 (4.77) ng/ml (p=0.015, Mann-Whitney U test); MIP-1α secretion was 1.98 (1.78) ng/ml in alcoholic hepatitis and 0.84 (1.10) ng/ml in controls (p=0.04, Mann-Whitney U test). For patients with alcoholic hepatitis there was a significant positive correlation between PBMC secretion of and serum concentrations of MCP-1 ($r=0.68$, $p<0.05$, Spearman’s rank test), and between PBMC secretion of MCP-1 and serum AST ($r=0.60$, $p<0.05$). However, there was no significant correlation between PBMC secretion of MIP-1α with MCP-1 secretion or serum AST.

**IDENTIFICATION OF CHEMOKINE mRNA**
In situ hybridisation of cytospin preparations using the probe for MIP-1α mRNA showed positive staining (mainly localised to monocytes) in patients with alcoholic hepatitis (fig 4).

**Discussion**
Our results suggest that secretion of MCP-1 is associated with disease activity in ALD. These findings complement previous observations of chemokine upregulation in the liver in ALD and suggest a role for chemokines in recruiting mononuclear cells to the liver following alcohol induced toxic injury. Previous reports of circulating IL-8 in alcoholic hepatitis have highlighted the importance of neutrophils in the pathogenesis of alcoholic hepatitis, however, while a neutrophilic infiltrate is the characteristic hallmark of alcoholic hepatitis, the inflammatory infiltrate includes mononuclear cells which play a vital role in inflammation and fibrogenesis. Thus beta chemokines such as MCP-1 are likely to play a central role in mediating these processes in ALD. We were unable to show increased serum concentrations of the beta chemokine MIP-1α in ALD despite showing enhanced PBMC secretion; it is possible that circulating concentrations were increased but below the range detectable by the assay.

The significance of raised circulating concentrations of MCP-1 remains unclear and while this may simply reflect increased tissue synthesis of MCP-1, circulating MCP-1 may also have immunomodulatory effects, including increased expression of adhesion molecules on circulating monocytes and promotion of proinflammatory cytokine secretion, thus amplifying the inflammatory cascade. Furthermore, circulating IL-8 has been shown to enhance neutrophil sequestration into the liver and MCP-1 may play a similar role in enhancing hepatic uptake of mononuclear cells. However, the dynamic effects of a sustained increase in circulating chemokines are not clear and it is possible that persistent ligand occupancy of leucocyte chemokine receptors from circulating chemokines may inhibit their transendothelial migration.

Our finding of a positive correlation between MCP-1 concentrations and serum creatinine probably reflects in part the renal excretion of chemokines; by virtue of its relatively small protein size, some excretion of MCP-1 is likely to occur from the kidneys. Measurable amounts are present in the urine of most healthy subjects and following this study we found high concentrations (1.34 and 1.36 ng/ml respec-
tively) in the urine of two patients with alcoholic hepatitis (but without renal failure), corresponding to at least twice the upper limit of normal renal values. Renal failure is therefore likely to increase circulating MCP-1 concentrations by a reduction in urinary excretion; however, in addition renal failure itself may promote synthesis of MCP-1 and other cytokines, including from intrarenal sources. 20 21

Increased secretion of MCP-1 from circulating monocytes, and increased MCP-1 concentrations in hepatic compared with peripheral (and portal) veins, suggest that MCP-1 synthesis is increased both peripherally and within the liver, respectively, in severe alcoholic hepatitis. While we found hepatic vein concentrations to be only modestly higher than peripheral concentrations, this difference was nevertheless significant and is remarkable in that patients were sampled in a "steady state" in the absence of any hepatic insult other than the ongoing inflammatory process. Thus, the increase in hepatic vein concentrations suggests synthesis within the liver.

Our observations support a direct role for MCP-1 in disease pathogenesis, presumably by recruiting and retaining monocytes and activated lymphocytes within the liver in severe alcoholic hepatitis. The fact that circulating monocytes showed increased spontaneous secretion of MCP-1 in alcoholic hepatitis suggests that recruitment of these cells into the liver would further elevate local concentrations and act as an amplifying-signal to enhance liver inflammation. The circulating monocytes might have been activated within the hepatic circulation during transit through the hepatic sinusoids or directly by soluble circulating factors.

The stimulus for chemokine production in ALD is not known but is likely to involve several signals. Proinflammatory cytokines (such as tumour necrosis factor alpha (TNF-α) and IL-1) and lipopolysaccharide are potent inducers of MCP-1; all these factors have been activated within the hepatic circulation in patients with alcoholic hepatitis, 22 23 and portal vein concentrations in alcoholic patients. MCP-1 synthesis following withdrawal of alcohol may induce a "rebound" increase in cytokine levels of the neutrophil chemotaxin interleukin-8 are elevated in severe alcoholic hepatitis, and tissue levels correlate with histological disease. 24 25


We are grateful to Dr Simon Olliff for help in obtaining hepatic and portal venous blood samples from patients undergoing radiological procedures. We are also grateful to our physician colleagues for allowing us to study patients under their care and to Dr Clare Morland and Janine Fear for practical help. This work was supported in part by grants from the Sir Jules Thorn Trust and the Endowment Fund of the former United Birmingham Hospitals.

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Gut 1999 45: 416-420
doi: 10.1136/gut.45.3.416

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