The effects of prednisolone and interferons on serum macrophage colony stimulating factor concentrations in chronic hepatitis B

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Backgrounds/Aims: Serum concentrations of macrophage-colony stimulating factor (M-CSF) are increased in parallel with hepatic inflammation. The aim of this study was to assess the immunologic significance of elevated M-CSF in patients with chronic hepatitis B virus infection.

Methods: The subjects included 20 asymptomatic HBV carriers and 45 patients with chronic hepatitis B, including 8 undergoing prednisolone treatment, 10 experiencing an acute exacerbation, and 12 undergoing daily administration of interferons.

Results: Serum concentrations of M-CSF significantly decreased during prednisolone administration, but significantly increased following prednisolone withdrawal, similar to the increase during acute exacerbation. Changes in the lipopolysaccharide-stimulated production of interleukin-1-beta and tumor necrosis factor-alpha by peripheral whole blood, or of interferon-gamma by peripheral blood mononuclear cells showed a similar pattern. Serum concentrations of M-CSF did not correlate with the titers of HBV-DNA or HBV-DNA polymerase activity. However, serum M-CSF peaked preceding seroconversion to HBe antibody in three HBe antigen positive patients. Exogenous interferon-alpha, -beta, or -gamma induced significant elevation in serum M-CSF concentrations, irrespective of changes in the serum alanine aminotransferase levels.

Conclusions: Increased serum M-CSF is closely associated with increased serum interferons and/or proinflammatory cytokines produced by peripheral blood cells during hepatic inflammation in chronic hepatitis B. This may be a consequence of the altered cytokine cascade resulting from the host immune response against hepatitis B virus.

Key words: Hepatitis B; Interferons; Macrophage-colony stimulating factor; Proinflammatory cytokines.
as tumor necrosis factor-alpha (TNF-alpha), interferon-gamma (IFN-gamma), or granulocyte macrophage-colony stimulating factor (GM-CSF) (13–15).

In contrast to other growth factors for monocytes, such as interleukin-3 or GM-CSF, circulating concentrations of M-CSF are constantly detectable in the serum (16–19). We have shown previously that the serum concentration of M-CSF rises in parallel with the serum alanine aminotransferase level in viral hepatitis (19).

In the present study, to assess the immunologic significance of the increase in serum M-CSF concentrations in hepatic inflammation, we examined the effects of prednisolone (PSL), interferon-alpha, (IFN-alpha), interferon-beta (IFN-beta), and IFN-gamma on the serum M-CSF concentration in patients with chronic hepatitis B. We also investigated the production of proinflammatory cytokines, such as interleukin-1-beta (IL-1-beta) and TNF-alpha by peripheral whole blood, or the production of IFN-gamma by peripheral blood mononuclear cells (PBMCs). To clarify the correlation between the serum concentration of M-CSF and the status of hepatitis B virus (HBV) infection, the titers of HBV-DNA and HBV-DNA polymerase activity and the presence of hepatitis B e antigen (HBeAg)/hepatitis B e antibody (HBeAb) in the serum were also examined.

**Materials and Methods**

**Patients and sera**

Sixty-five symptomatic or asymptomatic hepatitis B surface antigen (HBsAg) carriers (37 male and 28 female), ranging from 9 to 63 years old, were entered into this study. Informed consent was obtained from each patient and this study was approved by the human research committee of the hospital. The clinical and laboratory characteristics of the patients are summarized in Table 1. The presence of HBsAg and the predominance of HBeAg/HBeAb were assessed by commercially available radioimmunoassay kits (Abbott Laboratories, N Chicago, IL, USA). The titer of serum HBV-DNA was quantitated using HBV-DNA probe assay (20) and HBV-DNA polymerase activity was measured by the method of Kaplan et al. (21). All the patients were negative by second-generation assay for anti-HCV antibody (Abbott). Patients with a history of significant disease except for chronic hepatitis B were excluded from this study. None of the patients received any significant treatment for at least 6 months prior to entering this study, except for water, electrolytes, and vitamins. The asymptomatic HBV carriers (HBV ASC) were defined as patients with HBsAg showing no abnormal blood chemistries for at least a year. Liver biopsy was performed in patients showing abnormal liver tests except for five patients with severe disease prior to entering this study. Histologic diagnoses were made according to the previously published criteria of Desmet et al. (22). Although liver biopsy was not performed in five patients with severe chronic hepatitis B, their high ALT levels (more than 500 IU/l) confirmed the diagnosis. In this study, we used the term “acute exacerbation” to mean the state of hepatitis showing serum ALT levels more than 400 IU/l (more than 10-fold as compared with the upper limit of normal range).

Whole blood was drawn with a sterile syringe, transferred to a centrifuge tube, allowed to clot for 30 min at room temperature and centrifuged at 1500 g for 15 min at 4°C. One portion of the serum was used for serologic, virologic, and biochemical examinations, and 1 ml aliquots were stored in microtubes at 20°C to be used in radioimmunoassay for M-CSF.

**Treatment with PSL and IFN**

Eight patients with HBeAg positive mild or moderate chronic hepatitis B received PSL. Oral administration of PSL was given at a daily dose of 40 mg for 3 weeks and then withdrawn, or 40 mg for the first week, 30 mg for the second week, 20 mg for the third week, then withdrawn. Phlebotomy was performed just prior to, 2 weeks after, and at the end of PSL administration, then 2 and 4 weeks following withdrawal of PSL. Twelve patients with HBeAg positive mild or moderate chronic hepatitis B received IFNs. Three million units of IFN-alpha (Sumitomo Pharm. Co., Osaka, Japan) were administered daily for 4 weeks intramuscularly in six patients. Three million units of IFN-beta (Mochida Pharm. Co., Tokyo, Japan) were administered daily for 4 weeks intravascularly in three patients. One million units of IFN-gamma (Otsuka Pharm. Co., Tokyo, Japan) was administered daily for 7 days intramuscularly in three patients prior to initiating IFN-alpha therapy for 4 weeks. Phlebotomy was performed just prior to, and 1, 3, 5, and 7 days after administration of IFNs.

**Production of proinflammatory cytokines by peripheral blood**

Peripheral blood was drawn into sterile syringes (Termo Inc., Tokyo, Japan) containing 20 U heparin/ml (Green Cross Inc., Tokyo, Japan) and used for the following experiments. The production of IL-1-beta and TNF-alpha was examined using the whole blood induction system as we have reported previously (23). In brief, 100 μl of whole blood was plated into
24-well dishes (Corning Glass Works, Corning, N.Y., USA) containing 1 ml of complete RPMI 1640 medium (Nissui Pharm. Co. Ltd., Tokyo, Japan) buffered with NaHCO₃, and supplemented with L-glutamine (Nissui Pharm.Co., Japan), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma Co., St. Louis, MO, USA) and 10 μg/ml of lipopolysaccharide (LPS from *E. coli* 026: B6, Difco Co., Detroit, MI, USA), then incubated for 24 h. The supernatants were aspirated and stored at -20°C until assay.

Because the production of IFN-gamma by peripheral whole blood was undetectable in our assay system (23), we measured the production of IFN-gamma by peripheral blood mononuclear cells (PBMCs) in the presence of the patients' sera. In brief, heparinized whole blood was diluted with phosphate buffered saline. The PBMCs then were prepared by gradient centrifugation using "Lymphoprep" (Nyegaard, Oslo, Norway). PBMCs thus obtained were resuspended in complete RPMI 1640 medium at a density of 1x10⁶ cells/ml containing 10 μg/ml of LPS. An aliquot of 1 ml supplemented with 100 μl of sera from the patients was incubated in 24-well culture dishes for 24 h. The supernatants then were aspirated and stored.

Radioimmunoassay and enzyme-linked immunosorbent assay

Radioimmunoassay of M-CSF was performed as reported previously (19). In brief, duplicate samples of the patients' sera were mixed with ¹²⁵I-labeled recombinant human (rh) M-CSF and rabbit antiserum against rhM-CSF, then incubated for 48 h at 37°C. The bound product was separated from free ¹²⁵I-labeled rhM-CSF by the addition of goat anti-rabbit IgG and 6% polyethylene glycol. The tubes were shaken and then centrifuged at 1000 g for 15 min at 4°C. The supernatants were aspirated and the precipitates were counted for 1 min in an automated gamma spectrometer. The detection limit for M-CSF was 0.1 ng/ml in this study. Intra- and inter-assay variations were less than 10%.

The concentrations of IL-1-beta, TNF-alpha, and IFN-gamma were measured by essentially the same method as previously reported (24). In brief, using a 96 well microplate (Nunc, Naperville, IL, USA) duplicate samples were incubated with anti-IL-1-beta, anti-IFN-gamma rabbit polyclonal antibodies, or anti-TNF-alpha monoclonal antibody overnight at 4°C. After blocking with 1% bovine serum albumin, either the serum or serial dilutions of rhIL-1-beta, rhTNF-alpha, and rhIFN-gamma standards were added and incubated at 4°C for 24 h. After washing 3 times, peroxidase-conjugated polyclonal rabbit anti-rhIL-1-beta, and anti-rhIFN-gamma antibodies (diluted to 1/1000), or monoclonal anti-rhTNF-alpha antibody (diluted to 1/4000) were added to the wells and incubated for 24 h at 37°C. After washing 3 times, 100 μl of enzyme substrate (1 μg/ml O-phenylenediamine on 0.1 M sodium citrate buffer) were added. Five minutes later, the reaction was stopped by adding 100 μl of H₂SO₄ to each well of the microplate. The absorbance at 492 nm was determined by an automatic microtiter reader (Multiscan; Labosystem, Finland). The sensitivity of detection was 20 pg/ml for each cytokine.

The concentration of N-terminal propeptide of type III procollagen (PIIIP) was determined by a commercially available radioimmunoassay kit (RIAgnostic PIIIP, Behringwerk AG, Marburg, Germany) and that of type IV collagen 7s (IVcol-7s) also was determined by a commercially available radioimmunoassay kit (Nippon DPC Corp., Tokyo, Japan) according to the manufacturer's instructions. These two parameters were used to ascertain the hepatic necro-inflammatory process by the degree of hepatic connective tissue turnover.

Statistical analysis

Data are expressed as medians and/or ranges. The nonparametric Mann-Whitney U test and two-sample t-test were used for the comparison of data between the two groups. Wilcoxon's matched pairs test was used to compare the two variable parameters. The correlation between two parameters were calculated by the linear regression test. A *p*-value <0.05 was considered to be significant.

Results

The clinical backgrounds of 65 patients with chronic hepatitis B who entered into this study are summarized in Table 1. The serum level of M-CSF in each group of patients is presented in Fig. 1. The median concentration of M-CSF in healthy volunteers was 1.90 ng/ml, with a range of 1.65 to 2.32 ng/ml, which was not significantly different from the M-CSF concentrations of HBeAg positive HBV ASC or HBeAb positive HBV ASC. The M-CSF concentrations in HBeAg positive HBV ASC (median 2.00 ng/ml, range 1.72-2.29 ng/ml) were not significantly different from the M-CSF concentrations in HBeAb positive HBV ASC patients [1.95 (1.75-2.56) ng/ml], although the titers of HBV-DNA and HBV-DNA polymerase activity were significantly higher in HBeAg positive HBV ASC (HBV-DNA; 6815
TABLE 1
Clinical profiles of patients with chronic hepatitis B

<table>
<thead>
<tr>
<th>Sex (F/M)</th>
<th>Controls</th>
<th>HBV asymptomatic carrier</th>
<th>Chronic hepatitis B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8/12</td>
<td>5/8</td>
<td>6/6</td>
</tr>
<tr>
<td>Age (years)</td>
<td>34 (25-44)</td>
<td>26 (13-39)</td>
<td>36 (28-60)</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>12 (8-14)</td>
<td>21 (12-32)</td>
<td>51 (25-73)</td>
</tr>
<tr>
<td>DNA (pg/ml)</td>
<td>N.D.</td>
<td>6815 (2130-9980)</td>
<td>784 (0-10000)</td>
</tr>
<tr>
<td>DNA-p (cpm)</td>
<td>N.D.</td>
<td>3227 (8-6670)</td>
<td>1574 (0-3884)</td>
</tr>
</tbody>
</table>

**Chronic hepatitis B**

- **Mild**
  - eAg(+) (12)
  - eAb(+) (5)
  - eAg(+) (13)
  - eAb(+) (5)

- **Severe**
  - eAg(+) (10)

**Sex**
- (F/M)

**Age**
- (years)

**ALT**
- (IU/l)

**DNA**
- (pg/ml)

**DNA-p**
- (cpm)

**M-CSF**

- (2130–9980) pg/ml versus 0(0-0) pg/ml, HBV-DNA polymerase activity 3227 (8-6670) cpm versus 0 (0–0) cpm, p<0.001]. Moreover, concentrations of M-CSF did not correlate with the titers of HBV-DNA (r=0.215, N=65, NS) or the HBV-DNA polymerase activity (r=0.189, N=65, NS). Levels of M-CSF increased in proportion to the degree of hepatic inflammation as judged by the grade of liver injury and the serum ALT level (r=0.754, N=65, p<0.001). In patients with HBV ASC, mild chronic hepatitis B, and moderate chronic hepatitis B, serum concentrations of M-CSF were not significantly different between the patients with HBeAg and those with HBeAb (Fig. 1).

The serum concentration of M-CSF was examined in ten patients during acute exacerbation. As shown in Fig. 2, serum concentrations of M-CSF significantly increased during acute exacerbation of hepatitis (serum levels of ALT more than 400 IU/ml), as compared with the concentrations in the relatively stable phase (levels of ALT less than 100 IU/ml) prior to and following the exacerbation [4.92 (3.96–6.93) ng/ml versus 2.46 (1.94–3.08) ng/ml, and 2.39 (1.99–3.02) ng/ml, p<0.001, respectively]. During PSL administration, serum concentrations of M-CSF significantly decreased in comparison with the concentrations prior to PSL administration [2.19 (1.79–2.72) ng/ml and 2.00 (1.65–2.20) ng/ml versus 2.74 (2.25–3.29) ng/ml, p<0.001] (Fig. 3A). Four weeks after PSL withdrawal, the peak serum concentrations of M-CSF coincided with the increase in the serum ALT levels. Serum concentrations of M-CSF in this phase, 4.13 (2.20–5.44) ng/ml, were comparable to the concentrations during acute exacerbation [4.92 (3.96–6.93) ng/ml], but significantly higher than the concentrations prior to [2.74 (2.25–3.19) ng/ml] and dur-

![Fig. 1. Serum concentrations of M-CSF in different groups of patients and controls.](image-url)

- C: healthy controls.
- I: HBeAg+ve asymptomatic carriers.
- II: HBeAb+ve asymptomatic carriers.
- III: HBeAg+ve mild chronic hepatitis.
- IV: HBeAg+ve moderate chronic hepatitis.
- V: HBeAg+ve severe chronic hepatitis.

Data are expressed as ranges.

(a) is not significantly different from controls, (b) is significantly (p<0.05), and (C) is significantly (p<0.01) higher than controls.
Fig. 2. Time course analysis of the increase in serum M-CSF concentrations in 10 patients with acute exacerbation of chronic hepatitis B. Serum concentrations of M-CSF significantly (p<0.001) increased during the acute exacerbation (ALT levels more than 400 IU/l) as compared with the concentrations before and after the exacerbation (ALT levels less than 100 IU/l).

The LPS-stimulated production of proinflammatory cytokines, such as IL-1-beta and TNF-alpha by peripheral whole blood and that of IFN-gamma by PBMCs, was examined serially in eight patients who received PSL (Fig. 3B). The concentrations of these cytokines in the culture supernatants were expressed as the percent (%) increase/decrease in comparison with the values prior to PSL administration in each patient. During prednisolone administration, median values significantly decreased [IL-1-beta; 54 (33–72)% and 47 (24–63)%], TNF-alpha; 60 (22–81)% and 57 (36–75)%, IFN-gamma; 73 (35–110)% and 82 (56–105)%, p<0.01, respectively]. However, they significantly increased during the PSL withdrawal phase [IL-1-beta; 228 (158–281)% and 182 (117–266)%, TNF-alpha; 208 (122–274)% and 220 (120–316)%, IFN-gamma; 164 (89–192)% and 135 (92–184)%, p<0.01, respectively]. In a similar fashion, the serum levels of ALT, PIIP, and IVcol-7s decreased after starting PSL administration, but increased following PSL withdrawal (Fig. 3C).

In three patients, an exacerbation of hepatitis was followed by seroconversion to HBeAb. The time course of a representative patient who received PSL is shown in Fig. 4. Following an increase in the serum M-CSF concentration, or coinciding with a peak, the titer of HBV-DNA and HRV-DNA polymerase activity rapidly decreased to undetectable levels, followed by the appearance of HBeAb. Following seroconversion, concentrations of M-CSF declined in parallel with the normalization of ALT levels.

We examined the effects of IFNs (IFN-alpha, IFN-beta, and IFN-gamma) on the serum M-CSF concentration in vivo (Fig. 5). The serum concentration of M-CSF was significantly increased at the 3rd day of
Fig. 4. Time course study of serum M-CSF (●), ALT (○), HBV-DNA (▲), HBV-DNA polymerase activity (△), in a representative patient who experienced a seroconversion to HBeAb.

Fig. 5. The effects of in vivo administration of IFN-alpha, -beta, -gamma on the serum concentrations of M-CSF. IFN-alpha (3 MU), IFN-beta (3 MU), or IFN-gamma (1 MU) were administered daily for 7 days. Phlebotomy was performed on the 1st, 3rd, 5th, and 7th day following administration of IFNs. Values are expressed as ranges. Median values prior to IFN administration are expressed as 100%.

IFN administration with all forms of IFNs (p<0.01). Serum concentrations of M-CSF continued to be increased until the end of the 7th day. Although the doses and kinds of IFNs differed, no significant difference was noted in the concentration or the pattern of increase of serum M-CSF in our study. The increase in the concentration of serum M-CSF was independent of changes in serum ALT, HBV-DNA, and HBV-DNA polymerase activity (data not shown).

Discussion
In the present study, we have extended our previous finding that the serum concentration of M-CSF rises in parallel with hepatic inflammation (19) by classifying the patients with chronic HBV infection according to the grading of chronic hepatitis activity, and by serially examining patients with chronic hepatitis B during acute exacerbation and during PSL administration followed by withdrawal, prospectively. The administration of PSL suppressed the serum concentration of M-CSF with a decrease in the level of ALT, PIIP, and IVcol-7s. The production of IFN-gamma, IL-1-beta, and TNF-alpha by peripheral blood cells was also suppressed by administration of PSL. In contrast, the serum concentration of M-CSF significantly increased during the PSL withdrawal phase, coinciding with the increase in the levels of ALT, PIIP, and IVcol-7s, and with the enhanced production of IL-1-beta, TNF-alpha, and IFN-gamma by peripheral blood cells. In that phase, an increase in the serum concentration of M-CSF also coincided with a decrease in the level of HBV-DNA and HBVDNA polymerase activity.

These results suggest that the serum concentration of M-CSF is associated with the production of proinflammatory cytokines by peripheral blood cells and with the hepatic necroinflammatory process. A direct anti-viral effect of M-CSF on HBV is unlikely, because the concentrations of M-CSF were unrelated to the titers of HBV-DNA and HBV-DNA polymerase activity. Moreover, serum concentrations of M-CSF were similar between HbeAg positive ASC showing high titers of HBV-DNA or high HBV-DNA polymerase activity and HBeAb positive ASC having undetectable levels of HBV-DNA and HBV-DNA polymerase activity.

We speculate that the increased serum concentrations of M-CSF in hepatic inflammation may be induced by proinflammatory cytokines and/or interferons produced in vivo. Our finding that exogenous administration of IFNs enhanced the serum concentration of M-CSF in vivo, irrespective of changes in the levels of ALT, HBV-DNA, or HBV-DNA polymerase activity supports this hypothesis. In fact, it has been reported that the production of M-CSF is regulated by several proinflammatory cytokines in many kinds of cells. In monocytes, the production of M-CSF is enhanced by IL-1-beta, TNF-alpha, and IFN-gamma (25,26). Similarly, it is enhanced by IL-1-beta, TNF-alpha, IL-6, or IFN-gamma in bone marrow stroma (27) or fibroblasts (28), mesangial cells (29,30), or in astrocytes (31). In vivo, IL-1-alpha and TNF-alpha elevate the serum concentration of M-CSF in mice (32,33), and IFN-alpha also enhances the serum concentration of M-CSF in hepatitis C patients (34).
Although the mechanisms regulating M-CSF production in vivo are not well understood (10), we postulate that the serum concentration of M-CSF depends on the production of M-CSF in compartments such as the liver or bone marrow, and on circulating peripheral blood monocytes. In chronic hepatitis B patients with acute exacerbation, activated monocytes recruited to the inflamed liver may be one of the sources of increased M-CSF concentrations in the serum, considering our previous result that the number of liver infiltrating M-CSF positive mononuclear cells is increased in patients with acute hepatitis (19). Moreover, serum concentrations of M-CSF are correlated with the number of liver macrophages in patients with chronic hepatitis (unpublished data). However, because fat storing cells or hepatocytes also secrete M-CSF in vitro (35,36), production of M-CSF by these types of cells in vivo cannot be neglected. Further study of the expression of M-CSF mRNA in the liver tissue is necessary to solve this issue.

In studies using HBV transgenic mice, antigen-independent recruitment of macrophages or lymphocytes amplifies an inflammatory process initiated by antigen-specific cytotoxic T cells in the liver by inducing the production of proinflammatory cytokines (37). In acute exacerbation of chronic hepatitis B, increases have been reported in the mitogen stimulated production of IFN-gamma by PBMCs and in the hepatic mRNA expression of IFN-gamma (38,39). The production of TNF-alpha increases both in PBMCs and in liver infiltrating mononuclear cells in parallel with the hepatic inflammation of chronic hepatitis (40-42). We speculate that both in the peripheral blood cells and in the inflamed liver, increased production of proinflammatory cytokines such as TNF-alpha or IFN-gamma produced by lymphocytes and/or monocytes might induce the production of M-CSF by monocytes in a paracrine or an autocrine manner. We showed in this study that LPS-stimulated production of IFN-gamma by PBMCs, or of IL-1-beta and TNF-alpha by peripheral whole blood increased during the PSL withdrawal phase in parallel with the increased serum concentration of M-CSF. This result thus supports our hypothesis.

There are conflicting reports concerning the effects of M-CSF on the production of proinflammatory cytokines by monocytes/macrophages (43-45); however, there seems to be a general consensus that production of M-CSF is enhanced by several proinflammatory cytokines and IFNs in many kinds of cells in vitro or in vivo (25-36). In our study, three types of IFNs increased the serum concentration of M-CSF in vivo. The enhanced serum concentration of M-CSF in hepatitis B patients may be a partial consequence of enhanced production of IFNs in vivo. A recent report (46) showing that the serum level of IFN-gamma is correlated with active hepatic inflammation in hepatitis B patients supports our hypothesis. IFN-alpha, which is produced during HBV infection in the peripheral blood cells and within the liver (47,48), also enhances the production of IFN-gamma by PBMCs in hepatitis B patients (49).

In the present study, we have shown that the increased serum M-CSF concentrations in patients with chronic HBV infection is associated with the increased levels of IFNs and proinflammatory cytokines produced by peripheral blood cells during hepatic inflammation, which may be a consequence of the altered cytokine cascade resulting from host immune response against HBV. We are not sure whether or not the increased M-CSF has any pathophysiological significance; however, it is reasonable to think that a cytokine network supports proliferation of macrophages/monocytes in the liver in parallel with the severity of the hepatic necroinflammatory process in patients with chronic hepatitis B.

References
M-CSF in hepatitis B


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