Induction of Interleukin-12 Production in Chronic Hepatitis C Virus Infection Correlates with the Hepatocellular Damage

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Interleukin (IL)-12 plays an essential role in host defense against infectious diseases. Serum IL-12 concentration and blood mononuclear cell production with or without specific interferon (IFN)-γ priming were investigated in 65 chronic hepatitis C virus (HCV) patients and 25 healthy donors. HCV patients had higher serum IL-12 levels (P = .004) and produced higher amounts after IFN-γ priming (P < .001) than donors. A subset of patients did not produce IL-12: They had lower serum levels (P = .032) and showed signs of liver piecemeal necrosis less frequently (P = .011). Patients with greater liver necroinflammatory activity produced more IL-12 than patients with minimal or mild activity and donors (P < .01). During IFN-α therapy for 16 HCV patients, individuals with end-of-treatment alanine aminotransferase normalization and clearance of viremia had higher serum levels and produced more IL-12 than those who did not (P < .05). These results suggest a role for IL-12 in the immunopathogenesis and outcome of HCV infection.

Interleukin (IL)-12 is a heterodimeric glycoprotein of 70 kDa (p70) comprising two disulfide-bonded subunits with molecular masses of 40 kDa (p40 chain) and 35 kDa (p35 chain). IL-12, a cytokine produced by antigen-presenting cells in response to diverse stimuli, initiates cell-mediated immunity and promotes predominance of T helper type 1 cells [1, 2]. Together with a secondary stimulus, interferon (IFN)-γ influences specifically the monocytic expression of IL-12 [3]. IL-12 can prevent or reduce mortality to lethal doses of the herpes simplex virus or the murine cytomegalovirus in mice; indeed, animals cured by IL-12 are immune to reinfection [2]. Also, IL-12 can prevent or reverse retrovirus-induced immune deficiency. By enhancing resistance to intracellular pathogens by T cell–independent mechanisms, endogenous IL-12 plays an essential role in the host defense against several infectious diseases [1, 2].

T cell activation is important to determine the liver disease outcome in chronic hepatitis C virus (HCV) infection [4, 5]. HCV-specific cytotoxic T lymphocytes are present in persistently infected patients; this response, however, is stronger in low-viremic patients and in those with a more active liver disease [6, 7]. Thus, altered expression of IL-12 may be relevant in the pathophysiology of human HCV infection. In chronic hepatitis C, the goals of the therapeutic intervention are clearance of viremia and amelioration of the liver disease [8]. In this work, we studied the serum IL-12 levels and the peripheral blood mononuclear cell (PBMC) production in a cohort of persons with persistent HCV infection. The relationships between the levels of IL-12 and host and virus factors and IL-12 induction in relation to the therapeutic outcome following IFN-α treatment were investigated as well.

Materials and Methods

Patients. Sixty-five consecutive patients (40 men, 25 women; mean age, 41 years [range, 19–65]) with chronic HCV infection were included in this study. They were positive for anti–HCV (InnoteqHCV-AbIII confirmed by Inno-LiaHCV-AbIII; Innogenetics NV, Zwijnaarde, Belgium) and for HCV RNA in serum as detected by reverse transcription and nested polymerase chain reaction [9]. All patients had signs of chronic hepatitis in a liver biopsy; specimens were examined under code by 2 pathologists (with observer concordance) and scored according to the system of Knodell et al. [10], following international criteria [11]. Other causes of liver disease were excluded. None of the patients had received previous antiviral or immunomodulating therapy. Blood samples were taken for the baseline IL-12 measurements. HCV RNA was quantitated in serum by the Amplicor HCV Monitor assay (Roche Diagnostic Systems, Branchburg, NJ). HCV typing was done by restriction fragment length polymorphism analysis of reverse transcription and nested polymerase chain reaction products, as described [9].

The effect of IFN-α treatment on IL-12 levels was studied in 16 patients who were administered 5 million U of IFN-α2b (Schering-Plough, Kenilworth, NJ) every other day for 6 months: 5 had end-of-treatment biochemical and virologic response (normal alanine aminotransferase [ALT] values and negative for HCV RNA), of whom only 2 had a sustained response 6 months after completion of therapy; the remaining 11 were nonresponders (5 had normal
ALT values but continued to have detectable HCV RNA, and 6 had persistent abnormal ALT values and detectable HCV RNA). Blood samples were collected during treatment within 48 h following IFN-α injection; IL-12 was measured at the start and end of therapy. Twenty-five healthy unpaid blood donors with normal liver function tests and without markers of viral hepatitis (15 men, 10 women; ages 21–65 years) served as controls.

Cell isolation and culture. PBMC were isolated by gradient sedimentation (Seromed; Biochrom, Berlin, Germany) from fresh, heparinized venous blood, washed twice with PBS, and suspended in Dulbecco’s MEM (Life Technologies Gibco BRL, Paisley, UK) supplemented with 5% heat-inactivated fetal bovine serum (Imperial Laboratories, Andover, UK), 20 mM HEPES, 2 mM glutamine, and antibiotics. The cell viability was assessed by trypan blue dye exclusion. PBMC (2.0 × 10^6 viable cells/mL) were seeded in 12-well tissue culture clusters (Costar, Cambridge, MA) at 37°C in a humidified atmosphere containing 5% CO₂. Cultures were maintained with medium alone or stimulated with 10 µg/mL lipopolysaccharide (LPS; Sigma, St. Louis) for 48 h, or cells were primed with 100 ng/mL IFN-γ (Promega, Madison, WI) for 16 h followed by LPS stimulation as above [3]. At the end of culture, supernatants were collected, centrifuged, filtered, aliquoted, and stored at −80°C until use.

Detection of IL-12. To specifically detect the heterodimeric IL-12 p70, an ELISA was used as described previously [12] (reagents provided by M. K. Gatley, Hoffmann-La Roche, Nutley, NJ), with minor modifications. Undiluted serum samples or culture supernatants (100 µL) and recombinant human IL-12 as standard (at concentrations of 12.5–1600 pg/mL) were assayed in duplicate. The IL-12 dose-absorbance response was linear between the standard concentrations tested. The sensitivity of the assay was 1 pg/mL, calculated as the minimum detectable quantity that is 3 SD above the mean absorbance of 20 duplicate negative controls (zero standard). The test has intra- and interassay coefficients of variation of <6% and 14%, respectively. Samples whose absorbance values fell outside the upper range of the curve were diluted 10-fold and retested.

Statistical analysis. The Mann-Whitney test or Wilcoxon signed rank sum test was used for comparison of results in independent groups or paired data in the same group, respectively. Data were correlated by using Spearman’s rank correlation coefficient. Dichotomous variables were compared by the χ² test (or Fisher’s exact test, where applicable).

Results

The serum levels of IL-12 were significantly higher in patients with chronic HCV infection than in healthy donors (mean IL-12 pg/mL ± SE = 946 ± 235 vs. 595 ± 284; P = .004, Mann-Whitney test). IL-12 was produced spontaneously (medium alone) at low levels in PBMC cultures from 12 (18%) of 65 patients and 2 (8%) of 25 donors (P, not significant [NS]). Normally, LPS stimulation did not modify production of IL-12 compared with medium alone in patients (mean pg/mL ± SE = 8.3 ± 2.4 vs. 4.5 ± 2.2; P, NS) and in donors (1.8 ± 0.6 vs. 1.7 ± 0.4; P, NS); there was no significant difference between patients and donors (figure 1A). IFN-γ priming, followed by LPS stimulation (IFN-γ/LPS), significantly increased IL-12 production in PBMC from patients and donors (P < .001). Patients with chronic hepatitis C produced significantly higher amounts of IL-12 after IFN-γ/LPS-induction than did donors (mean pg/mL ± SE = 77.4 ± 19.0 vs. 7.5 ± 3.6, P < .001; figure 1A). The proportion of monocytes was similar among patients and donors (mean % cells ± SE = 5.01 ± 0.23 vs. 5.02 ± 0.52, respectively). The basal and stimulated IL-12 production was independent of the proportion of monocytes (rₛ = −.11).

PBMC cultures from 18 (28%) of 65 HCV patients still did not produce IL-12 after IFN-γ/LPS stimulation; similarly, PBMC from 6 (24%) donors did not respond to IFN-γ/LPS stimulation. The serum IL-12 level was significantly lower in these 18 HCV patients than in the remaining 47 with chronic hepatitis C (mean IL-12 pg/mL ± SE = 854 ± 358 vs. 1138 ± 295; P = .032 by Mann-Whitney test).

Serum IL-12 levels did not correlate with patient demographic data (age: rₛ = −.14; time of known disease: rₛ = .05), ALT values (rₛ = −.01), liver histology (rₛ = −.14), HCV RNA concentration (rₛ = −.14), and the HCV genotype 1b (mean ± SE = 1002.9 ± 34.3 pg/mL) compared with non-1b type (1a, n = 2; 2a, n = 1; 3a, n = 2; 4, n = 2) patients (538.0 ± 88.5 pg/mL; P, NS). Similarly, PBMC secretion of the IFN-γ/LPS-inducible IL-12 did not correlate with clinicodemographic data, the viremia level (rₛ = −.06), or the HCV genotype (mean ± SE = 71.7 ± 2.7 pg/mL in HCV type 1b vs. 136.0 ± 38.9 pg/mL in non–1b type HCV; P, NS). Patients who had moderate to severe liver necroinflammatory activity produced significantly higher levels of the IFN-γ–induced IL-12 (mean ± SE = 152.2 ± 44.7 pg/mL, n = 13) compared with HCV patients with mild (83.5 ± 27.6 pg/mL, n = 35) or minimal (42.7 ± 14.7 pg/mL, n = 17) histologic activity and healthy donors (P < .01; figure 1B). In addition, IL-12 production was significantly correlated with the histologic score of piecemeal necrosis (rₛ = .460, P < .001); indeed, 8 of 18 patients whose PBMC did not produce IL-12 had no signs of piecemeal necrosis in the liver biopsy compared with 6 of 47 who produced IL-12 (χ², 7.7; P = .011). The score of liver fibrosis correlated with the IFN-γ–induced IL-12 production (rₛ = .348, P = .005). Finally, histologic activity was independent of the proportion of monocytes (rₛ = .03; P, NS).

The baseline serum IL-12 levels (figure 2A) and the PBMC production of the IFN-γ/LPS-inducible IL-12 (figure 2B) were higher, although not statistically significant, in 5 of 16 patients with ALT normalization and loss of HCV RNA after IFN-α treatment compared with the remaining patients. At treatment cessation, however, serum IL-12 concentrations were significantly higher (P < .05) in the 5 responder patients than in the nonresponders who continued to have detectable HCV RNA, with either normal (n = 5) or abnormal (n = 6) ALT values (figure 2A). On the other hand, in general, IFN-α treatment
Figure 1. A. Production of IL-12 by cultured peripheral blood mononuclear cells (PBMC) from patients with chronic HCV infection (filled bars) and healthy donors (open bars). PBMC were cultured with medium alone, stimulated with lipopolysaccharide (LPS), or primed with interferon (IFN)-γ followed by LPS stimulation; IL-12 heterodimer p70 was measured in supernatants. Results are expressed as mean ± SE. * Significantly higher than medium alone or LPS stimulation alone (P < .001, by Wilcoxon signed rank test); ** significantly higher than healthy donors (P < .001, by Mann-Whitney test). B. Analysis of IFN-γ/LPS-induced IL-12 production by PBMC in healthy donors and patients with chronic HCV infection according to score of liver necroinflammatory activity classified as minimal, mild, and moderate to severe (see [10] for details). Results are expressed as mean ± SE. * Significantly higher than in patients with minimal or mild necroinflammatory activity and with healthy donors (P < .01, by Mann-Whitney test); ** significantly higher than in healthy donors (P < .01, by Mann-Whitney test).

decreased the PBMC production of the IFN-γ/LPS-inducible IL-12. At the end of therapy, IL-12 production by PBMC remained significantly (P < .05) higher in responder patients (end-of-treatment biochemical and virologic response) compared with nonresponders (HCV RNA detectable, with normal or abnormal ALT values) (figure 2b).

Discussion

In this study, we investigated the serum concentrations and the PBMC production of IL-12 in patients with chronic HCV infection. Chronic hepatitis C patients had significantly higher serum levels of IL-12 than healthy donors. IL-12 p70 is rapidly produced after infection; however, IL-12 has a relatively long serum half-life compared with other cytokines, probably due to its higher molecular mass [1, 2]. Thus, the likely explanation is that IL-12 accumulates in serum following induction and release from the target site of infection. Spontaneous secretion of detectable quantities of IL-12 was observed in PBMC from a minority of patients and donors, and LPS stimulation failed to significantly increase IL-12 production. The latter may not be due to an immune defect of our patients, because it was also observed in donors. More likely, IL-12 p70 expression requires specific triggering by IFN-γ, together with a secondary stimulus like LPS, as recently suggested [3]. In fact, LPS stimulation alone results in overproduction of only the IL-12 p40 chain, whereas formation of the p70 heterodimer requires coexpression of the p40 and p35 chains, the latter being specifically regulated by IFN-γ priming [3]. Accordingly, IL-12 production increased significantly in patients and donors after IFN-γ/LPS induction. However, HCV patients produced significantly more IFN-γ/LPS-inducible IL-12 than donors did, possibly due to the chronic inflammatory process. This finding suggests that the ability of PBMC to produce IL-12 in response to specific stimuli is preserved in patients with HCV infection, contrary to what has been reported in human immunodeficiency virus–infected patients, whose PBMC show an impaired IL-12 production associated with its immunodeficiency [13]. A subset of patients and donors did not produce IL-12, even with specific nonviral stimulatory signals. One explanation is that PBMC produce IL-12 at levels below the sensitivity of the assay. However, it cannot be ruled out that these
cases may have a disturbed IL-12 inducibility. Monocytes, but also other cell types, might be defective in IL-12 production. This issue and the fact that HCV antigen–specific stimulation does not induce IL-12 production in infected individuals [14] are intriguing aspects deserving further investigation.

In this study, we have not found any relationship between serum levels or PBMC production of IL-12 and a patient’s characteristics, the HCV genotype, or the viremia, suggesting that IL-12 induction is not related to HCV replication. IL-12 is involved in the development of cellular responses determining viral clearance from the infected cells [15] but may have a role in the immunopathogenesis of chronic hepatitis C as proinflammatory cytokine [1, 2]. Indeed, the IFN-γ–primed IL-12 production correlated significantly with the liver necroinflammatory activity, although not with ALT values, probably because serum ALT levels do not accurately reflect the level of inflammatory changes in chronic hepatitis C [8]. On the other hand, the association between IL-12 production and the liver fibrosis in our patients, although it suggests a possible involvement of IL-12 in the fibrogenic process, requires confirmation.

**Figure 2.** Individual IL-12 levels in 16 patients with chronic hepatitis C treated with interferon (IFN)-α. Patients were classified as end-of-treatment responders (normal alanine aminotransferase [ALT] values and loss of HCV RNA) or nonresponders (if they remained HCV RNA positive, with normal or abnormal ALT values). IL-12 in serum (A) or IFN-γ/lipopolysaccharide-induced IL-12 production by peripheral blood mononuclear cells (B) was measured before (baseline) and after (end) 6 months of treatment with IFN-α. Horizontal bar refers to median value. *Significantly higher than in nonresponders (P < .05, by Mann-Whitney test).
Finally, pretreatment IL-12 PBMC production, or its serum concentrations, did not make it possible to distinguish the subset of patients who responded to therapy. During IFN-α treatment, serum IL-12 levels and mononuclear cell production decreased in most patients. However, the amount of serum IL-12 and the IFN-γ/LPS-induced IL-12 production by PBMC was significantly higher in patients with end-of-treatment response, suggesting that IFN-γ–induced IL-12–dependent immune responses may be relevant for the outcome of the chronic HCV infection. Because all but two of the responders had a posttreatment hepatitis relapse, or the viremia reappeared, it was not possible to establish a definitive link between IL-12 induction and a sustained response to IFN-α treatment; this issue will be studied in a larger cohort.

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References


