In Vivo and In Vitro Induction of MxA Protein in Peripheral Blood Mononuclear Cells from Patients Chronically Infected with Hepatitis C Virus

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To test whether (HCV) persistence is related to interferon (IFN) hyporesponsiveness, peripheral blood mononuclear cells from 29 patients and 11 controls were studied for MxA protein expression. In vitro, only IFN-α (P < .001) and interleukin-2 (P < .05) induced MxA protein expression above unstimulated levels. Forty patients were treated with IFN-α2b. Patients showed higher basal levels of MxA protein (P < .02) and 2',5'-oligoadenylate synthase (2-5A) activity (P < .05) than controls. During therapy, MxA protein levels (P < .001) and 2-5A activity (P < .05) increased; after 1 month, MxA levels remained high, whereas 2-5A activity declined to initial levels. Increases in MxA were inversely correlated with decreases in serum alanine aminotransferase levels, and MxA induction was greater among virological responders. Thus, the IFN system seems to be activated in chronic HCV infection, but HCV appears to modulate these two components of the IFN system differentially. These results suggest that an inefficient response may contribute to virus persistence and affect the therapeutic outcome.

The interferon (IFN) system is activated in response to viral infection and plays an important role in the host’s defense response [1, 2]. Type I IFNs (IFN-α/β) induce different proteins with antiviral capacity, such as 2',5'-oligoadenylate synthetase (2-5A), P1 kinase, IP-10, indolamine 2,3-dioxygenase, and Mx proteins [3]. MxA and 2-5A are considered to be indicators of IFN actions and are, in part, responsible for the antiviral state induced by the presence of INF [4, 5]. In humans, there are 2 Mx genes, Mx1 and Mx2, encoding, respectively, the MxA and MxB proteins. MxA protein is found in the cytoplasm of cells and has a GTPase activity. MxA also has an antiviral effect, which is dependent on the GTPase activity [6]. MxA inhibits replication of several RNA viruses, such as orthomyxoviruses, rhabdoviruses, paramyxoviruses, and bunyaviruses [7–10]. In human peripheral blood mononuclear cells (PBMC), macrophages are the principal producers of MxA protein [11, 12].

Hepatitis C virus (HCV) is a member of the Flaviviridae family [13]. Its genome consists of a single positive-stranded RNA of about 9700 nucleotides encoding a polyprotein of 3010–3011 amino acids [13]. HCV infection has a high rate of persistence and usually evolves to chronicity in 70%–80% of cases [14]. Nowadays, IFN-α is the therapy that gives the best results, although a high proportion of patients do not respond to IFN-α therapy or show only a transient response [15]. Although it has been shown that the IFN system is poorly activated [16] during the acute phase of HCV infection, the capacity of PBMC from chronically HCV-infected patients to respond to type I IFNs has not been analyzed extensively. Therefore, we have tested the hypothesis that HCV persistence is related to an altered IFN responsiveness and that MxA and 2-5A may be involved in IFN action against HCV. Thus, we performed a cross-sectional study of the in vitro MxA responsiveness in chronically HCV-infected patients’ PBMC cultured with IFN-α and other cytokines, with healthy donors serving as controls. In addition, we analyzed in vivo human MxA and 2-5A steady levels and their longitudinal evolution during treatment with IFN-α.

Material and Methods

Patients Sixty-seven consecutive patients (34 men and 33 women; mean age, 46 years [range, 22–61 years]), with chronic HCV infection for an average of 7.2 years, were included in this study. All of them were anti-HCV– and HCV RNA–positive in serum, with a mean (+SD) alanine aminotransferase (ALT) value of 107 ± 77 IU/mL (normal value, <43 IU/mL). The patients had chronic hepatitis that had been histologically proven according to international criteria [17, 18]: the liver necroinflammatory activity was minimal in 17 cases, mild in 42, and moderate in 8. None of the patients had liver cirrhosis, and none had superimposed hepatitis B, D, or G or human immunodeficiency virus type 1 infection.
Other causes of liver disease were excluded. None of the patients had received previous antiviral or immunosuppressive therapy. Blood samples were obtained at the time of the liver biopsy. As controls, 21 healthy subjects with similar demographics, normal ALT levels, and no viral hepatitis markers were included. Of the 67 patients, 40 were treated with IFN-α2b (Intron-A; Schering-Plough, Liberty Corner, NJ), 5 MIU 3×/week, for 6 months. Sequential samples from all patients were taken before and after treatment and, in 15 cases, during the course of therapy.

Methods. Anti-HCV was assayed in serum by a commercial ELISA (INNOTEST HCV Ab III; Innogenetics, Ghent, Belgium). HCV RNA was detected in serum by reverse transcription polymerase chain reaction, as previously described [19]. Quantitation of HCV RNA was performed with the Amplicor HCV monitor assay (Roche Diagnostics Systems, Branchburg, NJ), following the manufacturer’s instructions. HCV genotyping was done by restriction fragment length polymorphism analysis, as described [19].

Collection of samples. PBMC from controls and patients were isolated by Ficoll-Hypaque gradient sedimentation (SEROMED; Biochrom KG, Berlin, Germany) from fresh, heparinized venous blood, washed twice with PBS, and suspended in RPMI 1640 (Serva Feinbiochemica, Heidelberg, Germany) supplemented with 10% fetal bovine serum (Imperial Laboratories, Andover, UK), 20 mM HEPES, 2 mM glutamine, and antibiotics. To study MxA expression in vivo, 1 aliquot of freshly isolated PBMC was lysed immediately with lysis buffer, as previously described [20], and the cell extract was stored frozen until its use. Another aliquot of fresh PBMC from 29 of the 67 patients and 11 of the 21 healthy donors was cultured to analyze MxA responsiveness in vitro. These mononuclear cells were seeded onto 96 well plates (Costar, Cambridge, MA) at a density of 2.0 × 10⁶ cells/well in 200 µL of RPMI 1640. Cells were cultured for 24 h at 37°C in a humidified atmosphere containing 5% CO₂, either unstimulated or stimulated with 10, 100, or 1000 IU/mL of recombinant human IFN-α (rhIFN-α) 2a (E. Hoffmann–La Roche, Basel, Switzerland); 10, 100, or 1000 IU/mL of recombinant human interleukin (rhIL)-2 (Chiron-Cetus, Emeryville, CA); 1, 10, or 100 ng/mL of rhIL-12 (R&D Systems, Minneapolis); 10 ng/mL of recombinant human granulocyte colony-stimulating factor (G-CSF; Amgen, Thousand Oaks, CA); 10 ng/mL of recombinant human macrophage CSF (M-CSF; Chiron-Cetus); and 10 ng/mL of recombinant human granulocyte-macrophage CSF (GM-CSF; Schering-Plough, Kenilworth, NJ). After the end of the culture period, cells were harvested and treated with lysis buffer, as described above.

Immunoblot assay for human MxA protein. The immunoblot assay for detection and quantitation of human MxA protein was performed exactly as described elsewhere [21]. Briefly, aliquots of lysed PBMC were boiled with sample buffer and electrophoresed in 10% SDS-PAGE. At the end of the run, the gel was blotted to an Immobilon-P membrane (Millipore, Bedford, CA). Human MxA protein was detected with a mouse-specific monoclonal antibody [22] and a secondary rabbit anti-mouse peroxidase-conjugated antibody (Dakopatts, Løstrup, Denmark). The membrane was revealed with the detection reagent of the enhanced chemiluminescence (ECL Amersham, Buckinghamshire, UK). The absorption of the human MxA protein band was recorded with a laser densitometer (Molecular Dynamics, Sunnyvale, CA), and the area under the curve was integrated. The amount of MxA protein in each sample was measured as described [21].

2-5A activity. The total activity of the enzyme 2-5A was measured in aliquots of lysed PBMC with a sensitive RIA (Eiken Chemical, Tokyo, Japan). Briefly, the sample was adsorbed and activated by use of poly (I) poly (C) agarose, and after that 2-5A was produced by using ATP as substrate. Then the sample was incubated with [32P]labeled 2-5A, the anti–2–25A serum, and a secondary antibody. The mixture was centrifugally separated, and the pellet was measured with a scintillation counter. The activity of 2-5A is calculated from the standard curve included with the kit in each run. The assay range is 10–810 pmol/dL, and the intra-assay coefficient of variation is <15%.

Statistical analysis. To analyze our data, we used the non-parametric Wilcoxon rank-sum test for paired results, Mann-Whitney’s test for independent groups, and Spearman’s correlation coefficient.

Results

Basal levels of MxA expression. PBMC from untreated patients with HCV infection had greater expression of MxA protein than healthy donors in basal conditions (mean MxA units, 3.00 ± 0.61 SEM vs. 1.02 ± 0.29 SEM, P < .02; figure 1). In addition, mononuclear cells from HCV patients had greater basal activity of the enzyme 2-5A than those from healthy donors (mean ± SEM, 2267 ± 887 vs. 432 ± 47 pmol/dL, respectively, P < .05; figure 1). There was no correlation between expression of MxA protein and 2-5A activity (r = .15, P not significant). There were no differences in cell subset counts between patients and controls, and there was no correlation between the proportion of monocytes in the sample taken and MxA expression (P = .78). The level of expression

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<th>MxA units</th>
<th>2-5A (pmol/50000 cells)</th>
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<td>HCV patients</td>
<td>Healthy donors</td>
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<td>P &lt; .02</td>
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Figure 1. Basal levels of MxA protein and 2,5'-oligoadenylate synthase (2-5A) activity in peripheral blood mononuclear cells from 67 patients with untreated chronic hepatitis C virus (HCV) infection (white bar) and 21 healthy donors (black bar). Bars represent mean ± standard error (statistical analysis done by use of Wilcoxon rank sum test).
of MxA protein did not correlate with ALT values ($r = .03$), serum HCV RNA concentration ($r = .09$), HCV genotype ($r = .05$), or γ-globulin level ($r = .04$). No correlation between levels of MxA protein and 2-5A activity or between levels of MxA protein and the different scores of histological activity (portal inflammation, piecemeal necrosis, or lobular cytolysis) was demonstrated. However, untreated patients with greater liver necroinflammatory activity (from minimal to severe) tended to express more MxA protein and less 2-5A activity (figure 2), although the differences were not statistically significant.

**MxA responsiveness in vitro.** In cultured PBMC from healthy subjects, only IFN-α induced MxA expression above basal levels ($P < .001$), whereas the rest of the cytokines tested—IL-2, IL-12, M-CSF, G-CSF, and GM-CSF—were not capable of inducing MxA protein expression above basal levels (figure 3). In contrast, all the cytokines induced MxA protein in PBMC from HCV patients, but there were statistically significant differences only when the basal and cytokine-stimulated levels were compared, after induction with IFN-α ($P < .001$) and IL-2 ($P < .05$), at all doses tested. PBMC from patients reached statistically significant greater levels of MxA protein than healthy subjects ($P < .001$) with all the cytokines used, except with IFN-α.

**MxA expression during IFN therapy.** For 3 months before IFN therapy, the MxA protein levels remained nearly constant. During the first month of treatment and thereafter, PBMC from patients with chronic HCV infection showed a clear induction of the MxA protein ($P < .001$; figure 4A). High levels of MxA continued during therapy (up to 3 months), and there was a peak of induction at month 6 in comparison with samples taken at month 3 ($P < .05$), which returned to baseline levels during the post-treatment follow-up. With respect to 2-5A, the enzyme levels remained low before initiation of treatment; showed an increase after the start of treatment, with a peak of activity at the 1st month ($P < .05$); and declined to near baseline levels during the rest of treatment ($P < .05$; figure 4B). During pretreatment and until month 2 of therapy, MxA expression and 2-5A activity did not correlate significantly ($P = .28$). However, an inverse correlation (patients with high levels of MxA protein but low levels of 2-5A activity) was obtained after month 2 and until the end of treatment ($r = -.76$; $P = .039$).

At the end of treatment, 21 (52%) of the 40 patients treated with IFN showed a biochemical response (ALT normalization), and 9 (22%) showed a biochemical and virological response (ALT level normalization and HCV RNA negativity). There were no statistically significant differences in pretreatment variables, levels of MxA, 2-5A activity, ALT values, or HCV RNA concentration between patients with and without ALT normalization at the end of treatment and at follow-up. However, there was an inverse correlation ($r = -.58$; $P = .001$) between the increase in MxA expression and decrease in serum ALT levels (figure 5), although there was no correlation with a decrease in HCV RNA concentrations ($r = .12$; $P$ not significant). At the end of therapy, MxA expression was significantly induced ($P < .001$; figure 6) in comparison with pretreatment levels in all patients, regardless of their biochemical and virological response to IFN ($P = .24$; figure 6). However, patients with
Figure 4. Individual levels of MxA protein expression (top) and 2'-5'-oligoadenylate synthase (2-5A) activity (bottom) in peripheral blood mononuclear cells from 15 patients with chronic hepatitis C infection. Samples were collected at the start of treatment, during therapy, and at posttreatment follow-up. Line represents mean.

Discussion

In this study, we investigated MxA expression in blood mononuclear cells from patients with chronic HCV infection. Compared with healthy subjects, who had a low MxA basal expression, HCV patients exhibited greater baseline expression of MxA protein. This result was not due to differences in the monocytes subset (the primary source of MxA expression), because the proportion of monocytes was similar in patients and controls. Instead, since MxA protein is induced by IFN α/β, mononuclear cells may be activated to produce IFN as a consequence of the persistence of HCV infection. Indeed, double-stranded RNA and viruses are activators of IFN-regulated genes such as 2-5A, which is thought to be an indicator of the endogenous IFN production in vivo [6, 23, 24]. Accordingly, the levels of 2-5A were also higher in patients than in controls, in agreement with previous reports [25]. Thus, our results suggest that the high MxA expression in chronic HCV infection might be due to the activation of the IFN system, at least in PBMC. We did not find a correlation between MxA and 2-5A levels in our patients. The explanation of this result is not clear, but it is possible that the expression of particular viral gene products might differentially regulate MxA and 2-5A responsiveness in individual patients, although this remains to be proven.

When mononuclear cells were stimulated with IFN-α in vitro, MxA production was increased in a dose-dependent manner both in chronically HCV-infected patients and in controls. Although patients reached higher levels of MxA protein at all doses of IFN-α tested, there was no statistically significant difference between patients and healthy donors. This result indicates that the inducibility of the IFN-regulated MxA gene is preserved in patients with chronic HCV infection, in contrast to the impaired response recently described in HBV patients [21]. This may be related to differences in the immunoregulatory mechanisms in chronic HBV and HCV infections [26, 27]. Thus, it has recently been demonstrated [21] that IL-2 is able to stimulate the synthesis of MxA protein in PBMC from chronically HBV-infected patients too, but to a lesser extent than in HCV patients, as observed in this study. It is likely that IL-2 acts via the induction of IFN-α [28], and the presence of endogenous IFN, as suggested by the high levels of MxA protein and 2-5A activity present in mononuclear cells from HCV patients, might prime PBMC to be more sensitive to the action of other cytokines, which could enhance the synthesis of more IFN. This observation is relevant in regard to the possible role of IL-2 in the treatment of chronic hepatitis C, as reported recently [29].

During IFN therapy, MxA levels increased significantly, and they declined only after cessation of IFN-α treatment. This end-of-treatment biochemical and virological response showed a higher induction of MxA protein, although the difference was not statistically significant.

Figure 5. Regression analysis of the correlation between individual MxA protein and alanine aminotransferase (ALT) values of patients with chronic hepatitis C virus infection before and at the end of interferon treatment (statistical analysis done by Spearman’s correlation coefficient).
result confirms in vivo the data obtained in vitro, in that HCV patients are highly responsive to IFN-α with respect to MxA production. In contrast, 2-5A activity increased only transiently during IFN-α treatment, declining before the end of therapy. This observation is in agreement with previous reports [30, 31]. The difference between MxA expression and 2-5A activity in our patients, and the different kinetics shown during IFN treatment, might have different explanations. Apparently the system of the 2-5A synthetase, in contrast to the response of MxA protein, is exhausted during the IFN treatment. This suggests a different mechanism of activation in the two systems. One possibility is that double-stranded RNA may differentially activate the diverse isoforms of 2-5A synthetase [32]. Another explanation is that the 2-5A isoforms may exhibit different dose responses to IFN-α and also different kinetics of expression, depending on cell-specific factors [33]. In any case, it is likely that these factors influence 2-5A activity but do not affect other IFN-inducible genes, such as MxA. In future works, better understanding of the IFN signaling pathways will enable us to explain the different behaviors of MxA and 2-5A observed in this study.

Finally, the increase in MxA expression correlated with the decrease in serum ALT levels but not with the viremia levels. The induction of the protein might be related with a possible antiinflammatory effect that could ameliorate the liver disease, but the elucidation of the pathways involved in MxA responsiveness against HCV deserves future research.

In summary, the IFN system appears to be activated during chronic HCV infection. This activation seems to enhance the capacity of PBMC to respond in vitro to IFN-α and to other cytokines, such as IL-2 and possibly IL-12. HCV appears to modulate differently in two pathways of the antiviral action of the IFN system. If this is so, it may explain the outcome of chronic HCV infection and the response to IFN therapy. Our data suggest that there might be a molecular basis for IFN-α nonresponsiveness, and this should be investigated in the future.

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References