Role of the Receptor for the Globular Domain of C1q Protein in the Pathogenesis of Hepatitis C Virus-Related Cryoglobulin Vascular Damage


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Role of the Receptor for the Globular Domain of C1q Protein in the Pathogenesis of Hepatitis C Virus-Related Cryoglobulin Vascular Damage


Mixed cryoglobulinemia (MC) is a lymphoproliferative disorder observed in ∼10 to 15% of hepatitis C virus (HCV)-infected patients. Circulating, nonenveloped HCV core protein, which has been detected in cryoprecipitable immune complexes, interacts with immunocytes through the receptor for the globular domain of C1q protein (gC1q-R). In this study, we have evaluated circulating gC1q-R levels in chronically HCV-infected patients, with and without MC. These levels were significantly higher in MC patients than in those without MC and in healthy controls and paralleled specific mRNA expression in PBL. Soluble gC1q-R circulates as a complexed form containing both C1q and HCV core proteins. Higher serum gC1q-R levels negatively correlated with circulating concentrations of the C4d fragment. The presence of sequestered C4d in the vascular bed of skin biopsies from MC patients was indicative of in situ complement activation. In vitro studies showed that release of soluble gC1q-R is regulated by HCV core-mediated inhibition of cell proliferation. Our results indicate that up-regulation of gC1q-R expression is a distinctive feature of MC, and that dysregulated shedding of C1q-R molecules contributes to vascular cryoglobulin-induced damage via the classic complement-mediated pathway. The Journal of Immunology, 2009, 183: 6013–6020.

It has recently been shown that cold-precipitating immune complexes (ICs) comprise HCV core protein as ligand (7). HCV nucleocapsid devoid of enveloped proteins in the bloodstream of HCV-infected patients is a good indicator of the circulating viral load (8). It may be the result of overproduction during virogenesis (9) and has been reported to be secreted by transfected hepatoma cell lines in culture and in HCV transgenic mice (10). The core protein has been found in the serum of most HCV chronic carriers with active liver disease and in almost half of those with inactive disease (11). In addition, serum HCV core protein levels change following antiviral therapy and become undetectable in responsive patients (12). Cold-dependent insolubility of this protein seems to be the result of the host product reaction, including IgM molecules with rheumatoid factor (RF) activity capable of activating the complement cascade (7).

The complement system is highly activated in cryoglobulinemic patients (13). Normal mean C3 and C4 levels in the soluble phase correspond to very low amounts (if any) in the cryoprecipitate, suggesting the existence of two virtually distinct microenvironments in which complement is differently activated (1). Complement is a major interdependent regulator of IC size and composition. Complement binding to nascent ICs may decrease their size and maintain them in solution (14). Compared with the supernatant, significant differences of C1q and C1q binding activity have been shown in unsolubilized ICs (7). Efficient engagement of C1q protein by cryoglobulins may be an important pathogenetic mechanism involved in the cryoglobulin-related pathway. In this context, it has been recently demonstrated that HCV core protein interacts directly with the globular domain of C1q protein (gC1q-R) (15). HCVcore-gC1q-R interaction has been assumed to play a critical role in modulating the T cell immune response (16–17). Nonetheless, engagement of circulating HCV core protein with gC1q-R on the surface of B lymphocytes (18) provides the virus with a direct means of affecting host immunity.
gC1q-R is a 33 kDa acidic protein expressed on somatic cells (19). It binds to the globular heads of C1q and modulates complement activation. The wide expression of gC1q-R on the surface of both circulating blood (20) and endothelial cells (21) may favor its specific binding to HCV core protein-containing ICs. HCV core deposition has indeed been reported in the skin (22) and kidney (23) of cryoglobulinemic patients. Apart from its specific interaction with C1q, it binds with several cell proteins, namely kininogen (24), vitronectin (21), nucleus-related-like TFII B (25–26), lamin B receptor (27), splicing factor 2 (28), mitochondria-related-like cytochrome b2 (29), and BH3 (30). In addition to cell proteins, C1q-R interacts with several pathogenic bacterial and viral proteins, such as adenovirus core protein (31) and HIV rev (32–33), and may thus be a part of the system which imports proteins to the cell.

Because gC1q-R is proteolytically cleaved and released from the cell surface, and because this release is enhanced in tumor cells or in infected cells, it may play an important role in health and disease (34). Circulating gC1q-R may indeed affect numerous aspects of cell biology and subsequent physiology via both intracellular and extracellular pathways (35). The binding of kininogen to gC1q-R on the endothelial cell surface serves as a platform for the assembly and activation of the intrinsic coagulation cascade that leads to the generation of bradykinin, with subsequent infiltration of vascular tissue by proinflammatory cells and vasculitic damage. This implies that at inflammation sites, where it is abundantly expressed both in soluble form and on the cell surface, gC1q-R exacerbates inflammation by generating vasoactive peptides from the complement system and bradykinin from the contact system (36).

We thus set out to determine the serum levels of gC1q-R and to assess its role in patients with cryoglobulinemic vasculitis. Our findings indicate that the high levels of gC1q-R in MC patients parallel those of specific mRNA expression in blood mononuclear cells, and suggest that this protein is involved in the pathogenesis of cryoglobulin-related damage upon engagement of nonenveloped HCV core protein. They also demonstrate the occurrence of a core-dependent gC1q-R regulation mechanism that may be implicated in the development of cold-dependent IC formation.

Materials and Methods

Patients and controls

Thirty-two patients with MC were studied. The eligibility criterion was no complicated in the development of cold-dependent IC formation.

Soluble gC1q-R capture

gC1q-R was quantified with an ELISA procedure using a mouse monoclonal Ab (moAb) against the amino-terminus of human gC1q-R protein (moAb 60.11, residues 76–93) (21) as a capture Ab. Microtiter plates (Nunc) were first coated with 100 ng of each moAb 60.11 or nonimmune, species control IgG in carbonate buffer pH 9.5, and incubated overnight. After washing twice with TBS (20 mM Tris, pH 7.5 containing 150 mM NaCl), the unrestricted sites were blocked with 1% BSA (1 h, 37°C) and washed again (2× TBS) before addition of 100 µl of either concentrations of gC1q-R ranging from 0 to 500 ng/ml or a dilution (1/100 in PBS) of serum. After incubation (1 h, 37°C), the wells were washed and further reacted with 100 µl of HRP-conjugated moAb 60.11. After incubation (1 h, 37°C), the reaction was developed by the addition of tetramethylbenzidine and the color developed and read in a spectrophotometer at 450 nm. Irrelevant proteins included HCV NS3 and HBSAg used as controls.

We have also performed quantitation of gC1q-R using a different type of moAb in a sandwich ELISA, namely mouse moAb no.1160 (clone 74.5.2) directed against the carboxy-terminal residues (204–218) of the mature form of gC1q-R protein. However, overlapping results (not shown) were achieved.

Detection of nonenveloped HCV core in the serum

Free nonenveloped HCV core was detected as described elsewhere (7) with an ELISA kit provided by Ortho Diagnostics. Briefly, 100 µl serum samples were mixed with 50 µl of dissociating buffer containing 0.3% Tri-ton X-1, 1.5% 3-cholamidopropyl)dimethyl-ammnonium-1-propane sulfonate, and 15% sodium dodecyl sulphate, and incubated at 56°C for 30 min. They were then allowed to cool for 10 min. Duplicate wells were pipetted into each well and incubated at room temperature for 60 min under shaking at 900 rpm. Complexes formed on the microwell surface were detected by HRP-conjugated anti-core moAb (Fab). The calibration curve was constructed with control calibrations ranging from 0 to 800 pg/ml.

gC1q-R/HCV core complex preparation and detection

Mature gC1q-R (aa: 74–282) was expressed in E. coli (39). Recombinant HCV core protein (aa: 2–192), expressed in E. coli, was obtained from Sigma-Aldrich. These proteins were held at 10 µg/ml concentration in PBS. The equivalence point for the gC1q-R/HCV core complex was determined by immunoprecipitation in liquid medium (40). Mixed proteins were incubated at 37°C overnight. gC1q-R/HCV core complex was then separated from uncomplexed proteins by size-exclusion chromatography on Sephacryl-300 (Pharmacia).

gC1q-R/HCV core complex in the serum was detected in a sandwich ELISA with solid phase moAb 60.11 as the capture Ab. Bound complex was revealed by HRP-conjugated anti-core Ab (Fab). Color was then developed with o-phenylenediamine and hydrogen peroxide, and the product was read at 492 nm. Results were expressed as ng of gC1q-R bound per 10 µg total protein. Each reactive test was accepted if confirmed in a separate test. Specificity of the reaction included the following controls: a panel of 20 well-characterized normal subjects, 10 HBsAg chronic carriers, 10 HCV-negative patients with rheumatoid arthritis and high levels of RF activity, and 5 with autoimmune chronic active hepatitis. Furthermore, HCV-recombinant proteins (E2, NS3, NS4, NS5) provided by Dr. S. Moroney (Ortho Clinical Diagnostics, Raritan, NJ) were included. OD was always below the first standard point (1 ng).

Enzyme immunoassay for detection of circulating C4d

The serum C4d fragment was quantitated with a C4d fragment ELIA kit (Quidel), a quantitative sandwich enzyme immunoassay using a mouse moAb against human C4d precoated on a microplate. Serum samples diluted 1/10 in PBS containing 0.05% Tween 20 and 0.035% ProClin 300 were pipetted into the wells and incubated at room temperature for 30 min to allow C4d fragments to be bound by immobilized Ab. After washing away the bound substances, moAb directed against C4d fragment conjugated with HRP was added and again incubated at room temperature for 30 min. After further washings, stabilized 2–2’-azino-di-(3-ethylbenzthiazoline sulfinic acid)-diammonium salt was added and color developed in proportion to the amounts of bound C4d fragment. Quidel C4d kit standards were used to construct a standard curve. The sensitivity threshold of the test was 0.03 µg/ml.

PBL and proliferation assay

PBMC were purified by Ficoll-Isoaque (Pharmacia) and washed three times with RPMI 1640 (Invitrogen Life Technologies) containing penicillin (100 µg/ml), streptomycin (100 µg/ml), and amphotericin B.

To allow C4d fragments to be bound by immobilized Abs. After washing away the bound substances, moAb directed against C4d fragment conjugated with HRP was added and again incubated at room temperature for 30 min. After further washings, stabilized 2–2’-azino-di-(3-ethylbenzthiazoline sulfinic acid)-diammonium salt was added and color developed in proportion to the amounts of bound C4d fragment. Quidel C4d kit standards were used to construct a standard curve. The sensitivity threshold of the test was 0.03 µg/ml.
with PHA (final concentration: 4 µg/ml of glutamine, antibiotics, 5 mM HEPES buffer. Cultures were incubated following equation: K gene series of 2-fold dilutions of cDNA template. This crossing point was used as a reference. The amplitude coefficient calculated by determining the crossing point (number of cycles required to reach a set threshold) for a positive control. The expression levels were analyzed using the amplification coefficient calculated by determining the crossing point for RT-PCR patients (t). The reaction mixture was subjected to 35 cycles (60°C annealing temperature). PCR-generated products were confirmed by DNA sequencing.

Quantitative real-time RT-PCR for gC1q-R gene

Specific mRNA was quantified by real-time RT-PCR light cycle systems (Roche Applied Science). The expression level of gC1q-R gene was relative to the β-actin gene used as internal control. The final expression was formalized as the amplification coefficient calculated by determining the crossing point (number of cycles required to reach a set threshold) for a series of 2-fold dilutions of cDNA template. This crossing point was used to measure gene-specific RNA quantity and fold expression from the following equation: $K_c = 2^{ΔcT}$, where $K_c$ is the amplification coefficient, and $ΔcT$ is the (crossing point for RT-PCR patients) – (the crossing point from normal controls) for the gene. A less than 2-fold increase of RNA levels was not considered significant.

Immunomagnetic separation of serum proteins bound to gC1q-R

We used monodispersed magnetizable M-280 particles (Dynabeads; Dynal Biotech), 2.8 µm in diameter. These particles have a rugged surface with a nominal surface area of 1.4 m²/g, but a measured effective area of 5 m²/g. Their primary hydroxyl groups are activated by p-toluene sulfonyl chloride (42). gC1q-R was used to coat them at 3.0 mg per 10^9 Dynabeads/ml in PBS containing Tween 20, then with 0.5 M NaCl, and finally with 0.15 M NaCl.

Results

All of the 32 anti-HCV-positive patients were viremic (Table I). There were more female than male MC patients. Liver damage was histologically defined. Except for a moderate prevalence of HCV genotype 2 in HCV-related MC, no distinct distribution profile emerged.

Plasma levels of soluble gC1q-R were measured in the 32 HCV-related MC patients, in 20 patients with HCV-related CAH without MC, and in 20 healthy subjects. None were receiving antiviral or immunosuppressive agents. A significant increase of soluble gC1q-R levels was demonstrated in MC patients compared with the HCV-infected patients without MC and the healthy controls. The greatest increase (Fig. 1) was noted in HCV-related MC with a median level of 65.9 ± 25.3 ng/ml. These levels were four to six times higher than those in HCV-related CAH without MC (14.9 ± 2.0 ng/ml, $p < 0.001$) and in healthy controls (11.7 ± 2.9 ng/ml, $p < 0.001$).

The relationship between soluble gC1q-R levels and the immunological, virological, and histological parameters is illustrated in Table I.

### Table I. Epidemiologic, virologic, and histologic data of 52 chronically HCV-infected patients with or without mixed cryoglobulinemia

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HCV + with MC</th>
<th>HCV + without MC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>64.94 ± 10.46</td>
<td>63.66 ± 11.51</td>
</tr>
<tr>
<td>Sex (m/f)</td>
<td>5/7</td>
<td>12/8</td>
</tr>
<tr>
<td>HCV RNA positives (%)</td>
<td>32 (100)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>HCV genotypes (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>20 (62.5)</td>
<td>9 (45)</td>
</tr>
<tr>
<td>2</td>
<td>12 (37.5)</td>
<td>10 (50)</td>
</tr>
<tr>
<td>3</td>
<td>14 (45)</td>
<td></td>
</tr>
<tr>
<td>Liver histology (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic active hepatitis</td>
<td>26 (82.6)</td>
<td>14 (74)</td>
</tr>
<tr>
<td>Cirrhosis</td>
<td>5 (12.4)</td>
<td>6 (26)</td>
</tr>
<tr>
<td>Source of infection (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood/blood products</td>
<td>19 (60)</td>
<td>13 (68)</td>
</tr>
<tr>
<td>transfusions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>13 (40)</td>
<td>6 (32)</td>
</tr>
<tr>
<td>Duration of infection (yrs)</td>
<td>21 ± 7</td>
<td>23 ± 7</td>
</tr>
</tbody>
</table>

**Immunohistochemistry**

C4d and HCV core proteins were detected by indirect immunostaining. Frozen skin sections were incubated with rabbit polyclonal antiseraum to C4d using as immunogen synthetic peptide ETGDNLYGWSVTGQ, corresponding to amino acids 1223–1227 of human C4 protein obtained from Abcam. HCV core was detected by anti-C22–3 mAb produced in mice by using recombinant immunogen C22–3, yeast-expressed, superoxide dismutase-fused HCV core, sequence 1–120 recognizing amino acids 29–43 of the core protein, as described elsewhere (43). Primary Abs diluted in PBS containing 1% BSA and used at a protein concentration of 1 µg/ml were incubated with skin tissue sections for 4 h at 37°C. After extensive washings with PBS, sections were incubated for 3 h at 37°C with HRP-conjugated goat anti-rabbit immunoglobulins and HRP-conjugated rabbit anti-mouse Ig Abs (Jackson ImmunoResearch Laboratories). The adjacent tissue sections were tested with the same procedure as the negative controls, for which primary Abs were adsorbed by preincubation with respective Ags for 12 h at 37°C. In the absorption experiments, anti-C4d Ab was also preincubated with human complement C3 α-chain (aa. 1303–1363) and anti-HCV core Ab with HCV E2-encoded protein. An irrelevant Ab (anti-human chorionic gonadotropin) was used as a further control. Immunostaining was also performed without the primary Abs. Five or more sections were examined for each case. The positive controls consisted of C4d-positive renal allograft nephrectomy sections and HCV-infected ex-planted liver.

**Statistic analysis**

Kruskall-Walls’ ANOVA, Student’s t tests, and linear regression analysis using Spearman’s correlation coefficient were performed.

### Table 1. Epidemiologic, virologic, and histologic data of 52 chronically HCV-infected patients with or without mixed cryoglobulinemia
A positive correlation between circulating gC1q-R and plasma levels of RF activity and C1q concentration in MC patients was shown. No relation was observed with circulating viral load, levels of nonenveloped HCV core, liver histology activity index, grade of fibrosis, and alanine aminotransferase activity. As compared with the noncryoglobulinemic group, MC patients displayed significantly higher serum mean levels of IgM ($p < 0.006$), RF activity ($p < 0.001$), and C1q protein ($p < 0.01$) and significantly lower mean levels of circulating C4 ($p < 0.001$).

When formed at equivalence, gC1q-R/HCV core complex was used to quantify its levels in clinical samples. As shown in Fig. 2, compared with HCV-related CAH without MC, patients with HCV-related MC had significantly higher levels ($31.5 \pm 31.5$ vs $63.9 \pm 10.6$ ng/10$^9$262 g total protein, $p < 0.001$) of gC1q-R/HCV core complex.

Because the binding site of C1q on C1q-R is distinct from that of the HCV core, it was expected that these proteins could bind simultaneously to the soluble receptor. Serum proteins were therefore eluted from polystyrene beads coated with gC1q-R recombinant protein and partially characterized. As shown in Table III, HCV core protein was indeed demonstrated in all aliquots recovered from gC1q-R-coated immunomagnetic beads, indicating that soluble gC1q-R circulates in a complex form containing HCV core protein other than C1q.

Binding to C1q protein is the primary requirement for the complement activation via the classic pathway, as incontrovertibly demonstrated by measuring the complement fragments generated during proteolytic cleavage of the C4 fraction. The C4d fraction is now regarded as a reliable activation marker of complement. The median serum C4d levels in the healthy controls ($1.89 \pm 0.39$ g/ml) were significantly higher than those in the MC patients ($1.33 \pm 0.36$ g/ml, $p < 0.006$), and lower than those in patients with HCV-related CAH without MC ($4.12 \pm 0.34$ µg/ml, $p < 0.001$). Notably, high statistical significance was found between patients with and without MC ($p < 0.001$). Lower levels in MC patients may reflect diversion near the sites of C4 activation. Thus, it could be inferred that C4d fragments could be documented within different biological compartments, where C4 is involved in the immune complex-mediated reactions. Skin biopsy samples from patients with cryoglobulinemic vasculitis were therefore explored by immunohistochemistry for the presence of HCV core and C4d deposition. As shown in Fig. 3, both HCV core and C4d

![FIGURE 1](http://www.jimmunol.org/)

**FIGURE 1.** Serum levels of soluble gC1q-R in sera of different categories of patients and in the healthy subjects.

![FIGURE 2](http://www.jimmunol.org/)

**FIGURE 2.** Circulating levels of gC1q-R/HCV core complex in sera of chronically HCV-infected patients.

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**Table II. Mean virologic, histologic, and immunologic parameters and pair-wise correlation with gC1q-R serum concentrations**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>HCV$^+$ with MC</th>
<th>HCV$^-$ without MC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulating HCV-RNA (IU/mL)</td>
<td>543.378 ± 671.750</td>
<td>1,392.417 ± 2,510.500</td>
</tr>
<tr>
<td>Nonenveloped HCV core protein (pg/mL)</td>
<td>288 ± 112</td>
<td>305 ± 160</td>
</tr>
<tr>
<td>Severity of liver disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Activity index</td>
<td>6.1 ± 1.8</td>
<td>5.8 ± 2.1</td>
</tr>
<tr>
<td>Stage</td>
<td>2.1 ± 0.8</td>
<td>2.9 ± 0.5</td>
</tr>
<tr>
<td>ALT (n.v.: 30–65 IU/L)</td>
<td>69.5 ± 42.5</td>
<td>41.5 ± 35.1</td>
</tr>
<tr>
<td>Immunological features</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cryocrit (%)</td>
<td>9.6 ± 12.3</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Serum IgM (n.v.: 40–230 mg/dL)</td>
<td>490.5 ± 656.4</td>
<td>78.1 ± 49.4</td>
</tr>
<tr>
<td>RF activity (n.v.: ≤ 20 UI/mL)</td>
<td>3,541.1 ± 1,161.4</td>
<td>3 ± 3.7</td>
</tr>
<tr>
<td>Complement, C4 (n.v.: 20–40 mg/dL)</td>
<td>4.8 ± 4.8</td>
<td>26.8 ± 19</td>
</tr>
<tr>
<td>Complement, C3 (n.v.: 90–180 mg/dL)</td>
<td>102.9 ± 85.7</td>
<td>105.2 ± 15</td>
</tr>
<tr>
<td>Complement, C1q (n.v.: 21–39 mg/dL)</td>
<td>44.8 ± 27.3</td>
<td>30.7 ± 5.9</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; n.v., normal values.
were present in the vessels with staining from the lumen and endothelia to the lamina propria. Experiments aiming at the detection of C3 fragment or its analogues in skin tissues have not been performed.

It was found that 84% (16/19) of skin biopsies from HCV-related MC patients displayed HCV core and C4d deposits, whereas no evidence of the signal was found in 11 samples from non-MC patients, nor in those from five healthy subjects.

The influence of HCV core protein on C4d production is depicted in Fig. 4. Serum samples were incubated with scalar concentrations of HCV core protein fixed on microwells. C4d production increased in step with the HCV core increments. The shape of the activating curve was indicative of saturable action, because C4d increased up to \(14 \mu g\) HCV core protein, in keeping with a dose-dependent effect. It was also demonstrated that HCV core protein directly activates the complement cascade. C1q-depleted serum, when incubated with scalar concentration of HCV core protein, generated C4d product in a dose-dependent manner. This indicates that HCV core protein alone activates complement, likely via the mannan-binding lectin pathway, and that higher concentrations of gC1q-R strengthen the activation of classic-mediated pathway via C1q. This concept was further confirmed by adding recombinant gC1q-R protein to normal sera containing very low levels of soluble gC1q-R protein.

We also assessed the in vitro effect of HCV core on PBL from patients with and without MC and from healthy subjects. Isolated lymphocytes were stimulated with PHA in the presence and absence of HCV core, and the synthesis and secretion of gC1q-R in the culture supernatants were measured. Results showed 50–90% inhibition of proliferation by HCV core in response to PHA stimulation in all categories (Fig. 5). Surprisingly, gC1q-R concentrations were greatly increased in the supernatants of HCV-related MC patients (62 ± 22 ng/ml), whereas very small increments were demonstrable in the non-MC group (22.8 ± 2.8 ng/ml). In addition, no significant variations were found in healthy controls (7.0 ± 2.0 ng/ml). These results are strongly indicative of an intrinsic difference in the regulation and secretion of lymphocyte-derived gC1q-R by HCV core proteins in these categories.

Frequency of cell surface gC1q-R was then examined in PBL of different groups by FACS analysis. Notably, the percentage of gC1q-R-expressing cells was significantly higher in MC patients (56 ± 23%), as compared with HCV-related CAH without MC (14 ± 6.6%) and the healthy controls (4.2 ± 2.2%, \(p < 0.001\)).

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**FIGURE 3.** Localization of HCV core (A) and C4d (C) proteins in skin biopsies of HCV-positive MC patients. Reactivity of control sections (B and D) is shown for comparison. Core protein is mainly located within the vessel lumen, whereas C4d is expressed along the walls. Note complete negativity of control sections.

**FIGURE 4.** In vitro effect of HCV core protein on C4d production. Scalar concentrations of HCV core protein were applied in microwells and C4d generation was determined after incubation with HCV-positive serum samples (□). C1q-depleted human serum (■) obtained from Quidel (lot 904184) was also incubated and C4d generation was quantified. Serum samples from a healthy subject before (○) and after (●) gC1q-R protein enrichment (2 ng/µl) were also tested.

**FIGURE 5.** Inhibition of PBL proliferation by HCV core protein. **Upper panel,** Degree of inhibition of PHA-induced proliferation of PBL in different categories. **Lower panel,** Relative concentrations of gC1q-R in the PBL culture medium.

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**Table III. Reactivity of serum proteins eluted from microparticles coated with gC1q-R protein in different categories**

<table>
<thead>
<tr>
<th>Patients</th>
<th>No.</th>
<th>gC1q-R BINDING</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCV+ with MC</td>
<td>10</td>
<td>10 (100)</td>
</tr>
<tr>
<td>HCV+ without MC</td>
<td>10</td>
<td>10 (100)</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

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line with this higher gC1q-R expression in the PBL of HCV-related MC patients, quantitative real-time RT-PCR confirmed the occurrence of greater amounts of gC1q-R mRNA in their cells. As shown in Fig. 6, specific gC1q-R mRNA expression was 6- to 10-fold higher in PBL from MC patients than in the non-MC group and the healthy controls. It is notable that differential regulation of the PBL response to HCV core-mediated gC1q-R release of the soluble form was strictly related to increments of specific mRNA. In vitro experiments showed that gC1q-R mRNA expression progressed in a dose-dependent manner to the HCV core-induced suppression of cell proliferation. The magnitude of gC1q-R expression may thus be predetermined by intrinsic differences in the gC1q-R gene regulation in MC.

Immunological, virological, and clinical parameters were monitored during and after pegylated IFN-α and RBV combination therapy. The complete response obtained in 15 of /32 (47%) HCV-infected patients without MC and in healthy subjects. It is notable that differential regulation of the PBL response to HCV core-mediated gC1q-R release of the soluble form was strictly related to increments of specific mRNA. In vitro experiments showed that gC1q-R mRNA expression progressed in a dose-dependent manner to the HCV core-induced suppression of cell proliferation. The magnitude of gC1q-R expression may thus be predetermined by intrinsic differences in the gC1q-R gene regulation in MC.

Discussion

These data show that in MC patients, gC1q-R is shed in the plasma as a bioactive molecule with amounts significantly higher than those in HCV-infected patients without MC and in healthy subjects. They also demonstrate that in the MC patients, soluble gC1q-R circulates as a complexed form containing both C1q and HCV core protein.

HCV core binds the gC1q-R region spanning aa 188 to 259, while its interaction site encompasses residues 26–124 (15). Furthermore, the globular head complex of C1q hexamer, a highly positively charged molecule, binds the NH2 terminus portion of gC1q-R spanning aa 74 to 95 (44). Insights into the structural basis of these interactions may be derived from the reported crystal structure of gC1q-R. It has been made clear that three gC1q-R molecules form a doughnut-shaped, quaternary structure with a sizable central channel and an asymmetric charge distribution on the surface, including exposed acidic residues in the COOH-terminal portion of the molecule and in the NH2-terminal α-helical domain (45). These negatively charged residues constitute the binding site for the positively charged HCV core protein.

C4d is a low-molecular-weight fragment cleaved from the C4 fraction during activation of the classic complement pathway (46). It is found in the sera of most patients with rheumatoid arthritis (47). Curiously, its serum levels in MC patients are lower than those in HCV-infected patients without MC or in healthy subjects. This may be due to diversion of C4d fragments. Splitting of C4d, in fact, reveals a thiostere group that leads to the formation of a covalent bond beside the site of C4 activation (48). Our examination of skin biopsy samples showed that C4d deposits were present in almost all MC patients and none of those without MC. Thus, low circulating C4d levels in MC patients are likely attributable to sequestered fragments in the vascular bed, whereas patients without MC are the biological counterpart, because higher C4d levels are associated with the absence of intratissue deposits.

HCV core protein suppresses the host immune response by engaging gC1q-R on the surface of immune cells (15–16). We readily detected it in the plasma of HCV-infected patients with or without MC, and extensive inhibition of mitogen-stimulated proliferation of PBL by recombinant HCV core protein was demonstrated in both groups. In vitro experiments disclosed a unique property of MC patients in that, in step with HCV core inhibition of the PBL proliferative response, large amounts of soluble gC1q-R were released in the culture supernatants. Very small increments were noted in non-MC patients and no changes from basal levels were demonstrated in healthy subjects. This strongly indicates that mechanisms underlying gC1q-R synthesis and release from PBL are HCV core-mediated and negatively regulated by cell proliferation. No direct relation was found between levels of nonenveloped HCV core and those of soluble gC1q-R, probably

Table IV. Levels of gC1q-R and C4d fragment in the sera of 32 HCV-positive MC patients before and after pegylated IFN-α plus RBV combination therapy

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Responders (n = 15)</th>
<th>Nonresponders (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before (mean ± SD)</td>
<td>After (mean ± SD)</td>
</tr>
<tr>
<td>gC1q-R (ng/ml)</td>
<td>56.3 ± 22.4</td>
<td>24.6 ± 6.7</td>
</tr>
<tr>
<td>C4d (ng/ml)</td>
<td>1.26 ± 0.11</td>
<td>4.13 ± 0.3</td>
</tr>
</tbody>
</table>
due to the saturable-effect of binding sites. However, we do not yet know why gC1q-R expression levels differ in patients with MC as compared with those without. This may be the result of transcriptional control of gC1q-R gene. The underlying reasons for the difference are not clear, but genetic difference in host gene regulation could be an explanation. Higher levels of soluble gC1q-R are, in fact, demonstrable in both HCV-positive and -negative MC patients, and quantitative real-time RT-PCR assay for specific gC1q-R mRNA determined an expression which was 6- to 14-fold higher in cells from MC than in those from non-MC. These results paralleled those obtained from our investigation of gC1q-R expression on PBL. The percentages of cells expressing gC1q-R in MC patients were 3- to 4-fold higher than those in non-MC patients, indicating that synthesis and release of gC1q-R protein in PBL is dependent on up-regulation of the gC1q-R gene.

At variance from previous data showing that agents or proteins inducing cell proliferation increase the amount of gC1q-R in the surrounding milieu (21), the present results indicate that release of soluble gC1q-R is regulated by core-mediated inhibition of cell proliferation. They thus point to the existence of a new mechanism capable of modulating its expression.

Soluble gC1q-R may have a deep impact on the clinical features, in that it acts as a bridging molecule responsible for linking apoptotic cells (49), and may activate endothelial cells and platelets (50–51) and initiate intrinsic blood coagulation and the kinin-generating pathway (52). Our observations shed new light on the pathogenetic mechanisms underlying cryoglobulin-induced vascular damage. HCV core protein generates C4d in serum depleted of C1q protein, indicating that it directly activates complement cascade likely via mannan-binding lectin pathway. Thus, it can be concluded that, in the presence of high levels of circulating gC1q-R, HCV core protein exacerbates the inflammatory condition by combined and simultaneous activation of both complement pathways. Endothelial cells are then activated with consequent initiation of a local inflammatory reaction.

Consistent with this model is the feature of regression of cryoglobulinemic vasculitis following the antiviral therapeutic response. Clinical response is biologically characterized by significant decrement of soluble gC1q-R and increment of serum C4d fragment levels, strongly suggesting disengagement of C4 complement fraction and inhibition of the C1 activation pathway. Efficacy of IFN-α-RBV combination therapy in the form of a dramatic improvement of cryoglobulin-related signs and symptoms, remarkable decrement of the cryocrit, and drastic lowering of viral load resulted in significant lowering of soluble gC1q-R and, conversely, an increase of serum C4d concentrations.

Further investigations, however, are needed to determine the precise activating conditions that result in HCV core-mediated release of gC1q-R. Once released, it regulates complement activation, which leads to consumption and deposition of C4d fragment levels, strongly suggesting disengagement of C4 complement component which results in HCV core-mediated repression. Clinical response is biologically characterized by significant lowering of soluble gC1q-R and, conversely, an increase of serum C4d concentrations.

Disclosures

The authors have no financial conflict of interest.

References

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