A comprehensive study of the association between hepatitis C virus and glomerulopathy

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Abstract

Background. Hepatitis C virus (HCV)-related infection is commonly associated with a wide range of glomerulonephritides (GN) including membranoproliferative glomerulonephritis (MPGN). The causal link between HCV infection and renal disease has been postulated through the induction of cryoglobulinaemia and secondary GN. However, the detection of viral particles or genomes within the kidneys of HCV-infected patients has proved to be difficult. With that in mind, we have studied a population of Egyptian HCV-positive patients with associated GN in an attempt to detect viral particles, antigens or RNA within their kidneys.

Methods. Fifty patients were found to be HCV positive out of 303 who presented with a glomerulopathy between 1998 and 1999 at the Mansoura Urology and Nephrology Center, Egypt. Comprehensive investigations of these 50 patients were undertaken including an evaluation of their clinical, biochemical, histological, virological and immunological parameters. In addition, their kidney biopsy material was analysed by electron microscopy (EM) to detect viral particles, by immunohistochemistry to detect a viral core antigen and by RT–PCR to detect RNA. This was compared with 50 HCV-negative controls.

Results. Positivity for HCV antibodies was higher among patients with GN (38%) compared with healthy blood donors (16%). Genotype 4 was sequenced in 70% of the HCV-positive samples examined. MPGN was the most common type of GN accounting for 54% of patients. Extrarenal manifestations were absent in the majority (80%) of patients even though 54% had cryoglobulinaemia. EM revealed virus-like particles in 50% of biopsies. Immunohistochemistry failed to reveal HCV-related antigens in kidney sections. HCV RNA was detected in the cryoprecipitates in 66% of patients and 22% of frozen renal sections. Control sections were negative.

Conclusion. Our findings suggest a causal link between HCV and GN based on the observation of virus-like particles as well as viral RNA within the kidney sections of patients with HCV-associated glomerulopathies.

Keywords: cryoglobulinaemia; glomerulopathy; hepatitis C virus; membranoproliferative glomerulonephritis

Introduction

Hepatitis C virus (HCV)-related infection represents a major public health problem worldwide [1]. In Europe overall, the proportion of blood donors carrying anti-HCV antibodies ranges from 0.1 to 1.5%, with a north to south gradient and a higher prevalence in the Mediterranean countries. Canada and the USA have a prevalence of around 0.03% with a higher prevalence observed in the Far East (1–2%). In Africa, the range is wider with figures oscillating between 0.05 and 10%, exceeding 20% in some areas [2]. In Egypt, the disease has reached epidemic proportions with up to 21.9% of the population affected [3]. This has been recently attributed to the systematic treatment of the population in the 1960s and 1970s with parenteral administration of anti-schistosoma drugs [4].

Several extrahepatic syndromes have been associated with chronic HCV infection including mixed essential cryoglobulinaemia (MEC) and a wide range of glomerulonephritides (GN) [5]. Amongst the different types of GN affecting patients infected with HCV, membranoproliferative GN (MPGN) is thought to be the most common [6]. An association has been described between this nephropathy and the presence...
of cryoglobulinaemia [7]. In spite of such associations, a direct link between the actual HCV infection and the nephropathy has been hard to prove in view of the difficulty in identifying the HCV within the kidneys [8]. While Johnson and collaborators failed to identify HCV antigens or RNA in the glomerular lesions [6], Yamabe and his colleagues [9] have localized the C22 antigen of HCV to the glomerular lesions in patients with MPGN. Similarly, Stokes and coworkers have located HCV antigens or RNA in the glomerular lesions of HCV-positive patients co-infected with HCV and HIV [10].

With the above data in mind, we have studied a population of HCV-infected individuals with nephropathy in an attempt to identify the viral particles, their core antigen and RNA within their kidneys.

Subjects and methods

We studied systematically all patients presenting to the Mansoura Urology and Nephrology Center, Mansoura, Egypt, during a 2 year period (1998–1999), with urinary symptoms justifying a renal biopsy. A total of 303 patients were studied for the presence of an associated HCV infection. These patients were screened for HCV antibodies by a third generation ELISA technique (Abbott Diagnostics, Maidenhead, UK). Verification of HCV infection was detected by polymerase chain reaction (PCR) (Amplicor PCR Diagnostics, Roche Diagnostic System, Lewes, UK). We identified 50 HCV-positive patients. These patients had clinical and laboratory investigations including urinalysis, serum creatinine and electrolytes as well as liver function tests. Rheumatoid factor and cryoglobulin detection was carried out using standard methods. Additional virological studies included testing for hepatitis B virus antibodies by ELISA (Abbott Diagnostics) and testing for anti-HIV 1 and 2 antibodies by the Murex HIV 1 and 2 immunoassay. One hundred patients were prospectively biopsied, 133 had archival renal biopsy material and 70 patients had no renal biopsy material available for analysis.

In the 50 HCV-positive patients other causes of GN were excluded by testing for diabetes mellitus (fasting and 2 h post-prandial blood glucose), autoimmune diseases (serum autoantibodies) and schistosomiasis (schistosomal circulating cathodic antigen by the double antibody sandwich ELISA technique). Renal biopsy was carried out in all 50 HCV-positive patients.

Genotyping

HCV genotyping was performed for 10 patients, serum samples were analysed by reverse transcription polymerase chain reaction (RT–PCR) using primers specific for HCV core region and five NCR products were sequenced with the ABI prism DNA sequencing kit (PE Applied Biosciences, Warrington, UK).

Light microscopy

For light microscopy, renal specimens were fixed in formalin and embedded in paraffin before 4 μm sections were cut and stained with haematoxylin and eosin.

Electron microscopy

In eight patients (diagnosed by light microscopy to have MPGN in four, focal segmental glomerulosclerosis [FSGS] in two and mesangioproliferative GN in two), renal biopsy sections were examined by electron microscopy (EM). For EM, renal cores were preserved in 3% phosphate-buffered glutaraldehyde, diced into 1 mm cubes, rinsed in distilled water, transferred into 1% aqueous osmium tetroxide and embedded in TAAB Emix resin. Sections of 0.5 μm were cut, mounted on glass slides, stained in 1% aqueous toluidine blue in 1% sodium tetraborate for 15 s on a hot plate at 15°C, cooled and examined by light microscopy for assessable glomeruli before being sectioned with a diamond knife in a Leica Ultracut E ultramicrotome, with interface colour gold of approximately 95 nm. Sections were stained by immersion for 7 min in 50% alcoholic saturated uranyl water, 3 min in Reynolds lead citrate and three washes in distilled water, and examined by a Philips 400 transmission electron microscope.

Immunohistochemistry

Immunohistochemistry was carried out on formalin-fixed and paraffin-embedded renal sections as well as on frozen renal cores (10 sections from patients with MPGN type I, four type II, three FSGS, two membranous nephropathy (MN), one mesangioproliferative GN). For paraffin sections, paraffin was removed by xylene, tissue rehydrated by immersion in decreasing grades of ethanol and endogenous peroxidase activity was inhibited by freshly prepared 3% hydrogen peroxide in absolute methanol. Sections were incubated with blocking serum (Vectastatin ABC kit, Vector Laboratories, Inc., Peterborough, UK). Overnight incubation (18 h) at 4°C with the primary antibody (monoclonal antibody to HCV core AA 1–120, Biogenesys Ltd, Poole, UK) was undertaken at different dilutions (1:50, 1:100, 1:250 and 1:300) in order to choose an optimal antibody concentration. ABC reagent was added (reagent A: avidin DH, reagent B: biotinylated horseradish peroxidase H. Vectorstain ABC Kit, Vector Laboratories, Inc.) and colour development was by the addition of aminochrome-bazole (AEC reagent). The sections were counterstained with haematoxylin and eosin, and examined by light microscopy.

For frozen renal tissues, 5 μm cryosections were cut and stored at –80°C before staining and fixation in cold acetone at 4°C for 10 min before washing three times with phosphate-buffered saline (PBS) (5 min each). The rest of the steps were as described above for the paraffin-embedded blocks.

Different methods to enhance antigen retrieval were tried including heat (56°C), incubation with proteinase K in PBS 20 μg/ml at 37°C for 15 min and acid electroelution as described elsewhere [11].

Reverse transcription–polymerase chain reaction

RT–PCR was performed on cryoprecipitates, when available, paraffin-embedded renal sections as well as frozen renal tissues. In brief, for formalin-fixed paraffin-embedded sections, RNA was extracted by tissue dewaxing in xylene followed by alcohol washes. In preliminary experiments two different methods for extraction were tried.

(i) Guanidinium isothiocyanate method. Sections were mixed with 200 μl guanidinium isothiocyanate. Four different
choices were tried with short (15 min, at room temperature) and long (overnight, at 4°C) incubations.

(ii) Proteinase K digestion method. Proteinase K buffer (100 mM NaCl, 10 mM Tris pH 7.4, 25 mM EDTA pH 8) was autoclaved for 24 h with 5% sodium dodecyl sulphate. Ten millilitres of the buffer was prepared by adding 1 ml from each solution to 6 ml PCR water. The digest was done in 200 μl volume and different concentrations were tried (0.5–5 μg/ml).

RNA was extracted by the proteinase digestion method (100 μg/ml) not by the guanidinium isothiocyanate method.

For cDNA synthesis 4 μl RNA was heated to 65°C, chilled on ice, added to 3.5 Master Mix aliquots (First Strand cDNA Kit, Pharmacia) and incubated at 37°C. A second PCR with nested inner primers was performed utilising 1 μl of the first PCR product as DNA template. The amplification cycles were identical to the first one [12].

Statistical analysis

Results are given as median and confidence intervals. Mann–Whitney (non-parametric rank sum) tests, Fisher exact probability tests and χ² tests were applied as appropriate. A P value of <0.05 was considered significant.

Results

In the 303 patients (190 males and 113 females) who presented with renal symptoms, glomerulopathy was suspected clinically by urinary abnormalities. Some (n = 133) of these patients had a previous renal biopsy before their HCV status was determined. One hundred patients with either positive or negative HCV serology were prospectively biopsied and tested for HCV antibodies by a third generation ELISA. Positivity for HCV antibodies was higher among the study group (116 of 303; 38%) compared with healthy blood donors (16%), P <0.05. Demographic, biochemical and serological profiles of the 50 HCV-positive patients studied are given in Table 1.

Clinical manifestations

Among the 50 HCV-positive patients studied, 30 presented with proteinuria in the nephrotic range (60%), while 20 presented with non-nephrotic range. Hypoalbuminaemia (serum albumin <3.0 gm/dl) was present in 33 patients (66%). In 54% of patients, MPGN was diagnosed (MPGN type I in 18 patients, MPGN type II in nine patients) while 24% had FSGS, 18% mesangioproliferative GN, and 4% had MN. In the remaining HCV-negative patients MPGN (48%), FSGS (30%), MN (4%) or mesangioproliferative GN (18%) were diagnosed.

Despite the fact that 13 patients had elevated liver enzymes, clinical manifestations of hepatic disease were not frequent and only five had hepatomegaly. No other manifestations of liver disease were observed (Table 1).

At presentation, 27 patients tested positive for cryoglobulins; however, in 26 of them cryoprecipitates were typed and shown to contain both IgM and IgG. In 80% of the cryoglobulinaemic patients extrarenal manifestations of cryoglobulinaemia were absent. Arthralgias were present in 8%, purpura in 7% and peripheral neuropathy in 5% of the cryoglobulinaemia-positive patients.

Virological studies

In all patients, the diagnosis of HCV infection was confirmed by detection of HCV antibodies using third generation ELISA, and HCV RNA in serum by RT–PCR as a confirmatory test. The testing was also

Table 1. Demographic, biochemical and serological profile, and histological characteristics of the 50 patients studied

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Data</th>
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<tbody>
<tr>
<td>Gender (male/female)</td>
<td>35/15</td>
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<tr>
<td>Risk factors for HCV acquisition</td>
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<tr>
<td>Past history for hospital admission</td>
<td>27</td>
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<tr>
<td>Past history of blood transfusion</td>
<td>21</td>
</tr>
<tr>
<td>Past history of surgery</td>
<td>25</td>
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<tr>
<td>Clinical findings</td>
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<td>Arterial hypertension</td>
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<tr>
<td>Hepatomegaly</td>
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<tr>
<td>Biochemistry (median and confidence interval)</td>
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</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>1.2 (1.15–1.47)</td>
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<tr>
<td>Serum cholesterol (mg/dl)</td>
<td>220 (226.08–310.04)</td>
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<tr>
<td>Serum bilirubin (mg/dl)</td>
<td>0.5 (0.45–0.94)</td>
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<td>Serum total protein (mg/dl)</td>
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<td>Serum albumin (g/dl)</td>
<td>2.7 (1.86–4.57)</td>
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<tr>
<td>Proteinuria 24 h (g)</td>
<td>3.7 (3.66–6.00)</td>
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<tr>
<td>Alanine aminotransferase (IU/l)</td>
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<td>50</td>
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<td>HCV RNA</td>
<td>50</td>
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<tr>
<td>Histology</td>
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<tr>
<td>MPGN type II</td>
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<tr>
<td>FSGS</td>
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</tr>
<tr>
<td>MN</td>
<td>2</td>
</tr>
<tr>
<td>Mesangioproliferative GN</td>
<td>9</td>
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</table>

C3, third component complement; C4, fourth component complement.
undertaken on the cryoprecipitates from 25 patients, 17 of which were positive.

Genotyping was performed for 10 randomly chosen samples, in all of them RT-PCR products were detected. However, only seven PCR products had sufficient quantities of cDNA for sequencing. All were classified as type 4 according to Simmonds and colleagues [13].

**Detection of HCV antigen and RNA in tissue specimens**

**Immunohistochemistry.** Immunohistochemistry was done on frozen and formalin-fixed paraffin-embedded renal sections from 20 patients who were HCV positive. Pre-treatment of the sections with heat or trypsinization did not affect the outcome as the samples remained negative.

**Electron microscopy.** Immune complexes were detected in the subendothelial space and paramesangial areas (Figure 1a). Virus-like particles were located inside the electron-dense deposits in 50% of cases (Figures 1b and c). In the three HCV-negative control patients with MPGN, neither electron-dense deposits nor virus-like particles were detected.

**RT–PCR in renal sections.** In frozen renal biopsies of 21 cryoglobulinaemic patients, four were positive for HCV RNA (Figure 2a). In non-cryoglobulinaemic patients, five out of 21 were positive (Figure 2b). In each run positive and negative controls were included.

**Discussion**

Since HCV was identified, it has been recognized as a major cause of chronic hepatitis and cirrhosis [14]. In our study, the prevalence of HCV antibodies, detectable by a third generation ELISA, was 16% among healthy blood donors, which confirmed the high prevalence among the Egyptian population [3]. This is in agreement with the observations of Darwish and his colleagues [3] who reported a prevalence of 13.6%. Recent evidence points to the systematic treatment of the Egyptian population with parenteral anti-schistosoma injections in the 1960s and 1970s as a possible factor in the transmission of the infection and its current high prevalence rate in Egypt [4].

The seroprevalence of hepatitis C is thought to be greater in individuals with chronic GN compared with those suffering from other renal diseases [15]. The results of our study confirm this since we found a higher prevalence of HCV antibodies among patients presenting with glomerulopathy (38%).

In our study, light microscopy examination revealed a high incidence of MPGN. The high prevalence of cryoglobulinaemic and non-cryoglobulinaemic MPGN observed in our patients has been previously reported by others [6,8]. An association between HCV infection and FSGS has also been reported [16]. In fact, the high prevalence of FSGS in our series (24%) may reflect a real association between HCV and this type of renal lesion or it may reflect a selection bias. Only 4% of our patients had MN, with nephrotic-range proteinuria, mild elevation of liver enzymes, normal complement level, negative for both cryoglobulins and rheumatoid factor, which is consistent with previous observations [17]. Other forms of renal disease that have been previously linked to chronic HCV infection include acute exudative and proliferative glomerulonephritis and IgA nephropathy [18]. Of interest, the distribution and types of GN in the HCV-negative patients was comparable to those with seropositivity.

The prevalence of cryoglobulinaemia in our series of HCV-positive patients was 54%. However, despite the presence of cryoglobulinaemia, extrarenal manifestations were absent in the majority of patients (80%). It has been suggested that HCV infection is responsible for all cases of type II MEC and that the idiopathic (essential) type no longer exists [8,19]. In the two large studies of patients with type II MEC and MPGN the prevalence of HCV RNA was 81 and 100% [8,19]. Our data agree with Johnson and his colleagues [6], who found the prevalence to reach 62% in their patients. The same authors described a prevalence of 59% in a series of patients with HCV-associated nephropathy at presentation. However, an additional 26% developed cryoglobulinaemia during the subsequent months [20]. The same findings were described by Lunel and coworkers, who found 54% of patients with HCV-associated nephropathy to have type II cryoglobulinaemia [21].

In this study, we were not able to locate any virus-related proteins in formalin-fixed, paraffin-embedded or frozen renal biopsies. The negative renal results were in spite of the application of a variety of techniques for antigen retrieval on both paraffin-embedded and frozen sections. This failure to locate viral antigens in the kidney is in agreement with most of the reported literature [6,10,20] with few exceptions [11,22]. Sansonno and coworkers [11], in 12 patients with MPGN and cryoglobulinaemia, found HCV-specific immunoreactive deposits in 66.7% of kidney biopsy specimens. In fact, these authors used a panel of HCV monoclonal antibodies including anti-C22-3, anti-envelope glycoprotein2 (E2)/NS1 antibody, anti-C33 antibody and a mixture of anti-C100 and anti-NS5 antibodies [11]. This may explain their positive results.
compared with other studies, including our own, where only one antibody was tested. Although some investigators have been unsuccessful in identifying viral proteins in renal specimens [6,20,21], others reported the detection of such proteins in the renal tissues of patients with HCV-associated glomerulonephritis [10,22]. Clearly, the presence of virus-like particles within the glomeruli does not necessarily implicate it in the pathogenesis of renal disease; rather it may simply represent antigen-trapping within an area of tissue damage [6,10].

The most reliable method for the diagnosis of HCV infection is the detection of viral RNA sequences by RT–PCR in serum and tissue samples [1]. We were unable to detect HCV RNA in formalin-fixed, paraffin-embedded renal specimens. This may be explained by the fact that HCV is an RNA virus and is therefore more labile than DNA viruses, making it susceptible to damage by the process of fixation and prolonged paraffin embedding [23].

Interestingly, HCV RNA was extracted and amplified successfully in frozen renal tissues from nine of our patients (22%), both cryoglobulinaemic and non-cryoglobulinaemic, representing most of the histological classes included in our study (four MPGN, two MN, two FSGS and one mesangioproliferative GN). Failure to detect HCV RNA in the remaining samples could be explained by the presence of only a small amount of RNA, which may be below the detection sensitivity of the method used, or simply by the absence of HCV RNA in renal tissues. Our results for HCV RNA yield in renal tissues is much lower than those described by Stockes and colleagues [10], who were able to extract and amplify HCV RNA in 58% of their patients. However, their patients were co-infected with HIV raising the possibility that such a co-infection may lead to the over-expression of HCV RNA. Also the signals obtained by RT–PCR could be due to circulating HCV-infected leukocytes within the kidney [24].

Previous studies have shown that aggregates or single virus-like particles of about 45 nm in diameter were detectable in the endoplasmic cisternae and in the cytoplasm of hepatocytes of HCV-infected patients [25]. Similarly, virus-like particles were demonstrated in human T- and B-lymphocyte cell lines [26]. We have demonstrated virus-like particles in the paramesangial electron-dense deposits by EM in 50% of patients studied. These particles were comparable to those previously described in the paramesangial dense deposits in a patient with HCV-related proliferative glomerulonephritis [27]. Their characteristic features support their classification as HCV-related particles.

In conclusion, HCV infection is frequent among the Egyptian population affecting about 16% of the whole population. This prevalence is higher among patients...
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with chronic GN. There is a documented link between cryoglobulinaemic and non-cryoglobulinaemic MPGN and chronic HCV infection. A possible role for HCV infection in other forms of GN requires further investigation.

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