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RESEARCH

Brief definitive reports

A rapid direct microwave irradiation technique for demonstrating hepatitis C virus-associated C100 protein in formalin-fixed liver tissue

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Summary. Hepatitis C virus-associated C100 protein was demonstrated in formalin-fixed, paraffin-embedded liver tissue from seven patients with non-A,non-B post-transfusion-related chronic liver disease, using a monoclonal antibody, biotin-streptavidin immunostaining and microwave irradiation. C100 antigen expression was limited to hepatocyte cytoplasm but was found in cells with morphological evidence of virus-induced damage and in apparently healthy cells. Positive cells were scattered throughout lobules and cirrhotic nodules with no special relation to vascular or portal structures. No staining was found in bile duct epithelium and sinusoidal cells. The method is suitable for both retrospective studies and diagnostic purposes.

Key words: Chronic hepatitis – C100 antigen – Hepatitis C virus – Microwave irradiation – Paraffin-embedded tissue

Introduction

Hepatitis C virus (HCV) has been identified as a major cause of non-A,non-B virus acute and chronic hepatitis [2]. Currently diagnosis is based on the detection of serum antibody to a recombinant virus-specific 363 amino acid polypeptide, named C100. This is the product of a non-structural portion of the virus genome and represents a small proportion of the total viral protein [5].

Direct demonstration of HCV has not yet been possible, despite intensive research, probably because its circulating level is below the sensitivity threshold of current assay techniques [1, 4]. However, amplification of the HCV RNA by the polymerase chain reaction has enabled viral-specific sequences to be detected in both liver and plasma [3, 7].

By using monoclonal antisera against C100 protein in association with microwave irradiation, we have increased the sensitivity of current immunohistochemical methods and demonstrated HCV C100 antigen in formalin-fixed, paraffin-embedded liver tissue.

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Patients and methods

Seven patients (3 males, 4 females) aged 29–70 years (mean 51 years), with a history of symptomatic post-transfusion non-A,non-B acute hepatitis, were included in this study. All patients underwent transcutaneous liver biopsy for diagnostic purposes. Histology revealed chronic active hepatitis with cirrhosis in five patients and chronic active hepatitis without cirrhosis in two patients. Serum samples were obtained at the time of liver biopsy, and all were positive for anti-HCV antibody by enzyme-linked immunosorbent assay as confirmed by four antigen recombinant immunoblot assay. Both kits were obtained from Ortho Diagnostic Systems (Raritan, N.J., USA).

Four patients showed serological evidence of anti-hepatitis B virus antibodies (i.e. anti-HBs and anti-HBc); the remaining three were negative for all HBV-associated markers. No patient had circulating auto-antibodies (i.e. anti-nuclear antibodies, anti-mitochondrial antibodies and rheumatoid factor).

Liver biopsy tissue used in this study had been routinely fixed in 10% buffered formalin and embedded in paraffin wax; it was not especially prepared. Sections (5 μm) were cut, attached to glass slides coated with 0.01% poly *L*-lysine (Sigma, Milan, Italy) and dried overnight at 37°C. Deparaffinization was carried out with xylene (30 min × 2). Sections were then placed in absolute alcohol (10 min × 2), 95% alcohol (10 min × 2) and 70% alcohol (10 min × 2). After shaking off the excess liquid, the slides were rinsed in distilled water for 20 min and then placed in 0.05 TRIS-HCl-buffered saline pH 7.6 (TBS).

To block endogenous peroxidase the sections were incubated with 7.5% hydrogen peroxide for 20 min at room temperature (RT). Endogenous avidin-binding sites were blocked by incubating the sections with 1 mg/ml avidin (Sigma) in TBS for 20 min at RT. After washing with TBS, they were further incubated with 0.1 mg/ml biotin (Sigma) in TBS for 20 min at RT. To block non-specific binding sites, normal swine serum and bovine serum albumin diluted 1:20 were applied to the sections that were incubated in a moist chamber for 15 min at 37°C. Thereafter, specimens were covered with murine monoclonal antisera against C100 protein obtained from Dr. M. Nelles (Ortho). Products of the following four clones were used: 2C4G3 (IgG1), 20A6F3 (IgG2a), 22A5B12 (IgG2a), 18A1D5 (IgG1).

Pilot experiments indicated that the best results could be obtained by incubating with 2C4G3 at a concentration of 100 μg/ml for

2 h at 37 °C. After rinsing with TBS, specimens were incubated with a goat anti-mouse biotin-conjugated antiserum (Janssen, Beerse, Belgium) at a dilution of 1:1000 (5 μg/ml) for 30 min at 37 °C. Excess reagent was then removed and slides were washed in TBS. Specimens were then covered with streptavidin-horseradish peroxidase complex (streptavidin-HRP, Janssen) diluted 1 : 200 for 30 min at 37 °C. After several rinsings with TBS, sites of enzymatic activity were visualized with 3-amino-9-ethylcarbazole dissolved in *N*, *N'*-dimethylformamide, and hydrogen peroxide. Sections were then counterstained with Mayer's hematoxylin and mounted in aqueous medium.

In a parallel experiment, sections were heated by direct microwave irradiation using a commercially available oven (Biorad, model H2500). During this procedure, immunostaining incubation periods were shortened as follows: anti-C100 antiserum was reacted for 30 min, while the biotinylated antiserum and streptavidin-HRP complex were reacted for 15 min each.

Controls included: (1) analogous staining procedures on tissue sections from five normal livers and ten patients with non-HCV-related chronic active hepatitis, (2) replacement of the primary antibody with an unrelated monoclonal antibody (IgG1 from HCG clone 10A5H7 at a similar concentration), (3) omission of the biotinylated antiserum.

Abolition or a significant reduction of the positive signal was also obtained by pre-incubation of the anti-C100 antiserum with a suspension of purified C100 antigen (pHCV-23 from Abbott, North Chicago, Il., USA). An aliquot of the 2C4G3 clone was incubated overnight with an equal aliquot of recombinant C100 protein. Immune complexes were precipitated by polyethylene glycol and supernatant was tested on liver sections. Parallel tests included pre-incubation with other viral suspensions (i.e. HBcAg and HBsAg).

All liver sections were tested for the presence of HBsAg and HBcAg by the indirect immunoperoxidase method as described in detail elsewhere [6], and were found to be negative.

Results

The method used in this study enables C100 antigen to be detected in paraffin-embedded liver tissue. As indicated in Fig. 1, microwave irradiation significantly reduced the time necessary for the immunostaining reactions and enhanced the sensitivity of the technique. C100 antigen was detected in liver tissues of all seven patients with post-transfusion-HCV-related chronic liver disease, whereas the conventional procedure allowed detection in five patients only.

C100 antigen was only located in the cytoplasm of hepatocytes with a diffuse homogeneous pattern. No histochemical picture associated with membrane localization of antigen was demonstrable. No staining was found in bile duct epithelium or in sinusoidal cells. Topographical distribution of C100-antigen-positive cells was extremely variable; strongly positive zones alternated with completely negative areas or with sporadic cell positivity. No apparent relationship to either portal tracts or central veins was found. A coarse granular pattern of peroxidase staining was observed, associated with either hydropic, swollen hepatocytes containing small, apparently fat vacuoles, or morphologically intact hepatocytes (Fig. 2).

Cells containing C100 antigen were sometimes involved in cytolytic "foci" in very close contact with inflammatory cells, but were more frequently observed far from damaged areas.

Discussion

The advent of a specific test for the major agent of non-A,non-B hepatitis virus infection represented a fundamental breakthrough in the diagnosis of transfusion-associated non-A,non-B viral liver disease.

Type I non-A,non-B virus, now named HCV, has been shown to be a small, chloroform-sensitive agent, capable of inducing characteristic cytoplasmic tubular structures in human and experimentally infected chimpanzee liver [1]. Despite previous results showing the biological inactivation of the non-A,non-B tubule-forming agent by formalin and heat [8], we have demonstrated that C100 antigenicity is well preserved during formalin fixation and heating (58 °C – 60 °C) for paraffin embedding.

The results of our immunohistochemical study allow us to draw preliminary conclusions about the nature of HCV infection in chronic progressive liver disease. HCV-infected cells are not only morphologically damaged hep-

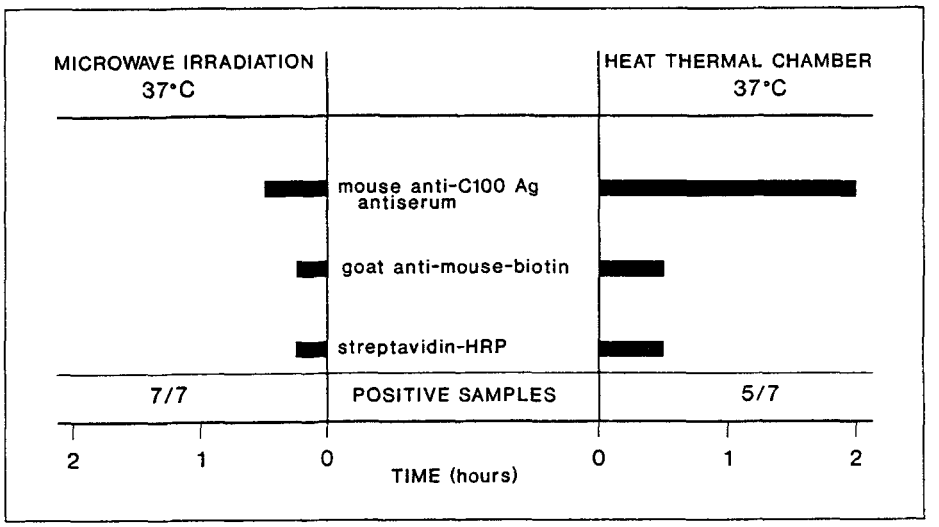

Fig. 1. The microwave irradiation procedure significantly shortens the incubation time of immunostaining and increases the sensitivity of the methods. *Streptavidin-HRP*, Streptavidin-horseradish peroxidase complex

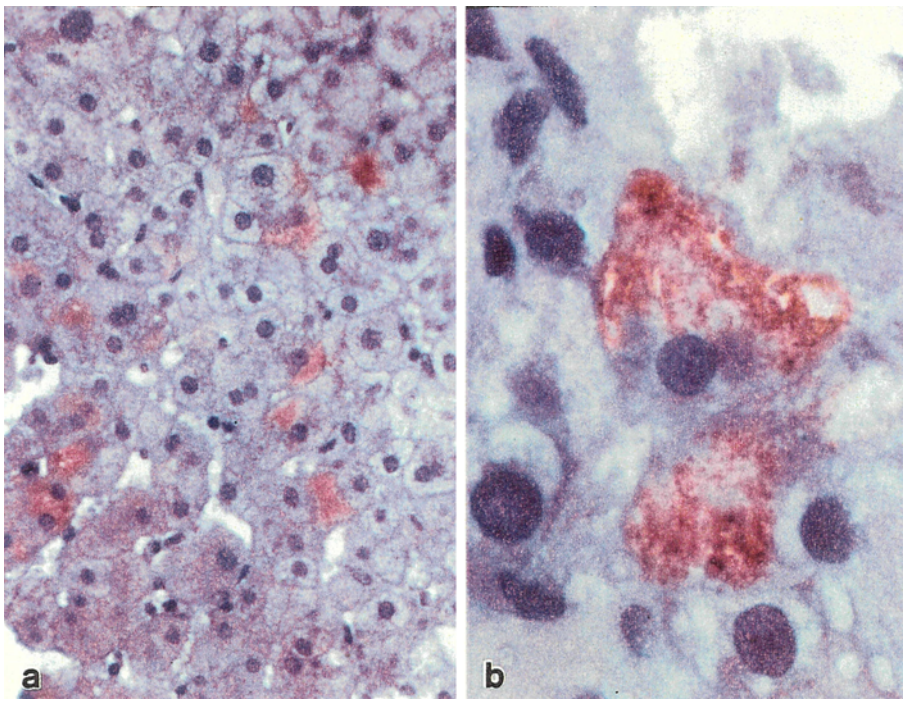

Fig. 2 a, b. Distribution of C100 antigen in liver tissue. **a** Apparently intact hepatocystes display the antigen within the cytoplasm (×100). **b** Higher magnification demonstrates a very coarse granular staining pattern within damaged cells (×600)

atocytes but apparently intact cells may give a positive signal, and their location within the lobular architecture is unrelated to vascular or portal structures. However, C100-positive hepatocytes may be found in damaged areas in very close contact with immunocytes, suggesting cell-mediated hepatocyte damage.

The possibility of locating C100 antigen within routinely prepared liver biopsies is important because it allows the retrospective investigation of stored material. Microwave irradiation increases the sensitivity of immunostaining, probably because it is a more efficient way of uniformly raising the temperature in small droplets of liquid.

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