Minireview

Ultrastructural Alterations and Expression of Cytoplasmic Antigen 48-1 in Hepatocytes in Association with Hepatitis C Virus Infection

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Hepatitis type C is an important disease of humans; it is responsible for more than 90% of the cases of posttransfusion non-A, non-B hepatitis (6, 14). The etiologic agent of this disease, however, remained elusive for almost two decades, although the viral nature of the disease had been demonstrated already in 1978 by inoculating the serum or plasma from the patients into chimpanzees (1, 29). In 1989, the genome of the virus was finally discovered by the recombinant cDNA approach and the agent was termed hepatitis C virus (HCV) (5). HCV was revealed to contain a plus-strand RNA of about 10 kilonucleotides with one large open reading frame encoding a protein of 3010 amino acids (7, 13, 31) and comparative sequence analysis indicated that the virus is distantly related to the flaviviruses and pestiviruses (16). A sensitive assay for detecting HCV RNA by reverse transcription/polymerase chain reaction (RT/PCR) (32) and immunodiagnostic assays for antibodies against recombinant HCV proteins have been developed (14). The results from the serological assays indicated that most cases of hepatocellular carcinoma in Japan (19), Italy (8), and Spain (4) were associated with HCV infection.

Before the emergence of these assays, chimpanzees (Pan troglodytes) were used for the characterization of the etiologic agent as the only reliable animal model for non-A, non-B hepatitis. The studies with chimpanzees have provided evidence that the virus is probably enveloped (9) and is approximately 30–60 nm in diameter (10). Additional research, however, has been hampered because of the limited availability of chimpanzees and the relatively low titer of the virus in clinical samples. To date, little is known about the replication and pathogenesis of this virus and the virion has not yet been visualized with certainty.

Previously, my colleagues and I reported the presence of characteristic ultrastructural alterations (cytoplasmic tubular structures), detected by electron microscopy (EM) (22), and induction of a cytoplasmic antigen, detected by immunofluorescence staining (IF) with a monoclonal antibody (48-1) (23), in the hepatocytes of chimpanzees infected with non-A, non-B hepatitis virus (now HCV). These findings were initially thought to be specific for HCV infection, but were later found
to be associated also with infections of other viruses, including hepatitis D virus (HDV) (12, 24). Both ultrastructural alterations and expression of the antigen reacting with 48-1 antibody (antigen 48-1) were subsequently shown to be indirect measures of viral replication and probably to represent a host response to the expression or action of interferon (IFN) (25).

This article reviews the characteristic ultrastructural hepatic alterations and expression of antigen 48-1 reported for chimpanzees in association with HCV infection.

1. Ultrastructural Alterations in Hepatocytes

During examination by EM of liver tissues from chimpanzees experimentally infected with HCV, we noticed the presence of peculiar structures in the cytoplasm of hepatocytes. They appeared circular in cross section and were composed of two parallel walls when cut longitudinally, indicating that the structures were tubular (Fig. 1A). The walls of the tubules were constructed of double-unit membranes with electron-dense material in between. The total thickness of the wall was 20–25 nm. The endoplasmic reticulum was contiguous with both the outer and inner membranes of the tubules (arrows in Fig. 1B). The appearance of these cytoplasmic tubular structures was temporally associated with hepatitis as determined by elevated transaminase level in the serum and histopathologic changes characteristic of viral hepatitis in liver biopsies. They were not detected in liver biopsies collected prior to virus inoculation, early in the incubation period, or during convalescence. In addition, liver biopsy specimens from chimpanzees with hepatitis A or B, or from normal chimpanzees were negative for the structures. Besides the tubular structures (also called type III alterations), three different types of ultrastructural changes were found in association with HCV infection, namely reticular inclusion bodies (Fig. 1C), convoluted membranes derived from smooth endoplasmic reticulums (Fig. 1D), and microtubular aggregates (Fig. 4A). They are also known as type I, II, and IV alterations, respectively (17). These structures were initially thought to be specific for HCV infection, but were later found in livers of HDV-infected chimpanzees as well. Furthermore, comparative studies revealed the occurrence of similar changes in cells infected with other RNA viruses (3); tubular structures morphologically identical to type III alterations seen in HCV-infected chimpanzees were found in lymphocytes of patients with AIDS (27) or with adult T cell leukemia (21). Bockus et al (2) demonstrated the structures similar to type III alterations by EM in cultured Daudi lymphoblastoid cells grown in medium containing recombinant alpha IFN, and they proposed that such alterations were the result of exposure of the cells to IFN.

2. Cytoplasmic Antigen 48-1

In 1985, before the discovery of the HCV genome, we attempted to obtain antibodies associated with non-A, non-B hepatitis (now hepatitis C) by using the Epstein-Barr virus (EBV) transformation method (23). This method is based on the fact that B lymphocytes from immune individuals can be transformed in vitro
by EBV infection into lymphoblastoid cell lines capable of producing specific antibodies (28). By using this method, successful production of antibodies against a variety of antigens has been reported. We started our experiments with lymphocytes from HCV-infected chimpanzees and established a lymphoblastoid cell line producing an antibody (48-1) which was shown by IF to react with livers from chimpanzees with hepatitis C. Figure 2 illustrates the procedure we employed for establishment and screening of lymphoblastoid cell lines. Peripheral lymphocytes

Fig. 1. Ultrastructural alterations observed in the cytoplasm of hepatocytes from an HCV-infected chimpanzee. A: Cytoplasmic tubular structures (type III alterations). B: Higher magnification of a tubular structure. The ER was contiguous with both the outer and inner membranes (arrow). C: Reticular inclusion bodies (type I alterations). D: Convoluted membrane structures (type II alterations).
from a chimpanzee, convalescent from infection with HCV, were infected with EBV (the multiplicity of infection was approximately 0.1) and cultured in microculture plates. Continuous cell cultures emerged with an average frequency of 96% within 4 weeks after the initiation of culture. At week 4, the culture supernatants were individually harvested and tested by IF for antibodies against sections of the liver tissue obtained from an HCV-infected chimpanzee. Antibody activity was detected in 19 of the 1,402 supernatants and one of them, 48-1 antibody, was chosen to be further characterized. Figure 3 shows positive IF by 48-1 antibody in hepatocytes of a chimpanzee infected with HCV. The granular staining was located in the cytoplasm and seen in almost every hepatocyte. Specificity of the antibody for hepatitis C was investigated by IF on liver biopsy specimens from chimpanzees with hepatitis A, B, C or D, or from normal chimpanzees. The 48-1 antibody reacted with liver biopsy specimens obtained from chimpanzees during acute or chronic hepatitis caused by HCV and HDV, but not with such biopsies from chimpanzees with hepatitis A or B, or from normal chimpanzees.

To determine ultrastructure where the cytoplasmic antigen reactive with 48-1 antibody is localized, immunoperoxidase EM was carried out on the IF-positive liver tissue from an HCV-infected chimpanzee. Peroxidase-reaction products, indicating the binding of the antibody, were observed on the aggregates of microtubules, type IV alterations (Fig. 4B).

To further clarify the nature of antigen 48-1 (the microtubular aggregate
antigen), we attempted to develop a radioimmunoassay, which would enable us to partially purify the antigen from the liver homogenate of HCV-infected chimpanzees. A higher degree of purification of the antigen, however, was hampered by the unsuitability of this antibody 48-1 for the necessary assays, such as Western blotting. Thus, the mouse hybridoma method was employed to obtain monoclonal antibodies directed against the same antigen (15). Utilizing a mouse monoclonal antibody newly obtained, the buoyant density of the antigen in sucrose was determined to be 1.12-1.13 g/ml, and a single protein having an Mr of 44,000 (p44) was obtained by Western blotting with the antibody, as shown in Fig. 5. The p44 antigen was further purified by reverse-phase high performance liquid chromatography and partial amino acid sequence, which does not resemble that of any proteins so far known, was obtained (11). The mouse monoclonal antibody was also used for immunoscreening to isolate a cDNA encoding the p44 from the hepatic cDNA library of a chimpanzee infected with HCV. The cDNA thus cloned (1.7 kilobases) contained an open reading frame encoding a 444 amino acid protein with an Mr calculated to be 50,468. The cDNA hybridized to a 1.9 kilobases mRNA obtained from chimpanzees infected with either HCV or HDV. It hybridized weakly to mRNA from hepatitis B virus (HBV)-infected chimpanzees but not to that from normal chimpanzees. Southern blot analysis revealed that p44 is a host protein encoded by gene of chimpanzees, and that an identical gene exists in human genome (30).
Fig. 4. A: Electron micrograph of microtubular aggregates (type IV alterations) in the cytoplasm of hepatocytes from an HCV-infected chimpanzee. B: Ultrastructural localization of the antigen reacting with 48-1 antibody revealed by immunoperoxidase EM. Peroxidase reaction products are seen in the microtubular aggregates.
3. Relationship of Antigen 48-1 to IFN

Schaff et al (20) observed type III alterations in Kupffer cells and macrophages from a patient with chronic active hepatitis B during treatment with recombinant alpha IFN. In addition, Bockus et al (2) reported that the recombinant alpha IFN induced in Daudi lymphoblastoid cells the formation of the structures identical to type III alterations. Their findings raised the question whether antigen 48-1 is also inducible by IFN, since we noticed that expression of this antigen is usually accompanied by appearance of type III alterations in hepatocytes of chimpanzees.

Fig. 5. Sucrose density gradient profiles of liver homogenates from (a) HCV-infected and from (b) uninfected chimpanzees. Antigen activities (●) were assayed by radioimmunoassay and sucrose densities (○) were determined by spectrophotometry. Protein concentrations (△) were determined by the method of Lowry et al (1951). (c) Fraction 10 from both (a) (lanes 1 and 3) and (b) (lanes 2 and 4) were analyzed by SDS-polyacrylamide gel electrophoresis (lanes 1 and 2) and Western blotting (lanes 3 and 4). [From Honda et al, 1990, J. Gen. Virol. 71].
An opportunity to investigate this possibility was afforded by studies of the effects of the IFN inducer and exogenous IFN on chronic HBV infection in chimpanzees. These studies were conducted in 1975 by Purcell et al. (18) at the National Institutes of Health, U.S.A. We studied liver biopsies from chimpanzees treated with an IFN inducer or exogenous IFN for the presence of the antigen by IF. As shown in Fig. 6, in two HBV carrier chimpanzees (Nos. 814 and 821) and one normal chimpanzee (No. 105) treated with IFN inducer, polyinosinic polyribocytidylic
acid poly L lysine carboxymethylcellulose (PICLC), the antigen became detectable in hepatocytes 2 weeks after the initiation of the treatment, remained detectable throughout the period of the treatment and disappeared within 4 weeks after the treatment was terminated. EM revealed that the liver biopsies positive for the antigen exhibited the hepatocyte cytoplasmic changes: convoluted membranes and microtubular aggregates. The antigen was not detected in any of the liver biopsies from non-treated control chimpanzees. Furthermore, liver biopsies obtained from an HBV carrier chimpanzee during treatment with exogenous human leukocyte IFN were found to be positive for the antigen as well. Thus, it is likely that expression of this antigen and induction of the ultrastructural alterations in chimpanzee hepatocytes infected with HCV or HDV may be a host response to IFN induced by these viruses.

4. Early Events in HCV Infection in Chimpanzees

The analysis of serial clinical specimens obtained from chimpanzees experimentally infected with HDV has revealed a relatively uniform relationship between peak replication of HDV and peak expression of cytoplasmic antigen 48-1; expression of the antigen in the liver was generally preceded by the appearance of increasing amounts of markers of HDV replication (hepatitis delta antigen in the liver and HDV RNA in the serum). The data, coupled with previous observations that

![Diagram of Ultrastructural Changes, Cytoplasmic Antigen, and Serum HCV RNA](image)

![Graph of ALT (IU/L) vs. Days, Weeks, Months](image)

*Fig. 7. Course of HCV infection in a chimpanzee. Symbols at top indicate that the specimen was positive (+) or negative (−) for HCV RNA detected by RT/PCR, 48-1 antigen detected by IF, and ultrastructural alterations found by EM. [From Shimizu et al, 1990, Proc. Natl. Acad. Sci. U.S.A. 87].*
antigen 48-1 becomes detectable in HCV-infected chimpanzees within one week after inoculation, led to the suggestion that de novo replication of HCV occurs very early in the incubation phase after experimental infection of chimpanzees. To investigate this hypothesis the precise time of appearance of (1) antigen 48-1 detected by IF, (2) ultrastructural alterations found by EM and (3) HCV RNA detected by RT/PCR was determined. As depicted in Fig. 7, HCV RNA became detectable in the serum as early as 3 days after the inoculation and remained positive throughout the period of aminotransferase elevation. Antigen 48-1 and ultrastructural alterations became positive in hepatocytes 3 and 6 days, respectively, after HCV RNA was first detected in the serum. Circulating anti-HCV (anti-C100) appeared 13 weeks after the inoculation. Thus, these data indicated that replication of HCV started at very early phase of the infection and potential infectivity continued for a long period until the appearance of anti-HCV (26).

Before the specific assays for HCV infection were established, the ultrastructural alterations and antigen 48-1 in hepatocytes were used as indirect markers in experimental HCV inoculation studies with chimpanzees. Research on antigen 48-1 is now being focused on its biological function and role in the viral infections, which would be important for understanding of the pathogenesis of HCV. As described in this article, expression of antigen 48-1 and induction of ultrastructural hepatic alterations in chimpanzees infected with HCV or HDV appeared to be a host response to IFN induced by these viruses, and IFN may play an important role in the regulation of replication of the viruses. This further suggests the possibility that IFN is an effector responsible for the previous observations that acute and persistent HCV infections in chimpanzees interfered with superinfection with HAV or HBV and that HDV superinfection in HBV chronic carriers caused suppression of replication of HBV. The mechanism of the antiviral action of IFN is still obscure. However, it has been established that IFN induces proteins and enzymes which may play an important role in the regulation of macromolecular synthesis in the IFN-treated cells. More studies are needed to determine whether the antigen we described could be such a mediator.


REFERENCES

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