Detection of hepatitis C virus RNA by in situ hybridization: a critical appraisal

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Summary. Despite the several papers that have appeared in the literature or have been communicated at scientific meetings, the detection of hepatitis C virus RNA by in situ hybridization seems a difficult goal to achieve. There have been conflicting reports on the type and proportion of hepatitis C virus-infected cells, the intracellular distribution of viral RNA and the topographical association with cell damage. As a consequence, some of the findings should probably be considered as non-specific and all protocols and data critically reviewed before a firm conclusion be made.

Key words: Hepatitis C virus – Viral hepatitis – Viral pathogenesis – In situ hybridization – Polymerase chain reaction

Despite the major advances in the past few years, the biology of HCV and the pathogenesis of the HCV-associated liver damage are largely unknown. This is partly due to the limited availability of experimental animal models (chimpanzees). Moreover, in vitro infection systems have been described only recently [10–12]. Thirdly, the low titer of the virus in clinical samples has hampered studies on the viral life cycle in vivo. This difficulty is reflected in the conflicting results that have been reported by those investigators who have attempted to localize HCV RNA in infected tissues by in situ hybridization (ISH). As of today, at least 15 groups have reported the detection of HCV RNA by ISH [13–27]. The data are, however, quite discrepant, and the scope of this review is to critically discuss their interpretation.

Introduction

Hepatitis C virus (HCV) is today recognized as the agent responsible for the vast majority of cases of the parenterally transmitted non-A, non-B hepatitis [1]. The discovery and characterization of HCV have followed an unconventional route, starting from direct cloning of the viral genome contained in the serum of a chronically infected chimpanzee [2]. These difficulties were primarily due to the low level of HCV replication in the infected host, both natural and experimental [3], and secondarily to the low immunogenicity of the viral proteins, making it difficult to characterize specific antigen-antibody systems associated with this virus [4].

The virion of HCV contains a plus-strand RNA of about 10 000 nucleotides [5]. Comparative sequence analysis has shown that HCV is distantly related to the flaviviruses and pestiviruses [6]. The viral genome can be detected in infected fluids by reverse transcription (RT) and polymerase chain reaction (PCR) assays, which have confirmed the very small amounts of HCV RNA in the infected host [7–9].

Significance of the detection of HCV RNA by ISH

From a general point of view, ISH protocols are aimed at identifying specific nucleic acid sequences in intact tissues. Therefore, ISH may assess the topographical correlations between the presence of a given nucleic acid and peculiar cytomorphological and immunocytochemical features [28]. In viral infections, ISH may be used to: (1) identify the tissue and cell tropism, (2) define the subcellular site of replication, (3) study the relationship with some host protein expression (e.g., adhesion molecules, proliferation markers) and with the cell damage (cytopathic effect, inflammatory infiltrate). ISH is therefore a powerful tool, together with other molecular biology techniques, to study the pathobiology of an infectious agent.

The major problem in the study of the tissue expression of HCV RNA by ISH is the low level of replication shown by this pathogen in the infected host. The intrinsic sensitivity limit of ISH makes it probably unsuitable for the detection of HCV RNA in all infected cells. This problem has not been overcome by the recent introduction of in-cell amplification procedures [24, 26], for reasons not completely understood. It should be stressed

that the analysis performed on total tissue RNA extracted and amplified through a combined RT/PCR procedure cannot give precise information about the replicative status of HCV in any given tissue. It has been shown, in fact, that RNA molecules corresponding to the putative replication intermediates of HCV can be found in serum at titers comparable to those in tissue extracts [29], an observation that should caution every investigator trying to ascertain whether HCV is actually replicating in the tissues under study. In other words, analysis of extracted, total RNA does not allow differentiation of the virion RNA present in the interstitium from the viral replicating RNAs found inside the cells. Therefore, ISH is the only appropriate method for evaluating viral replication at the single cell level.

A second problem lies in the fact that the true cell tropism of HCV is hypothetical. Comparison with the distantly related flavi- and pestiviruses suggests that HCV should infect hepatocytes, the mononuclear cells (including Kupffer cells) and, probably, the central nervous system cells [30, 31]. HCV RNA has indeed been detected by RT/PCR amplification of whole RNA extracted from peripheral blood mononuclear cells [32–38]. Again, direct evidence of the presence of HCV *inside* those cells is lacking. Besides, the spectrum of extrahepatic disorders related to chronic HCV infection in humans (especially skin and thyroid diseases) raises the possibility that HCV cell tropism may be much more varied than expected.

The third problem is that, so far, immunocytochemical detection of HCV proteins has been very difficult, and most observations, although convincing and mostly consistent, have not been independently reproduced [39–43].

In conclusion, availability of a sensitive, specific and reproducible procedure to detect HCV RNA by ISH would be of unquestionably great help to both the molecular virologist and the pathologist. Furthermore, several issues of clinical relevance are still to be resolved, e.g., recurrence of HCV infection in the transplanted liver, site of ongoing HCV replication in the apparently healthy HCV carrier, both spontaneous and interferon induced, and the putative role of HCV in the extrahepatic manifestations commonly seen in patients with acute and chronic hepatitis C. All these issues may be investigated using ISH, which seems, however, far from being routinely available.

Which cells are infected by HCV?

There is almost unanimous evidence that HCV infects the hepatocytes [13, 14, 16–27]. However, in a single, unconfirmed report [15] use of ¹²⁵I-labelled RNA probes representing about 35% of the HCV genome (a reportedly very sensitive approach) identified rare mononuclear cells as the only site of HCV replication. These cells were probably lymphocytes or macrophages close to portal tracts, and the signal was shown to be specific by appropriate experiments. This observation has not been confirmed. That mononuclear cells, together with hepatocytes, can contain HCV RNA has been reported only by

a few investigators [14, 22–25] who failed to further characterize the cell types involved. These results were obtained using differently labelled probes (both radioactive and digoxigenin): the objection that the staining of mononuclear cells is due to non-specific binding of ³⁵S-sulfur-labelled probes, as suggested elsewhere [44, 45], can therefore be dismissed. In only one instance [22] were the biliary epithelial cells also reported to be infected: this observation is intriguing, as HCV infection is often associated with biliary duct damage, but, again, unconfirmed by other, independent observers.

How many cells, on average, are infected and what is the overall proportion of positive specimens? Once more, the answer is not univocal. Oddly enough, many authors, using differently labelled probes, report a diffuse signal, often involving the vast majority of hepatocytes [13, 18, 20]. This is in striking contrast with the known low level of viremia, the detection of which invariably needs RT/PCR amplification. The existence of a restricted replicative cycle cannot account for this discrepancy, as HCV RNA must be amplified even when extracted from infected tissues. Moreover, despite the likely high sensitivity of the procedures used by these authors, one does not understand why the proportion of totally negative specimens can be as high as 60% [20], unless sampling bias is frequent when assessing intrahepatic HCV replication. This possibility may help to explain the large change (from 0% to 100%) in the proportion of stained hepatocytes seen in biopsy specimens taken 1 day apart during the acute phase of the experimental infection of chimpanzees [13].

Another question concerns the subcellular localization of HCV RNA during its replicative cycle. This is important not only for the virologist but also for the clinician: intranuclear HCV RNA may interact with the host DNA and thus play a direct role in carcinogenesis. Moreover, it might become even more relevant in the future when targeted antiviral drugs are available. Again, despite the majority of investigators reporting a cytoplasmic localization of HCV RNA, some authors have described nuclear staining, both with radioactive [14, 15] and non-radioactive [22] probes. Although most immunohistochemistry studies have localized HCV proteins in the cytoplasm of hepatocytes [39–43], a nuclear phase of the viral replicative cycle cannot be ruled out, as shown also for some flaviviruses [46–48], which have been otherwise reported to replicate predominantly in the cytoplasm [30, 49]. Hence, at least in this case, the different observations may not be mutually exclusive.

What is the relationship with cell damage?

This is a rather important issue, but the picture is again very unclear. In acutely infected chimpanzees, where the time-course of replication versus histopathology has been studied in detail [13], infected hepatocytes are healthy and necrotic areas do not contain detectable amounts of HCV RNA. However, the extent of intrahepatic replication of HCV seems to vary in parallel with the serum alanine aminotransferases: a direct damage to the hepa-

toocyte membrane has been hypothesized [13], but a sampling bias is also very likely. If HCV directly damages the liver cells, it may do so in the absence of morphological evidence of cell injury, as suggested for other viruses [50, 51]. Lack of cytopathic changes of the infected cells has been reported by almost all authors who have analyzed the histopathology in detail [14, 16–18, 22, 23]. In a single report [21], however, HCV RNA-positive hepatocytes often contained fat droplets, but the significance of this association was not evaluated and hence a precise cause-effect relationship cannot be established.

HCV-induced liver damage is probably due to the host immune reaction, as suggested by the finding that hepatocytes positive for HCV RNA were frequently associated with mononuclear cell infiltration [18, 19]. A very interesting correlation between biliary epithelial cell infection and damage was anecdotally reported [22]. All these observations await independent confirmation.

Some technical considerations

Since both biotin-labelled and radioactive probes may give non-specific staining [28, 44, 45], much attention has been devoted to the use of digoxigenin as a label. Digoxigenin is of plant origin and antisera raised against this hapten are unlikely to bind non-specifically to endogenous epitopes [52]. An alternative approach has been used by some Japanese authors [16, 17, 20, 23] who labelled a mixture of oligonucleotides with polythymidine tails, followed by ultraviolet-induced dimerization between two adjacent thymine rings, which creates an immunogenic structure [53]. Unfortunately, non-radioactive probes are also less sensitive than radioactive ones, and may therefore be unsuitable when the target copy number is low, such as in HCV infection. To overcome this problem, some gimmicks have been employed: use of mixtures of several oligonucleotides [16, 17, 20, 21, 23] or signal amplification by the alkaline phosphatase/anti-alkaline phosphatase antibody (APAAP) complexes [13, 54]. It is therefore surprising that convincing results were obtained [18, 19, 22] with a relatively insensitive approach, i.e., use of digoxigenin-labelled double-stranded DNA probes. A self-sustained signal amplification may be obtained with these probes if conditions are used that allow intermolecular network formation. However, especially with PCR-generated double-stranded DNA probes, background staining is a serious problem [27] and misinterpretation of results is likely.

The problem of the in situ PCR

The intracellular amplification of HCV RNA by RT/PCR was recently described [24]. The so-called in situ PCR technique was introduced a few years ago to successfully detect in situ DNA present in low copy number, such as in some retroviral infections [55, 56] and in human papillomavirus infection [57]. Two major techniques have been described: the *direct* in situ PCR, where the labelled nucleotides are directly incorporated in the am-

plicon, and the *indirect* procedure, in which the in-cell PCR amplification is followed by an in situ hybridization with a labelled probe [58, 59]. Direct procedures are not recommended, since non-specific, primer-independent incorporation of the label at sites of host DNA fragmentation (i.e., apoptosis) is very likely [58]. Pre-digestion with DNase has been suggested [24], but may not be sufficient to eliminate this possibility. Data should be viewed with great suspicion unless stringent specificity controls are reported. In an attempt to reproduce the procedure reported by Nuovo et al. [24], we showed that a perinuclear staining, similar to that claimed to be HCV RNA specific by Nuovo et al. [24], was both primer and cycling independent, suggesting that digoxigenin had labelled the host DNA. An indirect procedure has also been reported recently [26], but seems very insensitive.

Some suggestions for future work

In the author's opinion, in-cell amplification should be pursued with great caution and indirect procedures strictly adhered to. Use of multiple primer pairs [55] or co-amplification with house-keeping genes [60] may represent future, successful approaches, but the technical obstacles may be significant. Signal amplification procedures may still play an important role, especially those using APAAP complexes [54] or "branched" DNA systems [61], provided that technical improvements are developed to avoid background staining. Optimal fixation is crucial to combine good sensitivity, retention of the target RNA and morphological preservation; cryopreservation procedures seem to give the best results [18]. Above all, however, a bona fide, successful hybridization cannot fall short of a good amount of self-criticism.

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