Acta Histochem. Cytochem. Vol. 28 No. 4 341-348 (1995)

The Localization of HCV and the Expression of Fas Antigen in the Liver of HCV-related Chronic Liver Disease

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Received for publication June 26, 1995 and in revised form September 4, 1995

We have investigated the localization of HCV in the liver and the expression of Fas antigen, involved in hepatocellular death through apoptosis, to examine the relation between HCV infection and Fas antigen cellular expression. The localization of HCV was investigated by *in situ* hybridization (ISH), and the expression of Fas antigen was determined by immunohistochemistry using an anti-Fas monoclonal antibody, in 20 surgically-resected liver specimens from anti-HCV positive patients. The Methylgreen/Pyronin Y technique clearly showed well-preserved tissue RNA in 8 of 20 livers. ISH determined the co-localization of signals for plus and minus strands of HCV-RNA in the cytoplasm of hepatocytes. Fas antigen was more strongly expressed on the plasma membrane of the periportal hepatocytes than on the plasma membrane of the centrilobular hepatocytes, even when HCV-RNA was found to be widely distributed in the hepatocytes throughout the liver lobule. Furthermore, Fas antigen positive hepatocytes were frequently observed close to the area of piecemeal necrosis associated with a high incidence of apoptosis. The results suggested that the expression of Fas antigen does not occur by HCV infection alone, but probably requires several other factors.

Key words: Hapatitis C virus, *In situ* hybridization, Fas antigen

I. Introduction

The mechanism of hepatocellular damage by HCV is not yet clear. It has been suggested that HCV directly causes the cell damage, or that immunological processes after HCV infection are involved [15, 35].

In chronic hepatitis C, apoptosis is frequently recognized in the liver. Apoptosis has been defined as one major type of cell death which is different from necrosis (passive or accidental cell death) [10, 29], and is induced by several exotic signals transducted by transmembraneous proteins, such as Fas antigen, to the intracellular organelles [8, 16]. Acute lethal liver injury with histological changes involving apoptosis has been induced by an intraperitoneal administration of the anti-Fas antibody in mice [21]. Messenger RNA for Fas antigen has been found in the liver [27]. In addition, Hiramatsu *et al.* reported that HCV infection of hepatocytes was closely related to the Fas antigen expression[3]. These studies suggest a possible role of Fas antigen in liver injuries.

In 1992, an immunohistochemical study using monoclonal antibodies against core, envelope, and NS3 proteins of HCV demonstrated the localization of HCV in the liver [4]. Now, *in situ* hybridization (ISH) has been employed to reveal the localization of hepatitis A and B viruses [18, 25], Epstein-Barr virus [7, 19], and of HCV in the liver [1, 2, 9, 13, 14, 20, 24, 31, 32]. The localization of HCV-RNA positive cells has been classified in some patterns. However, they seemed to have no relationship between the localization of HCV-RNA positive cells and histological appearance.

ISH, used in the present study, is a method to investigate the localization of a specified genome in the tissue. Since conventional immunohistochemistry demonstrates the localization of a product, immunohistochemistry is not always able to demonstrate the producing cells, especially in the case when the product was transported from the producing area to a different area, or when the product is taken up by other cells. Thus, ISH provides a potency, which is not possible by immunohisto-

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chemistry, to determine the infected cells as well as the cells where the virus replicates.

Here we employed ISH and immunohistochemistry on the liver of anti-HCV positive patients to investigate the localization of the HCV-infected cell, comparing with the expression of Fas antigen on hepatocytes.

II. Materials and Methods

Materials

Surgically resected liver tissues were obtained from 25 patients, 24 of whom had liver tumors (23 primary hepatocellular carcinomas and one metastatic cancer from the colon), while the other one underwent laparotomy and a wedge biopsy for diagnostic purposes. Only the non-cancerous regions of these tissues were used in this study. Twenty patients were seropositive for anti-HCV using the second generation PHA test (Dainabot, Tokyo, Japan), and they were all seronegative for hepatitis B surface antigen. The histological diagnosis of the tissue was chronic hepatitis (CH) in 13, liver cirrhosis (LC) in 11, and normal liver in one (Table 1).

Methods

Specimens

Liver tissue was obtained by surgical resection, then cut into pieces immediately and fixed in 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS). After fixation, the tissues were immersed in 30% sucrose. Then the tissues were embedded in OCT compound (Miles Inc, Elkhart, USA) and frozen in acetone/dry ice. The frozen tissues were cut into serial sections (4 μm) using a cryostat (HM505E; MICROM Laborgeäte GmbH, Walldorf, Germany) and mounted on silanized slides (DAKO JAPAN CO., LTD., Kyoto, Japan). The specimens were dried completely and stored at $-80°C$.

Screening of RNA preservation in the specimens

The amount of tissue RNA, part of which was expected to be HCV-RNA, was evaluated by Methylgreen /Pyronin Y (MG/PY) technique [23] that could demonstrate preserved RNA as pinkish microgranules.

Table 1. *Clinical profile of the patients studied*

Case	Age	Sex	Serum				Liver		
			Anti-HCV	*HBsAg*	*HCV-RNA**	*ALT*	*Histology*	*MG/PY-s*	*HCV-RNA***
1	57	M	(+)	(−)	10^8	27	CH	(+++)	(+)
2	59	M	(+)	(−)	10^8	28	LC	(+++)	(+++)
3	57	F	(+)	(−)	10^8	27	LC	(++)	(+)
4	66	F	(+)	(−)	10^7	60	LC	(+++)	(+++)
5	66	M	(+)	(−)	10^6	49	CH	(++)	(++)
6	26	M	(+)	(−)	10^6	53	CH	(+++)	(+++)
7	62	M	(+)	(−)	10^5	40	LC	(++)	(+)
8	68	M	(+)	(−)	10^4	100	LC	(+++)	(+)
9	71	M	(+)	(−)	10^9	86	LC	(+)	(−)
10	64	M	(+)	(−)	10^8	66	CH	(+)	(−)
11	74	M	(+)	(−)	10^8	27	CH	(+)	(−)
12	58	M	(+)	(−)	10^7	21	CH	(+)	(−)
13	55	M	(+)	(−)	10^7	27	LC	(+)	(−)
14	69	F	(+)	(−)	10^7	16	LC	(+)	(−)
15	60	M	(+)	(−)	10^7	24	LC	(+)	(−)
16	69	M	(+)	(−)	10^3	30	CH	(+)	(−)
17	79	M	(+)	(−)	10^3	25	CH	(+)	(−)
18	59	M	(+)	(−)	10^2	46	CH	(+)	(−)
19	57	M	(+)	(−)	10^2	62	LC	(+)	(−)
20	58	M	(+)	(−)	10^2	68	LC	(+)	(−)
21	53	M	(−)	(+)	(−)	36	CH	(++)	(−)
22	61	M	(−)	(−)	(−)	15	CH	(++)	(−)
23	70	M	(−)	(−)	(−)	15	CH	(+)	(−)
24	77	F	(−)	(−)	(−)	37	CH	(+)	(−)
25	61	M	(−)	(−)	(−)	8	NL	(+)	(−)

Anti-HCV; antibody to hepatitis C virus by second generation PHA test, HBsAg; hepatitis B surface antigen, ALT; alanine aminotransferase (normal <40 IU/*l*), CH; chronic hepatitis, LC; liver cirrhosis, NL; normal liver, MG/PY-s; Methylgreen/pyronin Y stain (staining intensity; +: weak, ++: moderate, +++: strong)

*; Estimated by multicyclic reverse transcription polymerase chain reaction (MRT-PCR) method. (genome copies per *ml*)

**; Detected by *in situ* hybridization method. (positive hepatocyte; +: scattered, ++: focal, +++: lobular)

Preparation of probe

Three pairs of oligonucleotide DNAs were synthesized as nonradioactive probes [17], which were homologous or complementary to the sequences corresponding to −267～−223, −36～8, and 5657～5701 of the HCV RNA genome [31] (Japan Bio Service Co., Ltd., Saitama, Japan) (Table 2).

Thymine-Thymine dimerization of probes

Thymine-Thymine (T-T) dimerization of the probes was performed as previously described in detail by Koji *et al.* [12]. The probes were then irradiated at a dosage of 5000, 7000 and 10000 J/m^2 (XL-1000 UV CROSS-LINKER; SPECTRONICS CORPORATION, New York, USA). The optimal UV dosage was determined by the dot blot hybridization method [12, 32] to be 10000 J/m^2 (data not shown). The detection sensitivity of T-T dimerized probe was confirmed immunochemically using an anti-mouse thymine dimer antibody conjugated with horseradish peroxidase (anti-T-T Ab) (Kyowa Medex Co., Ltd., Tokyo, Japan). Various quantities of T-T dimerized probes (1 pg to 10 ng per spot) were applied on stripes of nitrocellulose filters. The filters were completely dried and baked at 80°C for 2 hr. The filters were then reacted with an anti-T-T Ab at the dilution of 1 : 80 overnight. Then, the filters were histochemically stained using 3,3′-diaminobenzidine (DAB)-H_2O_2 as substrate (data not shown). Binding specificity of the probes was confirmed by dot blot hybridization method before use (data not shown).

In situ hybridization

The ISH procedure was performed essentially as described elsewhere [12]. Removal of proteins from the specimens with 0.2 N HCl and proteinase K (1 μg/ml, 37°C, 15 min) (Sigma, Milwaukee, USA) and postfixation with 4% PFA/PBS was performed. After washing with glycine/PBS, the specimens were immersed in 40% deionized formamide (FA)/4 × standard saline citrate (SSC) until hybridization. They were then reacted with hybridization solution (final probe concentration; 6 μg/ml) overnight at 37°C in a moist chamber. Washing with 50% FA/2 × SSC for 1 hr 3 times and 50% FA/0.5 × SSC for 1 hr twice at 37°C dislodged the nonspecific hybrids. For visualization of the hybrids site, histochemical staining was performed as described above.

Detection of Fas antigen

For comparison with the results obtained by ISH and HE stain, Fas antigen was detected immunohistochemically by the avidin-biotin complex (ABC) method [6, 22] (Vectastain ABC KIT; Vector Laboratories, Burlingame, CA). The specimens were incubated with an anti-Fas monoclonal antibody [33] (clone UB-2; MBL; Nagoya, Japan) at the dilution of 1 : 200 overnight at 4°C. All specimens were counterstained with hematoxylin.

III. Results

Localization of HCV-RNA in the liver

MG/PY technique revealed that 8 to 20 liver specimens had well-preserved tissue RNA (Table 1, Fig. 1A). These eight liver specimens and some other specimens had been processed for ISH of HCV-RNA. In a study with the anti-sense probes, the positive signals of the plus strand HCV-RNA were localized in the cytoplasm of hepatocytes in all eight specimens (Fig. 1B, C). Regarding the distribution of HCV-RNA, three cases showed signals in most hepatocytes throughout the liver lobule, four cases in the hepatocytes dispersed throughout the lobule, and one case in the periportal hepatocytes (Table 1). In a study using the sense probes, signals for the

Table 2. *Oligonucleotide sequences used as probes*

HCV anti-sense probe
(−267～−223)
5′; TTA TTA-TCC TGG AGG CTG CAC GAC ACT CAT ACT AAC GCC ATG GCT AGA CGC-ATT ATT ATT; 3′
(−36～8)
5′; TTA TTA-GTG CTC ATG GTG CAC GGT CTA CGA GAC CTC CCG GGG CAC TCG CA-ATT ATT ATT; 3′
(5657～5701)
5′; TTA TTA-TTG CTG CAC AGA CCA CGC CGA CTA CGA GGG CTC CGG GCG AGA GGA-ATT ATT ATT; 3′
HCV sense probe
(−267～−223)
5′; TTA TTA-GCG TCT AGC CAT GGC GTT AGT ATG AGT GTC GTG CAG CCT CCA GGA-ATT ATT ATT; 3′
(−36～8)
5′; TTA TTA-TGC GAG TGC CCC GGG AGG TCT CGT AGA CCG TGC ACC ATG AGC AC-ATT ATT ATT; 3′
(5657～5701)
5′; TTA TTA-TCC TCT CGC CCG GAG CCC TCG TAG TCG GCG TGG TCT GTG CAG CAA--ATT ATT ATT; 3′

Three pairs of oligonucleotide probes were synthesized for ISH. Two pairs of them were derived from the 5′ non-cording region, the other was derived from nonstructural 4 region. ATT sequences were repeatedly added to each end of the probes to introduce T-T dimers.

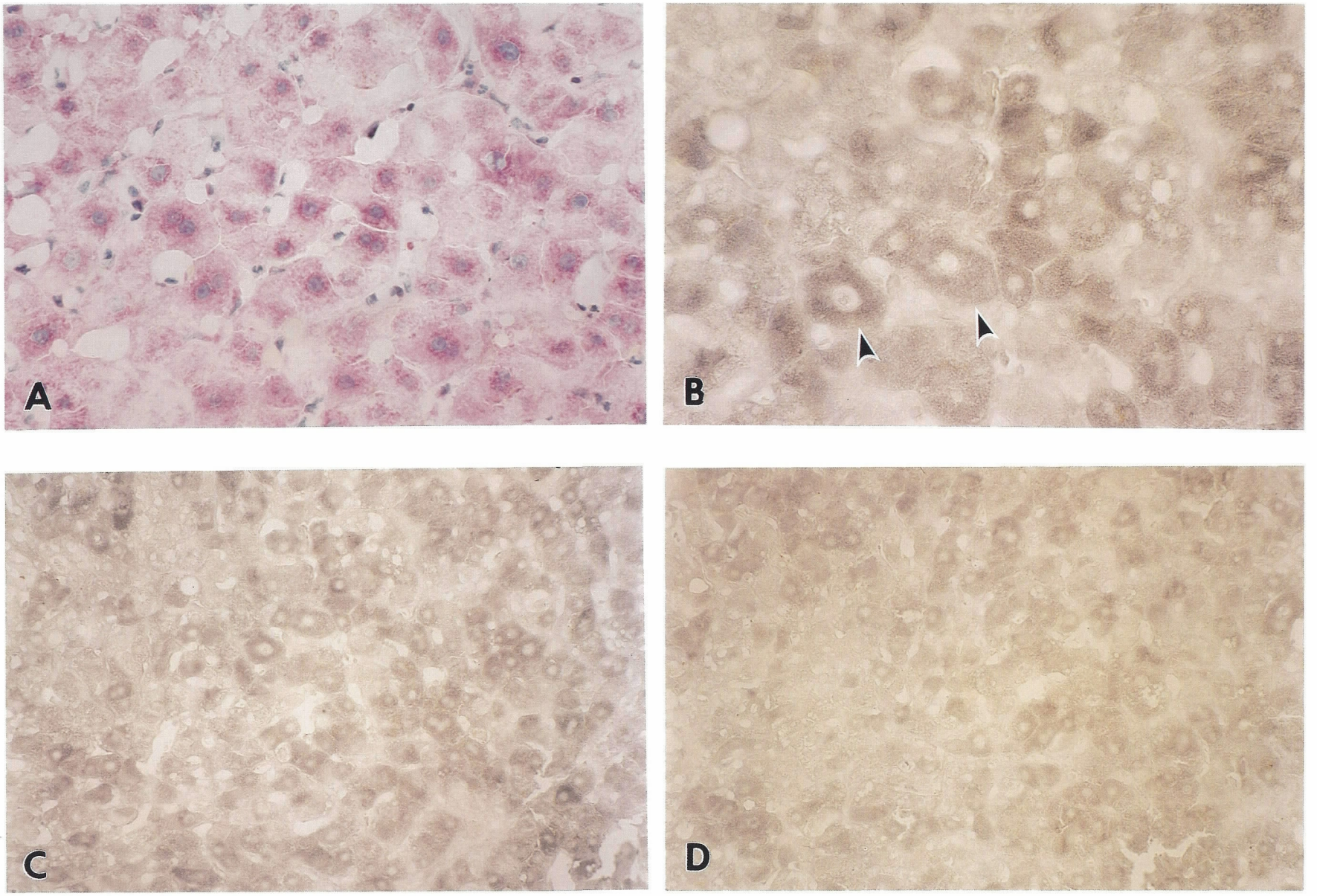

Fig. 1. Preservation of tissue RNA was demonstrated as pinkish microgranules by MG/PY technique (**A**: ×200). *In situ* localization of HCV-RNA in the liver tissue. Positive signals were localized in the cytoplasm of the hepatocytes (arrow) (**B**: ×200). Both the plus and minus strands of HCV-RNA genome were hybridized with T-T dimerized anti-sense (**C**: ×100) and sense (**D**: ×100) probes in serial sections. A similar distribution of positive cells was observed between them.

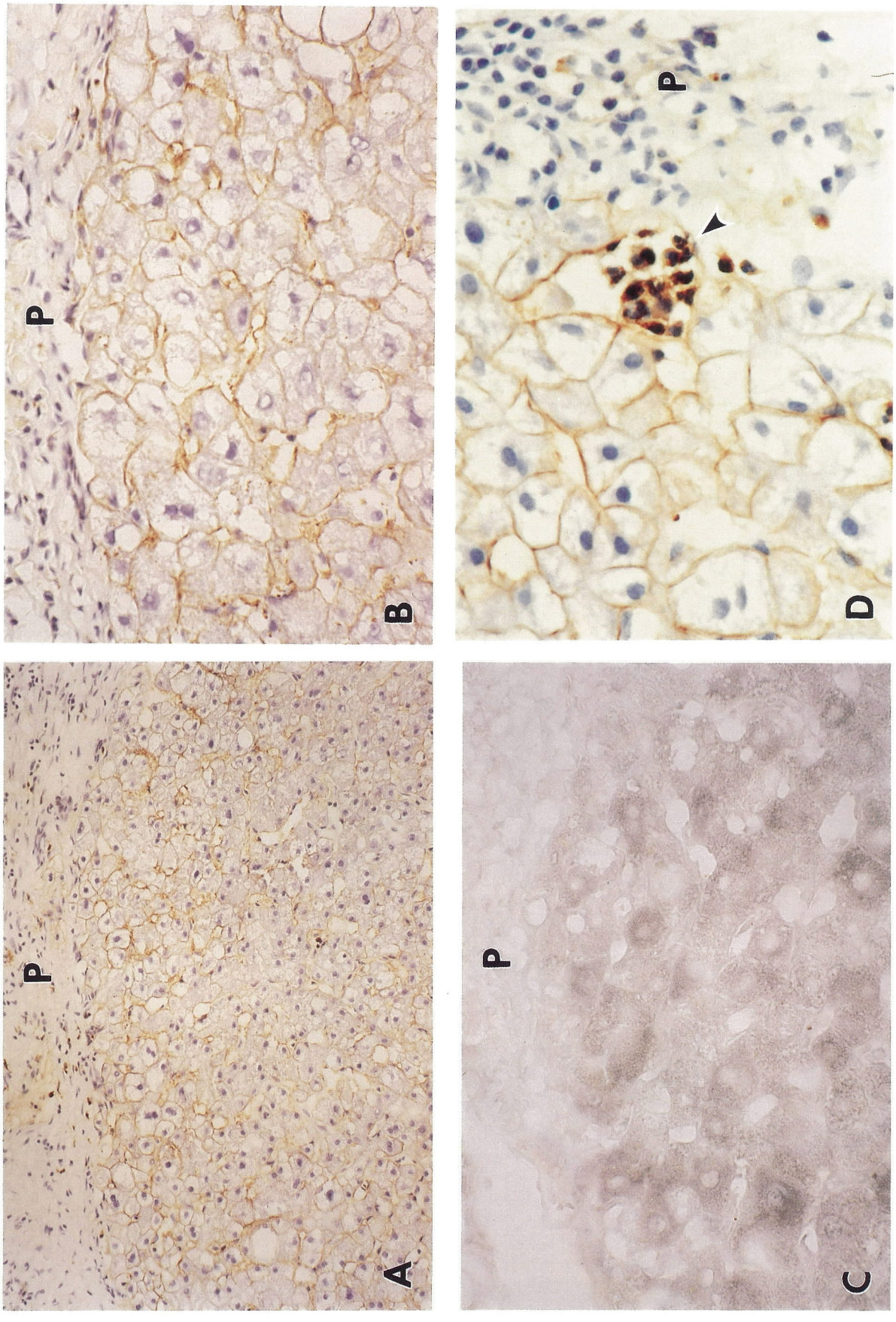

Fig. 2. Comparison of localization between the hepatocytes expressing Fas antigen (**A**: ×100, **B**: ×200) and HCV-RNA positive cells (**C**: ×200). Fas antigen was strongly expressed on the plasma membrane of the periportal hepatocytes. There was no clear relation between the Fas antigen expression and HCV-infection. Fas antigen was frequently seen to be strongly expressed on the plasma membrane of the hepatocytes close to the regions of piecemeal necrosis (arrow) (**D**: ×400). P: portal area.

minus strand HCV-RNA were noted in the cytoplasm of the hepatocytes similar to those for the plus strand HCV-RNA (Fig. 1D). Although the distribution of the signals for the minus strand HCV-RNA was similar to those for the plus strand HCV-RNA, the number of positive hepatocytes appeared to be fewer for the minus strand HCV-RNA than that for the plus strand HCV-RNA. Neither bile duct cells nor inflammatory cells in the portal area presented signals for HCV-RNA. Signal for HCV-RNA was not detected in a liver specimen of poorly-preserved tissue RNA evaluated by MG/PY technique and in the liver specimens from serum anti-HCV antibody negative patients. RNase treatment prior to the ISH procedure abolished positive signals for HCV-RNA.

Expression of Fas antigen

Fas antigen was demonstrated on the sinusoidal and lateral plasma membrane of hepatocytes. Fas antigen was more strongly expressed in the periportal hepatocytes than in the midzonal and pericetral hepatocytes (Fig. 2A, B). In some cases in which HCV-RNA in hepatocytes distributed throughout the liver lobule (Fig. 2C), Fas antigen expression was fairly localized in the periportal hepatocytes. In addition, Fas antigen was frequently seen to be strongly expressed on the plasma membrane of the hepatocytes close to the regions of piecemeal necrosis (Fig. 2D).

In summary, this study found the following results; 1) HCV-infected cells and HCV-replicating cells were revealed by ISH. 2) Fas antigen was expressed in some HCV-infected cells, and strongly expressed in the periportal hepatocytes in HCV-related chronic liver diseases. Moreover, the current study was the first report that described the combined studies of Fas antigen and HCV-infection demonstrated by ISH.

IV. Discussion

In the present study using the anti-sense probes for HCV-RNA and specimens of HCV-related chronic liver disease, the plus staind HCV-RNA (indicating the existence of HCV) was observed to exist in the cytoplasm of hepatocytes, as previously reported [1, 2, 9, 13, 14, 20, 24, 31, 32]. Using the sense probes for HCV to demonstrate the localization of the minus strand HCV-RNA, representing the replication product of HCV, the signals for the minus strand HCV-RNA with low intensity were present in hepatocytes having a distribution similar to that of the plus strand HCV-RNA-positive hepatocytes. This finding may indicate that HCV replicates in most HCV-infected cells. However, in contrast to previous studies [1, 9, 13, 31], neither the plus strand HCV-RNA nor the minus strand HCV-RNA appeared in bile duct cells or infiltrated mononuclear cells in the portal area. Several controversial results concerning HCV-RNA in these two kinds of cells have been reported. The discrepancy between the two results might have resulted from the difference of the tissue-sampling methods, needle biopsy and surgical resection at laparotomy.

In this study, signals of HCV-RNA were observed in 8 to 20 (40%) of anti-HCV positive patients, in contrast to 30 to 88% in previous studies. [2, 13, 14, 24]. Signals of HCV-RNA could be detected only in the specimens which showed strong pinkish microgranules by MG/PY technique. Conversely, specimens of twelve cases with poor staining by MG/PY technique showed no signals of HCV-RNA. It is quite essential for ISH of HCV to use specimens with well-preserved total RNA (selected specimens using MG/PY technique), due to the very small amount of HCV-RNA content in a cell. Furthermore, to examine the quality of preserved tissue RNA, a new method reported by Yoshii *et al.* [34] could be used.

ISH of HCV provided new information concerning the localization of HCV infection and replication. The potential of this technique in predicting the efficacy of interferon, prior to the treatment of chronic hepatitis C, should be evaluated in the near future.

Fas antigen is a transmembraneous protein with a structure similar to tumor necrosis factor (TNF) receptor, nerve growth factor (NGF) receptor, and B cell CD40 antigen, and Fas antigen plays a role in mediation of apoptotic signals to the cell nucleus [8]. The present study demonstrated that Fas antigen was observed on the plasma membrane of the hepatocytes, as reported previously [26, 30]. The hepatocytes were intensely stained for Fas antigen in the periportal area, where piecemeal necrosis was frequently observed by using HE stain [11], even when HCV-RNA positive cells were distributed throughout the liver lobule. These findings suggested that Fas antigen was not expressed by HCV infection alone but by HCV infection and other factors. Although a potent factor for inducing Fas antigen expression has not been fully determined, it may be possible to assume that the periportal hepatocytes are exposed to a higher concentration of cytokines in the portal blood or cytokines released from infiltrating cells because periportal hepatocytes were located close to infiltrating inflammatory cells in the portal area. Contrary to the present results, Hiramatsu described that HCV-infected (HCV core protein positive) hepatocytes were found at or near the area with cells with Fas antigen expression [3]. The discrepancy between his and our results cannot be explained. However, the differences in the method for detecting localization of HCV and of the tissue-sampling methods may be the major factor.

Fas antigen expression was found on the hepatocytes in and near the area of piecemeal necrosis, therefore apoptosis might be involved in the piecemeal necrosis. Since apoptosis was not always observed in the periportal hepatocytes with expressed Fas antigen, then Fas antigen expressing cells do not always progress to apoptosis, and other anti-apoptotic factors such as bcl-2 might rescue the cells from apoptosis [5, 28]. Therefore, in HCV-related chronic liver diseases, other factors are suggested to be required for apoptosis in Fas antigen-expressing hepatocytes.

V. Acknowledgments

We are grateful to Dr. Michio Sata and the doctors of our laboratory for their helpful advice. We also thank Dr. Kurohiji (First Department of Surgery, Kurume University School of Medicine) and Dr. Bekki (Koga Hospital) for providing us with the opportunity to study material from patients under their care. We also thank Mr. Takashima for technical assistance.

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