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Intracellular expression of the proliferative marker Ki-67 and viral proteins (NS3, NS5A and C) in chronic, long lasting hepatitis C virus (HCV) infection

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Abstract: Hepatitis C virus (HCV) continues to represent the main causative agent of the hepatitis, which leads to chronic transformation of the process in 60-80% individuals. It remains unclear how far cellular expression of HCV proteins *in vivo* may represent an index of progression of the disease and of proliferative activity in the liver in chronic hepatitis C. Aim of the studies included detection and subcellular localization of three HCV proteins (NS3, NS5A and C) in liver biopsies from adults (n=19) with chronic, long lasting hepatitis C as related to hepatocyte proliferative activity. The immunocytochemical ABC (avidin biotin-peroxidase complex) technique was applied, alone or associated with the ImmunoMax technique. Results of the immunocytochemical tests were compared to histological alterations in liver biopsies, proliferation index and with selected clinical data. A significantly higher expression of NS3 protein was noted, as compared to expressions of NS5A and C proteins. In all the patients, cytoplasmic localization of all proteins dominated over nuclear localization ($p<0.05$). At the level of electron microscopy, protein localization in endoplasmic reticulum (ER) membranes, mitochondria, perinuclear region and/or in hepatocyte cell nucleus was observed. No direct relationships could be demonstrated between expressions of HCV proteins and of Ki-67 antigen. No correlations could also be demonstrated between cellular expression of any HCV protein on one hand and grading or staging, alanine transaminase (ALT), serum level of HCV RNA or alpha-fetoprotein (AFP) on the other. However, positive correlations were disclosed between proliferative activity of hepatocytes on one hand and patient's age, grading and staging on the other. Advanced hepatic fibrosis correlated also with serum levels of AFP. The studies were supplemented with data on subcellular localization of HCV proteins. Moreover, they indicated that in HCV infection grading and staging, proliferative activity of hepatocytes and serum AFP level represent more valuable indices of the disease progress than those provided by cellular expression of three potentially oncogenic HCV proteins *in vivo*.

Key words: Chronic hepatitis C virus (HCV) infection - Proliferative marker Ki-67 - HCV proteins (core, NS3 and NS5A) subcellular localization

Introduction

Hepatitis C virus (HCV) continues to represent the main causative agent of the hepatitis, which leads to chronic transformation of the process in 60-80% individuals, except those infected with genotype 2 HCV in Africa, which is cleared by 53% of individuals [1]. The infection is induced by a virus carrying a positive-sense single-stranded RNA genome that is about 10 kb in length and encodes for a precursor polyprotein of about 3,000 amino acids that is then cleaved into structural and nonstructural proteins [1,2]. Chronic hepatitis C is one of the major risk factor for the development of liver cirrhosis and hepatocellular carcinoma (HCC), but the mechanisms underlying liver damage and the carcinogenic process are unclear [2]. In recent years, studies *in vitro* have been focused on effects of HCV genome products in control of hepatocyte proliferation [3-7]. The potential members of the oncogene group which might be involved in development of

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HCC include at least three proteins of hepatitis C virus (HCV), *i.e.* C (core, capsid), NS3 and NS5A proteins [2,4,8]. All the three proteins might undergo translocation to cell nucleus acting as transcription activators [2,9]. Involvement of the proteins was described in cell cycle control, mediated by interactions with p21, p53 proteins and cyclins [3,5-7,10,11]. Some authors suggest that steatosis poses an additional risk for HCC [12]. HCV core protein plays an important role in the development of hepatic steatosis in HCV infection by stimulating expression of lipogenic enzyme genes and fatty acid uptake associated protein [13]. Recent *in vitro* studies provided a molecular explanation for HCV genotype 3-specific lipid accumulation [14]. Apart from C protein, a direct role in neoplastic transformation of hepatocytes *in vivo* and *in vitro* plays also NS3 protein [6,15]. NS3 in a dose-dependent manner specifically inhibits activity of *p21/WAF1* promoter. The effect is not cell specific and manifests synergy with the effect of C protein [6]. NS5A protein represents a component of the complex of viral replicase, localized on the cytosolic part of endoplasmic reticulum [1,2,16]. Post-translational modifications result in development of a protein form with lower molecular weight, capable of translocation to the cell nucleus. This protein form may act as a transcription activator. In the virus/host interactions the protein exhibits a multidirectional activity, influencing, among other, the cell growth control [3,16,17]. Several results point to inter-relationships between subcellular localization, molecular forms, concentrations of the proteins in hepatocytes of patients with chronic type C hepatitis and their effects on mechanisms linked to oncogenesis [15,18,19]. Controversies as to localization of HCV proteins in chronic type C hepatitis reflect, first of all, the difficulties in obtaining an ideal model for studies on replication of HCV. In most cases, both C protein and the non-structural proteins (NS2-NS5) are associated with cellular organelles in the cytoplasm (endoplasmic reticulum, mitochondria, Golgi apparatus) and lipid droplets [20-23]. Moreover, they have been localized in the perinuclear region and in the cell nucleus itself [15,24].

Ki-67 expression in tumours has been shown to be associated with prognosis in patients with hepatocellular carcinoma [25]. Application of MIB-1 antibodies for testing of proliferative activity in the liver suggested that HCV infection induced increased and abnormal hepatocyte proliferation, which might be related to the increased risk of HCC in patients with HCV-related liver damage [26]. Other HCC-associated variables in patients with chronic viral hepatitis and cirrhosis are: histological activity, platelet count and alpha-fetoprotein serum levels [27].

In this paper, in an attempt to further elucidate the cellular expression of three HCV proteins (NS3, NS5A and C protein) and hepatocellular proliferation, we assessed the potential relationship between cellular expression of the proteins and proliferative activity and selected clinical data in a new series of 19 patients with chronic, long lasting (approximately 20 years) hepatitis C.

Material and Methods

Human liver specimens. Studies were performed on 19 archival biopsies of liver, obtained from adult patients with documented chronic, long-lasting hepatitis C and with active replication of the virus, confirmed serologically (all the patients were anti-HCV and HCV RNA positive). The biopsies originated from patients of the Department of Infectious Diseases and Chair of Gastroenterology and Human Nutrition, Poznan University of Medical Sciences, in whom the biopsies were performed in 2005-2006. All the patients had negative serology for hepatitis B surface antigen (HBsAg) and antibodies to human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), and human immunodeficiency virus (HIV) by standard enzyme immunoassays (ELISA). Other cases of liver damage (*e.g.* 1-antitrypsin deficiency, Wilson's disease, alcohol dependency) were ruled out. All the patients had abnormal serum alanine aminotransferase (ALT) levels (normal level below 40 IU/l) for at least 6 months. All the patients were seropositive for antibody to HCV by ELISA (HCV version 3.0 AXYM System, Abbott). Quantitation of HCV RNA was conducted in 10/19 patients using AMPLICOR HCV^{TM} test, version 2.0 (ROCHE, Mannheim, Germany) with sensitivity of 600 IU/ml. The test was standardized against the WHO International Standard for HCV RNA. HCV genotyping was carried out with INNO LiPa HCV II Assay (Innogenetics, Zwijnaarde, Belgium) in 11/19 patients. Before liver biopsy none of the patients was subjected to anti-viral therapy. At the time of biopsy the mean age of patients (13 men and 6 women) was 46.7 ± 4.0 years (range: 19 to 74 years). At the time of liver biopsy, the mean duration of HCV infection was 19.3 ± 2.3 years.

The control group consisted of ten fragments of livers devoted to organ grafting and obtained from serologically HCV, HBV, CMV and EBV negative donors. Written informed consent was obtained from each patient before liver biopsy, and approval for the study was granted by the institution's Ethical Committee.

Tissue preparation. Biopsy specimens were fixed in 10% formalin, embedded in paraffin for purposes of light microscopy. Histopathological lesions were evaluated, following the classical H+E staining as well as silver technique and tri-chromate technique, according to Masson and periodic acid-Schiff with diastase pretreatment. At least 10 sections were prepared from each biopsy or from control liver fragments. Each tissue specimen was evaluated basing on a numerical scoring system for the grade of portal/periportal necroinflammation (G1=0-4), for the grade of lobular necroinflammation (G2=0-4) and for the stage of fibrosis (S=0-4), as proposed by Scheuer [28], and their sum (final grading, G1+G2). The histological lesions considered to be characteristic of chronic hepatitis were also evaluated. Histological evaluation was performed independently by two experienced histopathologists. For electron microscopy, the material was fixed in 2% glutaraldehyde (transmission electron microscopy) or 4% (v/v) paraformaldehyde containing 0.2% (v/v) glutaraldehyde in 0.1M phosphate buffer (pH=7.3) (immunoelectron microscopy). The embedded in epon ultrathin sections were subjected to a conventional electron microscopy and ultraimmunocytochemistry [22].

Immunocytochemistry. For immunocytochemistry 5 μm thick sections were cut and mounted onto SuperFrost/Plus microscope slides. Immunocytochemistry, using the mouse anti-NS3, anti-NS5A proteins (NOVOCASTRA Labs.), and anti-capsid C protein

(CHEMICON International, Inc.) monoclonal antibodies was performed according to classical ABC (avidin-biotin-peroxidase complex) technique alone or in association with the ImmunoMax technique [22,29]. For testing proliferative activity of hepatocytes mouse anti-human Ki-67 monoclonal antibodies (clone MIB-1) were used in dilution of 1:2 (DAKO). In ABC and ImmunoMax techniques microwave-oven pretreatment for antigen retrieval was used. In cases of three applied monoclonal antibodies against HCV proteins the dilution of 1:50 proved to yield optimum results. In ImmunoMax technique, the key reaction involved 8 min. incubation with biotinylated tyramine (1:50) at room temperature (PerkinElmer Life Sciences, Inc.). This was followed by another application of streptavidin complex. The colour reaction was evoked with the horse radish peroxidase substrate, 0.05% DAB in 0.05 M Tris-HCl buffer, pH=7.6, supplemented with 0.001% H_2O_2. Positive reaction manifested, in at least three sequential sections, as a dark brown or black precipitate in the cell nucleus and/or cytoplasm. Control reactions employed control sera of the respective species in 0.05 M Tris-HCl, pH=7.6, supplemented with 0.1% BSA and 15 mM sodium azide (negative control).

Appropriate positive control for Ki-67 antigen expression involved 5 tonsils with inflammatory lesions.

Immunoelectron microscopy. At the electron microscope level, the immunocytochemical tests were preceded by examination of NS3, NS5A and C protein expression in semithin sections, using ABC and ImmunoMax techniques. Ultrathin section were subjected to labelling with 15 nm colloidal gold-streptavidin (Biocell Int., Cardiff, UK). In each case, mouse MAbs were used in 1:50 dilution. The negative control of the immunocytochemical reaction at the level of electron microscopy involved substitution of primary antibodies by a normal serum obtained from the same animal species as the primary antibodies. Moreover, the company which produced the antibodies (NOVOCASTRA, CHEMICON) warranted specificity of the antibodies (ELISA tests, results of Western blot analysis).

Semiquantitative evaluation of the results and statistics. The contents of NS3, NS5A and C proteins in liver biopsies obtained using ImmunoMax technique were calculated by the semiquantitative technique and reported as follows: 0=0% positive cells; 1=individual positive cells (<10%); 2=10-25% positive cells; 3=26-50% positive cells; 4=51-75% positive cells; 5=76-100% positive cells) [30], evaluating 10 microscopic fields at magnification of 400×. The final result represented mean score for the 10 fields. Expression of the antigens was evaluated separately in the cytoplasm and in cell nucleus. Expression of Ki-67 proliferation-associated antigen (only clearly labelled cell nuclei were considered), was calculated taking into account mean proportion of immunopositive cells in 10 light microscope fields. Positive nuclear staining was evaluated by counting a total of at least 1000 cells. Expression was evaluated using the semiquantitative scale of Gatter *et al.* [31], in which the score of 1 corresponded to up to 10% positive cells; the scores of 2, 3 and 4 corresponded to 11-25%, 26-50% and ≥51% positive cells, respectively. Fatty degeneration of liver was also semiquantitatively appraised, scoring 0 when no steatosis was noted under a light microscope, and annotating grades 1 or 2 when, respectively, <30% of hepatocytes or 30-70% of hepatocytes were affected [32].

All the preparations were examined under an OLYMPUS B-2 light microscope, at magnification ×400.

In order to determine statistical significance of variations in the expression intensity of the proteins (the results obtained in ImmunoMax technique) and Ki-67 expression, we first calculated the mean values of staining scores for liver biopsy groups. The means were compared using the Mann-Whitney U test for non-parametric independent data and the Wilcoxon test for non-parametric dependent data. Correlations between data rows were determined employing Spearman's rank correlation index.

Table 1. Epidemiological, biochemical and virological features of chronic HCV infected patients.

Genotype 1b HCV	8/11 (73%)
Gender (male/female)	13/6
Age (mean±SEM, yrs)	47±4
Duration of HCV infection (yrs)	19±2
ALT (mean±SEM, U/L)	100±15
AST (mean±SEM, U/L)	91±12
HCV RNA (mean±SEM, IU/mL)	601354±371017
AFP (mean±SEM, IU/mL)	8.6±4.3

Table 2. Histopathological scoring of chronic HCV infected patients.

Category	Score (mean±SEM)
Portal/periportal activity (G1)	1.9±0.2
Lobular (acinar) activity (G2)	2.1±0.2
Final grading of inflammation (G1+G2)	4.0±0.4
Stage of fibrosis	2.6±0.3
Fatty degeneration of liver	1.2±0.2

Results

Epidemiological, biochemical and virological data of patients are summed up in Table 1.

Liver histology

All 19 liver biopsy samples of anti-HCV-positive patients were diagnosed as manifesting histological criteria typical of chronic type C hepatitis. Patients with stage 3 and 4 fibrosis were combined into a group with advanced fibrosis. The average stage of fibrosis was 2.6±0.3. The average portal/periportal activity (G1) was 1.9±0.2 and lobular (acinar) activity was 2.1±0.2; the sum of grading (G1+G2) was 4.0±0.4 (Table 2). In histological patterns large groups of lymphoid cells in the dilated portal spaces were observed in individual patients. Infiltrates consisting of individual lymphoid cells or of their groups as well as traits indicative of fibroblast proliferation were observed also within hepatic lobules. Numerous biopsies demonstrated enlarged hepatocytes, often with the evident fatty degeneration, involving the entire cell. Numerous cells were observed with swollen, partially vacuolised cytoplasm and individual cells showed lysis. Cells with traits of cholestasis were also observed. The most significant lesion in the entire material involved pathology of hepatocyte nuclei (their variable size and irregular shape, altered chro-

Table 3. Histological findings and semiquantitative appraisal of Ki-67, NS3, NS5A and C proteins in liver biopsies of patients with chronic hepatitis C. Designations: G1 - portal/periportal activity; G2 -grading in lobules; S - staging; [a] - score according to [28]; fatty degeneration score according to [32]; [b] - scoring system according to [31]; [c] - score: 0=0% cells; 1=individual positive cells (<10%); 2=10-25% positive cells; 3=25-50% positive cells; 4=50-75% positive cells; 5=75-100% positive cells) [30]; the results are given in two numbers of which the first denotes expression in cell nucleus and the other - cytoplasmic expression in hepatocytes; nt - not tested.

Patient/age (yr)/sex	Scoring of histological diagnosis[a]				Antigens			
	G1	G2	S	steatosis	Ki-67[b]	NS3[c]	NS5A[c]	C protein[c]
1. JZ, 57/F	3	3	4	2	2	0/5	1/1	0/0
2. SA, 20/M	1	1	1	0	1	0/5	0/5	0/0
3. KJ, 72/M	3	3	3	2	3	0/4	0/3	0/3
4. SH, 48/M	2	3	2	1	1	0/3	0/2	0/3
5. WJ, 27/F	3	3	4	1	3	0/5	0/2	0/3
6.KP, 24/M	1	1	1	1	2	0/5	0/4	0/2
7. AJ, 60/M	3	3	4	1	3	0/5	0/3	0/1
8. SJ, 55/M	1	3	1	1	2	0/5	0/3	0/1
9. FA, 34/M	1	1	1	2	2	0/4	0/1	0/1
10. WM, 54/M	0	1	3	1	1	0/1	0/1	0/1
11. NA, 65/M	2	2	3	0	2	0/5	0/5	0/3
12. WW, 37/M	2	3	2	2	2	0/5	0/1	1/2
13. SL, 53/M	3	2	3	2	3	0/5	1/2	1/1
14. KD, 27/M	0	1	1	0	1	0/5	0/4	0/3
15. UD, 19/F	1	0	1	1	1	0/4	1/1	1/2
16. PJ, 55/F	3	2	4	1	3	1/5	nt	3/4
17. SJ, 51/F	2	3	4	1	nt	0/5	nt	1/4
18. SB, 56/F	2	2	4	2	nt	0/4	nt	1/4
19. RE, 74/M	3	3	4	2	nt	0/4	nt	0/4

matic arrangement, numerous nucleoli, lesions in nuclear envelope, frequent binary cell nuclei, variable staining).

In ultrastructure, numerous damaged mitochondria, with a markedly more lucid, fine granular, swollen matrix were frequently observed, with absent or short cristae, the membranes of which formed numerous horse-shoe shaped structures, frequently in a direct contact with ER cisterns. The ER cisterns were frequently widened, contained fine fibrillar material and manifested only focal presence of ribosomes. Cell nuclei differed in size. Occasionally they were markedly enlarged, with irregularly less compact chromatin, large active nucleoli and formation of perinucleolar network of filamentous structures. The altered cell nuclei contained also sets of tubular structures.

In control liver biopsies, nonspecific changes were noted, with individual cells of inflammatory infiltrate in portal spaces, with no traits of liver fibrosis.

Immunocytochemical localization of HCV proteins in the liver using ABC technique

Using ABC technique alone only NS3 protein was detected in all patients (n=19) but the reaction product was poorly visible. In each case demonstration of the reaction product required that the high temperature antigen unmasking technique was employed. Microscope analysis demonstrated cytoplasmic staining in a few hepatocytes near blood vessels or a diffuse cytoplasmic staining in many hepatocytes in the entire field of hepatic lobule. Using the classical ABC technique, neither NS5A nor C protein could be detected in any of the patients.

Immunocytochemical localization of proteins in the liver using ABC technique combined with ImmunoMax method

NS3 protein. Biotinylated tyramine amplification of ABC technique permitted to detect NS3 protein in all

the examined liver biopsies (n=19). Cellular localization of the protein resembled that documented using the classical ABC technique. In the entire group of the patients, a definite prevalence of cytoplasmic as compared to nuclear localization of NS3 protein was noted (Fig. 1A and 1B). The reaction product was much more evident than that obtained with the use of unamplified ABC technique. The nuclear plus cytoplasmic signal of NS3 protein presence was observed in individual hepatocytes in a single liver biopsy specimen (Table 3). No priviledged localization of NS3 protein in hepatic lobules could be disclosed although the immunopositive hepatocytes frequently were present close to blood vessels or were present in groups. Presence of NS3 protein was demonstrated also in cells of hepatic sinusoids (macrophages, endothelial cells) and in individual cells of inflammatory infiltrates in portal spaces of individual patients. In the entire group of patients, more cells with a cytoplasmic presence of NS3 protein were detected than cells with nuclear localization of the protein (p=0.0001) (Table 4).

NS5A protein. The ImmunoMax technique permitted to detect NS5A protein in all the examined liver biopsies (n=15). As a rule, a cytoplasmic localization of the protein was observed, which focally manifested a relatively high intensity (Fig. 2A and 2B). In the entire group of patients, more cells with a cytoplasmic presence of NS5A protein were detected than cells with nuclear localization of the protein (p=0.001) (Table 4). In three patients the nuclear localization was observed only in individual cells (Fig. 2C). It should be stressed that detection of NS5A protein in the studied clinical material was possible only when the ImmunoMax amplification was employed.

C protein. Presence of C protein was demonstrated in 17/19 patients. A cytoplasmic plus nuclear as well as perinuclear expression of the protein was disclosed in whole group of patients (Fig. 3A and 3B). The reaction product was noted also in the cytoplasm of individual cells in hepatic sinusoids and inflammatory infiltrates of portal spaces. It should be stressed that detection of C protein in the studied clinical material was possible only when the ImmunoMax amplification was employed. In the entire group of patients, more cells with a cytoplasmic presence of C protein were detected than cells with nuclear localization of the protein (p=0.0004) (Table 4). Nuclear localization was observed only in individual cells and in 6/19 biopsy specimens.

NS3 and NS5A protein *vs.* C protein. Significantly higher numbers of cells with NS3 protein expression were detected as compared to cells with C protein expression (p=0.0006) or cells containing NS5A protein (p=0.002). No significant differences could be disclosed between expression of C protein and that of NS5A protein in the liver biopsy specimens (Table 4). All the control reactions and reactions in control biopsy specimens yielded negative results.

Table 4. Comparative immunocytochemical intensity of NS3, NS5A and C protein in nuclear *vs.* cytoplasmic localization in patients chronically infected with HCV (mean±SEM).

Proteins	Patients (n=19)	
	Nuclear localization	Cytoplasmic localization
NS3 (4.4±0.2)	0.1±0.1	4.4±0.2
	p=0.0001	
NS5A (2.5±0.4)*	0.2±0.1	2.5±0.4
	p=0.001	
C (2.2±0.3)**	0.4±0.2	2.2±0.3
	p=0.0004	

* - p=0.002 for NS3 *vs*. NS5A; ** - p=0.0006 for NS3 *vs*. C protein.

Table 5. MIB-1 (Ki-67 antigen) labelled hepatocytes in patients with or without cirrhosis by Scheuer score (mean±SEM).

	No. of patients	MIB-1 cells/1000[a]
No cirrhosis	10	1.6±0.2
Cirrhosis	6	2.7±0.2
p value		0.01

[a] - score according to [31]; 1=up to 10% positive cells; 2=11-25% positive cells; 3=26-50% positive cells; 4=51-75% positive cells.

Ki-67 antigen. Expression of Ki-67 proliferation-associated antigen was detected in liver biopsies of all the patients. In most cases an intense nuclear reaction was detected in hepatocytes and in individual cells of inflammatory infiltrates (Fig. 4). In the semiquantitative scale the mean cellular expression was 2.0±0.2. No correlation could be noted between expressions of Ki-67 antigen and of HCV proteins. However, a significantly higher expression of Ki-67 antigen was documented in patients with liver cirrhosis as compared to patients with no cirrhosis (p=0.01) (Table 5). Significant positive correlations were detected between expression of the antigen and patient's age (r=0.507; p=0.045), portal/periportal grading (G1) (r=0.817; p=0.0001), lobular activity (G2) (r=0.529; p=0.03), final grading (G1+G2) (r=0.754; p=0.0007), and staging (r=0.644; p=0.007). A negative correlation was noted between proliferative activity and duration of the infection (r=-0.766, p=0.03).

In the negative control material individual MIB-1-positive cells were noted, as compared to numerous MIB-1-positive lymphocytes in tonsillar lymphoid follicles.

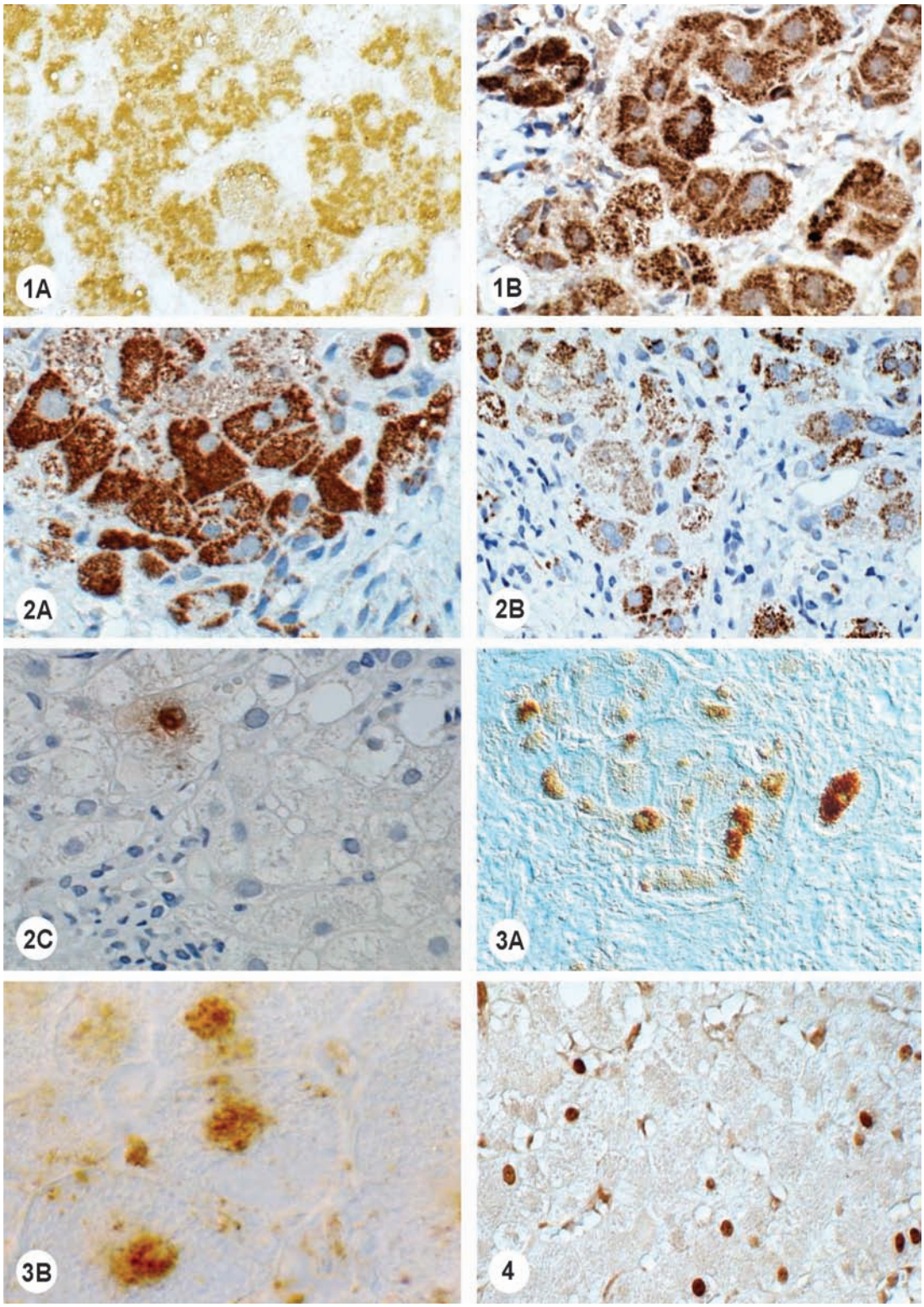

Fig. 1. Fragments of hepatic lobules from patients with chronic, long lasting HCV infection. (**A**) Immunocytochemical localization of NS3 protein in hepatocytes in a semithin section. (**B**) NS3 protein in the cytoplasm of liver cells in stage 3 fibrosis. ABC-ImmunoMax technique. Hematoxylin counterstained (B). Original magnification ×400. **Fig. 2**. Fragments of hepatic lobules from patients with chronic, long lasting HCV infection. (**A**) Very strongly immunopositive hepatocytes with NS5A protein and (**B**) NS5A protein dispersed between inflammatory infiltrate cells, hepatocytes in the same patient as that in Fig. 1B. (**C**) Nuclear localization of NS5A protein in another patient with stage 4 fibrosis. ABC-ImmunoMax technique. Hematoxylin counterstained. Original magnification ×400. **Fig. 3**. Fragments of hepatic lobules from patients with chronic, long lasting HCV infection. (**A**) Immunocytochemical cytoplasmic localization of C protein. (**B**) Nuclear localization of C protein. ABC-ImmunoMax technique. Hematoxylin counterstained (A). Original magnification ×400 (A), ×1000 (B). **Fig. 4**. MIB-1 (Ki-67 antigen) positive nuclei of hepatocytes in patient with stage 3 fibrosis. ABC technique. Original magnification ×400.

Immunoelectron microscopy

Application of ABC technique to semithin sections confirmed expression and cellular localization of all proteins and a more intense signal using ImmunoMax technique (Fig. 1A). At the ultrastructural level, application of colloidal gold permitted to document the presence of NS3 and NS5A proteins frequently localized in dilated ER cisternae, and also of enlarged mitochondria and cytoplasm (Fig. 5A-C). C protein was also mainly localized in dilated ER cisternae, mitochondria, perinuclear region and the cytoplasm of hepatocytes (Fig. 5D-F). We also observed individual cell nuclei labeled with immunogold, mostly detecting C protein (Fig. 5E and 5F).

Semiquantitative evaluation of NS3, NS5A and C protein expression as compared to selected histopathological and clinical data

In none of the patient significant correlation between amounts of detected proteins and grading/staging, ALT activity, AFP level, the length of infection or HCV RNA level could be disclosed. Only in the case of NS5A protein a negative relationship could be documented between expression of the protein and fatty degeneration of the liver ($r=-0.594$, $p=0.02$). No significant reciprocal relationships could be detected between expressions of three HCV proteins in individual patients.

Other correlations

The mean serum level of AFP in the studied patients was 8.6±4.3 IU/ml. A significant positive correlation was detected between serum concentration of AFP and advancement of fibrosis (staging) ($r=0.692$, $p=0.01$). No relationships could be noted between serum AFP level and ALT activity, G1, G2, final grading (G1+G2) or duration of the infection ($p>0.05$).

Discussion

Role of HCV proteins in oncogenesis of HCC remains unclear. Studies at the tissue level continue on expression of viral proteins and/or of HCV RNA, which might participate in neoplastic transformation [4,5,6]. The last decade brought several experimental proofs for participation of particularly three HCV proteins, i.e. core (C), NS3 and of NS5A in the process of hepatocellular neoplastic transformation [2,5,6,16]. Debates continue on cellular and subcellular localization of HCV proteins [9,15,19,21,23]. Until now studies performed by our team on expression of two HCV proteins (C and NS3) in the chronic HCV infection in children, point to prevalence of cytoplasmic as compared to nuclear localization of the two proteins in hepatocytes, which has been confirmed by ultrastructural studies. Presence of the two proteins in a cell used to be accompanied by a significant dilatation of endoplasmic reticulum cisternae and altered mitochondria. The subcellular localization in the cell nuclei and/or the perinuclear region has been observed more frequently for C protein than for NS3 [22].

More frequent cytoplasmic than nuclear localisation of the three HCV proteins observed by us at the level of light microscopy is consistent with the literature data even if the studies differ in the applied antibodies and in tissue processing (paraffin *vs.* cryostat sections) [33,34]. Reports have also been published on unsuccessful attempts to detect C protein and NS3 protein using non-human antibodies [35]. In other studies a prevalence of nuclear localization of HCV antigens has been detected in patients with chronic type C hepatitis [24]. C protein has been detected most frequently inside cytoplasm and in association with ER membranes [36,37], but also in lipid droplets [21,23] and in mitochondria [18,38]. Immunofluorescence studies have shown that the C protein colocalized with the E2 glycoprotein as well as with a cellular ER membrane marker. There are also available reports which do not confirm the mitochondrial localization of C protein [21]. At the beginning, the nuclear localization of C protein has been excluded *in vivo* [23], suggesting only a potential for perinuclear localization although subsequent studies have demonstrated two forms of C protein and one of them (21 kDa) has been present both in the cytoplasm and in the cell nucleus [9]. The nuclear localization has been confirmed also by other authors [9,18,24]. Moreover, the nuclear localization of C protein pertains an incomplete protein molecule [19,39]. Relatively recent studies have identified a novel bipartite nuclear localization signal (NLS), which requires two out of three basic-residue clusters for efficient nuclear translocation of C protein, possibly by occupying binding sites on importin-α [18]. On the other hand, no nuclear localization of C protein could have been confirmed by immunofluorescence studies using a confocal microscope and the Huh-7 cell line [21]. Earlier studies of our team have demonstrated C protein both in hepatocyte cytoplasm and in individual cell nuclei. The two localizations have been confirmed by electron microscopy [22].

Our present ultrastructural observations indicate that C protein in cytoplasm is present first of all in dilated cisternae of ER and frequently in mitochondria. Thus, we can confirm the suggestion on the role of C protein in disturbing function of mitochondria, which leads to augmented levels of free oxygen radicals and, thus, to lipid peroxidation also *in vivo* [38]. Our earlier studies in HCV infected children [22] and our present data on livers of patients with long-lasting HCV infection have demonstrated mitochondrial localization of also NS3 protein, but significance of such localization has not been closer recognized in *in vitro* studies. NS5A protein detected by our team for the first time *in vivo* has been observed mainly in the cytoplasm of hepatocytes and in

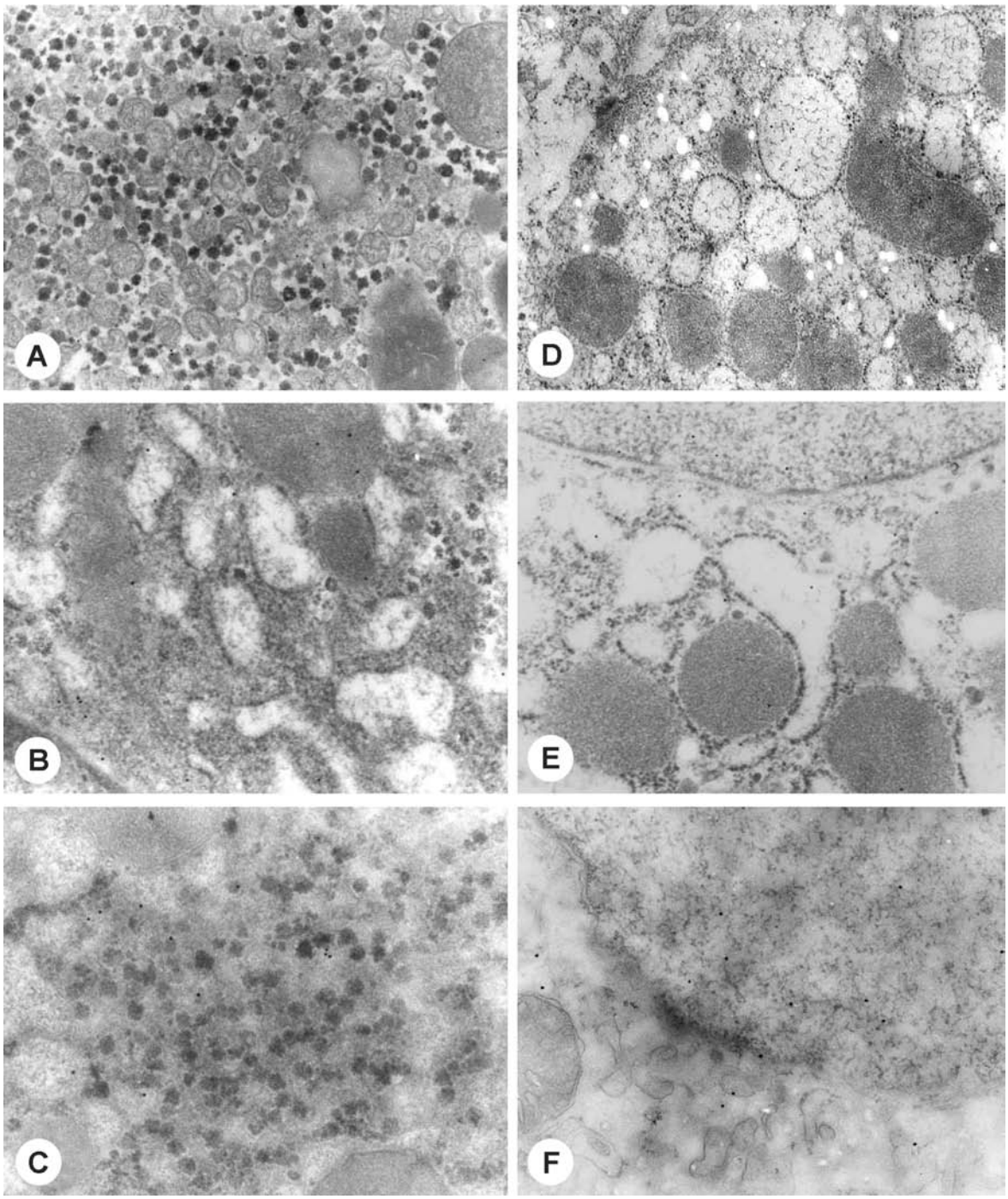

Fig. 5. Ultrastructural localization of NS3 (**A, B**), NS5A (**C**) and C proteins (**D-F**) in hepatocytes of patients chronically infected with HCV. (**A**) Gold particles overlay mainly endoplasmic reticulum, cytoplasm and mitochondria. (**B**) Gold particles overlay mainly dilated ER membranes surrounding mitochondria, mitochondria and cytoplasm. (**C**) Gold particles overlay mainly cytoplasm and ER. (**D-E**) Gold particles overlay dilated ER surrounding mitochondria, mitochondria and cell nucleus structures. Streptavidin-colloidal gold. Original magnification (A) ×20 000; (B) ×25 000; (C) ×30 000; (D) ×15 000; (E) ×20 000, (F) ×25 000.

individual cell nuclei of infected hepatocytes. All the proteins which we discuss in this study have also been noted in individual mononuclear cells of inflammatory infiltrates. In this study a significantly more pronounced expression of NS3 protein has been demonstrated, as compared to expressions of NS5A and C protein, with no differences between the latter two expressions. On the average, slightly above 50% cells have been found positive for NS3, which has exceeded proportions observed by other authors (1-20%) [36]. Moreover, detection of the remaining HCV proteins (C and NS5A) has been observed only when the ImmunoMax technique has been applied. This corroborates our earlier results on detection of HCV proteins [22]. In a given

patient, no correlation could have been disclosed between expressions of the proteins. Also, the expression has shown no significant relationship to proliferative activity, evaluated by expression of Ki-67 antigen nor to histopathological variables such as grading/staging and liver steatosis, except for the negative correlation observed between expression of NS5A protein on one hand and fatty degeneration on the other. We cannot confirm the suggestions on the inducing role of C protein in lipid droplet accumulation *in vivo* [13], and the association of NS5A and C proteins with lipid droplets and apolipoprotein A1 [40]. Among our 11 patients with HCV genotyping, 73% had HCV-1b infection. Some recent *in vitro* observations suggest that liver steatosis is related to genotype 3 HCV [14]. Expression of the studied HCV proteins has failed to correlate with age of the patients, ALT activity, serum HCV RNA and AFP levels, or duration of the infection. We observed the significant positive correlations have been noted between proliferative index of hepatocytes and staging in the organ. Thus, more pronounced expression of Ki-67 antigen has been observed in patients with liver cirrhosis, as compared to patients with less advanced fibrosis. In studies of other authors positive correlations were documented between proliferative activity and staging, but at the stage 4 of fibrosis decreased expression of Ki-67 was noted, suggesting an impairment of hepatic proliferation in cirrhosis [41]. Our results have demonstrated no relationship between hepatocyte proliferative index and serum AFP level, confirming the results of other authors [41]. Similarly to present results, other authors detected positive relationship between serum AFP levels and extent of fibrosis in the liver [32,41,42]. Finally, this study has shown positive correlation between expression of Ki-67 antigen on one hand and patient's age and grading in the liver on the other. This is consistent with observations of other authors who in HCV infected patients demonstrated positive correlations between numbers of Ki-67-positive cells on one hand and ALT levels, Knodell index [26], older age, more severe necroinflammation, and more severe fibrosis [41] on the other. In patients with HCV more numerous Ki-67-positive cells were detected in the periportal area and in the poorly proliferating perivenular area (zones 2 and 3) [26]. In our results no differences in expression of Ki-67 antigen could have been detected between various regions of the hepatic lobule. Similarly, no correlation could have been detected between Ki-67 tissue expression and ALT activity. Similar results were obtained by Canchis *et al.* [39] although some other authors have detected such a relationship [26,43]. The negative correlation between proliferative activity evaluated by expression of Ki-67 antigen and duration of infection has been surprising. In studies of other authors on HCCs, patients with anti-HCV antibody had a lower MIB-1 labeling index and better prognosis than HCC patients negative for the antibody. However, there was no significant correlation between anti-HCV antibody and other proliferative parameters [44]. We cannot comment on results of such studies since we have not examined patients with hepatocarcinoma. Nevertheless, it is worth stressing that advancement of fibrosis represents a more accurate index of the disease progress than duration of the infection. As many as two of the studied by us variables (tissue expression of Ki-67 and serum AFP level) have shown a direct relationship to staging. Other authors have shown that also increased serum alpha-fetoprotein levels were one of the HCC-associated variables in patients with virus-related hepatitis [27].

In conclusion, our microscopic analysis demonstrated cellular expression of NS3, NS5A and C proteins mainly in the cytoplasm of infected hepatocytes in our patients. Only in individual cells the nuclear presence of the three proteins was noted. It should be stressed that detection of all studied HCV proteins was possible in our archival, paraffin-embedded material obtained from immunocompetent patients with long lasting liver disease, independently of their detailed clinicopathological data. As compared to the classical ABC technique, higher sensitivity of the ImmunoMax-amplified technique was demonstrated, employing monoclonal antibodies for detection of even small amounts of viral protein in the tissue. Application of the technique significantly augmented the potential for diagnosis of chronic type C hepatitis in routinely sampled and fixed biopsy material. Our results supplemented the data on subcellular localization of HCV proteins. Moreover, they demonstrated that proliferative activity of hepatocytes *in vivo*, intensity of fibrosis, grading and serum AFP level provide a more accurate index of progression in HCV-induced liver disease as compared to cellular expression of HCV proteins.

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