Liver Cell Apoptosis in Chronic Hepatitis C Correlates With Histological But Not Biochemical Activity or Serum HCV-RNA Levels

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In hepatitis C virus (HCV) infection, mechanisms responsible for liver cell damage are still poorly understood and both necrosis and apoptosis may be operative. By using terminal deoxynucleotydil transferase-mediated d-UTP-biotin nick-end labeling (TUNEL) we have evaluated and quantified apoptosis in liver biopsy specimens from 61 patients with chronic hepatitis C. All patients had detectable apoptotic cells in the liver. Presence of increased apoptotic activity was confirmed in selected cases by electron microscopy and by DNA gel electrophoresis. The amount of liver cell apoptosis expressed as apoptotic index, ranged between 0.01% to 0.54% and showed a positive correlation with histological activity grading ($P < .0005$) and with the amount of infiltrating CD8-positive cells ($P = .01$). Apoptosis did not correlate with transaminase levels or with HCV load and genotype. These results support the concept that immune-mediated apoptosis may play a role in the pathogenesis of chronic hepatitis C and indicate that this type of reaction may occur in the absence of significant alanine transaminase (ALT) elevation, thus explaining the lack of correlation between biochemical activity and liver histological damage. (Hepatology 2000;31:1153-1159.)

Hepatitis C virus (HCV) is a major cause of chronic liver disease, cirrhosis, and hepatocellular carcinoma[1] worldwide. The infection has a high propensity to chronicity and the majority of HCV carriers have histological evidence of liver inflammation and chronic damage, although with a very wide spectrum of severity and progression rate.[2] The mechanisms leading to liver cell injury, inflammation, and fibrosis in chronic hepatitis C, are not fully understood. Both immune-mediated reactions and more direct cytopathic effects of HCV and of its proteins may be involved. Evidence has been provided that apoptosis of liver cells may play a significant role in the pathogenesis of hepatitis C.[3-5] Apoptosis is an active, genetically programmed phenomenon of cell death characterized by a unique sequence of events, with morphological features distinct from necrosis.[6] Three pathomorphologic features are suggestive of increased apoptosis in the liver of patients with chronic hepatitis C including (1) presence of shrinkage and fragmentation of nucleus/cytoplasm in areas of piecemeal necrosis, (2) presence of acidophilic bodies, and (3) focal cell drop out in the liver lobule.[7,8] Increased expression of Fas, one of the most important members of the tumor necrosis factor family receptors able to transduce the apoptotic signal to programmed cell death, has been described in chronic hepatitis C. Hepatic up-regulation of Fas was found to correlate with more severe inflammation[9] and with ongoing HCV infection.[10] Parallel activation of T lymphocytes expressing Fas ligand was detected in liver infiltrating mononuclear cells, allowing transduction of the apoptotic death signal to Fas-bearing hepatocytes[3] and to proinflammatory activated cells that continuously migrate from extrahepatic sites.[11] In addition, both structural and nonstructural HCV proteins have been shown to interact with apoptosis mediators and possibly modulate the active cascade of events leading to programmed cell death. The core protein has shown both proapoptotic or antiapoptotic activities, depending of the experimental conditions and type of cell used,[12-14] whereas both the NS3[15] and the NS5A[16] proteins were shown to have antiapoptotic effects. In this study we have quantified apoptosis by the transferase-mediated d-UTP-biotin nick-end labeling (TUNEL) technique in the liver of 61 patients with chronic hepatitis C. The degree of liver cell apoptosis obtained was then correlated with the degree of inflammation and fibrosis in the liver, as well as with transaminase, serum HCV-RNA level, and genotype. Apoptosis was also investigated in selected specimens by electron microscopy and by DNA agarose gel electrophoresis. To assess the relationship between apoptosis and liver cell proliferation, liver biopsy specimens were also investigated using the Ki-67 equivalent MIB-1 antibody, which identifies cycling cells in formalin-fixed and archival tissue.[17]

PATIENTS AND METHODS

Patients

Liver biopsy specimens were obtained from 61 patients with chronic hepatitis C. The diagnosis was based on the presence of anti-HCV and HCV RNA in serum for at least 6 months. There were 38 men and 24 women, mean age being 42 ± 10 years (range, 18-64 years). The main clinical, biochemical, virological, and histological features in the 61 patients at the time of liver biopsy are described in

Abbreviations: HCV, hepatitis C virus; TUNEL, transferase-mediated d-UTP-biotin nick-end labeling; ALT, alanine transaminase; TBS, Tris-buffered saline; ABC, avidin-biotin complex; AI, apoptotic index; HAI, histological activity index.

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Table 1. Alanine transaminase (ALT) levels were normal in 5 cases (8%) and elevated in 56 (92%). Two cases (3%) had normal liver histology or only minor inflammatory changes, whereas 31 (51%) had mild chronic hepatitis, 21 (35%) had moderate/severe chronic hepatitis, and 7 (11%) had cirrhosis. Thirty-three (57%) patients were infected by HCV 1, 17 (29%) by HCV 2, and 8 (14%) by HCV 3.

Patients had no other causes of liver disease, and cases with alcohol abuse, autoimmune or metabolic disorders, and coinfection with hepatitis B virus or human immunodeficiency virus were excluded.

Liver specimens of uninfected patients, obtained during cholecystectomy (7 cases) or for the staging of mediastinal or laterocervical Hodgkin's disease (13 cases) were used as control. A mild cholestasis, without active inflammation, was identified in 6 of 7 livers derived from cholecystectomy, whereas the remaining liver specimens showed essentially normal morphology.

Histological Assessment

Liver biopsy specimens (length range 7-10 mm) were fixed in 10% neutral buffered formalin, progressively dehydrated in alcohol, and paraffin embedded. Thin sections (approximately 4 μm) were stained with hematoxylin and eosin, with Van Gieson, with diastase-Periodic Acid-Schiff and with Prussian blue. Grading of necroinflammatory activity (0 to 18) and staging of fibrosis (0 to 4) were semiquantitatively assessed using the Knodell scoring system, as modified by Desmet et al.[19] The following histological parameters were also considered: steatosis (absent = 0; mild = 1; marked = 2), bile duct lesions (present or absent), and follicular lymphomonocytic infiltrate (present or absent). Bile duct damage was defined as vacuolated acidophilic cytoplasm with nuclear pyknosis and the presence of inflammatory cell migration into or between the epithelial cells. Follicular lymphomonocytic infiltrate was defined as lymphoid follicles with germinal centers, or lymphoid aggregates lacking a germinal center in the center of portal tracts or as a part of a diffuse portal infiltrate.

Immunohistochemistry

For MIB-1 immunolocalization in the second staining sequence, sections were treated with 0.6% H_2O_2 for 30 minutes, to block endogenous peroxidase, and were heated in 0.01 mol/L citrate buffer (pH 6) in a microwave oven for 2 minutes at 700 W and for 4 minutes at 300 W. The slides were allowed cooling to room temperature, rinsed in Tris-buffered saline (TBS), and incubated with rabbit normal antiserum (Dako, Gloustrup, Denmark) for 30 minutes. The MIB-1 monoclonal antibody (Immunotech, Marseille, France) was applied at 1:50 dilution in TBS overnight at 4°C in a humidified chamber. A biotinylated monoclonal rabbit anti-mouse (Dako) at 1:200 dilution in TBS for 30 minutes was used as secondary antibody. The specimens were then stained using the avidin-biotin complex (ABC) horseradish peroxidase technique (Dako) according to the manufacturer's instructions, using diaminobenzidine as chromogen. Lymph node sections were used as positive controls.

The presence of CD4 or CD8 T lymphocytes in biopsy specimens was evaluated on paraffin sections. As the primary antibody, anti-human T cell CD4 mouse monoclonal antibody (IgG clone 1F6, 1:20; Novo Castra, Newcastle, UK) or anti-human T cell CD8 mouse monoclonal antibody (IgG, clone C8/144B, 1:100; Dako) were applied. Sections were incubated for 1 hour at 37°C with monoclonal antibodies and then detected using the ABC horseradish peroxidase technique. The specimens were classified into 3 groups according to the intensity of CD8 T lymphocyte infiltration: 1 = no/minimal infiltration, 2 = moderate infiltration, and 3 = marked infiltration. To confirm the specificity of immunostaining for CD4 and CD8 antigens, negative controls were prepared using mouse monoclonal IgG (1:400, Dako) as the primary antibody.

TABLE 1. Clinical, Histological, and Virological Characteristics of Patients With Chronic Hepatitis C

Sex (M/F)	38/23
Age	
Mean ± SD	42 ± 10 yrs
Range	18-64 yrs
Disease duration	
Mean ± SD	6 ± 5 yrs
Range	1-16 yrs
ALT levels	
Normal (No)	5
Elevated (No.)	56
Mean value ± SD (IU/L)	150 ± 107
Range	58-440
Histology	
Normal liver	2
Mild chronic hepatitis	31
Moderate/severe chronic hepatitis	21
Cirrhosis	7
HAI	
Mean ± SD (range)	6 ± 3 (1-15)
Genotype	
HCV 1	33
HCV 2	20
HCV 3	8

Assessment of Liver Cell Apoptosis

In Situ *End Labeling.* Terminal deoxynucleotydil TUNEL[18] was used to detect DNA fragmentation. Proteins were stripped from the nuclei of tissue sections by incubation with proteinase K (20 μg/mL; Boehringer Mannheim, Mannheim, Germany) for 10 minutes at room temperature. Terminal deoxynucleotidyl transferase (TdT) (0.3 e.u./μL) and digoxigenin-11-UTP (Boehringer Mannheim) in TdT buffer (30 mmol/L Trizma base, pH 7.2, 140 mmol/L sodium cacodylate, 1 mmol/L cobalt chloride) were added to the sections before incubation in a humid atmosphere at 37°C for 60 minutes. Before diaminobenzidine staining, the sections were incubated with peroxidase-conjugated Fab fragments of anti-digoxigenin antibody (Boehringer Mannheim) in phosphate buffer (1:50).

All solutions were prepared using DNAse-free distilled water and molecular biology grade reagents to avoid false positive results due to DNAse contamination.

Sections pretreated for 10 minutes with 0.7 mg/mL DNAse I (Boehringer Mannheim) in sodium cacodylate buffer (pH 7.2) served as positive controls. After thorough washing in distilled water, the slides were processed according to the above protocol. TdT was omitted for the negative controls. TUNEL evaluation was performed without knowledge of the patient's clinical and histopathologic data.

To identify cellular localization of the TUNEL signal double immunocytochemistry with alkaline phosphatase visualization was combined with the above technique. Hepatocytes were characterized using mouse monoclonal antibody anti-cytokeratin 18 (1:50; Boehringer Mannheim) as primary antibody.[20] To check for nonspecific binding, the primary antibody was omitted in controls.

A further double immunohistochemistry was combined with alkaline phosphatase TUNEL using 5-bromo-4 chloro-3 indolyl phosphate/nitro blue tetrazolium (NBT, Biogenex, San Ramon, CA) as chromogen and ABC for CD8 antibody.

At least 5,000 hepatocytes in each specimen were evaluated for DNA fragmentation. The proportion of labeled cells counted, using a microscope with an eyepiece grid, was expressed as a percentage of the total number of cells and was defined as the apoptotic index (AI). Stained hepatocytes at the edge of biopsy specimens were not counted and the number of hepatocytes present in a 7-mm biopsy specimen was chosen as a maximum common reference number. Apoptotic bodies were not considered in apoptotic index calculation as this would result in an overestimation of the number of apoptotic cells.

Ultrastructural Assessment

Small liver fragments were fixed in Karnowsky's solution (2% PF, 2.5% GTA in Milloning pH 7.3) for 24 hours, post-fixed with 1% osmium tetraoxide (Milloning pH 6.8) for 1 hour, progressively dehydrated in alcohol, and then embedded in epon.

Semithin sections were stained with 0.1% toluidine blue for light microscopical examination. Ultrathin sections were counterstained with uranyl acetate and lead citrate for transmission electron microscopy (Hitachi H-7000; Hitachi Ltd, Tokyo, Japan).

DNA Extraction and Electrophoresis

For DNA ladder analysis DNAs were obtained from frozen liver tissue by addition of 0.5 mL of lysis buffer (1.25% sucrose, 1% sodium dodecyl sulfate, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, 20 mmol/L Tris-HCl, pH 7.4 and 200 µg/mL proteinase K) at 37°C for 16 hours. DNA was extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with 5 mol/L NaCl (1/25 vol/vol) and 3 volumes of ethanol at −70°C and resuspended in Tris ethylenediaminetetraacetic acid buffer. To avoid overlapping bands of RNA, each DNA sample was treated for 30 minutes at 37°C with 100 µg/mL of RNAse/DNAse free (Boehringer Mannheim). Equal amounts of DNA from each specimen were incubated at 65°C for 10 minutes before electrophoresis on 1.8% agarose gel at 50 V for 3 hours. The gels were then stained with ethidium bromide, and DNA was viewed with ultraviolet transillumination.

Positive controls consisted of DNAs extracted from liver specimens of Landrace X large white pigs 7 days after ischemia/reperfusion injury (induced by clamping the hepatic hilum).[21]

Virological Assessment

Serological Markers. The presence of anti-HCV antibodies was determined by commercially available enzyme-linked immunosorbent assay kits (Ortho Diagnostics, Raritan, NJ), and confirmed by recombinant immunoblot assay 2 or 3. Hepatitis B surface antigen and antibodies to human immunodeficiency virus were detected by enzyme-linked immunosorbent assay tests (Abbott Diagnostics, North Chicago, IL).

HCV RNA. To evaluate HCV RNA in serum, home made nested polymerase chain reaction was performed following transcription using primers derived from the highly conserved 5′ untranslated region. The sensitivity of this assay was 10^3 to 10^4 genomes/mL.[22]

The amount of HCV RNA in serum samples obtained at the time of liver biopsy, was quantified by the bDNA assay (Quantiplex, 2.0; Chiron Corporation, Emeryville, CA) following the manufacturer's instructions.

HCV Genotype Determination. HCV genotype was determined using a previously standardized method.[23] Briefly, polymerase chain reaction amplified products of the 5′ untranslated region were spotted in parallel on nylon filters and hybridized with type-specific probes derived from the three major genotypes (genotype 1, 2, and 3)[24] which were labeled with dUTP fluoresceine. After washing under stringent conditions, the reaction was detected by an enhanced chemiluminescent method (Amersham International plc, Little Chalfont, Buckinghamshire, UK) on autoradiographic films.

Statistical Analysis

The results were analyzed using the Kruskal-Wallis ANOVA median test, regression analysis, χ^2, and the Fisher's exact test.

RESULTS

Detection of Apoptosis in the Liver

TUNEL Analysis. In the uninfected control liver specimens, none or rare TUNEL positive hepatocytes were observed (Table 2). By light microscopy with conventional hematoxylin and eosin staining Councilman bodies and pycnotic nuclei were seen in 55 out of 61 patients with chronic hepatitis C, and in all 61 biopsy specimens TUNEL stained nuclei could be detected. Only cells with strong staining of shrunken nuclear chromatin were considered positive. Some apoptotic cells and bodies showed enhanced nuclear membrane staining with reduced or absent staining of the remainder of the nucleus. Occasionally, apparently normal nuclei were also labeled. Apoptotic cells and bodies were seen both in the periportal and in the intralobular areas (Fig.1A and B). Most of the TUNEL-positive nuclei were cytokeratin 18–positive cells, confirming the parenchymal origin of the cell (Fig. 1C). Apoptotic bodies derived from hepatocytes (DNA fragments surrounded by cytokeratin 18 membrane positive) could be identified only when phagocyted by Kupffer/macrophagic cells. The apoptotic hepatocytes frequently occurred in conjunction with T lymphocytes, in particular with CD8-positive cells (Fig. 1D) and occasionally were "naked," with no adjacent lymphocytes. The apoptotic index of hepatic cells ranged from 0.01% to 0.54% (mean ± SD, 0.17 ± 0.13). Condensed and fragmented as well as strongly TUNEL-positive nuclei were also observed in few lymphocytes, particularly in areas with more diffuse inflammatory infiltrate (data not shown).

TABLE 2. Histological and Apoptotic Findings of Control Patients

Patient No.	Diagnosis	Liver Histology	Mean AI
7	Hodgkin's lymphoma (LC)	Normal morphology (7/7)	0
6	Hodgkin's lymphoma (M)	Normal morphology (6/6)	0.02
7	Cholecystitis	Mild cholestasis (6/7)	0.04

Abbreviations: LC, laterocervical; M, mediastinal.

Electron Microscopy and DNA Gel Electrophoresis. Nine biopsy specimens were examined by electron microscopy and in all of them ultrastructural cellular alterations typical of apoptosis were detected. In one case (AI 0.18%) early apoptotic features were observed with irregular peripheral subnucleolemmal condensation of granular osmophilic chromatin (Fig. 2A). Apoptotic cells/bodies were frequently seen near lymphocytic cells, in areas of increased collagen deposits (Fig. 2B) and in sinusoidal spaces (Fig. 2C). In one sample (AI 0.5%) nucleo-cytoplasmic changes, typical of necrosis, such as swollen mitochondria with sporadic flocculent densities and slight nuclear swelling accompanied by heterochromatin dissolution, were also seen (Fig. 2D).

Six biopsy specimens could be examined by DNA gel electrophoresis, and in 5 of them extracted DNA showed a high molecular weight band and a series of smaller fragments whose size ranged between multiples of 180 to 220 bp units, with a "ladder" pattern of DNA fragmentation, which is considered a biochemical marker of cellular apoptosis. Only one case showed a single band of high molecular weight DNA without DNA fragmentation.

Correlation Between TUNEL-Positive Cells and Liver Morphological Features. As shown in Fig. 3, a significant correlation was seen between the AI and necroinflammatory grading ($P <$ .0005). The AI correlated also with the presence of follicular lymphomonocyte infiltrates ($P <$ .005), with the score of CD8-positive cells ($P =$ .01), but not with the steatosis score and the presence of bile duct lesions (Table 3). Although mean AI values did not significantly differ between patients with lower or higher fibrosis stage, a fibrosis stage of ≤2 was

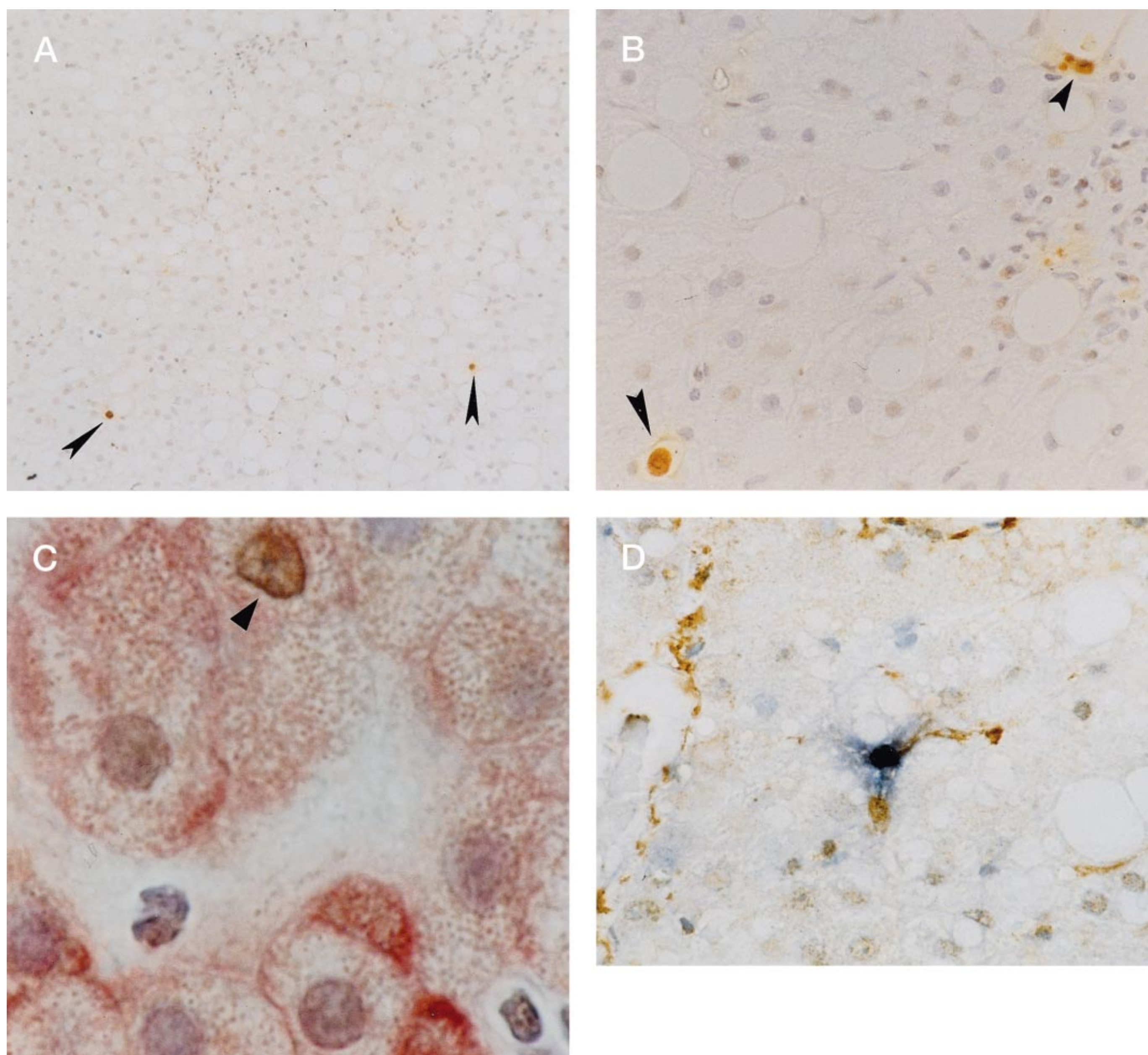

FIG. 1. *In situ* detection of apoptosis in chronic hepatitis C. (A) Intralobular area: Hepatocytes with fragmented DNA are strongly stained brown (*arrow*). TUNEL; original magnification ×40. (B) Periportal area: the positive nucleus (*arrow*) indicates nuclear injury, although not representing typical apoptotic nucleus. Aggregated DNA fragments appear in the portal zone (*small arrow*). TUNEL; original magnification ×200. (C) Double labeling: red stain for cytokeratin 18–positive hepatocytes. The nucleus of one hepatocyte is intensely TUNEL stained (*arrow*) indicating that this cell is apoptotic. Original magnification ×520. (D) Double labeling: apoptotic hepatocyte, depicted by blue nuclear labeling (NBT) and brown cytoplasmic staining for CD8 lymphocytes. Original magnification ×200.

found in 37% of the patients with AI less than 0.1% but only in 12% of those with AI greater than 0.1% ($P < .005$). In most cases, TUNEL-positive hepatocytes were seen both in the periportal and in the intralobular zones, often near infiltrating lymphocytes, mainly consisting of CD8-positive cells (Fig. 1D).

Proliferation Activity. Immunohistochemical Ki-67 expression was not seen in parenchymal cells in any cases, whereas it was positive in some interstitial cells, such as Kupffer cells or inflammatory cells, particularly within lymphoid aggregates.

Correlation of Apoptosis With Biochemical and Virological Parameters. As shown in Figs. 4 and 5, the AI did not correlate with HCV RNA or ALT levels. Mean AI was 0.20% ± 0.16% in 33 patients infected with genotype 1, 0.13% ± 0.09% in 18 patients with genotype 2, and 0.18% ± 0.11% in 8 patients with genotype 3. Interestingly, AI correlated with the histologic activity score (HAI) in patients with genotype 1 and in those with genotype 2, but not in patients with genotype 3, as 6 out of 8 of them had high AI with low HAI score.

DISCUSSION

In this study, direct evidence for variable degree of liver cell apoptosis in the liver of patients with chronic hepatitis C has been obtained. Apoptosis was measured by *in situ* end

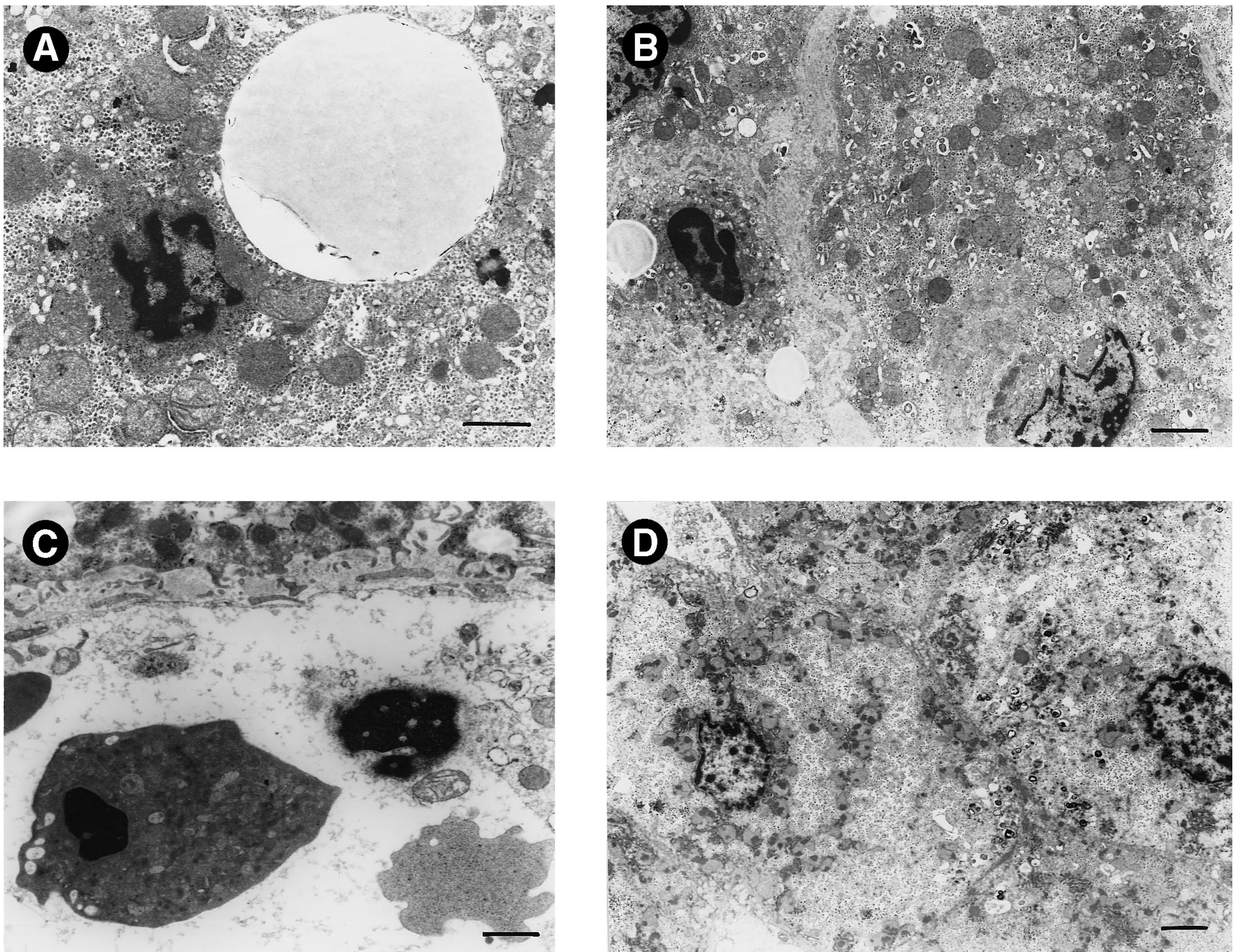

FIG. 2. Ultrastructure of apoptotic hepatocytes in chronic hepatitis C. (A) Hepatocyte with slight chromatin condensation and margination indicating an early stage of apoptosis. (B) Hepatocytes with chromatin condensation and margination are seen close to collagen fibers and (C) in the sinusoidal space. (D) Hepatocytes show marked mitochondrial swelling with sporadic flocculent densities and nuclear changes with heterochromatin dissolution.

labeling of fragmented DNA with TdT and with digoxigenin-dUTP, an accepted method for the detection of the apoptotic process. It has been reported that this technique might overestimate apoptosis because it might include necrotic and autolysed cells.[25] However, recent studies using TUNEL and laser scanning confocal microscopy have confirmed its validity in identifying and quantifying apoptosis.[26,27] Early and late ultrastructural features of programmed cell death were observed in all the liver biopsy specimens examined. Indeed, apoptosis was the most frequent form of hepatocellular damage observed in these cases, whereas in only one biopsy the electron microscopy pattern was more consistent with more prominent necrosis rather than apoptosis. By DNA gel electrophoresis, the classic "ladder pattern" of DNA fragmentation was observed in 5 out of 6 cases confirming that apoptosis was occurring at a significant rate, because it is well known that DNA gel electrophoresis is not a very sensitive method for apoptosis detection and it is not able to detect the large DNA fragments formed in the initial stages of apoptosis.[28] Hepatocyte loss by apoptosis should be considered an important pathological sign, because of the negative balance of liver cell proliferative activity. The observed lack of liver cell proliferation is in agreement with previous findings describing absence or low proliferative index in patients with chronic hepatitis C.[29,30] A significant correlation was found between the amount of apoptosis, expressed as apoptotic index, and necroinflammatory activity in the liver. This finding, and the positive correlation also with the number of CD8-positive cells, support the hypothesis of an immune-mediated mechanism of apoptotic liver cell damage in chronic hepatitis C, as already suggested by previous reports describing immunohistochemical and molecular detection of apoptosis related proteins.[3-5,9] Presence of apoptosis was detected not only in liver cells, but also in lymphocytes, especially in areas with more diffuse lympho-histiocytic infiltrates, confirming the dynamic process of chronic inflammation.[11]

Although cytotoxic T lymphocytes are known to induce apoptosis via the Fas ligand/Fas antigen system, other apoptosis mediators released by activated T cells, such as tumor necrosis factor α, interferon gamma, and the granzyme/perforin system, could be involved in the process. With their

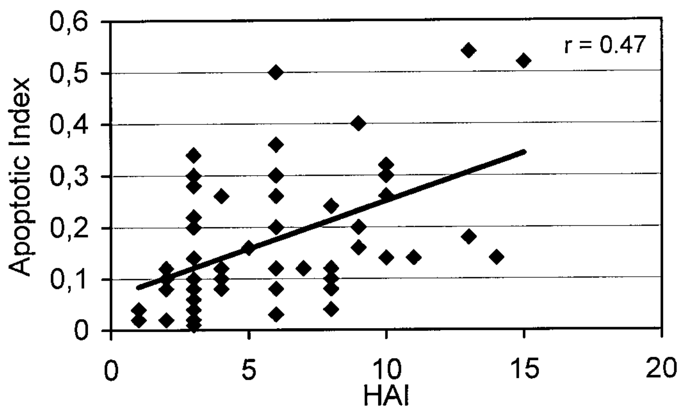

FIG. 3. Correlation between apoptotic index and necroinflammatory activity score (HAI) determined in the same liver biopsy specimen in patients with chronic hepatitis C. Regression line is shown in the graphic area.

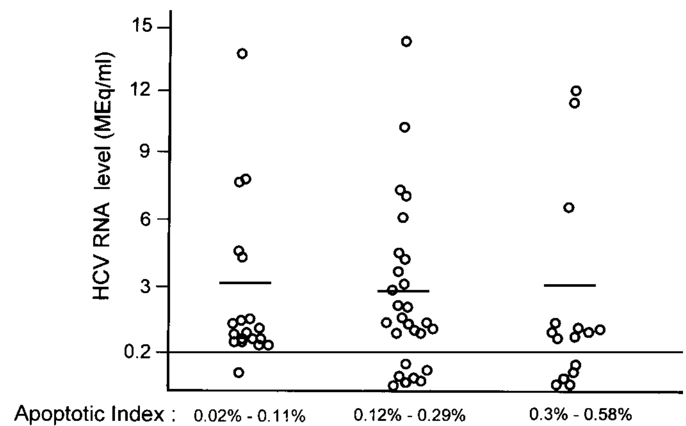

FIG. 4. HCV RNA levels in serum of patients with different ranges of apoptotic index. *Horizontal bar* within each group indicates mean value of positive samples. The value 0.2 MEq/mL represents the sensitivity limit of the assay.

multiple effects, including direct cytotoxicity, induction of expression of human leukocyte antigen (HLA) molecules and increased susceptibility of natural killer cells, these cytokines may be crucial for the development of chronic hepatitis. Apoptotic hepatocytes were not only detected in close contact with inflammatory (CD8-positive) cells, but could be also observed in samples with increased deposition of collagen fibers, as documented by electron microscopy.

The correlation between apoptosis and transaminase levels was weak if any. Thus, chronic liver damage and hepatocyte cell loss by apoptosis can occur in HCV-infected patients without overt biochemical changes, explaining the progressive nature of liver disease that can be seen in asymptomatic carriers with persistently normal transaminases. Silent disappearance of death cells, removed by neighboring cells or tissue macrophages is currently viewed as the main way of destruction of apoptotic cells.[31] However, release of transaminases in serum after an apoptotic stimulus in the liver can occur in some circumstances and it has been considered as the result of secondary inflammation and necrosis, excited by the apoptotic process itself.[32]

TABLE 3. Relationship Between AI and Liver Histological Parameters

Liver Histological Features	AI (mean ± SD)	P
Fibrosis stage		
0-2	0.14 ± 0.2	NS
3-4	0.18 ± 0.13	
CD8 score		
1	0.1 ± 0.08	
2	0.24 ± 0.13	.01*
3	0.24 ± 0.12	
Steatosis score		
0	0.26 ± 0.06	
1	0.17 ± 0.14	NS
2	0.17 ± 0.15	
Follicular lymphomonocytic infiltrate		
Absent	0.12 ± 0.09	.003
Present	0.21 ± 0.14	
Bile duct lesions		
Absent	0.15 ± 0.12	NS
Present	0.19 ± 0.14	

*$P = .01$ for CD8 score 1 vs. 2 and 3.

Modulation of hepatocyte sensitization to apoptotic stimuli by HCV proteins has been proposed, and antiapoptotic activity has been ascribed to NS5A protein, because of its capability to repress the interferon-induced double stranded RNA-activated protein kinase (PKR) *in vitro*,[16] whereas for the core protein both proapoptotic and antiapoptotic properties have been reported in different experimental conditions.[12-14,33] In the present study no correlation was observed between extent of liver cell apoptosis and amount of HCV RNA in serum. It has been reported that viral load in serum might reflect viral replication in the liver,[34,35] and therefore, although viral assessment was not performed in liver specimens, the virus *per se* does not seem to play a pivotal role. Concerning the influence of the infecting genotype, no significant relationship was found between viral type and degree of apoptosis. However, although the number of patients infected by genotype 3 was small, a high apoptotic index, not correlated with high AI, was more frequently observed in this subgroup of patients. Further studies should clarify whether intrinsic characteristics of this genotype could be directly associated with apoptotic liver cell death.

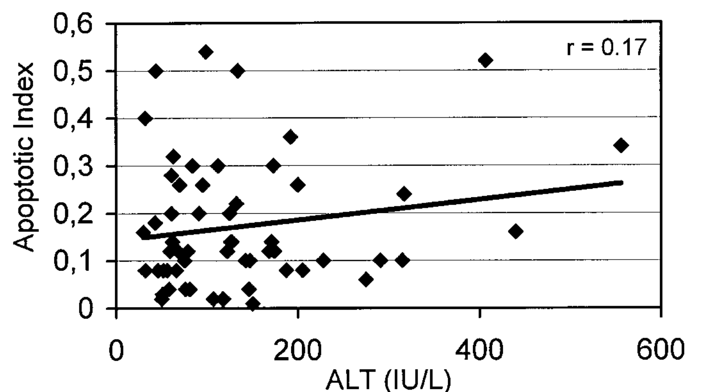

FIG. 5. Relationship between apoptotic index and ALT values obtained at the same time of liver biopsy in patients with chronic hepatitis C. Regression line is shown in the graphic area.

REFERENCES

1. Alter MJ, Margolis HS, Krawcynski K, Judson FN, Mares A, Alexander WJ, Hu PY, et al. The natural history of community-acquired hepatitis C in the United States. N Engl J Med 1992;327:1899-1905.
2. Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. Lancet 1997;349:825-832.
3. Hayashi N, Mita E, Iio S, Takehara T, Kasahara A, Fusamoto H, Kamada T. Fas system and apoptosis in viral hepatitis. J Gastroenterol Hepatol 1997;12 (suppl):S223-S226.
4. Ando K, Hiroishi K, Kaneko T, Moriyama T, Muto Y, Kayagaki N, Yagita H, et al. Perforin, Fas/Fas ligand, and TNF-alpha pathways as specific and bystander killing mechanisms of hepatitis C virus-specific human CTL. J Immunol 1997;158:5283-5291.
5. Lau JYN, Xie X, Lai M, Wu PC. Apoptosis and viral hepatitis. Semin Liv Dis 1998;18:169-176.
6. Williams GT, Smith CA. Molecular regulation of apoptosis: genetic controls on cell death. Cell 1993;74:777-779.
7. Searle J, Harmon BV, Bishop CJ, Kerr JFR. The significance of cell death by apoptosis in hepatobiliary disease. J Gastroenterol Hepatol 1987;2: 77-96.
8. Kerr JFR, Cooksley WGE, Searle J, Halliday JW, Halliday WJ, Holder L, Roberts I, et al. The nature of piecemeal necrosis in chronic active hepatitis. Lancet 1979;2:827-828.
9. Hiramatsu N, Hayashi N, Katayama K, Mochizuki K, Kawanishi Y, Kasahara A, Fusamoto H, et al. T. Immunohistochemical detection of Fas antigen in liver tissue of patients with chronic hepatitis C. HEPATOLOGY 1994;19:1354-1359.
10. Okazaki M, Hino K, Fujii K, Kobayashi N, Okita K. Hepatic Fas antigen expression before and after interferon therapy in patients with chronic hepatitis C. Dig Dis Sci 1996;41:2453-2458.
11. Nuti S, Rosa D, Valiante NM, Saletti G, Catarozzolo M, Dellabona P, Barnaba V, Abrignani S. Dynamics of intra-epatic lymphocytes in chronic hepatitis C. Enrichment for $V\alpha24^+$ T cells and rapid elimination of effector cells by apoptosis. Eur J Immunol 1998;28:3448-3555.
12. Ruggieri A, Harada T, Matsuura Y, Miyamura T. Sensitisation to Fas-mediated apoptosis by hepatitis C virus core protein. Virology 1997;29:68-76.
13. Zhu N, Khoshnan A, Schneider R, Matsumoto M, Dennert G, Ware C, Lai MMC. Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. J Virol 1998;72:3691-3697.
14. Murasawa H, Hijkata M, Chiba T, Shimotohno K. Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-κB activation. J Virol 1999;73:4713-4720.
15. Sakamuro D, Frukawa T, Takegami T. Hepatitis C virus nonstructural protein NS3 transforms NIH 3T3 cells. J Virol 1995;69:3893-3898.
16. Gale M, Kwieciszewski B, Dossett M, Nkao H, Katze MG. Antiapoptotic and oncogenic potentials of hepatitis C virus are linked to interferon resistance by viral repression of the PKR protein kinase. J Virol 1999;73:6506-6516.
17. Catoretti G, Becker M, Key G, Duchrow M, Schluter C, Salle J, Gerdes J. Monoclonal antibodies against recombinant parts of Ki-67 antigen (MIB1 and MIB3) detect proliferating cells in microwave processed formalin-fixed paraffin sections. J Pathol 1992;168:357-363.
18. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell-death in situ via specific labelling of nuclear DNA fragmentation. J Cell Biol 1992;119:496-501.
19. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PS. Classification of chronic hepatitis: diagnosis, grading and staging. HEPATOLOGY 1994;19:1513-1520.
20. Moll R, Franke WW, Schiller DA, Geiger B, Krepler R. The catalogue of human cytokeratins; patterns of expression in normal epitelia, tumorous and cultured cells. Cell 1982;31:11-24.
21. Calabrese F, Valente M, Pettenazzo E, Ferraresso M, Burra P, Cadrobbi R, Cardin R, et al. The protective effects of L-arginine after liver ischemia/reperfusion injury in a pig model. J Pathol 1997;183:477-485.
22. Pontisso P, Gerotto M, Ruvoletto MG, Fattovich G, Chemello L, Tisminetzky S, Baralle F, et al. A. Hepatitis C genotypes in patients with dual hepatitis B and C virus infection. J Med Virol 1996;48:157-160.
23. Tisminetzky S, Gerotto M, Pontisso P, Chemello L, Prescott LE, Rose KA, Baralle F, et al. Comparison of genotyping and serotyping methods for the identification of hepatitis C virus types. J Virol Meth 1995;55:303-307.
24. Pontisso P, Ruvoletto MG, Nicoletti M, Tisminetzky S, Gerotto M, Levrero M, Artini M, et al. Distribution of three major hepatitis C virus genotypes in Italy. A multicentre study of 495 patients with chronic hepatitis C. J Vir Hepat 1995;2:33-38.
25. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R. *In situ* detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. HEPATOLOGY 1995;21:1465-1468.
26. Olivetti G, Abbi R, Quaini F, Kajstura G, Cheng W, Nitahara JA, Quaini E, et al. Apoptosis in the failing human heart. N Engl J Med 1997;336: 1131-1141.
27. Kimura K, Sasano H, Shimosegawa T, Kato K, Noguchi T, Mochizuki S, Sawai T, et al. Ultrastructural and confocal laser scanning microscopic examination of TUNEL-positive cells. J Pathol 1997;181:235-242.
28. Walker R, Kokileva L, LeBlanc J, Sikorska M. Detection of the initial stages of DNA fragmentation in apoptosis. BioTechniques 1993;15:1032-1040.
29. Nagao T, Kondo F, Sato T, Nagato Y, Kondo Y. Immunohistochemical detection of aberrant p53 expression in hepatocellular carcinoma: correlation with cell proliferative activity indices, including mitotic index and MIB-1 immunostaining. J Pathol 1995;26:326-333.
30. Dutta U, Kench J, Byth K, Khan MH, Lin R, Liddle C, Farrel G. Hepatocellular proliferation and development of hepatocellular carcinoma: a case-control study in chronic hepatitis C. J Pathol 1998;29:1279-1284.
31. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995;267:1456-1462.
32. Lawson JA, Fisher MA, Simmons CA, Farhood A, Jeaschke H. Parenchymal cell apoptosis as a signal for sinusoidal sequestration and transendothelial migration of neutrophils in murine models of endotoxin and Fas-antibody–induced liver injury. HEPATOLOGY 1998;28:761-767.
33. Ray RB, Meyer K, Ray R. Suppression of apoptotic cell death by hepatitis C virus core protein. Virology 1996;226:176-182.
34. Coelho-Little E, Jeffers LJ, Bartholomew M, Reddy KR, Schiff ER, Dailey PJ. Correlation of HCV-RNA levels in serum and liver of patients with chronic hepatitis C. J Hepatol 1995;22:508.
35. De Moliner L, Pontisso P, De Salvo GL, Cavalletto L, Chemello L, Alberti A. Serum and liver HCV RNA levels in patients with chronic hepatitis C: correlation with clinical and histological features. Gut 1998;42:856-860.