Apoptosis and Hepatitis C Virus Infection in Renal Transplant Recipients

Ioanna Delladetsima, MD,1 Mina Psychogiou, MD,2 Paraskevi Alexandrou, MD,1 George Nikolopoulos, MSc,2 Kostantinos Revenas, MD,3 Angelos Hatzakis, MD,2 and John Boletis, MD3

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Abstract

Hepatocellular injury in renal transplant recipients with hepatitis C virus (HCV) infection remains unclear. The suppressed immune response, in combination with increased viremia levels, provides a unique setting for the study of a potential HCV-induced apoptotic process.

Liver biopsy specimens from 59 HCV-infected renal transplant recipients were examined histologically. DNA fragmentation was detected by the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate–nick end labeling assay, and the CD8 T-cell count was assessed immunohistochemically.

A low apoptotic index (0-2.5) was observed in 31 cases, a moderate index (2.6-5) in 16, and a high index (>5) in 12. Apoptotic cell death correlated significantly with viremia because it was demonstrated by higher HCV-RNA levels in cases with a high number of apoptotic cells (odds ratio, 2.96; 95% confidence interval, 1.0-8.5; \(P = .04\)). No correlation was found between the apoptotic index and hepatitis necroinflammatory activity, CD8 cell count, fibrosis stage, immunosuppressive therapy, or genotype.

In HCV-infected renal transplant recipients, apoptotic cell death seems to be associated with high viral load, thus providing indications of viral interference in the pathogenetic process.

Apoptosis seems to have a significant role in the pathogenesis of liver damage in hepatitis C virus (HCV) infection. There are indications of an immune-mediated pathway induced by the Fas/Fas ligand (FasL) and perforin systems. Up-regulation of Fas expression on hepatocytes was found in HCV-infected livers and was shown to correlate with the severity of necroinflammation. Participation of infiltrating T lymphocytes expressing FasL and enhancement of Fas messenger RNA transcript have also been demonstrated in the liver tissue of patients with chronic hepatitis C. Another factor implicated in the pathogenesis of liver cell death is the virus itself, which seems to modulate the apoptotic pathways by interaction with apoptosis mediators. Based on data provided from in vitro studies, the multipotential core protein combines proapoptotic and antiapoptotic properties, NS3 and NS5 proteins have been related to antiapoptotic effects, and E1 and E2 proteins may induce an apoptotic response.

The contribution of apoptosis and underlying mechanisms in liver injury in HCV-infected transplant recipients has been the subject of investigation in a small number of studies. Information is limited and refers to findings in liver allografts, whereas in the setting of renal transplant, data are lacking.

We studied the extent and degree of liver cell apoptosis in liver injury and its association with possible pathogenetic factors in liver biopsy specimens of renal transplant recipients with HCV infection. The suppressed immune response, in combination with increased viremia levels, provides a unique setting for the study of a potential HCV-induced apoptotic process. In this context, the obtained apoptotic index was correlated with hepatitis necroinflammatory activity, CD8 cell count, and hepatitis fibrosis and with virologic parameters such as viral load and HCV genotype.
Materials and Methods

The examined material consisted of 59 liver biopsy specimens from renal transplant recipients with HCV infection; biopsies were performed a mean (SD) of 4.7 years (3.6 years) after transplantation. There were 36 men and 23 women with a mean (SD) age of 41.3 years (11.3 years). At the time of liver biopsy, all patients were anti-HCV positive. Samples were tested by a second-generation enzyme-linked immunosorbent assay (Abbott Labs, Wiesbaden, Germany) or by a third-generation assay (HCV 3.0 Test System, Ortho, Emeryville, CA) depending on the availability of the immunoassays during the study period. All anti-HCV positive samples were confirmed by an immunoblot assay (INNO-LIA HCV Ab III, Innogenetics NV, Zwijjaarbe, Belgium). Quantitative assessment of HCV RNA was performed by using a branched DNA-enhanced label amplification assay (b-DNA 3.0, Bayer, Tarrytown, NY) in serum samples obtained at the time of liver biopsy. HCV genotyping was done by using a commercially available assay (INNONlIPA HCV II, Innogenetics NV).

Liver biopsy was performed in the presence of anti-HCV irrespective of the levels of liver function tests. Liver biopsy specimens were fixed in buffered formalin solution and processed according to the routine protocol. Histologic evaluation of chronic hepatitis included grading and staging based on the scoring system proposed by Ishak et al. No. Grades 1 through 3 were ascribed to minimal chronic hepatitis, grades 4 through 7 to mild, and grades 8 through 12 to moderate. The fibrosis stage was assessed by using a trichrome stain.

Liver cell apoptosis was assessed by using the terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling method using the TdT-FragEL DNA fragmentation detection kit (Oncogene, San Diego, CA) according to the manufacturer’s instructions. All liver biopsy specimens included at least 1,000 hepatocytes corresponding to approximately 10 high-power fields (HPF; ×400). DNA fragmentation was evaluated by co-observation by 2 pathologists (I.D. and P.A.), and labeled liver cells were counted in the entire section. Outside liver cell plates and within sinusoids, cells showing DNA fragmentation were considered of hepatocellular origin when their nuclei were larger than lymphocytes, not elongated, or surrounded by condensed cytoplasm. Free or phagocytosed small nuclear fragments were not calculated. The apoptotic index was defined by the mean number of apoptotic liver cells per 10 HPF and was classified into 3 numeric categories ranging from 0 to more than 5 cells. CD8 T lymphocytes were identified immunohistochemically by using a monoclonal mouse antihuman CD8 antibody, clone DK25 (DakoCytomation, Glostrup, Denmark), and visualizing the staining with the streptavidin–biotin method. The CD8 cell count was evaluated by following the same method applied for DNA fragmentation.

Patients were receiving immunosuppressive therapy by the following regimens: azathioprine and methylprednisolone, 5 patients; cyclosporine and methylprednisolone, 5 patients; and azathioprine, cyclosporine, and methylprednisolone, 49 patients. No patient received anti-HCV treatment after transplantation.

The \( \chi^2 \) test and 1-way analysis of variance were performed to explore possible relationships between variables. The nonparametric Kruskal-Wallis test was also used. In multivariate analysis, apoptosis was used as a dependent variable with 3 ordered categories, and ordinal logistic regression was applied. Test results were considered statistically significant when the \( P \) value was less than .05. Analyses were conducted in STATA statistical software, version 8.0 (StataCorp, College Station, TX).

Results

Study findings are shown in Table II. Histologic evaluation revealed no significant changes in 8 (14%) and chronic hepatitis in 51 (86%) liver biopsy specimens. Minimal hepatitis was diagnosed in 32 cases, mild in 17, and moderate in 2. The stage of fibrosis was 0 (no fibrosis) in 25 cases, 1 (minimal) or 2 (mild) in 17, 3 (moderate) or 4 (severe) in 7, and 6 (cirrhosis) in 2. CD8 was evaluated in 49 cases, and the mean (SD) value was 38.3/10 HPF (32.9/10 HPF; range, 1-120/10 HPF).

DNA fragmentation in liver cells was detected in 50 (85%) of 59 cases, with a median value of 2.17 (interquartile range, 0.66-5). A low apoptotic index (0-2.5) was assessed in 31 cases (53%), a moderate index (2.6-5) in 16 (27%), and a high index (>5) in 12 (20%). It is interesting that 9 cases with no significant changes or minimal hepatitis exhibited a high apoptotic cell count, whereas in 9 cases with mild and 2 with moderate hepatitis, the apoptotic index was low.

In univariate analysis, no significant association was found between the apoptotic index and hepatitis grade, lobular activity, periportal hepatitis, or stage, whereas there was a trend toward correlation with the CD8 cell count.

Apoptotic cell death correlated significantly only with HCV RNA viremia because it was demonstrated by higher HCV RNA levels in cases with a high number of apoptotic cells (\( P = .04 \)) Figure I. When we fitted an ordinal logistic regression with apoptosis as the dependent variable and HCV RNA levels as an independent factor and after adjusting for factors that may act as confounders (CD8 and grade), the only significant correlation we obtained was between apoptosis and HCV RNA levels with an odds ratio of 2.96 (95% confidence interval, 1.103-8.503; \( P = .04 \)). Thus, the predicted odds of having a higher apoptotic index are nearly 3 times the odds of being in a lower category for a 1-unit increment in the base-10 logarithmic scale of HCV RNA levels.
No association was found between the apoptosis rate and alanine aminotransferase (ALT) values, whereas a strong correlation was noted between ALT values and hepatitis grade \((P = .006)\). There was no relationship between apoptosis and HCV genotype, patient age, or immunosuppressive regimen.

### Discussion

In 59 renal transplant recipients with HCV infection, apoptotic liver cell death constituted a phenomenon frequently participating in HCV-related liver damage. Although the mean count was low, apoptotic cells were encountered in the majority of cases, even in liver biopsy specimens with an almost normal histologic appearance. In addition, the number of apoptotic cells did not show any significant correlation with the degree of necroinflammatory activity or CD8 count. It is
noteworthy that a high apoptotic index was found in 9 (21%) of 43 cases showing no significant changes or minimal hepatitis, whereas in 11 (69%) of 16 cases with increased necroinflammatory activity, the apoptotic index was low.

This discrepancy diminishes the pathogenetic role of the immune response and points toward additional determinants involved in the apoptotic process. The correlation of apoptosis with high viremia levels, irrespective of hepatitis grade, lobular activity, and CD8 T-cell count, enhances this hypothesis and provides evidence of virus involvement. In favor of this assumption are experimental data confirming that the contribution of HCV proteins in the apoptotic mechanisms is possible. In vitro studies using different cell lines have shown that the HCV core protein binds to the cytoplasmic domain of tumor necrosis factor receptor 1, lymphotixin-β receptor, and Fas.\(^\text{10,11,26}\) Cells expressing the HCV core protein were prone to apoptotic death mediated by ligands of tumor necrosis factor receptor family members, indicating indirect cytoxicity through an immun-mediated pathway.\(^\text{10,11,27}\) Other experimental data suggest a direct apoptotic effect of HCV E1 and E2 proteins, the latter through a mitochondrial-related caspase pathway.\(^\text{19,20}\) Moreover, there is increasing information that a number of viruses are directly involved in the apoptotic process.\(^\text{28}\)

In the setting of liver transplantation with HCV infection, immune-mediated mechanisms and viral toxicity were implicated in the pathogenesis of apoptosis. Consistent with our findings was the detection of apoptosis in histologically normal allografts\(^\text{23,24}\) that was found in higher values than in specimens of patients with chronic active hepatitis who had not undergone transplantation.\(^\text{24}\) Similar to the observations in immunocompetent patients,\(^\text{5,6}\) increased apoptosis was associated with the expression of Fas and FasL in recurrent HCV hepatitis in liver grafts,\(^\text{22}\) and a correlation was observed between Fas antigen expression, intensity of lobular activity, and apoptotic index.\(^\text{21}\) In addition, the up-regulation of Fas messenger RNA was attributed to intrahepatic viral replication, thus implicating viral proteins in the regulation of Fas transcription, although this correlation was independent of the degree of liver cell apoptosis.\(^\text{21}\) The frequent contact of CD8 lymphocytes with hepatocytes bearing HCV suggested antigen-dependent immune response, whereas the increased number of apoptotic cells during the acute phase of hepatitis in the presence of massive infection indicated virus-related cytotoxic damage.\(^\text{24}\)

In our study group, there was a trend toward correlation between the CD8 cell count and apoptosis, pointing toward an immunopathogenic relationship. The lack of statistical significance could be ascribed to diminished effectiveness of cytotoxic T lymphocytes ensuing from immunosuppression. Within this concept, strong immunosuppressive therapy differentiates the findings in this group of patients from findings in liver transplant recipients who need a much lower level of immunosuppression.

What becomes evident from our results is that in HCV-infected renal transplant recipients, apoptotic liver cell death may occur in the absence of inflammation, thus restricting the significance of immune-mediated mechanisms in pathogenesis. Moreover, there are indications of a direct proapoptotic viral effect closely associated with high viremia levels. Poorly restricted virus replication is a reasonable process to lead to increased intracellular viral load, to the infection of a higher fraction of hepatocytes, and to high HCV RNA levels from the continuous release of viral particles into the circulation. Without excluding the contribution of the immune response, our findings can be explained by the aforementioned interconnection and permit the speculation of an apoptotic process promoted or enhanced by virus interference, especially when intracellular virus accumulation has reached a certain threshold. This hypothesis warrants further support by investigation of HCV expression and quantification of HCV RNA in liver tissue, although serum HCV RNA levels constitute a reliable index of the HCV RNA replication rate in the liver.\(^\text{29}\)

The fact that in immunocompetent patients no correlation was identified between liver cell apoptosis and the level of serum HCV RNA\(^\text{6,30}\) can be explained by their lower viremia compared with that encountered after kidney transplantation.\(^\text{31}\) It is plausible that a more efficient immune response prevents HCV from reaching cytotoxic levels by suppression of its replication and/or by more rapid elimination of infected cells.\(^\text{32}\)

In our study, apoptosis did not correlate with ALT levels, indicating that cell injury may occur in the absence of biochemical activity. The same observation is reported in the literature\(^\text{3,6}\) and can be reasonably attributed to the type of cell death, defined by membrane-bound bodies with intact organelles and plasma membrane. In contrast, the lytic type of necrosis most probably accounts for the identified significant association of ALT values with the grade of hepatitis.

Poor and controversial data exist for liver transplant about the possible involvement of HCV genotype in apoptotic liver cell death. One study related marked apoptosis to genotype 1b,\(^\text{23}\) whereas in another, no relationship was found.\(^\text{21}\) In the present study, HCV genotype lacked a significant association with the degree of apoptosis.

In transplant recipients, a modification of apoptosis by immunosuppressive drugs seems possible.\(^\text{33,34}\) However, our results could not identify any influence of the immunosuppressive regimen on liver cell apoptosis. An analogous experience has been reported in liver allograft recipients.\(^\text{21,23}\)

The role of apoptosis in fibrogenesis remains unclear. Based on experimental studies, it is postulated that an interactive relationship exists between apoptotic cell death and fibrogenesis.\(^\text{35}\) The phagocytosis of apoptotic bodies by stellate and Kupffer cells produces fibrogenic stimuli, whereas a fibrotic microenvironment induces proapoptotic
gene expression. Our findings did not provide any indications of a pathogenetic interrelationship between apoptosis and fibrosis because there was no correlation between the degree of apoptosis and hepatitis stage.

In renal transplant recipients with HCV infection, liver cell apoptosis seems to be an important factor in liver damage in cases with high viral loads, thus providing indications of viral interference in the pathogenetic process.

From the Departments of 1Pathology, Laiko General Hospital and 2Hygiene and Epidemiology, Athens University Medical School; and the 3Transplant Center, Laiko General Hospital, Athens, Greece.

Address reprint requests to Dr Delladetsima: Mikras Asias 75, Athens, Greece, 11527.

References