10.1053.paor.2000.0262 available online at http://www.idealibrary.com on IDEAL

ARTICLE

Apoptosis-related Proteins, BCL-2, BAX, FAS, FAS-L and PCNA in Liver Biopsies of Patients with Chronic Hepatitis B Virus Infection

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While the elimination of hepatitis B virus (HBV) is a common phenomenon at the end of the acute phase of disease, the persistence of HBV is characteristic for chronic hepatitis (CHB). Recent evidence indicates that the elimination of HBV is achieved by FAS/FAS-L induced apoptosis of infected hepatocytes. The aim of this study was to test the hypothesis that HBV persistence in the hepatocytes of CHB patients is due to the delayed onset of apoptosis caused by altered FAS/FAS-L interactions between lymphocytes and hepatocytes. The expression of FAS, FAS-L, BAX, BCL-2, ICE and PCNA in the liver biopsies of 55 patients (14 HBsAg positive, 20 patients with alcoholic hepatopathy, 21 patients with other hepatopathies) was tested by immunohistochemistry. Apoptosis of hepatocytes was evaluated by morphological as well as by TUNEL method. The results were correlated with a grading/staging score and analysed statistically using a one way analysis of variance and the Duncan test. Significantly higher numbers of BAX positive hepatocytes were observed in HBsAg positive patients when compared to control groups. Similarly, both BAX and FAS positive lymphocytes were more frequent in HBsAg positive patients. FAS-L positive lymphocytes and hepatocytes were numerous in all patient groups. Increased numbers of BAX positive hepatocytes in CHB may reflect the increased readiness of these cells to undergo apoptosis. However, the increased numbers of both BAX and FAS positive lymphocytes in CHB suggest that these cells may be particularly sensitive to FAS-L mediated apoptosis potentially resulting in lowered viability of these lymphocytes. This may explain, at least in part, the defective removal of virus-infected cells in chronic hepatitis. However, we cannot rule out the possibility that survival of hepatocytes during CHB may be due to other mechanisms such as defects in apoptosis activation triggered by CD40, defects involving DNase and/or other caspases downstream in the apoptotic cascade within these cells, or to defects in CTL function. (Pathology Oncology Research Vol 6, No 2, 130–135, 2000)

Keywords: apoptosis, hepatitis B virus, FAS, FAS-L

Introduction

It is generally accepted that the hepatitis B virus (HBV) does not directly cause the pathological effects of acute and chronic necrotizing inflammatory liver disease. This conclusion is supported by the fact that HBV infection in approximately 90–95% of cases results in transient liver disease followed by viral clearance.[18,51] It is evident that an immune response mediated by cytotoxic T lymphocytes (CTLs), either by the elaboration of various cytokines, or by direct interaction of these cells with HBsAg-positive hepatocytes, is primarily responsible for the associated liver disease.[3,11,16,17,19,25,31,35,38,41,43,44] There are two separate mechanisms by which CTLs induce apoptosis in target cells. The first of these involves the perforin-granzyme pathway leading to secretion of the lytic protein, perforin, and of various serine proteases (granzymes), each of which

Received: Febr 28, 2000; *accepted:* April 20, 2000

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 1219-4956/00/020130+06 $ 12.00/0

is stored in specific CTL granules and the consecutive release of which induces DNA fragmentation.[5,28,45] The second mechanism involves interaction between the FAS ligand and it's cell surface receptor, FAS. Both FAS and FAS ligand are members of the TNF receptor and ligand superfamily[6,34] and their interaction leads to the initiation of apoptosis by the activation of various caspases.[10,30,37] It has been suggested that the interaction between FAS ligand, expressed on activated CTLs, and FAS protein located on HBV-infected hepatocytes, plays an essential role in the development of acute and fulminant hepatitis[24,42] or cirrhosis.[13] However, other proteins may mediate the control of apoptosis. Among these the Bcl-2 gene family are one of the most important. Members of this family are either inducers (BAX, BAD, BAK, BID, BIK, BCL-X_S) or inhibitors (BCL-2, BCL-X_L) of apoptosis.[14,26,39] It has recently been shown that BCL-2 expression by mouse hepatocytes protects them from FAS-mediated apoptosis, suggesting the potential for alternative approaches to the prevention of hepatic failure due to viral hepatitis in man.[27]

Chronic hepatitis associated with viral persistence and potentially serious complications such as cirrhosis and hepatocellular carcinoma develops in 5–10% of patients infected with HBV. A number of viruses, including HBV, have developed strategies that enable them to persist inside host cells and escape immune control. Clonal deletion of virus-specific T cells, mutation of viral gene regions encoding epitopes critical for T cell recognition, inhibition of intracellular antigen processing and induction of T cell anergy,[36] are among the most important. Another mechanism, the maintenance of an immunoprivileged state within infected hepatocytes might be relevant to the long-term survival of HBV. FAS/FAS ligand interactions could play an important role in this process by the induction of apoptosis and the elimination of FAS-bearing CTLs,[15,47] as has been demonstrated in the prevention of graft rejection,[4,49] in testicular and melanoma cells[20,50] and in hepatocellular carcinoma cells.[46]

The role of FAS in chronic hepatitis has been studied by Mochizuki et al,[32] who showed that FAS expression by hepatocytes closely correlated with the activity of viral hepatitis in patients infected with HBV. However, with exception of the study of Luo et al[29] who detected FAS-L protein in hepatocytes and infiltrating lymphocytes in the HBV-related chronic liver disease, there are no data on the expression of the FAS ligand during chronic HBV infection. We can speculate that an increase in the FAS ligand expression by HBV-infected hepatocytes could contribute to the development of an immunoprivileged state by the induction of apoptosis in FAS bearing CTLs. To support this hypothesis and to enhance understanding of the FAS/FAS ligand role in HBV infected hepatocytes we analyzed by immunohistochemistry the expression of BCL-2 and BAX proteins in chronic HBV infection in relation to FAS/FAS-L expression. We also assessed proliferative activity by analysis of PCNA expression and the frequency of apoptosis. Furthermore, we compared the results with expression of the same proteins in patients with alcoholic and other hepatopathies.

Materials and Methods

Formalin fixed, paraffin-embedded liver biopsies from 14 HBsAg seropositive patients, 20 patients with alcoholic hepatopathy and 21 patients with other hepatopathies were used for the immunohistochemical detection of FAS, FAS-L, BCL-2, BAX, ICE and PCNA. Histopathological grading and staging were performed according to Ishak et al.[23] The presence of HBsAg in hepatocytes was also detected by histochemical means (Orcein stain). At the time of biopsy all patients were tested for serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyltransferase (GMT), alkaline phosphatase (ALP) and for prothrombin time (Quick), albumin (ALB) and total serum protein (TSP).

A standard immunoperoxidase technique using biotinylated secondary anti-mouse or anti-rabbit antibodies, followed by streptavidin peroxidase[9] was used for the immunohistochemical detection of PCNA, BCL-2, BAX, ICE, FAS and FAS-L proteins (see *Table 1*). The primary antibody was omitted from negative controls. As positive controls we used anaplastic astrocytoma stained with anti-PCNA, follicular lymphoma stained with anti-BAX and anti-BCL-2, bile ducts stained with anti-FAS and activated CTLs in hepatitis stained with anti FAS-L. The grade of immunopositivity in each case was scored semi-quantitatively at a magnification of 400x in the following range: 0=10% of cells positive; I=11–29% cells positive; II=30–59% cells positive; III=60% or more cells positive. The immunopositivity score was verified by measurement of the percentage of positive cells within specimens using the computerised image analysis system LUCIA M (Laboratory Imaging, Prague). A case was classified as positive if the percentage of positive cells was greater than 11% (grade score I–III). The expression of proteins was assessed in both hepatocytes and lymphocytes. Determination of apoptosis was achieved by the use of TUNEL kit for *in situ death*

Table 1 **Primary antibodies**

Antigen (clone)	*Dilution*	*Antigen retrieval*	*Source*
PCNA (PC10)	prediluted	yes (3 x 5 min.)	MOÚ Brno
BCL-2 (124)	1 : 20	yes (2 x 5 min.)	Dako
BAX (N-20)	1 : 40	No	Santa Cruz
FAS (N-18)	1 : 50	No	Santa Cruz
FAS-L (N-20)	1 : 50	No	Santa Cruz
ICE p10 (C-20)	1 : 50	yes (1 x 5 min.)	Santa Cruz

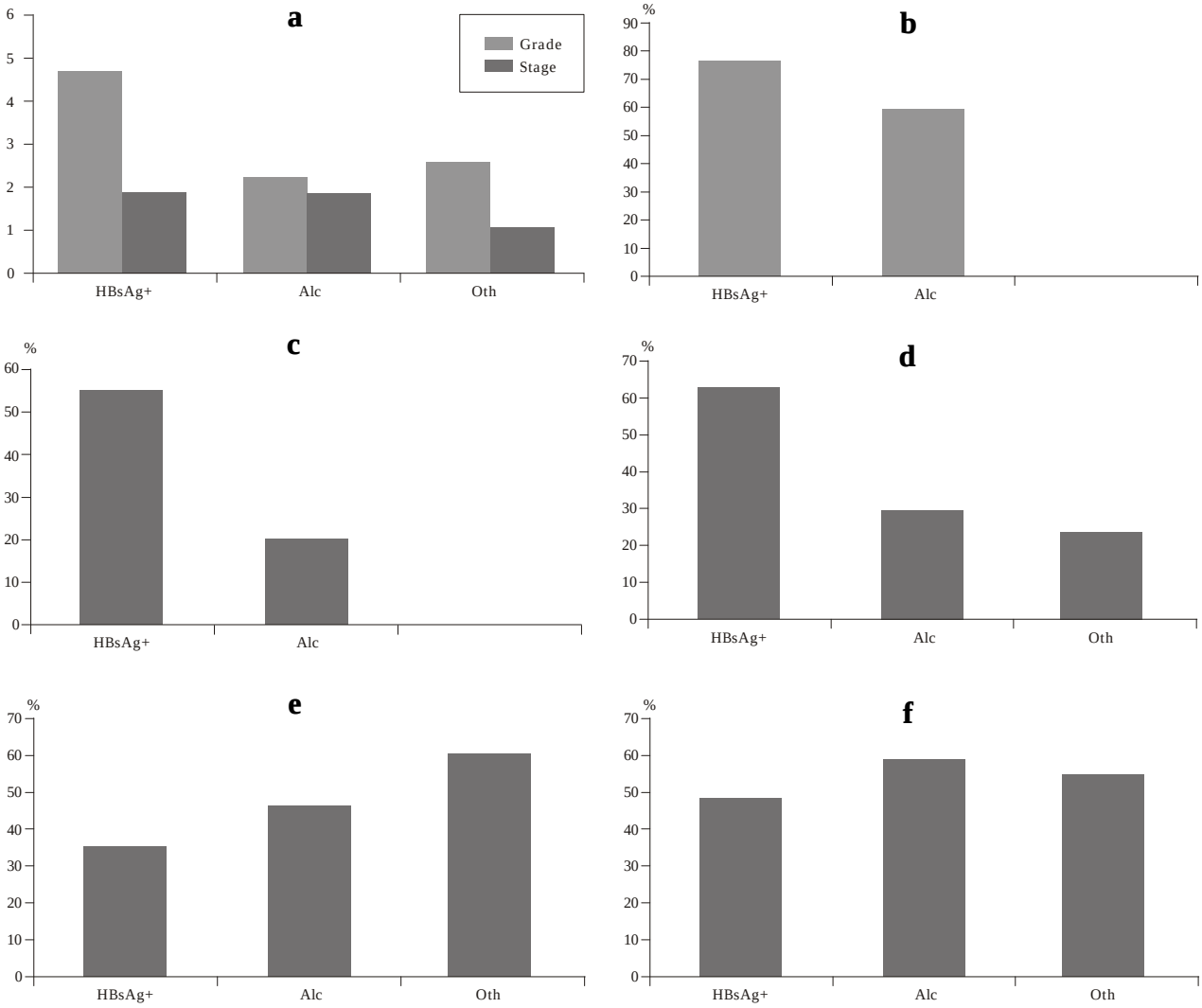

*Figure 1. Statistically significant differences observed between patient groups; **a)** Grade/stage of disease (according to Ishak); **b)** percentage of BAX positive hepatocytes and; **c)** infiltrating lymphocytes within patient groups **d)** percentage of FAS positive infiltrating lymphocytes. **e)** Percentage of FAS-L positive hepatocytes and **f)** lymphocytes. Abbreviations; Alc = alcoholic hepatopathy; Oth = Other hepatopathies.*

detection (Roche, cat. No. 1 684 809). The tissue samples were firstly digested by proteinase K for elimination of the DNA masking nucleoproteins. By the use of TdT (terminal deoxunucleotidyl transferase) the biotinylated deoxyuridin was incorporated into the DNA break points and then visualized using the avidin-peroxidase system. The cells were estimated at a magnification of 400x with an eyepiece microsquare micrometer (Olympus Optical). Tunel index (TI) was estimated as a percentage of TUNEL positive cells. The results of HBsAg-positive patients, those with alcoholic hepatopathy and patients with other hepatopathies were compared with the biochemical data and with the grade and stage. Statistical analysis was performed using a one way analysis of variance, by multifactorial range analysis (Duncan test) and by the Chi-square test.

Results

HBsAg-positive patients were characterised by a significantly higher grade score than the other groups (average score 4.79 in the HBsAg-positive group, versus 2.35 in patients with alcoholic hepatopathy, and 2.43 in those with non-specific hepatopathies, p=0.02) (*Figure 1a*). BCL-2 expression in hepatocytes as well as in adjacent lymphocytes of all groups was generally low (47 negative cases, 87%), whereas the levels of PCNA expression in hepatocytes was comparatively high (33 positive cases, 60%) (*Figure 2*). The expression of ICE was absent both in hepatocytes and lymphocytes in virtually all cases.

We found significantly higher numbers of BAX positive hepatocytes (11/14 cases, 78% versus 12/20 cases, 60%)

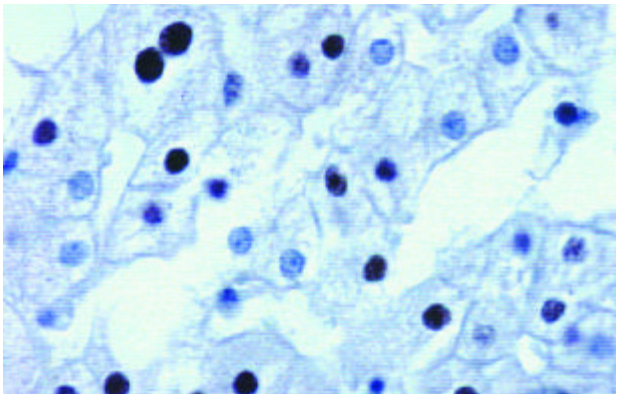

Figure 2. PCNA expression in hepatocytes from a HBsAg positive patient.

and lymphocytes (8/14 cases, 57% versus 4/16 cases, 25%) from the HBsAg-positive group ($p<0.01$) compared to patients with alcoholic hepatopathy. There were also significantly higher numbers of FAS positive lymphocytes in the HBsAg-positive group (9/14 cases, 64%, $p<0.05$) (*Figure 1b-d, Figure 3*). FAS-L positive hepatocytes and lymphocytes were relatively frequent in all patient groups (31/55 cases- 56%) and no significant difference in these numbers was observed (*Figures 1e-f*).

We also analysed the relationship between expression of the various apoptosis regulating genes in all patients. In hepatocytes the number of BAX and PCNA positive cells was positively correlated (coeff. 0,31, $p=0.02$), as were the number of BAX and FAS positive cells (coeff. 0,54, $p<0.001$), and the number of PCNA and FAS-L positive cells (coeff. 0,49, $p<0.001$). However, there was an inverse relationship between the number of BAX and BCL-2 positive cells (coeff. 0,39, $p<0.001$).

The TUNEL index (TI) of hepatocytes was higher in the HbsAg-positive group (20,7 %) compared to group with non-specific hepatopathies (12,8 %) and to group with alcoholic hepatopathy (4,4 %), however, these results were statistically non significant.

The cohort of patients was also divided into two groups according to Ishak`s grade (grade score >4; grade score <4) and stage (stage >3, stage <3). Liver biochemistry correlated well with grade and stage (increased ALT in 14/18 cases of high grade, 77% versus 19/37cases of low grade, 45%; increased AST in 14/18 of high grade cases, 77% versus 14/37 cases of low grade, 37%) (*Figure 4*), however we did not find any correlation between grade or stage and expression of apoptosis regulating genes (data not shown).

Discussion

Recent work suggests that, in patients with chronic hepatitis B (CHB), treatment by interferon can stimulate a specific CTL response that leads to viral clearance and resolution of disease.[40] There is growing evidence to implicate FAS/FAS-L interactions as important mediators of apoptosis in a variety of situations including graft rejection, tumors, autoimmune diseases and inflammation.[4,7,12,20,34,46,47,49,50] The Fas ligand is expressed on activated CTLs and induces apoptosis in FAS bearing cells.[48] This mechanism is believed to operate in hepatitis B virus infection where it is responsible for the associated liver disease and is supported by studies on HbsAg transgenic mice where FAS dependent apoptosis by CTLs induces acute liver disease.[35,43] Therefore, defects in FAS/FAS-L proteins might be relevant to the persistence of HBV in hepatocytes during CHB. In addition, FAS-ligand bearing hepatocytes in CHB may also induce apoptosis in CTLs expressing FAS. Such a mechanism may contribute to CTL escape of Hepatitis B virus infected hepatocytes.

At the present time an adequate in vitro model for the study of the possible involvement of the FAS ligand in the development of chronic hepatitis B does not exist. There-

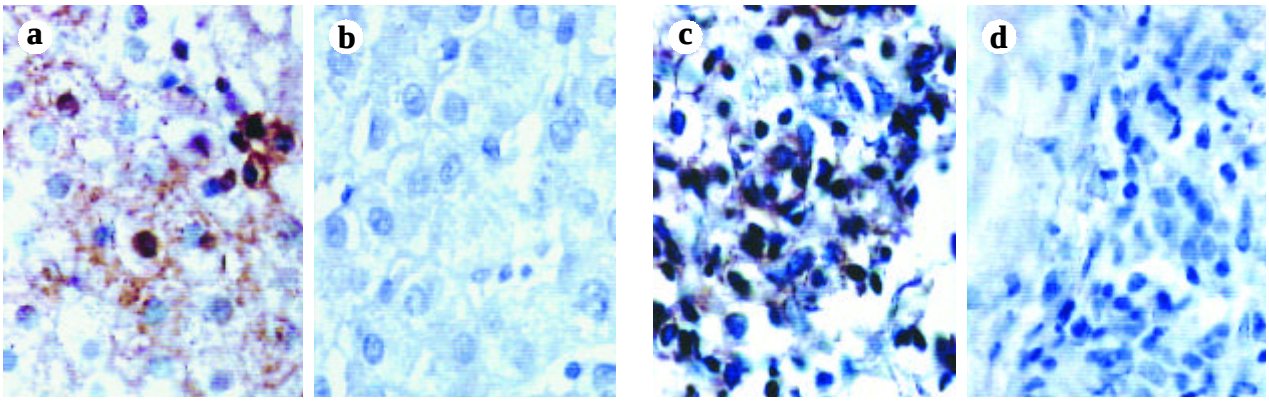

Figure 3. a) BAX expression in hepatocytes from HBsAg positive patient and, b) negative BAX immunostaining in hepatocytes from a patient with alcoholic hepatopathy, c) FAS expression in lymphocytes from HBsAg positive patient and, d) negative FAS immunostaining in lymphocytes from a patient with alcoholic hepatopathy.

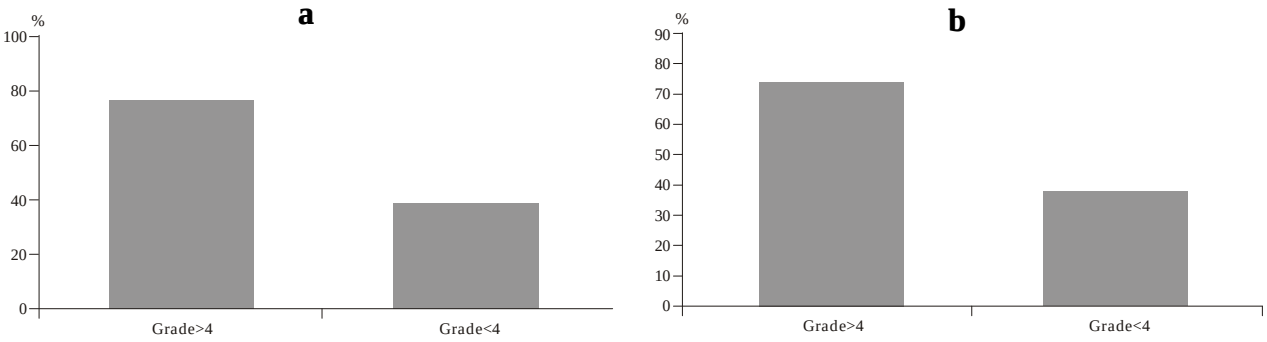

Figure 4. Percentage of HBsAg patients with increased, a) ALT, b) and AST, in relation to grade.

fore, based on our extensive experience, we believe that immunohistochemistry is the best choice of method by which we can support of our hypothesis. We have shown increased numbers of BAX positive hepatocytes in HbsAg-positive patients compared to the control groups suggesting that these cells may be susceptible to apoptosis. This is also supported by finding of higher TUNEL index of hepatocytes in HBsAg-positive patients. However, we have also shown that infiltrating lymphocytes from HBsAg positive cases more frequently express BAX and FAS. Taken together with the finding that the FAS-ligand is also expressed by hepatocytes, these results support the contention that apoptosis of CTLs induced by FAS-L bearing hepatocytes could be important in the persistence of infected hepatocytes. A similar mechanism of FAS bearing cells destruction which facilitate local tumor invasion was recently described in vitro by Yoong et al.[52] However, we did not find significant differences in the numbers of FAS-ligand expressing hepatocytes between patient groups. We might suggest that induction of FAS-ligand expression on hepatocytes could be a reaction not only to HBV infection but also to various non-specific agents.[21,33]

The infrequent expression of the anti-apoptotic protein, BCL-2, by hepatocytes and lymphocytes in the majority of cases, is further support that these cells may be susceptible to apoptosis. On the other hand, with the exception of the BAX protein, we did not test other members of BCL-2 family to validate this finding. Moreover apoptosis can be mediated in a BCL-2 independent fashion.[1] In addition, the high level of expression of PCNA in damaged liver cells suggests the beginning of liver regeneration. It must, however, be mentioned that the assessment of proliferative activity by Ki-67 analysis is more accurate, however estimation of proliferation by analysis of PCNA expression can also give valid results.[8] The absence of ICE expression in our series might suggest that this protein is not important in the apoptotic pathway of hepatocytes.

In summary, we have provided evidence to suggest that FAS/FAS-L interactions are important not only in the generation of liver cell damage in CHB but also potentially in the induction of apoptosis in CTLs leading to persistence of virus-infected hepatocytes. However, we cannot exclude the possibility that the survival of HBV-infected hepatocytes in CHB is due to other mechanisms like viral inhibition of apoptosis triggered by CD40/Fas interactions[2] or defects of downstream factors involved in the apoptotic cascade, such as DNAase enzymes and/or other caspases.

Acknowledgements

This work was supported by the GACR No. 301/96/KO47, and by the Grant of MSTM No. VS 96154 and No. J14/98 151100001.

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