

# Comparative Immunohistochemical Detection of Caspase-3 Activation in Liver Biopsy Specimens of Patients with Mono and Mixed Infection

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## ABSTRACT—

**Background:** Apoptosis is a genetically programmed form of a cell death that plays a major role in pathogenesis of chronic liver diseases. As Caspase-3 activation is required to produce apoptotic chromatin condensation and DNA fragmentation it is used in the study of apoptosis.

**Subjects and methods:** Liver biopsies of patients with heroin abuse and coinfection of tuberculosis, chronic hepatitis C, human immunodeficiency virus (TB, HCV, HIV) were investigated. For comparison liver biopsies of patients with chronic hepatitis B (HBV) were used. All biopsies were performed according to routine medical program, using standard Mengini procedure. Material was fixed in formalin, embedded in paraffin and cut 7 micrometers in thickness. For immunohistochemical detection endogenous peroxidase activity was blocked with 3% hydrogen peroxide and the sections were stained using Immunohistochemistry kit according to the manufacture's guidelines. Cleaved Caspase-3 (Asp175) served as primary antibody. Biotinylated secondary antibody and streptavidin-conjugated peroxidase were used for detection using DAB as chromogen. Nuclei were counter stained with Harris Hematoxylin. Negative controls did not contain primary antibodies. In addition to the immunohistochemical study of Caspase-3 numerical score was established for each biopsy specimen both for grading of necroinflammatory activity (Knodell et al.) and stage of fibrosis (French METAVIR).

**Results:** Our investigation showed that positive reaction of Caspase-3 strongly varies in different liver biopsies; activation is revealed in nuclei and cytoplasm of hepatocytes and some Kupffer cells as well as in endotheliocytes of sinusoids. In portal tracts the activation occurs in some endotheliocytes of interlobular portal veins, in cells of lymphohistiocyte infiltrates. In liver biopsies of patients with coinfection the Caspase-3 expression was noticed in 30-50% of nuclei, whereas in liver biopsies of patients with HBV mono infection the index did not exceed 10%. Index of histological activity according to Knodell of patients with coinfection reached 12-15 balls, fibrosis stage according to METAVIR – F2; in patients with HBV these indexes were respectively 4 balls and F-1.

**Conclusions:** Our study demonstrated the expression of Caspase-3 was significantly higher in cases of chronic hepatitis with more severe histological necroinflammatory activity. Especially high activation of Caspase-3 was in liver biopsies of patients with coinfection and heroin abuse. Our results suggest high level of apoptosis in liver biopsies of such types of patients.

**Keywords—** Apoptosis, Biopsy, Caspase-3, Immunohistochemistry, Mixed infection, Tuberculosis, Viral hepatitis

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## 1. INTRODUCTION

Recent years active study about various aspects of apoptotic hepatocytes in the liver during viral hepatitis has been investigated [1-8] as apoptosis is one of the defense mechanisms in viral infection [9]. Pro-apoptotic and anti-apoptotic signaling proteins have both essential roles in apoptosis. In particular, apoptotic effectors include caspases [10]. Caspases are a family of proteases which play an important role in development and regulation of apoptosis and inflammatory processes [11-12]. The term “caspase” is an English abbreviation of cysteine-dependent aspartate specific protease. Caspases are produced in inactive form and activated by different apoptotic signals of pro-caspases [13-15]. In particular it is assumed that caspases regulate the final stages of apoptosis, i.e. cutting of specific substrates in the cell [16]. According to some studies the activation of Caspase-3 triggers a cascade of enzymatic changes leading to morphological manifestations of apoptosis, including chromatin condensation and DNA fragmentation [17-18].

Increased activation of caspases is described in liver diseases [19-23]. Caspase activation has been detected in the serum of patients with chronic hepatitis C virus (HCV) and the metabolic syndrome (non-alcoholic fatty liver disease, NAFLD) [23].

Previously we studied the characteristics of apoptosis in the liver of patients with chronic hepatitis viral hepatitis C by TUNEL method [24-30]. In addition, immunohistochemical analysis were carried out to study the expression of the oncogene Bcl- 2, which is considered as one of the important factors suppressing apoptosis in acute and chronic liver damages [31].

The aim of the present study was the comparative immunohistochemical investigation of Caspase-3 in liver biopsy specimens in the cases of monoinfection (chronic hepatitis B) as well as during mixed infections (tuberculosis, viral hepatitis C, and human immunodeficiency virus).

## **2. SUBJECTS AND METHODS**

Liver biopsies were collected from 5 male patients with chronic viral hepatitis B (HBV) and from 5 male patients with mixed infection - combination of tuberculosis (TB), chronic viral hepatitis C (HCV) and immunodeficiency virus (HIV). Patients were 30-50 years old. All biopsies were performed using the standard Mengini procedure. The material was fixed in 10% formalin, embedded in paraffin; sections 7 micrometers in thickness were cut. For morphological examination and semi-quantitative analysis the sections were stained with hematoxylin-eosin.

Liver biopsies of the patients with chronic hepatitis B (HBV) were used for comparison. All the biopsies were performed according to routine medical program, using standard Mengini procedure. Material was fixed in formalin, embedded in paraffin and cut into sections of 7 micrometers in thickness. For immunohistochemical detection of Caspase-3 expression endogenous peroxidase was blocked with 3 % hydrogen peroxide, thereafter the sections were stained according to standard procedure by the manufacturer using kit (Cell Signaling Technology, Inc., USA). Cleaved Caspase-3 (Asp175) served as the primary antibody (Cell Signaling Technology, Inc., USA). The nuclei were counterstained with Harris-hematoxylin. The negative control contained no primary antibody (Figure 7).

For semi-quantitative analysis histological activity index was determined (IGA) according to Knodell [32], fibrosis (F) was evaluated according to Ishak (1995) and the stage and activity of fibrosis (A) according to French METAVIR [33]. A quantitative analysis of cellular elements labeled by caspase-3 (mononucleated and binucleated hepatocytes, sinusoidal lining cells) was performed. The counting was carried out in different parts of the lobules and the percentage of labeled elements out of the 100 hepatocytes was determined.

Photos of the slices were made by microscope Leica DM 2500 connected with the digital camera Leica DFS 320 R2. Photomicrographs were electronically stored and analyzed further by computer.

## **3. RESULTS**

### **3.1. Distribution of Caspase- 3 in the Liver Biopsies of Patients with Chronic Viral Hepatitis B (HBV)**

Liver biopsies of the patients with mild and moderate activity of HBV were used for immunohistochemical analysis. In this group, biopsy was mostly characterized by minor or moderate expansion of portal areas, sometimes with partial damage of the borders of the liver plates. In portal areas lymphocytic infiltrates could be find slightly. Typically the dramatic tissue damages were near the central veins expanding to the sinusoids in the middle part of the hepatic lobules. In some hepatocytes large fat vacuoles were present. Polymorphism of the nuclei of hepatocytes was expressed predominantly in the middle parts of the lobules (Figure 1), whereas some vacuolated nuclei were located near the portal tracts.

By immunohistochemistry (IHC) Caspase-3 activity was detected in the nuclei of hepatocytes (Fig.1). In IHC study for Caspase-3 chromatin become intensely dark brown color, however at the same time characteristically nucleolus remained unpainted (Figure 2, arrows). Interestingly, in the nucleated hepatocytes only one nucleus of the pair was stained (Fig. 2, arrows). The expression of Caspase-3 in the nuclei of hepatocytes varied within different parts of the lobule. The greatest number of immunoreactive hepatocytes was determined in the middle parts of the lobule (Fig.1, 2). In the central parts of the lobules and around portal areas the activity of Caspase-3 was less expressed (Fig.3). The cytoplasm of some hepatocytes colored mottled-brown. In these hepatocytes at high magnification cytolemma (karyotheca) distinctly contoured and in the cytoplasm of hepatocytes displayed rod-shaped pinkish-green granules, possibly mitochondria.

Immunoreactive hepatocytes had not any morphological signs of apoptosis (Figure 1, 2). The number of individual hepatocytes or groups with morphological signs of apoptosis in did not exceed 0.1 - 0.5 %. At the same time the number of all hepatocytes immunoreactive for Caspase-3 (with and without morphological features of apoptosis) was 6 - 8%; among them mononucleated hepatocytes formed 2.8 % to 3.6%, while the amount of labeled binuclear hepatocytes

ranged from 3.2 % to 4.4 %. Besides the hepatocytes, brown appearance of the nuclei and the cytoplasm of cells lining the sinusoids revealed mainly also Kupffer cells (Figure 2). The number of sinusoidal immunoreactive Caspase - 3 cells varied in different parts of the lobules from 0.5 % to 1.5 %. In portal zones only single connective tissue cells were stained (Fig. 3). In this, monoinfected (HBV), patient group inflammatory changes were typically mild. Only in one case Caspase -3 was weakly expressed in the epithelial cells of interlobular bile ducts.

Semiquantitative analyzes indicated that the histological activity index by Knodell was less than 3 points and fibrosis stage by Ishak - F 1. The activity and stage of fibrosis by French METAVIR - A1 and F1 respectively.

### **3.1. Distribution of Caspase-3 in the Liver Biopsies of Patients with Mixed Infection (Tuberculosis, HCV, HIV)**

Compared to HBV monoinfected patients the biopsies of patients with the mixed infection group showed significantly more inflammatory and necrotic changes. There were typical changes: expansion of portal area, damage of limiting plate, development of peace-meal and bridging necroses. In the portal tracts there was an expressed lymphocytic infiltration. Inside the liver lobes many small focal infiltrates revealed. Sinusoids often contained the lymphocytes. Immunohistochemical expression of Caspase-3 in this group was significantly higher when compared with the monoinfection group. Caspase-3 was expressed in the central zone of liver lobules (Fig. 4, 5). In the peripheral parts of the lobules the number of Caspase-3 labeled hepatocytes was significantly lower (Figure 6). Both nuclei were usually stained in t binucleated hepatocytes. However, the number of binucleated hepatocytes was significantly lower than in the patients group with monoinfection.

Unlike in the case of monoinfection, expression of Caspase -3 was significantly higher in the nucleus and cytoplasm of the sinusoidal cells (Figure 6). The contours of the cytoplasm of these cells were in reddish-brown colors. Massive nuclear staining of inflammatory cells in the portal areas occurred in liver biopsies of the patients with mixed infection (Figure 6). Approximately 50% of lymphocytes nuclei expressed Caspase -3; similarly the nuclei of the cells in interlobular infiltrates could be also characterized by activation of Caspase-3. Caspase-3 expression was observed in isolated cholangiocytes of the bile duct epithelium and in separate endothelial cells of portal venules in the part of portal tracts (Figure 6). The number of hepatocytes expressed by Caspase-3 varied in different parts of the lobes from 15% to 45%. Sinusoidal linings expressed Caspase-3 from 15 - 20%.

Semi-quantitative analyzes indicated that the histological activity index by Knodell was 15 points, fibrosis by Ishak - F 4. Activity and stage of fibrosis by French METAVIR - A3 and F3, respectively.

## **4. DISCUSSION**

Despite the large number of publications the pathogenesis of chronic viral hepatitis has remained as the subject of actual debate. Studies in the recent years have given interesting results with unified mechanism of cell death - apoptosis [34, 3]. Features of hepatitis virus proteins and activation of caspases in hepatocytes are discussed [35].

Caspase -3 in patients with chronic hepatitis C has been discussed the mostly frequently [19-21, 5, 37-39]. Caspase-3 activity has been studied also in serum [24, 39].

Data on morphological characteristics to identify Caspase -3 in the liver biopsies of patients with chronic viral hepatitis are very contradictory. In some of the previous studies Caspase-3 activity has not been found in the liver biopsies [38] or the activity has found out as relatively low, and 1.2 % of the hepatocytes [40]. Sarfaz et al [38] observed that Caspase-3 activity is not detectable in the hepatocytes and was present only mainly in the sinusoidal linings and in inflammatory cells. However in other studies [19-21, 40] the high activity of Caspase -3 is described in the hepatocytes of patients with chronic liver diseases.

In this paper, the expression of Caspase-3 was detected in the hepatocytes as well as in the sinusoidal linings, in the inflammatory cells of the infiltrates of the portal zones, in interlobular foci of infiltration in the bile duct epithelium and in vascular endothelium. The expression of Caspase- 3 in one way or another is detected in all cellular elements of biopsies, which confirms their relationship to the development of both pathological and protective responses.

At present there are rather contradictory evidences about the relationship of Caspase-3 and the degree of inflammation and fibrosis in liver biopsy specimens. Some authors [21, 37, 41] have noted a correlation between the level of activation of Caspase-3 and the degree of histological activity. At the same time when analyzing serum Caspase-3 [39] no statistically significant difference between the activity of Caspase -3 activity and the degree of histological activity or fibrosis have been found. Comparison of the activity of Caspase-3 during monoinfection (HBV) and mixed infections in the present study clearly indicates the existence of such a relationship. Inflammatory necrotic changes during monoinfection were mild: Histological Activity Index (HAI) was no more than 3 points and the stage of fibrosis - F1. Hepatocytes labeled by caspase-3 were respectively 6-8%. When mixed infections IGA reached 15 points, fibrosis stage - F4. Number labeled caspase -3 by hepatocytes ranged from 15 to 45% in different parts of segments. Moreover strong Caspase -3 activation was detected also in other cells of the liver comprising sinusoidal wall and lymphocytic infiltrate in the portal tract and intralobular necrosis.

Previously we have carried out immunohistochemical analysis of apoptosis in liver biopsies of patients with chronic viral hepatitis C [24-30, 42-43]. For identification of apoptotic cells was used TUNEL method, based on the labeling of the free ends of DNA. The study showed that the apoptotic index ranged from 0.02 % to 1.2 %. Other authors have also noted that in assessing the TUNEL method apoptotic index was relatively low, not exceeding 0.5 % of viral hepatitis [3].

Our present data on the amount of labeled Caspase-3 showed significantly stronger expression of Caspase-3 in hepatocytes during mixed infection (15-45 %) compared to mono-infection (6-8%). Interestingly during our immunohistochemical study of Caspase-3 hepatocytes without morphological features of apoptosis were also stained, which is very evident in the micrographs (Figure 1-2). Some authors suggest that activation of Caspase-3 identifies the earlier stages of apoptosis [20, 44]. One reason for discrepancies between the low number of TUNEL- positive cells and high number of cells, reflecting activation of Caspase-3, can be the time difference of biochemical changes in apoptosis. DNA fragmentation was detected apparently in the later stages of apoptosis.

It is believed that monitoring of Caspase -3 can serve as a marker of early liver damage liver diseases [20], useful in determining the effectiveness of therapy and therapeutic strategy selection activities [45] which opens up new diagnostic capabilities in chronic liver disease, including viral hepatitis.

## 5. CONCLUSIONS

1. In the liver biopsies of the patients with mono-infection (HBV) and mixed infection (TB, HCV, HIV) expression of Caspase-3 was detected not only in hepatocytes but also in endothelial cells and Kupffer cells, in lymphocytic infiltration in the portal areas and in intralobular focal necrosis in epithelium of the bile ducts
2. There are differences in the expression of Caspase-3 in mono- and binucleated hepatocytes.
3. Quantitative analysis revealed significant differences in the expression of Caspase-3 in biopsies of patients with mono (HBV) and mixed infections (tuberculosis, HCV, HIV).
4. Expression of Caspase-3 depends on the extent of necrosis, inflammation and fibrosis.

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#### **SIGNATURES TO THE FIGURES:**

**Figure 1:** Liver biopsy from the patient with chronic hepatitis B (HBV) infection. Immunohistochemical detection (IHC) of Caspase-3 activation. Expression of Caspase-3 predominates in liver cell nuclei. Only single nucleus labeled inside in binucleated hepatocytes. Ob.x40.

**Figure 2:** Liver biopsy from the patient with chronic HBV infection. IHC of Caspase-3 activation. Expression of Caspase-3 is typical for the hepatocytes without any morphological signs of apoptosis. Ob.x40.

**Figure 3:** Liver biopsy from the patient with chronic HBV infection. IHC of Caspase-3 activation. Minimal expression of Caspase-3 in region of portal zone. Ob.x40.

**Figure 4:** Liver biopsy from the patient with mixed infection (TB, HCV, HIV). IHC of Caspase-3 activation. Moderate number of hepatocytes nuclei positive for Caspase-3. Expression of Caspase-3 in the wall of sinusoids. Central zone of liver lobule. Ob.x 20.

**Figure 5:** Liver biopsy from the patient with mixed infection (TB, HCV, HIV). IHC of Caspase-3 activation. Expression of Caspase-3 in liver nuclei and sinusoids in the middle zone of liver lobule. Ob.x 40.

**Figure 6:** Liver biopsy from the patient with co-infection (TB, HCV, HIV). IHC of Caspase-3 activation. High expression of Caspase-3 in nuclei of portal infiltration. Ob.x40.

**Figure 7:** Liver biopsy from the patient with chronic HBV infection. Negative control containing no primary antibody (Caspase-3). Ob.x4.