Dynamic of liver cell population in biopsies of patients with chronic hepatitis B

Master Thesis

Supervisors: Piret Hussar, M.D., D.M.Sci
Martin Kärner, M.Sc
Galina Filimonova, D.M.Sci
Viljar Jaks, PhD

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ABBREVIATIONS

AIF – Apoptosis-inducing factor

Anti-HBc – Antibody to hepatitis B core antigen

Anti-HBe – Antibody to hepatitis B e antigen

Anti-HBs – antibodies to surface antigen of the hepatitis B virus

Bcl-2 – B-cell lymphoma 2

CHB – Chronic hepatitis B

HBCAg – Hepatitis B core antigen

HBeAg – Hepatitis B e antigen

HBsAg – Hepatitis B surface antigen

HBV – Hepatitis B virus

HCV – Hepatitis C virus

HDV – Hepatitis D virus

HIV – Human immunodeficiency virus

IFN-α – Interferon α

IgG anti-HBc – IgG antibodies to the hepatitis B core antigen

IgM anti-HBc – IgM antibodies to the hepatitis B core antigen

IHC – Immunohistochemistry

MOMP – Mitochondrial outer membrane permeabilization

PCD – Programmed cell death

WHO – World Health Organization
INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem. Approximately one third of the world’s population has serological evidence of past or present infection with HBV and 350-400 million people are chronic HBV surface antigen (HBsAg) carriers. The spectrum of disease and natural history of chronic HBV infection are diverse and variable, ranging from an inactive carrier state to progressive hepatitis B (CHB), which may evolve to cirrhosis and hepatocellular carcinoma (HCC). HBV is the leading cause of cirrhosis and hepatocellular carcinoma (HCC) worldwide. HBV-related end stage liver disease or HCC are responsible for over 0.5–1 million deaths per year and currently represent 5–10% of cases of liver transplantation.

HBV is mainly transmitted through perinatal infection, sexual contact, blood and contaminated needles from drug abusers. It can be transmitted through the direct contacts or skin wounds between patients and surgeons or dentists, or indirectly through hemodialysis, razors, toothbrushes, and acupuncture therapy.

During the chronic hepatitis a lot of morphological changes appear in well organized liver structure. A hallmark of chronic hepatitis is portal inflammation (portal hepatitis), mainly consisting of lymphocytes. The severity of portal inflammation may vary from one patient to another and from one portal tract to another. A variable degree of lobular changes (lobular hepatitis), including focal and confluent necrosis, apoptosis and inflammation, completes the picture of necroinflammatory changes in chronic hepatitis.

The Bcl-2 family of proteins play an important role in the control of apoptosis. It has emerged as a key regulator of apoptosis, because it can protect cells from death induced by a number of injuries including radiation, chemotherapy, or growth factor deprivation. Apoptosis during the virus hepatitis can be a result of the direct action of the virus or as the defined immune reaction. Since in the dead cell the replication of the virus becomes
impossible, the "interest" of virus is to stop apoptosis and to preserve hepatic cells alive. It has been shown that some virus proteins are coded to possess anti-apoptotic activity, suppressing the functions of the protein p53 and enhancing the expression of Bcl-2, so the apoptosis is inhibited and virus can whether replicate or remain in inactive state in human hepatocytes.

The aim of this study was to perform morphometrical analysis of liver biopsies of patients with chronic viral hepatitis B and to detect localization of apoptosis in liver biopsies of patients chronically infected with HBV and to evaluate Bcl-2 oncoprotein expression in biopsies of patients with HBV.
1. REVIEW OF LITERATURE

1.1. Features of morphological structures and functions of the human liver

The liver is one of the largest, most important, and least appreciated organs in the body. The liver is organized into lobules which take the shape of polygonal prisms. Each lobule is typically hexagonal in cross section and is centered on a branch of the hepatic vein (central vein). Within each lobule, hepatocytes are arranged into hepatic cords separated by adjacent sinusoids. At the corners between adjacent lobules are the so-called portal areas (portal canals, portal triads). These are regions of connective tissue which include branches of the bile duct, the portal vein, and the hepatic artery. Along the central axis of each lobule runs a central vein, which is a branch of the hepatic vein (SIUC. School of Medicine. Anatomy / David King http://www.siumed.edu).

Figure 1. Vascular structure of a hepatitic lobule. HC-hepatocyte, BD-bile duct, PV-portal veinule, APA-arterioportal anastomoses, HA-hepatic arteriole, CV-centrolobular vein (Julien Santi-Rocca et al., 2009).
Under the low power of the light microscope, normal liver is seen to have a regular structure based on portal tracts and efferent veins. The smallest portal tracts contain portal venules, hepatic arterioles and small interlobular bile ducts. Blood from both venules and arterioles passes through the sinusoidal system to reach efferent hepatic venules (central venules). From these, the blood drains into successively larger veins to reach the intestine by way of the common bile duct (Jay H. Lefkowitch, 2010).

**Figure 1.1** Normal adult liver. A small portal tract contains a portal venule (V), interlobular bile duct (B) and arteriole (A). Needle biopsy, H&E.

Near or at the margins of the small portal tracts, the bile canaliculi, formed as spaces between adjacent hepatocytes, communicate with the canals of Hering (Theise N.D., *et al.* 1999; Saxena R., Theise N. 2004; Roskams T.A., *et al.* 2004). These are lined partly by hepatocytes
and partly by biliary epithelial cells. From the canals of Hering bile drains into bile ductules (or cholangioles), lined entirely by biliary epithelium. Bile ductules converge from hepatic lobule onto portal tract and connect bile canaliculi to the interlobular bile ducts. Neither canals of Hering nor ductules are easily seen in normal liver, but they may become apparent in disease.

The hepatic sinusoids are lined by specialised endothelial cells, which form an incomplete, porous barrier allowing easy exchange of materials between blood and hepatocytes. Within the sinusoidal lumen lie the Kupffer cells, specialised hepatic macrophages. They are most numerous near portal tracts (Jay H. Lefkowitch, 2010). Hepatocytes are polygonal cells with well-defined cell borders. Each cell contains one or more nuclei. Polyploidy and variation in nuclear size are normal characteristics of adult human liver.

Hepatocyte cytoplasm is normally rich in glycogen. In sections stained with haematoxylin and eosin the cytoplasm appears granular and often pale-staining centrally, where glycogen and endoplasmatic reticulum predominate. A few fat vacuoles and occasional apoptosis may be seen in the absence of obvious disease. Many different proteins can be demonstrated in or on the hepatocytes, in keeping with the liver's many metabolic functions. These include secreted proteins such as albumin and cell surface proteins such as adhesion molecules (Hinchcliffe S.A., et al. 1996).

Within the hepatocytes, aggregated near the bile canaliculi and most abundant in perivenular areas, there are fine yellow-brown granules of lipofuscin pigment. Lipofuscin is a normal constituent of adult liver, increasing in amount with age but also sometimes found in children. The granules represent lysosomes containing materials which cannot be further degraded.

**Functions of liver:**
• formation and secretion of bile;
• storage of glycogen, buffer for blood glucose;
• synthesis of urea;
• metabolism of cholesterol and fat;
• synthesis and endocrine secretion of many plasma proteins, including clotting factors;
• detoxification of many drugs and other poisons;
• cleansing of bacteria from blood;
• processing of several steroid hormones and vitamin D;
• volume reservoir for blood;
• catabolism of hemoglobin from worn-out red blood cells.

1.1.2. Changes in the structure of liver during chronic viral hepatitis

Chronic hepatitis is a necroinflammatory process that may be complicated by fibrosis. A hallmark of chronic hepatitis is portal inflammation (portal hepatitis), mainly consisting of lymphocytes (Ishak K.G. 2000; Scheuer P.J., Lefkowitch J.H. 2006). The severity of portal inflammation may vary from one patient to another and from one portal tract to another. A variable degree of lobular changes (lobular hepatitis), including focal and confluent necrosis, apoptosis and inflammation, completes the picture of necroinflammatory changes in chronic hepatitis.

As for fibrosis, the process usually starts in the portal tracts (which become enlarged) and proceeds with the formation of fibrous septa that may lead to the onset of cirrhosis.

“Ground-glass” hepatocytes are hallmark of chronic hepatitis B infection (Hadziyannis S. et al. 1973). They are liver cells with an eosinophilic, granular, glassy cytoplasm on light microscopy. The triad of lymphocyte nodular inflammation in portal tracts, steatosis and bile duct damage is considered highly characteristic of chronic hepatitis C
1.2. **Hepatitis B virus**

Hepatitis B is the leading cause of liver cancer in the world today and frequently leads to cirrhosis and liver failure, particularly in individuals with early life acquisition. Although significant progress has been made in the area of vaccination, universal vaccination of children and young adults has not yet been realized (WHO, 2004).

Hepatitis B virus belong to the family Hepadnaviridae and HBV is the smallest human DNA virus, carrying a genome only 3,200 nucleotides in length (Lay J.Y., Wright T.L., 1993). Hepatitis B virus has a partially double-stranded circular DNA genome harboring four overlapping open reading frames encoding the surface (S), core (C), polymerase (P), and X genes. The HBV genome efficiently uses its genetic information to encode four groups of proteins and their regulatory elements by shifting the reading frames over the same genetic material (Chisari F.V. et al., 1989). The HBV reverse transcriptase lacks a proofreading activity, which leads to high error rate and quick HBV evolving over time (Lay J.Y., Wright T.L., 1993).

The virus has a core antigen surrounded by a shell containing surface antigen (HBsAg) (Seeger C., Mason W.S., 2000). HBV contains numerous antigenic components, including HbsAg, HbcAg and hepatitis B e antigen (HBeAg). The immune response to HbsAg provides the immunity against HBV. Antibodies to HbcAg indicate infection, IgM antibodies to the hepatitis B core antigen (IgM anti-HBc) indicates recent infection and usually disappears within six months, while IgG antibodies to the hepatitis B core antigen (IgG anti-HBc) persists for life and indicates past infection. Antibody to HBsAg (anti-HBs) appears after clearance of HBsAg or after immunization. The presence of HBsAg for more than six months is defined as chronic HBV infection.

Although effective and safe vaccines for hepatitis B virus have been available for
more than two Decades, hepatitis B virus kills 600,000 to 1 million people annually worldwide (Kao J.H., Chen D.S., 2002). Approximately 15-40% of HBV carriers who acquire the virus early in life usually develop HBV-related cirrhosis or HCC (Fattovich G. et al., 2008).

1.2.1. Etiopathogenesis

HBV is transmitted parenterally by contaminated blood or other body fluids through blood vessels, skin or mucous membranes. The virus can be detected in all human body fluids, the virus concentration in the fluids is highest in the blood or serous exudates, and it is relatively low in saliva, semen, vaginal fluids (Margolis H.S. et al., 1997). In adolescents and adults in the countries with low and middle prevalence rates of hepatitis B, sexual contact is the major cause of HBV infection (Alter M.J., Margolis H.S., 1990). Infection through fecal-oral transmission does not occur. In injection drug abuser, using the same syringe among injection drug users at the same time is the main transmission route of hepatitis B infection (Hutin Y. et al., 2002). In addition, tattooing, ear piercing, acupuncture, dialysis and even using a syringe can be the source of infection (Goldstein S.T. et al., 2002).

Even when blood products are tested for HbsAg there is still a minor risk of transmission (Gerlich W.H., Caspari G., 1999). WHO estimates that globally about 6 million units of blood are not properly tested (Simonsen L. et al., 1999). Transmission by bone marrow and non-liver solid organ transplantation has been largely eliminated by screening donors for serum HBsAg.

Hepatitis B shows variable clinical manifestations ranging from asymptomatic HBV carriers to fulminant liver failure, and it becomes chronic, often progresses to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Wright T.L., Lau J.Y., 1993). When the virus persists longer than 6 months, it becomes a chronic state. The clinical course of hepatitis B is determined by the interaction of viral replication status and host immune response. Otherwise, it can be worsened by the factors such as alcohol and coinfection with other
viruses. The risk of progression to decompensated liver disease or the incidence of liver cancer increases with high viral replication status and the risk is higher in patients with cirrhosis than hepatitis (Liaw Y.F. et al., 2004).

1.2.2. The clinical picture of acute and chronic hepatitis B

At any given stage of hepatitis B, there is balance between immunologic control and viral replication. The triggering events for spontaneous reactivation and clinical relapse of hepatitis B are poorly understood, but are likely to be due to viral or host events that change this balance (Perrillo R.P., 2001).

Hepatitis B virus infection is defined by the presence of the virus in the infected host. Undetectable or low serum HBV DNA levels are associated with inactive disease. High serum HBV DNA may or may not be associated with active disease. A provisional threshold of $10^5$ copies/ml has been proposed to define high serum HBV DNA levels (Look A.S. et al., 2001).

Occult HBV infection is characterized by undetectable serum HBsAg but detectable HBV DNA in serum or liver.

In chronic hepatitis B, there is persistent hepatic inflammatory injury. Chronic infection is characterized by the persistence of serum HBsAg, anti-HBc and there is histological evidence of necroinflammation or elevated serum aminotransferase levels cannot be explained by another cause of liver injury.

In the inactive HBsAg carrier-state HBsAg and antibody to hepatitis B e antigen (anti-HBe) are present in serum, but serum aminotransferase levels are persistently normal and there is little or no necroinflammatory activity on liver biopsy. Such patients have either low or undetectable levels of HBV DNA in serum.

The usual age of patients at the time of diagnosis is 41-52 years. Preditors for progression to cirrhosis include the following: older age, detectable serum HBV DNA levels using non-PCR based methods, concurrent HCV infection, concurrent HDV infection,
concurant HIV infection, alcohol abuse, recurrent episodes of severe acute exacerbation with bridging hepatic necrosis, high fibrosis stage at presentation and severity of necroinflammation at diagnosis (Di Marco V. et al., 1999; Brunetto M.R. et al., 1989; Fattovich G. et al., 1988).

Perinatal infection from the infected mother is almost always asymptomatic, and evolves to chronicity in 90% of cases (McMahon B.J. et al., 1985). The risk of perinatal infection is about 90% in babies born to HBeAg-positive mothers and 10% in babies born to HBeAg-negative mothers (Chang M.H., 2000). The risk is related to the maternal serum HBV DNA level. Infection acquired in early childhood (1-5 years) is in general asymptomatic and evolves to chronically in about 30% of cases. Infection resolves with development of antibodies to surface antigen of the hepatitis B virus (anti-HBs) in >95% of adults (Tassopoulos N.C. et al., 1987).

The 5-year mortality rate is 0-2% for patients with chronic hepatitis B without cirrhosis, 14-20% for patients with compensated cirrhosis, and 70-86% after the occurrence of decompensation (Ikeda K. et al., 1998; Fattovich G. et al., 1991; Di Marco V. et al., 1999; Fattovich G. et al., 1988; Fattovich G. et al., 1995; Hui A.Y. et al., 2002). The development of hepatocellular carcinoma and the complications of cirrhosis are the main causes of death.

1.2.3. Co-infections

Patients with HIV co-infection and marked immune deficiency tend to have mild chronic hepatitis B but to have high levels of HBV DNA (Colin J.F. et al., 1999). Overall morbidity and mortality may be higher than in patients with only HIV infection because of the added risk of HBV-related liver disease (Puoti M. et al., 2000).

In most patients with co-infection with HCV, HBV replication is suppressed while HCV replication remains active. However, the opposite has also been observed. Patients with
HCV and HBV co-infection tend to have more severe chronic hepatitis, and cirrhosis and HCC are more common (Sagnelli E. et al., 2000). Patients with HCV co-infection and active HBV replication should be considered for interferon therapy, which is active against both HBV and HCV.

In HBV carriers, super-infection with HDV usually results in chronic hepatitis D, with suppression of HBV replication but persistence of HDV replication (Sakugawa H. et al., 2001). Co-infection with HDV and HBV causing acute hepatitis D is usually followed by complete recovery. Treatment is therefore not recommended.

1.2.4. Diagnosis

A combination of biochemical, serological and virological test, and histological features have been used to diagnose and classify HBV infection. Biochemical assays for aminotransferases are widely available (Piton A. et al., 1998; Prati D. et al., 2002). Qualitative serological assays for the detection of HBV antigens (HBsAg and HBeAg) and antibodies (anti-HBs, total and IgM anti-HBc, and anti-HBe) are also widely available and standardized (Decker R., 1998).

HBV DNA may be detected in serum using methods that employ DNA hybridization with or without signal amplification (Pawlotsky J.M., 2002).

Laboratory diagnosis of hepatitis B infection centres on the detection of the hepatitis B surface antigen HBsAg, as the most important means for ensuring blood safety. A positive test for the hepatitis B surface antigen (HBsAg) indicates that the person has an active infection (either acute or chronic). WHO recommends that all blood donations are tested for this marker to avoid transmission to recipients (http://www.who.int). Serum aminotransferase levels often reflect hepatic necroinflammation while derangement of serum bilirubin, albumin, prothrombin time and platelet count may provide evidence of cirrhosis.
Laboratory Tests:

- Serology for HBV viral antigens: HBsAg, HBcAg, HBeAg;
- Serology for anti-HBV antibodies: anti-HBs, anti-HBc, anti-HBe;
- Serum HBV DNA and viral load;
- Liver function tests (Misdraji, Y. et al., 2011).

It is envisioned that other methods in development that are more sensitive including real-time PCR amplification assays will be marketed in the future. Thus, positive results using new assays may be found among individuals previously considered, on the grounds of negative results in non-PCR-based assays, to be in the inactive HbsAg carrier-state (EASL International consensus conference on hepatitis B, 2003).

Biochemical and other laboratory parameters measured in peripheral blood not always reflect the severity of liver histology (Crovatto M. et al., 2000), therefore the assessment of a liver biopsy by an expert pathologist, in association with a clinician is accepted to be an integral part of the diagnosis and management of patients with HBV infection. Liver biopsy has been used for confirming the diagnosis of chronic hepatitis B, for identifying inter-current disease affecting the liver, and in grading the severity of necro-inflammation and the stage of fibrosis (Ferell L. et al., 2002). Liver biopsy remains the gold standard in the evaluation of patients with liver disease, particularly in patients with chronic liver diseases. In selected instances, liver biopsy is necessary for diagnosis but is more often useful in assessing the severity (grade) and stage of liver damage, in predicting prognosis, and in monitoring response to treatment (Dan L. Longo and Anthony S. Fauci., 2010).

The METAVIR score helps interpret a liver biopsy. When this biopsy is performed, pathologist need a reliable way to quantify what is seen under the microscope. Metavir system was designed for chronic HCV hepatitis (METAVIR Cooperative Study Group, 1996), but is
also used for hepatitis B. This scoring system assigns two standardized numbers: one to represent the degree of inflammation and the other the degree of fibrosis. The fibrosis is graded on a 5-point scale from 0 to 4. The activity, which is the amount of inflammation (specifically, the intensity of necro-inflammatory lesions), is graded on a 4-point scale from A0 to A3.

**Fibrosis score:**
F0 = no fibrosis
F1 = portal fibrosis without septa
F2 = portal fibrosis with few septa
F3 = numerous septa without cirrhosis
F4 = cirrhosis

**Activity score:**
A0 = no activity
A1 = mild activity
A2 = moderate activity
A3 = severe activity
(Kaplan M.M., Bonis P.A., 2009).

1.2.5. Changes in the structure of liver during chronic viral hepatitis B

- Acute hepatitis B
  - Hepatocytic swelling, mononuclear cell infiltrates, spotty necrosis, apoptotic bodies, confluent and bridging necrosis, collapse of hepatocytic cords, hepatocytic regeneration
- Chronic hepatitis B
  - Portal inflammation, interface hepatitis, lobular hepatitis, fibrosis
• Ground-glass hepatocytes
• Grading denotes inflammatory activity while staging indicates degree of fibrosis
• Fibrosing cholestatic hepatitis B
  - Unique histology and more progressive course following orthotopic liver transplantation and in other immunosuppression status (Misraji Y. et al., 2011).

In biopsies with acute HBV, lymphocytic lobular inflammation may show close contact with injured hepatocytes, with lymphocytes occasionally identified within the cell cytoplasm (Gary C. Kanel, Jacob Korula., 2011).

In biopsies with chronic viral hepatitis B „Ground-glass“ cells are present in 1/2 to 2/3 of the cases. These hepatocytes have a diffusely finely granular eosinophilic cytoplasm, sometimes with a thin „clear“ space adjacent to the cell membrane, and represent proliferation of the endoplasmatic reticulum synthesizing the 22-nm HBsAg particles. The liver cell nuclei can sometimes have a sanded, finely granular appearance as a result of the presence of HBcAg. Portal and lobular plasma cell infiltrates can be prominent at times. The degree of liver cell dysplasia and oxyphilic cellular changes are more frequently seen when compared to the other hepatotropic viruses. Development of hepatocellular carcinoma is frequent over time and may occur in both fibrotic and cirrhotic livers (Gary C. Kanel, Jacob Korula., 2011).

1.2.6. Treatment

The therapeutic endpoints for the management of chronic hepatitis B include sustained suppression of the HBV DNA level, normalization of the serum ALT level, histologic improvement, HBeAg clearance or seroconversion and HBsAg clearance or seroconversion (Liu C.J. et al., 2005).

Two types of drug therapy are in current use for HBV treatments interferon (IFN)-α,
which is used in both standard and pegylated forms, and nucleoside analogues, including lamivudine, entecavir, telbivudine, adevir dipivoxil and tenofovir disoproxil fumarate (Kurbanov F. et al., 2010). Treatment with alpha interferon results in an antiviral state due to induction and expression of intracellular genes and the functional activation of a variety of cellular proteins (Rang A. et al., 2002). Interferon alpha also stimulates cell mediated immune responses against HBV which in turn results in the destruction of infected hepatocytes. Three large phase III trials of pegylated interferon have been published (Janssen H.L. et al., 2005; Lau G.K. et al., 2005; Marcelin P. et al., 2004).

Interferon remained the only licensed treatment for hepatitis B until lamivudine became available in 1998. Since then, the use of nucleoside analog therapy has been preferred. In sharp contrast, nucleoside analogs are orally delivered and generally free of adverse events.

There is no specific treatment for acute hepatitis B. Care is aimed at maintaining comfort and adequate nutritional balance, including replacement of fluids that are lost from vomiting and diarrhoea. Some people with chronic hepatitis B can be treated with drugs, including interferon and antiviral agents. Treatment can cost thousands of dollars per year and is not available to most people in developing countries. Liver cancer is almost always fatal and often develops in people at an age when they are most productive and have family responsibilities. In developing countries, most people with liver cancer die within months of diagnosis. In high-income countries, surgery and chemotherapy can prolong life for up to a few years. People with cirrhosis are sometimes given liver transplants, with varying success (http://www.who.int/topics/hepatitis).

1.2.7. Prevention of HBV

Hepatitis B infection and its complications can be prevented by vaccination (Mahoney F.J. et al., 1997). Vaccination is the most effective method for decrease in the prevalence of HBV
infection. Hepatitis B vaccines have been evaluated in clinical trials to determine protective level of serum anti-HBs. Persons who respond to HBV vaccine with titers of anti-HBs 10 mIU/mL or greater are protected against acute hepatitis B and chronic infection (Jack A.D. et al., 1999). Antibody production rate is higher in the younger age at vaccination and lower for those older than 40 years of age.

Currently available vaccines are safe and their benefit outweighs their untoward effects. Minimal reactions are frequent, including local pain, mild and transient fever, mostly lasting only 24 h (McMahon B.J. et al., 1992). Pregnancy or breast feeding are not a contraindication to vaccination (Levy M., Koren G., 1991). Since 1997, hepatitis B vaccinations to newborns are being performed worldwide regardless of the prevalence of hepatitis B. Currently, vaccinations to all newborns are being performed and even in adults, catch-up vaccination is recommended in case of particularly recommended to high-risk groups of hepatitis B (Table 1) (Kwon S.Y., 2007).

**Table 1.** Groups of high risk for hepatitis B virus infection.

- Injection drug user
- Homosexually active men
- Persons at high risk of sexually transmitted HBV infection
- Household members and sexual partner of HBV carriers
- Patients of hemodialysis units
- Clients and staffs in mental health institutions
- Extended travel to areas of high endemicity
- Persons at occupational risk of HBV infection
- Patients who receive clotting factor concentrates

1.3. Analysis of mechanisms of apoptosis in chronic viral hepatitis

Apoptosis, also called programmed cell death (PCD), plays a key role in developmental biology and in maintenance of the steady state in continuously renewing tissues.
PCD is a selective process of physiological cell deletion (Ucker P.S., 1991; Wylie A.H., 1981).

Apoptosis is a form of cell death and is characterized by two major markers. The first is composed of morphological features such as reduction in cell volume, chromatin condensation, and nuclear fragmentation, resulting in apoptotic bodies. The other marker is DNA cleavage by a $Ca^{2+}/Mg^{2+}$-dependent endonuclease into oligonucleosomal-length fragments detected as a ladder pattern on gel electrophoresis (Kerr et al., 1972; Wyllie A.H. et al., 1993).

Induction of apoptosis: 1) through direct proapoptotic activity of specific virus proteins, which form during virus replication process: hepatitis virus B X protein (Kondo T. et al., 1997; Reed J.C., 1997); 2) by increase of the receptors on the cell membrane through which the signal for induction of apoptosis is transmitted, for example Fas (Honda M. et al., 2000), and increase of cells sensitivity towards various proapoptotic stimuli, in particular towards TNF-α (Tanaca M. et al., 1996).

One of the oncogenes regulating apoptosis is B-cell lymphoma 2 (Bcl-2). Bcl-2 is important as apoptotic inhibitor for development of liver tissue and under acute and chronic hepatitis. Bcl-2 family member proteins are anti-apoptotic molecules that are known to be overexpressed in most cancers. The proapoptotic Bcl-2 family members are Bax, tBid, Bak, Bax, Bik, Bok, Bim, Krk, Mtd and others. The anti-apoptotic subfamily comprises Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1(Bfl-1), and Bcl-B.

The major function of Bcl-2 family members is to control directly mitochondrial membrane permeability and thereby regulate release of apoptogenic factors from the intermembrane space into the cytoplasm (reviewed in Tsujimoto and Shimizu, 2000a,b; Green, 2000; Martinou and Green, 2001; Zamzami and Kromer, 2001). Apoptogenic factors known to be released include cytochrome c, Smac Diablo (Verhagen et al., 2000,
Du et al., 2000), apoptosis-inducing factor (AIF) (Susin et al., 1999), heat shock protein 60 (Xanthoudakis et al., 1999; Samili et al., 1999), and endonuclease G (Li et al., 2001). Smac Diablo and cytochrome c are involved in the activation of caspases. AIF and endonuclease G are thought to play roles in induction of caspase-independent apoptotic changes in nuclei. Anti-apoptotic members of the Bcl-2 family inhibit the release of these apoptogenic factors, whereas proapoptotic members promote it (Tsujimoto, 2002).

It is well documented that Bcl-2 functions through heterodimerization with proapoptotic members of the Bcl-2 family to prevent mitochondrial pore formation and prevent cytochrome c release and initiation of apoptosis (Vo and Letai, 2010). The mitochondrial (intrinsic) pathway is regulated by the Bcl-2 family and activated by mitochondrial disruption with subsequent cytochrome c release. Initiators of this pathway include UV irradiation and cytotoxic drugs. An ‘apoptosome’ is formed by the interaction of cytochrome c, Apaf-1, d-ATP/ATP and procaspase-9 with subsequent initiation of the caspase cascade (Kumar et al., 2005).

The intrinsic (mitochondrial) apoptotic pathway is controlled by the balance between anti-apoptotic proteins belonging to the Bcl-2 family and proapoptotic proteins bearing a single BH3 domain (Richardson and Kaye, 2008). In healthy cells, proapoptotic proteins, Bax, Bid and Bad reside in the cytosol. On initiation of apoptosis, these pro-apoptotic proteins translocate to the outer mitochondrial membrane, causing the mitochondria to lose membrane potential.

The “point of no return” in this pathway is defined by mitochondrial outer membrane permeabilization (MOMP), which leads to the release of cytochrome c (Chipuk et al., 2006). Bcl-2 family proteins regulate MOMP and thereby determine the cellular commitment to apoptosis. From the above discussion, it is clear that MOMP is a greatly organized process, principally controlled through interactions between
pro- and anti-apoptotic members of the Bcl-2 family (Tait S.W. and Green D.R., 2010).

Apoptosis during the virus hepatitis can be a result of the direct action of the virus or as the defined immune reaction. Since in the dead cell the replication of the virus becomes impossible, the "interest" of virus is to stop apoptosis and to preserve hepatic cells alive. It has been shown that some virus proteins are coded to possess anti-apoptotic activity, suppressing the functions of the protein p53 and enhancing the expression of Bcl-2 (Patel T., Gores G.G., 1995; Patel T. et al., 1998).

2. MATERIALS AND METHODS

2.1. Aims of the study

Perform morphometrical and immunohistochemical analysis of liver biopsies of patients with chronic viral hepatitis B.

2.2. Materials

Patients

In the study (10) HBV patients with different degree of fibrosis according to classifications by Ishak and META VIR participated (French META VIR Cooperative study group.,1994; Ishak K. et al.,1995).

Diagnosis of chronic HBV was established after careful examination of patients: anamneses of diseases and life, laboratory analyses, virological and morphological studies. During the formulation of diagnosis the classification of chronic liver diseases, accepted by the International Congress of Gastroenterologists (Los Angeles, 1994), was used. To refine the diagnosis as well as for detection the activity of pathologic processes in the liver, aspiration biopsy according to G. Menghini was taken from all patients (Menghini G., 1970; Menghini G. et al.,1975).
2.3. Methods

Histological treatment of biological objects: fixation, embedding, cutting and coloration of slides by hematoxylin and eosin.

2.3.1. Fixation

As soon as fresh tissues are removed from human, they begin to degenerate and break down because of the release of enzymes from tissues themselves and also due to the activity of any contaminating bacteria. The tissue loses its normal characteristic structure. In order to prevent such structural changes, tissues are immersed in chemicals which inactivate the autolytic enzymes and kill the bacteria. Such chemicals, called fixatives, also assist in maintaining cellular structure.

Many different chemical fixatives exist and usually act by cross-linking the proteins in the cells to preserve the *in vivo* structure. There are no perfect fixatives and many preserve only specific components such as the cytoplasm or the nucleous (Ratcliffe *et al.*, 1983).

2.3.2. Embedding

In order to prevent the collapse and distortion of the tissue during sectioning, it must be impregnated and surrounded by hard wax for support. A simple infiltration procedure in the xylene method:

1. From absolute alcohol → xylene (1 change) 2-6 h
2. 50:50 xylene:wax → in oven (1 change) 1h
3. Molten wax → in oven (3 changes) 1h each

The tissue is now ready for casting into a solid block of wax which can be trimmed and fitted into a microtome.
Celloidin is an alternative embedding medium which is particularly useful for cutting large objects and for hard and brittle material. Plastics, particularly epoxy resins, are increasingly being employed as the embedding medium for both light and electron microscopy (Ratcliffe et al., 1983).

2.3.3. Cutting and coloration of slides by haematoxylin and eosin

The embedded tissue is trimmed into the form of truncated pyramid, fixed to a specimen holder which fits on the microtome and sectioned with a sharp knife at approximately 5-8 μm.

Staining-coloration of slides is necessary to increase contrast between the various tissue and cell components. There is a whole range of different staining techniques available, but the most widely used method is the haematoxylin and eosin technique, which stains the nuclei dark blue with haematoxylin and the cytoplasm pink with the counterstain, eosin.

Short scheme of staining with haematoxylin-eosin:

1. 70 alcohol-water (1 min)
2. Haematoxylin (5 min)
3. Water (1 min)
4. Acid alcohol 2-3s, if necessary
5. Water (5-10 min)
6. Eosin (1 min)
7. Water (1 min)
8. Alcohol I (70 - 2 min)
9. Alcohol II (96 - 2 min)
10. Carbol-xylol (2 min)
11. Xylol (2 min)
12. Canada balsam

(Ratcliffe, 1983).

2.3.4. Avidin-biotin methods

These methods utilize the high affinity of avidin or streptavidin for biotin. Avidin has four binding sites for biotin. However, fewer than four molecules of biotin will actually bind. Currently, there are two avidin-biotin methods in use—the avidin-biotin complex (ABC) method and the labeled avidin-biotin (LAB) technique. Both methods require a biotinylated antibody as a link antibody. Biotinylation is a process, whereby biotin is covalently attached to the antibody. Open sites on avidin from ABC or LAB bind the biotin on the link antibody.

The sequence of reagent application is primary antibody, biotinylated secondary antibody, preformed avidin-biotin-enzyme complex (ABC) or enzyme-labelled avidin (LAB), substrate solution. Horseradish peroxidase and alkaline phosphatase are commonly used enzyme labels (Sally Ed, Naish J., 1989).

**Figure 2.** Avidin-biotin methods. Preformed avidin-biotin-enzyme complex (ABC) or enzyme-labelled avidin (LAB) reacts with biotinylated secondary antibody (DAKO Corporation).
2.3.5. Histology

The material for morphological studies was fixed with 10% formalin and demineralized with EDTA (ethylenediamine tetraacetic acid). Paraffin embedded slices with 5 μm in thickness were stained with hematoxylin and eosin. Routine histology was followed by immunohistochemistry (immunohistochemical methods for studying of mechanisms of apoptosis (Bcl-2), stereometric and semiquantitative methods.

2.3.6. Immunohistochemistry

As it has been shown that some virus proteins are coded to possess anti-apoptotic activity enhancing expression of Bcl-2 (Patel T., Gores G.G., 1995; Patel T. et al., 1998) immunohistochemical studies were carried out using monoclonal antibody Mouse anti-Bcl-2 (Invitrogen, CA, USA).

In short: formalin-fixed, paraffin embedded slices with a thickness of 5 μm were deparaffinized with xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase activity was blocked with 3% H2O2 and the sections were stained using Histostain-SP Kit (Broad Spectrum, Invitrogen, CA, USA) according to the manufacture guidelines (PROTOCOL 1.). Biotinylated secondary antibody and streptavidin-conjugated peroxidase were used for detection using AEC (3-amino-9-ethylcarbazole) as chromogen. Nuclei were counterstained with Harris haematoxylin. Negative control did not contain the antigen to be stained.

2.3.7. Methods of digital morphometric analysis

Using light computer microscope (Leica DM 2500) with attached digital camera (Leica DFC 320R) were made sequential serial microphotographs of liver biopsy, subsequently made microphotographs were glued together using Adobe Photoshop CS5. Glued liver biopsy
pictures were analysed using ACDSee Photo Manager 2009.

2.3.8. Stereometric analysis

Stereometric analysis is based on determination method of specific volumes of different structures (DeHoff R. and Rhines F., 1968). The calculation was carried out using standard graticule (400 squares) by microscope objective 400-times. In each field of sight a quantity of non-parenchymal liver structures - portal tracts, with nearby necroses, vessels, interlobular necroses (infiltrations) - were calculated. Other liver structures were studied together with parenchyma (hepatic cords and sinusoids). The relationship between parenchymal and non-parenchymal elements was calculated in percents.

The main stage of the study is to calculate the area of the characteristic elements of the biopsy. In short: visually define the boundaries of nonparenchymal cells (portal tracts infiltrates, vessels, etc.), label them with the tools Adobe Photoshop CS6 and fix the area occupied by them in absolute (pixels). Information about area occupied by the object, appear after highlighting it in the “Histogramm” and is expressed in pixels.

3. RESULTS AND DISCUSSION

Using Adobe Photoshop CS5 sequential serial microphotographs of liver biopsy were glued together (Figure 3). Further this glued photos were used to calculate the relationship between non-parenchymal and parenchymal liver structure in liver biopsies of patients with chronic viral hepatitis B.
Figure 3. Glued microphotographs of immunohistochemistry for Bcl-2 (400X) using Adobe Photoshop CS5.

The relationship between non-parenchymal and parenchymal liver structure shows great variability (0.9%-15.2965% for non-parenchymal and 78.9984%-99.1% for parenchymal elements) depending on severity of liver disease (Table 2).
**Table 2.** The relationship between parenchymal and non-parenchymal elements in percents.

<table>
<thead>
<tr>
<th>Number of biopsy</th>
<th>Quantity of non-parenchymal liver structures in percents</th>
<th>Quantity of parenchymal liver structure in percents</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>9,2537</td>
<td>90,7463</td>
</tr>
<tr>
<td>2.</td>
<td>8,7484</td>
<td>91,2516</td>
</tr>
<tr>
<td>3.</td>
<td>0,9</td>
<td>99,1</td>
</tr>
<tr>
<td>4.</td>
<td>8,888</td>
<td>91,1120</td>
</tr>
<tr>
<td>5.</td>
<td>21,0016</td>
<td>78,9984</td>
</tr>
<tr>
<td>6.</td>
<td>0,99</td>
<td>99,010</td>
</tr>
<tr>
<td>7.</td>
<td>12,6894</td>
<td>87,3106</td>
</tr>
<tr>
<td>8.</td>
<td>15,2965</td>
<td>84,7035</td>
</tr>
<tr>
<td>9.</td>
<td>10,9899</td>
<td>89,0101</td>
</tr>
<tr>
<td>10.</td>
<td>12,285</td>
<td>87,7150</td>
</tr>
</tbody>
</table>

All liver biopsy specimens were calculated using image analysing system consisting of microscope (Leica DM 2500) with attached digital camera (Leica DFC 320R). Detection of cells expressing Bcl-2 oncoprotein was based on immunohistochemical reaction- the primary antibody Mouse anti-Bcl-2 reacted with biotinylated secondary antibody (Histostain- Sp Kit., Broad spectrum, Invitrogen). Sections were counterstrained with Harris hematoxylin (Supplement 1). The nuclei of Bcl-2 positive hepatocytes stained in yellow-brownish colours (Figure 4). Positive reaction was detected inside hepatocytes nuclei, especially near the portal area or near the middle part of lobules. A lot of separate lymphocytes were indicated in the portal areas.
**Figure 4.** Photomicrograph of IHC showing high activity of Bcl-2 in cytoplasm (C) and nuclei (N) of hepatocytes. 200x (Black arrows).

Positive reaction of Bcl-2 oncoprotein strongly varies in liver biopsies during chronic hepatitis B. In patients with a lower level of apoptosis Bcl-2 positive cells located separately (*Figure 5*). In the patient group with a higher level of apoptosis Bcl-2 positive cells revealed more frequently in the zone of small interlobular infiltrations (*Figure 6*).
Figure 5. Photomicrograph of IHC showing low activity of Bcl-2 in separate cells. 200x (Black arrow).

Figure 6. Photomicrograph of IHC note expression of Bcl-2 inside cells of the portal area infiltration. 400x (Black arrow).

Positive reaction was expressed in nuclei of Kupffer cells and in the cytoplasm of sinusoidal
endotheliocytes (*Figure 7*).

*Figure 7.* Photomicrograph of IHC showing Bcl-2 expression in the nucleus of hepatocyte(N), in cytoplasm of some hepatocytes (C) and in Kupffer cells(K).  400x (Black arrows).

Bcl-2 expression was also noted in separate lymphocytes located in composition of portal areas infiltration.

In some biopsies was observed high activity of Bcl-2 in cytoplasm of hepatocytes, whereby positively stained cytoplasm occurred in grain form (*Figure 8*).
Figure 8. Photomicrograph of IHC showing activity of Bcl-2 in cytoplasm of hepatocytes. 400x (Black arrows).
Bcl-2 expression was indicated in the wall of sinusoids (Figure 9).

Figure 9. Photomicrograph of IHC showing activity of Bcl-2 in the wall of sinusoids. 200x (Black arrow).

Our investigation demonstrated high activity of expression of Bcl-2 in some cases of chronic hepatitis B. Bcl-2 positive Bcl-2 expression in composition of Kupffer cells and endotheliocytes was detected. Positive Bcl-2 reaction was also detected in hepatocytes nuclei and cytoplasm, at most near portal area. Some separate lymphocytes inside portal infiltration showed expression of Bcl-2. It seems that during chronic HBV infection lymphocytes themselves become objects of cytotoxic CTL action. There has been noticed correlation between the severity of disease and detection of positive Bcl-2 cells, what can be used for evaluation of the severity of the HBV and assessing the efficiency of therapy carried out.

Unlike previous studies we did not observed coloration of ducts or cholangiolar cells, as reported previously. We suppose that expression of Bcl-2 oncogen is very important in regulation of apoptosis in the liver of patients with chronic viral hepatitis B.
Hepatitis B is a potentially life-threatening liver infection caused by the hepatitis B virus. It is a major global health problem and the most serious type of viral hepatitis. It can cause both acute and chronic disease and puts people at high risk of death from cirrhosis of the liver and liver cancer. The hepatitis B virus is 50 to 100 times more infectious than HIV. Worldwide, an estimated two billion people have been infected with the hepatitis B virus and more than 240 million have chronic (long-term) liver infections. About 600 000 people die every year due to the acute or chronic consequences of hepatitis B.

HBV is mainly transmitted through perinatal infection, contact with the blood and contaminated needles or other body fluids of an infected person. It can be transmitted through the direct contacts or skin wounds between patients and surgeons or dentists, or indirectly through hemodialysis, razors, toothbrushes, and acupuncture therapy.

Long-standing chronic hepatitis of varying severity leads to structural changes, ultimately resulting in cirrhosis, which is characterized by a complete loss of normal architecture by the formation of fibrous septa and regeneration nodules.

During the chronic hepatitis a lot of morphological changes appear in well organized liver structure. A hallmark of chronic hepatitis is portal inflammation (portal hepatitis), mainly consisting of lymphocytes. The severity of portal inflammation may vary from one patient to another and from one portal tract to another. A variable degree of lobular changes (lobular hepatitis), including focal and confluent necrosis, apoptosis and inflammation, completes the picture of necroinflammatory changes in chronic hepatitis.

The Bcl-2 family of proteins play an important role in the control of apoptosis. Bcl-
2 is a key regulator of apoptosis, because it can protect cells from death induced by a number of injuries including radiation, chemotherapy, or growth factor deprivation. Viral hepatitis apoptosis may be a result of both the direct damaging effects of viral infection (cytopathic effect) or indirect, resulting from the immune response. The "interest" of virus is to stop apoptosis and to preserve hepatic cells alive because in the dead cell the replication of the virus becomes impossible. It has been shown that some virus proteins are coded to possess anti-apoptotic activity, suppressing the functions of the protein p53 and enhancing the expression of Bcl-2.

The results of this study show that localization of apoptosis in liver biopsies of patients chronically infected with hepatitis B highly varies between patients with different severity of disease. The relationship between non-parenchymal and parenchymal liver structure in patients with chronic HBV shows also great variability (0.9%-15.2965% for non-parenchymal and 78.9984%-99.1% for parenchymal elements).

Positive Bcl-2 reaction was detected in hepatocytes nuclei and cytoplasm. Some separate lymphocytes inside portal infiltration showed expression of Bcl-2, especially near the portal area or near the middle part of lobules. Also positive reaction was detected in the wall of sinusoids and in composition of Kupffer cells and endotheliocytes. If during chronic HBV as a result of apoptosis infected cells die then the induction of apoptosis would contribute to the elimination of these cells. Conversely, if the main cause of apoptosis are autoimmune lesions, it is advisable to inhibit apoptosis. There is possibility of developing new strategy for chronic HBV therapy, based on the regulation of the level of apoptosis in hepatocytes.
ACKNOWLEDGEMENTS

I would like to express my gratitude to my supervisors Piret Hussar, M.D., D.M.Sc., Martin Kärner, M.Sc., Galina Filimonova, D.M.Sc., Viljar Jaks, PhD. for their useful comments, support, engagement through the learning process of this master thesis and for their patience and motivation. Furthermore I would like to thank laboratory technician Mare Tamm for introducing me to the topic as well for the support and practical advises. Also, I would like to thank the participants in my survey, who have willingly shared their precious time during the process of interviewing. I would to thank my family, I am grateful for their constant support and help. I would like to express my gratitude for the support of the Estonian Ministry of Education and Research and the Foundation Archimedes for Krstjan Jaak scholarship for my partial foreign studies in Russia at the Department of Histology and Cytology, St.-Petersburg’s Medical Academy and at the Laboratory of Computer Microscopy, St.-Petersburg’s State University.
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PUBLICATION

Kroonilist B hepatiiti põdevate patsientide maksarakkude uuring

Maria Žuravskaja

KOKKUVÕTE

B-hepatiit on potentsiaalselt eluohulik maksa infektsioon, mida põhjustab hepatiit B viirus. See on suur globaalne terviseprobleem ja kõige ohutumalt täüpi viirushepatiit. See võib põhjustada ägedat ja kroonilist haigust, millega kaasneb suur risk surra maksatsirroosi või maksavähki. B-hepatiidi viirus on 50-100 korda nakkatamisohutlikum kui HIV. Ülemaailmselt, hinnanguliselt kaks miljardit inimest on nakatunud hepatiit B viirusega ning rohkem kui 240 miljonit põeb kroonilist (pikaajalist) maksa infektsiooni. Umbes 600 000 inimest sureb igal aastal ägeda või kroonilise B-hepatiidi tagajärjel.

HBV edastatakse perinataalse infektsioonina emalt lapsele, kokku puutel verega ja nakatatud noelte või muude kehavahede kaudu nakatunud inimest. Haigus võib levida otse kontaktide kaudu või naha haavade kaudu patsiendi ja kirurgi või hambaarsti vahel või kaudselt läbi hemodialüüsi, žilettide, hambaharjade ja akupunktuuri.


Bcl-2 perekonna valkude on oluline roll apoptoosi kontrollis. Bcl-2 on peamine apoptoosi regulaator, sest see võib kaitsta rakke surma põhjustavate vigastuste eest. Viirushepatiidi puhune apoptoos võib olla tingitud nii otsesest viirusinfektsiooni kahjulikust mõjust kui ka kaudselt immuunreaktsioonide tulemustest. Viiruse "huvides" on lõpetada
apoptoos ja säilitada maksarakke elusena, sest surnud rakus muutub viiruse replikatsioon võimatuks. On näidatud, et mõned viiruse valgud on kodeeritud omama antiapoptootilist tegevust, surudes alla p53 valgu funktsioone ja suurendades Bcl-2 ekspressiooni.

Antud uuringu tulemused näitavad, et apoptoosi lokaleerumine maksabiopsias kroonilise B-hepatiidiga patsientidel on äärmiselt erinev ja sõltub haiguse raskusest. Suur erinevus esineb ka maksa mitte-parenhümatoossete ja parenhümatoossete struktuuride seoses kroonilise B-hepatiidiga patsientidel (0,9% - 15,2965% ja 78,9984% - 99,1% elementidel).

Positiivset Bcl-2 reaktsiooni leiti maksarakkude tuumades ja tsütoplasmas. Samuti ekspresseerus Bcl-2 üksikutes lümfotsüütides portaalsete infiltratsioonis, eelkõige portaalala ja loobulite keskosa lähedal. Lisaks tuvastati positiivset reaktsiooni sinusoidide seintes, Kupfferi rakkudes ja endoteeliotsüütides.

Kui kroonilisest B-hepatiidist tingitud apoptoosi tulemusena nakatunud rakud surevad, siis apoptoos aitab neid rakke kõrvaldada. Tulevikus on võimalus arendada uus strateegia kroonilise B-hepatiidi raviks, mis põhineks maksarakkude apoptoosi taseme reguleerimisel.
### SUPPLEMENTS

**PROTOCOL (1)**

Histostain®-SP Kits (Broad spectrum)

Invitrogen® LAB-SA Detection System

<table>
<thead>
<tr>
<th>1. PREPARATION OF SLIDES:</th>
</tr>
</thead>
<tbody>
<tr>
<td>➢ Paraffin sections are deparaffinized with xylene and rehydrated in a graded series of ethanol.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2. PEROXIDASE QUENCHING SOLUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>➢ Add 1 part of 30% hydrogen peroxide to 9 parts of absolute methanol (PEROXIDASE QUENCHING SOLUTION) Mix well.</td>
</tr>
<tr>
<td>➢ Submerge slides in PEROXIDASE QUENCHING SOLUTION for 10 minutes.</td>
</tr>
<tr>
<td>➢ Wash with TRIS 2 min., 3 times.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3. SERUM BLOCKING SOLUTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>➢ Add 1 drop (50 μL) or enough to completely cover tissue, of SERUM BLOCKING SOLUTION to each section. Incubate at room temperature for 10 min.</td>
</tr>
<tr>
<td>➢ Drain or blot off solution. DO NOT RINSE.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>4. PRIMARY ANTIBODY</th>
</tr>
</thead>
<tbody>
<tr>
<td>➢ Apply 2 drops (100 μL) or enough to completely cover tissue, of PRIMARY ANTIBODY (previously prepared dilution 1:100 primary antibody:TRIS) to each section.</td>
</tr>
<tr>
<td>➢ Incubate in moist chamber for 60 min.</td>
</tr>
<tr>
<td>➢ Rinse with TRIS for 2 min., 3 times (washing off the unreacted primary antibody).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>5. BIOTINYLATED SECOND ANTIBODY</th>
</tr>
</thead>
<tbody>
<tr>
<td>➢ Apply 1 drop (50 μL) or enough to completely cover tissue, of BIOTINYLATED SECOND ANTIBODY to each section. Incubate for 10 min.</td>
</tr>
<tr>
<td>➢ Rinse with TRIS for 2 min., 3 times.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>6. ENZYME CONJUGATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>➢ Apply 1 drop (50 μL) or enough to completely cover tissue, of ENZYME CONJUGATE to each section. Incubate for 10 min.</td>
</tr>
<tr>
<td>➢ Rinse with TRIS for 2 min., 3 times.</td>
</tr>
</tbody>
</table>
7. AEC CHROMOGEN

- Add 1 drop of Reagent 3A, 1 drop of Reagent 3B, and 1 drop of Reagent 3C to 1 mL distilled or deionized water. Mix well. Protect from light and use within one hour.
- Apply 1 drop (50 μL) or enough to completely cover tissue, of AEC CHROMOGEN to each section. Incubate for 10 min.
- Rinse well with distilled water.

8. COUNTERSTAIN

- Counterstain the slides with 2 drops or 100 μL of HEMATOXYLIN. Wash slides in tap water. Put slides into TRIS until blue (approximately 30 seconds). Rinse in distilled water.

9. MOUNTING SOLUTION

- Dip slides in xylene.
- Apply 2 drops or 100 μL of CLEAR MOUNT to the slide and mount with coverslip.
- Let the slides dry for 30 min.

**Supplement (1)**

Harris hematoxylin:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematoxylin crystals</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Alcohol, 100%</td>
<td>50.0 ml</td>
</tr>
<tr>
<td>Ammonium or potassium alum</td>
<td>100.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000.0 ml</td>
</tr>
<tr>
<td>Mercuric oxide (red)</td>
<td>2.5 gm</td>
</tr>
</tbody>
</table>

Dissolve the hematoxylin in the alcohol, the alum in the water by the aid of heat. Remove from heat and mix the two solutions. Bring to a boil as rapidly as possible. (Limit this heat to less than 1 minute and stir often). Remove from heat and add the mercuric oxide slowly. Reheat to a simmer until it becomes dark purple, remove from heat immediately and plunge the vessel into a basin of cold water until cool. The stain is ready for use as soon as it cools. Addition of 2-4 ml of glacial acetic acid per 100 ml of solution increases the precision of the nuclear stain. Filter before use.
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„Dynamic of liver cell population in biopsies of patients with chronic hepatitis B“, supervised by Piret Hussar, Martin Kärner, Galina Filimonova, Viljar Jaks.

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