

Investigation of Molecular Mechanisms Involved in Hepatitis B Virus Associated B-cell Non- Hodgkin Lymphoma (B-NHL)

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Abstract

The Non-Hodgkin Lymphoma (NHL) is a heterogeneous group of malignancies ranked as the most common haematological cancer worldwide, more than 544 000 new cases being reported in 2020. More recent epidemiological studies have shown an increased risk of several types of B-cell Lymphoma development, the most prevalent being diffuse large B-cell lymphoma (DLBCL) and follicular lymphoma (FL) subtypes, in patients infected with hepatitis B virus (HBV). These patients are more difficult to treat and their survival rate is lower compared to uninfected people. HBV is known as a hepatotropic virus, although viral HBV-DNA has been identified in extrahepatic tissues, such as kidney, pancreas or peripheral blood mononuclear cells (PBMC). However, the mechanistic relationship between HBV infection and lymphoid cancer is not known. Preliminary data have shown that NTCP, the specific HBV receptor is expressed in normal B cells. After exposure to HBV viral particles, B cells become capable of producing viral core antigens (HBcAg). Antiviral roles and/or cancer-promotion functions in B-cells of APOBEC3 deaminases overexpression remain to be determined.

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Introduction

NHL starts in B and T lymphocytes, or Natural Killer (NK) cells at different stages of their differentiation, 86% of the total NHLs are represented by the malignant transformation of B lymphocytes and the remaining 14% are associated with T and NK cells (1). In Romania, 30.8% of NHL patients are HBV carriers, while the infection rate in the general population is about 6.3% (2). Another study performed in a group of 200 patients with NHL-B has shown that the prevalence of HBV infection was 15%; however, as no systematic virologic screening was performed in all patients, this percentage may be underestimated (3). Moreover, a high prevalence of HBV infection (17.7%) has been recently reported in Romanian patients with splenic marginal area lymphoma (SMZL) (4). To understand the role of HBV in lymphomagenesis, we investigated whether HBV infection is possible in B lymphocytes and the functional consequences caused by HBV in these cells.

Methods

Cell lines and HBV infection

IM-9 (ATCC), a human lymphoblastoid B cell line was maintained in RPMI (Gibco) medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose, 50 units/ml penicillin, 50 µg/ml streptomycin and 10% serum fetal bovine (FBS). HepaRG (INSERM, France) a human liver cell line that retains many characteristics of primary human hepatocytes was maintained in William's E medium (Gibco) supplemented with 5 µg/ml insulin, 5x10⁵ M hydrocortisone hemi succinate, 50 units/ml penicillin, 50 µg/ml streptomycin and 10% FBS. After DMSO treatment, used to sustain hepatocyte differentiation in vitro, cells were incubated with 4% polyethylene glycol (PEG) and a viral inoculum containing 800 genomic equivalents/cell for 18 hours at 37°C. At the end of incubation, B lymphocytes were incubated with trypsin mixture, washed three times with culture medium and maintained in appropriate culture medium supplemented with 1.8% DMSO for another 12 days. For the inhibition of HBV entry, cells were incubated for 3 hours with Myrcludex 1µM.

MTS assay

Cell viability and cytotoxicity was determined by using a colorimetric method (CellTiter 96® AQueous One

Solution Cell Proliferation Assay, Promega). An equal number of IM-9 cells (7x10⁵) untreated or treated with DMSO were cultivated in a 96-well plate with culture media. The absorbance obtained was measured with the Mithras LB940 Microplate Reader.

Immunofluorescence

The expression of HBcAg was detected by incubation with anti-HBc (dilution 1:100) and donkey anti-mouse Alexa Fluor 594 secondary antibodies (red). Sequentially cells were incubated with anti-NTCP (dilution 1:50) primary antibodies, followed by incubation with goat anti-rabbit Alexa Fluor 488 secondary antibodies (green).

RNA extraction and real-time reverse transcription PCR (RT-PCR)

Total RNA was isolated and analysed from an equal number of cells (1x10⁶) using Trizol (Life Technologies) according to the manufacturer's specifications. Transcripts coding for NTCP, APOBEC3A-H, as well as the reference genes GAPDH and TPB, were quantified by real-time PCR using the SensiFast SYBR No-ROX One-Step Kit (Bioline) and the Rotor Gene 6000 system. Data was analysed using the data interpretation program Rotor Gene 3000 software (Qiagen).

Results

In order to determine whether the immune cells are permissive and susceptible to HBV infection, first we investigated if lymphocytes B express NTCP, the functional receptor of HBV. NTCP receptor was detected and its expression is comparable to those detected in the HepaR-liver cell line (figure 1A). An adapted protocol used for the infection of hepatocytes- like cell line in vitro with HBV was performed on both cell lines (IM-9 and HepaRG). Unlike adherent hepatic cells, the IM-9 cell line grows in suspension, and for this reason we tested several conditions of dimethyl sulfoxide (DMSO) treatment to establish a possible cytotoxicity given the long-term treatment required. Therefore, an optimal concentration of 1.8% DMSO was established by cytotoxicity tests (figure 1B).

Productive infection can be quantified after several cycles of replication, at approximately 7–8 days post-infection (pi). Detection of intracellular viral core proteins HBcAg has been obtained on day 11 pi by immunostaining (figure 1C).

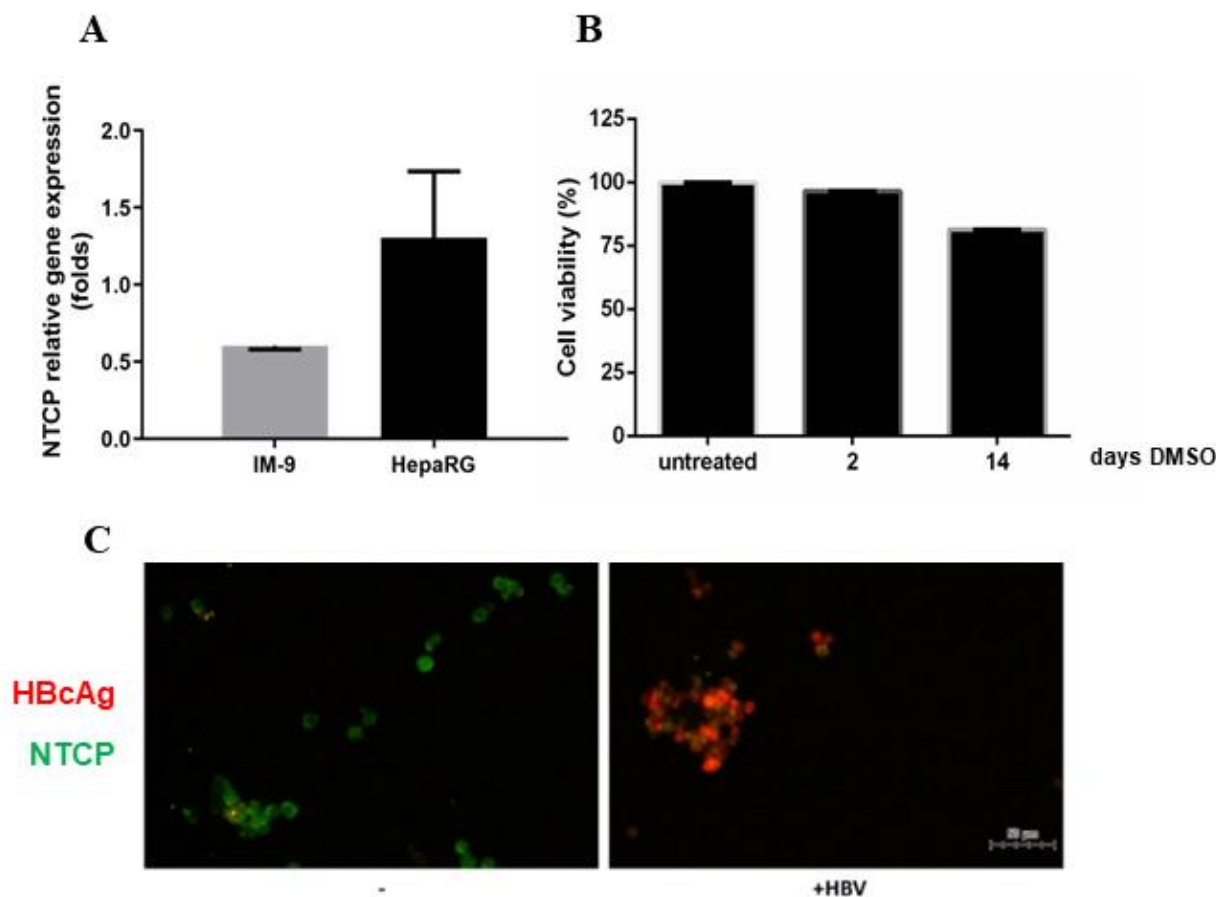


Figure 1. Investigation of HBV „infection” in normal B lymphocytes. A Evaluation of endogenous NTCP expression in B cell line compared with hepatic HepaRG cell line. The values obtained were normalized to the reference gene GAPDH. B Evaluation of DMSO cytotoxicity of IM-9 cells treated with 1.8% DMSO. C Immunofluorescence labelling of HBcAg (red) and NTCP receptor (green) in IM-9 cells on day 12 pi.

In addition to antiviral effects by degradation of HBV cccDNA in HBV-infected patients (5), APOBEC3 mutagenic enzymes may have oncogenic roles when mistarget or misregulate host genome rather than viral genomes (6). The most potent inducers of APOBEC3 expression are interferons (IFNs) (7). A high level of secreted IFN- γ was observed in supernatants of IM-9 cells with HBV “infection” (figure 2A). The expression levels

of APOBEC3 transcripts (A-H) were assessed in lymphocytes B in the presence of HBV or uninfected cells. Expression of APOBEC3 transcripts was detected by RT-PCR, and overexpression of A3A, A3D, and A3F mRNAs was identified, especially at the onset of HBV exposure (figure 2B). These data may correlate with the increased level of IFN- γ in cells exposed to HBV.

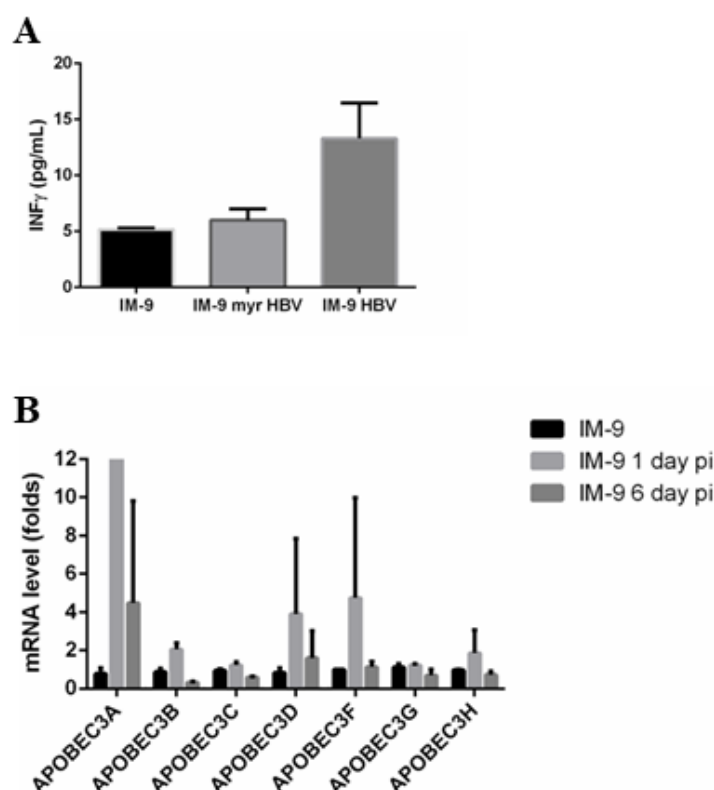


Figure 2. Inflammatory Cytokine Levels and APOBEC3 Family Expression. A IFN- γ concentration in IM-9 cells infected with HBV at 12 day pi by ELISA quantification. B Evaluation of endogenous APOBEC3 (A-H) transcripts in IM-9 cells by RT-PCR analysis. The values were normalized to TPB reference gene.

Discussion

The presence of the NTCP receptor and detection of HBV core antigen (HBcAg) in B cells exposed to viral particles suggest a potential HBV lymphotropism. APOBEC3 expression at the onset of HBV infection may be correlated with both an inhibitory effect on viral DNA synthesis and/or an oncogenic role. To establish the role of APOBEC3 in pathogenesis, in-depth investigations are needed in which viral markers will be analysed in correlation with enzyme expression.

References

1. Ninkovic S, Lambert J. Non-Hodgkin lymphoma. *Medicine*. 2017;45:297–304.
2. Cucuianu A, Patiu M, Duma M, Basarab C, Soritau O, Bojan A, Vasilache A, Mates M, and Petrov L. 1999. “Hepatitis B and C Virus Infection in Romanian

Conclusions

Starting from epidemiological data that suggest a potential role of HBV in the occurrence and development of B-cell non-Hodgkin's lymphomas (8), in this work we investigated the molecular basis of the interaction between HBV and B cells. These preliminary results open the possibility of identifying some potentially therapeutic strategies.

Non-Hodgkin's Lymphoma Patients.” *British Journal of Haematology* 107 (2): 353–56.

3. Török-Vistai T, Bojan A, Cucuianu A, and Zsoldos A. 2013. “Primary Non-Hodgkin Lymphoma of the Orbit Presenting with Massive Bilateral Periorbital Tumors.” *Clujul Medical* (1957) 86 (4): 380–82.

4. Fetica B, Achimas-Cadariu P, Pop B, Dima D, Petrov L, Perry AM, Bharat N Nathwani, et al. 2017. "Non-Hodgkin Lymphoma in Romania: A Single-Centre Experience." *Hematological Oncology* 35 (2): 198–205.
5. Lucifora J, Xia Y, Reisinger F, Zhang K, Stadler D, Cheng X, Sprinzl MF, Koppensteiner H, Makowska Z, Volz T, Remouchamps C, Chou WM, Thasler WE, Huser N, Durantel D, Liang TJ, Munk C, Heim MH, Browning JL, Dejardin E, Dandri M, Schindler M, Heikenwalder M, Protzer U. Specific and nonhepatotoxic degradation of nuclear hepatitis B virus cccDNA. *Science*. 2014;343:1221–1228.
6. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, et al. (2013). Signatures of mutational processes in human cancer. *Nature* 500, 415–421.
7. Covino DA, Gauzzi MC, and Fantuzzi L (2018). Understanding the regulation of APOBEC3 expression: current evidence and much to learn. *J. Leukoc Biol.* 103, 433–444. doi: 10.1002/jlb.2mr0717-310r
8. Li M, Gan Y, Fan C, Yuan H, Zhang X, Shen Y, Wang Q, Meng Z, Xu D, and Tu H. 2018. "Hepatitis B Virus and Risk of Non-Hodgkin Lymphoma: An Updated Meta-Analysis of 58 Studies." *Journal of Viral Hepatitis* 25 (8): 894–903. <https://doi.org/10.1111/jvh.12892>.