Hepatitis B Virus Infection of Normal Hepatic Stem/Progenitor Cells

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Background/Aims: Whether the hepatitis B virus (HBV) infects normal hepatic stem/progenitor cells (NSCs) and if so, whether such infections play a role in the pathogenesis of HBV-induced chronic liver disease (CLD) and/or hepatocellular carcinoma (HCC) remains to be determined. The objectives of this study were to determine whether HBV infects NSCs and whether such infections alter NSC activity in a manner likely to contribute to the development of CLD and/or HCC. **Methods:** Liver biopsies from five hepatitis B surface antigen (HBsAg) positive patients were co-stained for HBcAg and HBx and the stem cell markers EpCAM, Oct-4 and Nanog. In addition, primary NSCs derived from healthy human livers were exposed to HBV contaminated serum *in vitro*. Supernatant and/or cellular HBsAg, HBcAg and HBV-DNA expression were documented over the subsequent 30 days of culture. Pro- and anti-inflammatory cytokine expression, membrane potential differences (PDs), proliferative and telomerase activities of HBV-infected NSCs were also documented. **Results:** Markers of HBV infection were present within the NSC population of all five biopsy specimens. *In vitro*, HBV markers appeared within three days of exposure, peaked in expression after 10–15 days and remained positive thereafter for the duration of cell viability. There were no consistent changes in HBV-infected NSC pro- or anti-inflammatory cytokine expression, membrane PDs, proliferative or telomerase activities. **Conclusions:** Although the results of this study need to be confirmed, they suggest that HBV infects human NSCs but in the short term, do not alter those NSC features or activities associated with CLD and/or HCC. (J CLIN EXP HEPATOL 2019;9:34–42)

The hepatitis B virus (HBV) is a well-established cause of chronic liver disease (CLD) and hepatocellular carcinoma (HCC) in humans.¹ Amongst the various mechanisms involved in disease progression to these outcomes are viral-induced enhanced expression of pro-inflammatory cytokines within the liver, depolarization of hepatocyte membrane potential differences (PDs), increased cell proliferative activity and alterations in telomere lengths resulting from altered telomerase activity.^{1–4}

Hepatic stem or progenitor cells are a small (<1%) subpopulation of resident liver cells that have the capacity to self-replicate and differentiate into either hepatocytes or cholangiocytes.⁵ They can be identified by their expression of various stem cell markers including Ep-CAM, Oct-4 and Nanog.⁶ Recently, we hypothesized that HBV infection of normal (non-malignant) hepatic stem cells (NSCs)

might provide important insights regarding the pathogenesis of CLD and HCC.⁷

The principal objective of the present study was to determine whether HBV infects human NSCs. Thus, liver biopsy tissues derived from chronic HBV carriers were co-stained for HBV (core and x antigens) and NSC (EpCAM, Oct-4 and Nanog) markers. We also isolated NSCs from resected liver tissues of uninfected (HBV seronegative) individuals, exposed the cells to HBV contaminated sera *in vitro* and monitored culture supernatants and cells for evidence of viral replicative activity. A secondary objective was to determine whether HBV infection of NSCs contributes to the pathogenesis of CLD and HCC. Here, we documented pro-inflammatory cytokine expression, NSC PDs, proliferation and telomerase activities. We also documented the effects of hepatitis B immunoglobulin (HBIG) in preventing HBV infection of NSCs and nucleotide analog treatment (Tenofovir) on viral replicative activity within infected NSCs.

METHODS

Immunohistochemistry of Liver Biopsies

Randomly selected, pre-treatment liver biopsies from five adult hepatitis B surface antigen (HBsAg) positive patients were deparaffinized, washed with phosphate-buffered saline (PBS) plus 0.1% triton X buffer and incubated

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Abbreviations: d: days; IU: international units; ml: milliliters; Neg: negative; SN: supernatant; UND: undetected

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overnight with primary monoclonal mouse or polyclonal rabbit antibodies to HBcAg (Dako, Carpenteira California 1:500), anti-HBx (Abcam, 1:100), anti-EpCAM (Millipore 1:100), anti-Oct-4 (Abcam 1:500) and anti-Nanog (Abcam 1:500). Sections were then washed and incubated with secondary antibodies; donkey anti-mouse Cy3 and goat anti-rabbit FITC (Jackson labs) prior to counterstaining with DAPI (Invitrogen). Clinical and virologic features of the five patients are provided in Table 1.

NSC Isolations

NSCs were isolated from healthy human liver tissues adjacent to metastatic lesions or benign hepatic tumors in HBsAg negative adult patients undergoing surgical resections for their tumors. Magnetic bead separations based on positive EpCAM expression and characterization of these cells as somatic stem/progenitor cells were carried out as described by Neurauter et al. and modified by Bautista et al.^{8,9} Briefly, liver tissues were dissected and digested in 1 µg/ml of type 4 collagenase (Sigma-Aldrich, Oakville, Ont, CAN) solution at 37 °C for 15–30 min. Contaminated red blood cells were lysed with ammonium chloride solution (STEM-CELL Technologies, Miltenyi Biotec, San Diego, CA, USA) on ice for 5 min. CD45⁺ leukocytes and Annexin V⁺ apoptotic cells were removed by autoMACS-pro cell separator and magnet beads (Miltenyi Biotec). EpCAM-positive and -negative cells were enriched by autoMACS-pro cell separator and CD326 (EpCAM) MicroBeads (Miltenyi Biotec). Between passages 4 and 10, NSCs were separated from mature hepatocytes by magnetic bead separation based on EpCAM expression and then replenished after subsequent magnetic separations after 2 weeks.

HBV Infection of NSCs *In Vitro*

Freshly isolated EpCAM positive NSCs were exposed to 1 ml aliquots of serum containing HBV genotype C (10⁶ IU/ml) for 48 h (day-2) prior to extensive washing and 0.1% trypsin in order to remove cell adherent virus (day 0). Evidence of HBV infection within NSCs consisted of testing cells or supernatants for HBsAg, HBcAg and HBV-DNA expression on days 3, 5, 10, 15, 25 and 30.

(i) HBsAg testing

Quantitative HBsAg titers were determined at the National Microbiology Laboratory in Winnipeg, Canada by the HBsAg II quant assay (Roche Diagnostics, Laval, Quebec, Canada) using the cobas e411 immunoanalyzer. Specimens were assayed in triplicate without the initial pre-dilution step. The range of measurement for undiluted specimens is 0.05–130 IU/ml.

(ii) HBV-DNA testing

HBV-DNA testing, performed on supernatants and DNA isolated from NSCs included both qualitative real time PCR detection of HBV-DNA as well as quantitative HBV-DNA measurements. In brief, HBV-DNA was extracted from 200 µl of material using QIAamp DNA extraction reagents (Qiagen, Mississauga, Ontario, Canada) with the DNA eluted in 60 µl elution buffer and stored at –20 °C. Qualitative detection of HBV-DNA in supernatants and cellular DNA was performed by real time PCR (RT) of 3 distinct HBV genomic targets; the surface/polymerase overlapping region, the Enhancer I region and X/Enhancer II region. A positive finding with two or more RT targets defined a positive HBV-DNA result. Real time PCR reactions were prepared using QuantiTect Virus Master Mix (Qiagen) and FAM-labeled probes (Integrated DNA Technologies, Coralville, IA, USA) and were run on an Applied Biosystem 7500 Fast Real Time PCR System (Life Technologies, Burlington, Ontario, Canada). Quantitative HBV-DNA measurements were determined using the RealStar HBV PCR Kit 1.0 assay (Altona Diagnostics, Hamburg, Germany) run on the 7500 Fast Real Time PCR System. The lower limit of quantification for the RealStar HBV PCR assay is 10 IU/µl.

(iii) Immunofluorescence

HBcAg and EpCAM expression within isolated NSCs exposed or not exposed to HBV were documented by confocal microscopy using immunofluorescence. NSCs were grown on glass coverslips and processed for single-labeled or dual-labeled immunofluorescence assay as described above for biopsy specimens using HBcAg and EpCAM followed by Alexa 488 and Cy3 secondary antibodies and counterstained with DAPI for nuclei visualization. Coverslip preparations were mounted in fluorescent mounting medium and viewed with an Olympus Confocal IX and with an epifluorescence Z2 Zeiss microscope (Carl Zeiss Canada, Toronto, Ontario, Canada). A ZEN program was used to collect and analyze images. Fluorescence was examined on a Zeiss Axioskop2 fluorescence microscope with image capture using Axiovision 3.0 software (Carl Zeiss).

(iv) NSC cytokine, membrane potential differences, proliferative and telomerase activities

Pro-inflammatory (IL-2, IL-6 and TNF-α) and anti-inflammatory (IL-10) cytokine release by NSCs were documented by

Table 1 Clinical and Virologic Features of Five HBV-infected Patients with Hepatocellular Carcinoma.

Patient #	Age (yrs)	Gender	HBsAg	HBeAg	Anti-HBe	HBV-DNA	AFP
1	72	M	Pos	Neg	Pos	4.6×10^4	27
2	78	M	Pos	Neg	Pos	1.7×10^6	785
3	76	M	Pos	NA	NA	NA	479
4	53	M	Pos	Neg	Neg	UN	444
5	63	M	Pos	Neg	Pos	1.8×10^6	5

Abbreviations: HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B “e” antigen; Anti-HBe, antibody to hepatitis B e antigen; HBV-DNA, hepatitis B virus DNA; AFP, alpha fetoprotein; M, male; NA, not available; UN, undetectable.

ELISA analysis of supernatants at the subsequently identified peak of HBV infection (days 10–15). ELISAs were performed using internal standards calibrated against recombinant human cytokine (Preprotech Inc., Rock Hill, NC, USA). Sample concentrations of cytokine were calculated from a minimum of three points falling on the linear portion of titration curves calibrated against recombinant cytokine standards serially diluted on each plate. NSC PDs were documented by microelectrode impalement of cells on days 0, 4 and 7 as described previously by the authors.¹⁰

Proliferative and h-TERT activities were documented using commercially available kits as per the manufacturer's instructions on days 10–15 of infection (BrdU Cell Proliferation ELISA Kit, Cell Biolabs Inc., San Diego, CA, USA and Telomerase PCR ELISA^{PLUS}, Roche Diagnostics, Gmbh, Mannheim, Germany respectively).

- (v) Hepatitis B Immunoglobulin Neutralization Assay
In a separate set of experiments, high titer, commercially available hepatitis B immunoglobulin (HBIG) was administered to cultured NSCs prior to HBV-infection (Sigma-Aldrich, Oakville, Canada). The HBIG consisted of monoclonal anti-HBs of the IgG1 subclass against epitopes within the alpha HB3 region of HBsAg. The concentration employed was 1 mg/ml containing 98% human immunopurified IgG. EpCAM positive NSCs were seeded in coverslips and cultured with and without HBIG for 3 days prior to being exposed to 1 ml aliquots of serum containing HBV (10^6 IU/ml) for 48 h. Cells were then washed, cultured and stained for HBcAg expression on days 10–15 as described above. Cells exposed to HBV and no HBIG served as controls.
- (vi) Antiviral treatment of HBV-infected NSCs
HBV-infected NSCs (on day 5 of infection) were treated daily with 0.1–10 μ M of Tenofovir and viral loads in the supernatants and cells determined at various time intervals for seven days thereafter (days 5–12 of infection).

The University of Manitoba Human Ethics Research Board committee did not consider the study as human subject research.

RESULTS

Patient Findings

To determine whether HBV markers can be found in human liver NSCs we stained liver biopsy tissues from five randomly selected patients with chronic HBV infections for HBcAg and HBx as well as the stem cell markers EpCAM, Oct-4 and Nanog. As shown in Figure 1 for a representative patient, HBcAg expression was evident in NSCs from all five patient biopsies. In the majority of cases, HBcAg expression was both nuclear and cytoplasmic in distribution and tended to be positive in 30–50% of EpCAM⁺ cells. Similar results for HBcAg and EpCAM staining were obtained for HBx and the other stem cell markers: Oct-4, and Nanog (data not shown).

HBV Infection of NSCs *In Vitro*

To document HBV infection of NSCs *in vitro*, HBsAg testing of supernatants, HBcAg immunofluorescence of

Figure 1 Immunofluorescence staining of a representative liver biopsy for HBcAg and EpCAM expression in a patient with chronic HBV infection. HBcAg expression is stained green, EpCAM expression: red and cell nuclei: blue. The box highlights a cell staining positive for HBcAg and EpCAM using confocal microscopy. Similar results were obtained with HBx, Oct 4 and Nanog staining (not shown). Scale bar 100 μ m.

NSCs and HBV-DNA expression in supernatants and NSCs were documented. HBsAg was detected in days 10 and 15 supernatants of NSCs exposed to HBV (HBsAg concentrations of 0.375 and 0.082 IU/ml respectively) but negative at earlier and subsequent time samples. Immunofluorescence staining for HBcAg protein in NSCs was positive by day 3 and remained positive for the remainder of the 30 days of infection (Figure 2). The results of HBV-DNA testing of supernatants and cellular DNA on days 3, 5, 10, 15, 25 and 30 following exposure to HBV are provided in Table 2 and Figure 3. HBV-DNA was detected in both the supernatants and cellular DNA derived from NSCs by day 3 of infection. In supernatants, HBV-DNA levels peaked between days 10–15 while in NSCs the peak occurred earlier (day 5 of infection). In both supernatants and cellular DNA, HBV-DNA levels were lower on day 30 when the viability of cultured NSCs tended to wane.

NSC Cytokine, Membrane Potential, Proliferation and Telomerase Activity

The release of pro- and anti-inflammatory cytokines influence HBV-induced progression to fibrosis and cirrhosis.¹¹ Recent data indicate that NSCs express these cytokines and could thereby alter the natural history of chronic HBV.^{12,13} Thus, we documented the release of the pro-inflammatory cytokines IL-1, IL-6 and TNF- α as well as the anti-inflammatory cytokine IL-10 by HBV-infected NSCs. Aside from an increase in IL-6 which was not accompanied by significant increases in other pro-inflammatory cytokines (IL-2 and TNF- α), pro- and anti-inflammatory

Figure 2 Immunofluorescence staining for HBCAg (green) in EpCAM positive (red) NSCs following 0–30 days of culture. HBCAg can be observed as early as day 3 and persists throughout the 30 day culture period. DAPI, blue staining identifies cell nuclei.

Table 2 Hepatitis B Viral DNA in the Supernatants and Cellular DNA of Human, Hepatic Stem Cells Exposed to Hepatitis B Virus *In Vitro*.

Sample	IU/ μ L	IU/ml	Log ₁₀ IU/ml
SN d3	0.152468905	4.6E+02	2.7
SN d7	11.85062599	3.6E+04	4.6
SN d10	292.6731262	8.8E+05	5.9
SN d16	200.9280548	6.0E+05	5.8
SN d25	8.693920135	2.6E+04	4.4
SN d30	0.009514319	2.9E+01	1.5
Water control	UND	Neg	
Cells d3	1.677366495	5.0E+03	3.7
Cells d5	1895.015137	5.7E+06	6.8
Cells d10	73.50504303	2.2E+05	5.3
Cells d16	81.33956909	2.4E+05	5.4
Cells d25	31.83733368	9.6E+04	5.0
Cells d30	1.374098063	4.1E+03	3.6
Water control	UND	Neg	

Abbreviations: SN, supernatant; d, days; IU, international units; ml, milliliters; UND, undetected; Neg, negative.

cytokine release by HBV-infected NSCs were similar to those of control (uninfected) NSCs (Figure 4).

Changes in cell membrane PDs and in particular, a depolarized state is thought to play an important role in the malignant transformation of non-malignant cells including stem/progenitor cells.⁸ In the present study, NSC PDs depolarized (became less negative) following exposure to HBV however, the depolarized state was transient with PD values returning to baseline by day 5 of infection (Figure 5).

Increased proliferative activity renders cells more susceptible to undergoing genomic mutations and thereby, malignant transformation.¹⁴ The risk is enhanced in the setting of short telomere lengths as occurs with decreased telomerase activity.¹⁵ Thus, proliferative and telomerase activities were documented in HBV-infected NSCs. The results (Figures 6 and 7 respectively) revealed NSC proliferative and telomerase activities remained largely unchanged following HBV infection.

HBIG Neutralization

HBIG is a well-established means of providing passive immunity to susceptible individuals by inhibiting HBV uptake by hepatocytes. Whether HBIG also prevents infection of NSCs has yet to be reported. In the present study, pre-treatment of NSCs with HBIG had no protective effect on HBV infection in that NSCs exposed to HBV stained positive for HBCAg on day 15 of infection.

Response to Anti-Viral Therapy

The response of HBV-infected NSCs following exposure to a range of Tenofovir concentrations over a 7 day period are provided in Figure 8. There were no dose-dependent decreases in viral loads after 2, 4 or 7 days of exposure to this agent.

DISCUSSION

The results of this study indicate that in the setting of chronic hepatitis B, human hepatic NSCs commonly

Figure 3 HBV-DNA expression in supernatants (SN) and normal stem cells (NSCs) at various times following infection with hepatitis B virus (HBV) *in vitro*. Expression peaked at day 5 in NSCs and day 10 in supernatants.

express markers of HBV infection. The results also suggest that HBV can infect NSCs *in vitro* and maintain infection for much of the duration of NSC viability. However, such infections do not result in the acquisition of features and/or activities of NSCs associated with CLD or HCC. Finally, HBIG does not prevent HBV infection of NSCs and in the short term, exposure to a potent antiviral agent does not significantly decrease HBV viral loads in this liver cell population.

There are paucity of reports describing the presence of HBV markers within non-induced, human hepatic NSCs. In a study by Arzumanyan et al., the investigators stained tumor and adjacent non-tumor tissues from HBV infected individuals and documented HBx expression in EpCAM, Oct-4, Nanog, β -catenin and E-cadherin positive stem/progenitor cells (both in tumor and adjacent non-tumor tissues).¹⁶ In the non-tumor tissues, HBx and EpCAM co-staining was positive in the majority of cases. In the only other report documenting HBV markers within the stem

cell population of the liver we described positive co-staining for both HBx and HBcAg antigen in EpCAM, Oct-4, Nanog and Notch positive cells present within the tumor and adjacent non-tumor tissue of a chronic HBV carrier with HCC.¹⁷ However, in neither study could it be ascertained whether the HBV markers expressed in NSCs resulted from reprogramming of mature hepatocytes toward features of “stemness” or direct HBV infection of resident NSCs.

The question as to whether HBV directly infects NSCs remains unresolved. Arguing against such infections are previous reports describing undifferentiated, pluripotent stem cells being resistant to HBV infection *in vitro*, while supportive findings included HBV infection of bone marrow derived mesenchymal stem cells and multipotent adult stem cells induced to differentiate into hepatocyte lineages.^{18–23} If confirmed by others, the results of the present study indicate that HBV can infect human NSCs and remain replicatively active for the duration of time these cells remain viable in culture.

Figure 4 Pro- and anti-inflammatory cytokine release by HBV infected NSCs. Histograms show ELISA quantification of cytokines IL-2, IL-6, TNFα and IL-10 in non-infected NSCs and HBV infected NSCs (HBV-NSC). Only the increase in IL-6 expression in HBV-NSCs was significant ($P < 0.05$).

Figure 5 Membrane potential differences (PDs) in HBV infected NSCs. Histograms shows mean PDs from NSC controls (no exposure to HBV-contaminated serum) and following exposure to HBV on days 0, 4 and 7 thereafter. Although exposure to serum containing HBV resulted in significant depolarization ($P < 0.001$) the effect was transient and PD values returned to baseline by days 5 and 7.

The CLD associated with chronic HBV infection occurs within a pro-inflammatory environment wherein components of the innate and adaptive immune systems target HBcAg expressing hepatocytes.¹¹ Recently, it has been suggested that in addition to lymphocytes and macrophages, hepatic NSCs influence this inflammatory milieu.^{12,15} However, in the present study, aside from an isolated increase in IL-6 cytokine release by HBV-infected NSCs, there were no

significant changes in pro- or anti-inflammatory cytokine expression arguing against NSCs being the principal cell population responsible for the proinflammatory environment. Clearly, co-culture experiments are required to determine whether HBV-infected NSCs alter the release of these cytokines by innate and adaptive immune system cells.

Malignant or cancer stem cells have been previously documented to have depolarized cell membrane

Figure 6 NSC proliferative activity as determined by BrdU staining in HBV-infected NSCs on day 0 and day 10. There were no significant differences in proliferative activity between HBV-infected NSCs at time 0 and day 10 of infection.

Figure 7 NSC telomerase activity as determined by hTERT levels in uninfected NSCs (controls) and HBV-infected NSC cytoplasm and nuclei. There were no significant differences in hTERT activity in either the cytoplasm or nuclei of uninfected and HBV-infected NSCs.

potentials, increased proliferative activity and high telomerase levels.^{8,14,15} In the present study, none of these features developed in HBV-infected NSCs. Future studies in which NSCs are cultured for periods beyond 30 days are required to more definitively determine whether HBV infection of NSCs ultimately results in the acquisition of these features and/or their predisposition to malignant transformation.

It is now well established that HBV utilizes the sodium taurocholate cotransporting polypeptide to infect human hepatocytes.²⁴ There are also data implicating other hepatocyte proteins in the uptake process.²⁵ Regardless of which transporter is involved, HBIG has been documented to be a safe and effective means of preventing HBV infections in humans.²⁶ The results of the present study suggest that such protection is not afforded the NSC population of the liver. This finding could help to explain why passive immunoprophylaxis with HBIG can on occasion fail and why recurrent infections can occur (despite the presence of anti-HBs) when the host becomes immune compromised. However, if HBV infects NSCs and HBIG does not protect NSCs from such infections it remains unclear why HBV infections do not occur more frequently in HBV-exposed, HBIG recipients. Here, one might speculate that HBV resides within the NSC population of the liver but given their low prevalence within the liver cell population (<1% of all liver cells), such infections are not clinically or serologically apparent. Future studies documenting the presence or absence of HBV markers within NSCs of HBV exposed subjects administered HBIG but with no clinical or serologic evidence of infection are required to resolve this issue.

Tenofovir is considered first-line therapy for chronic HBV due to its prompt and often complete inhibition of HBV replicative activity. However, in this study, Tenofovir had no appreciable effect on HBV viral loads in NSCs following seven days of exposure to a range of drug

Figure 8 The effects of antiretroviral therapy with tenofovir on HBV-infected NSCs. The three different colored lines represent days 2, 4 and 7 of tenofovir treatment. Viral loads were not significantly decreased at any concentration (0.1 μ M to 10 μ M).

concentrations. One interpretation of this finding is that Tenofovir is ineffective in this cell population, perhaps as a result of increased multidrug resistance protein activity which is a feature of stem cell populations.²⁷ If so, NSCs may represent an additional reservoir for HBV during antiviral treatment and thereby, contribute to the relapses that often occur when treatment is held or discontinued. Another possibility is that seven days of exposure was too short a period of time to appreciate an antiviral effect. Arguing against this interpretation are the results of previous studies indicating that HBV-viral loads tend to decrease significantly within the first week of initiating antiviral therapy.²⁸

There are a number of limitations to this study that warrant emphasis. First, the number of liver tissues studied ($N = 5$) was small and therefore, the results might have been different if patients with different HBV genotypes or viral loads had been employed. Second, although attempts were made to ensure the purity of NSCs isolated from healthy human liver tissues, one cannot rule out the possibility that a small number of “contaminating” hepatocytes, lymphocytes, differentiated stem cells or unwashed viral particles might have been responsible for the positive *in vitro* findings. Finally, as indicated earlier, the inability to maintain NSC viability beyond 28 days significantly impaired attempts to determine whether HBV infection of NSCs ultimately results in their malignant transformation.

In conclusion, in this study, HBV infection of human NSCs was a common histologic finding and preliminary data suggest that HBV can infect NSCs *in vitro*. Whether such infections impact on the natural history of HBV-induced CLD and/or HCC will remain unclear until

methods are developed to maintain human NSCs in culture for prolonged periods of time.

AUTHORS' CONTRIBUTIONS

G.Y. Minuk: study conception and design, drafting of manuscript; W. Bautista: performed experiments, analysis and acquisition of data, revision of manuscript; C. Osiowy: virology testing and revision of manuscript; J. Klein: analysis and interpretation of data, revision of manuscript.

CONFLICTS OF INTEREST

The authors have none to declare.

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