Non AIDS complications and treatment optimizations for HIV-1 infected Thai adult patients with and without TB or hepatitis
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CHAPTER 5

Increased intrahepatic apoptosis but reduced immune activation in HIV-HBV co-infected patients with advanced immunosuppression

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Increased intrahepatic apoptosis but reduced immune activation in HIV-HBV co-infected patients with advanced immunosuppression

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Objective: To determine if intrahepatic immune activation is increased in HIV-hepatitis B virus (HBV) co-infected patients compared to HBV mono-infected patients and whether this reduced following HBV-active antiretroviral therapy (ART) in HIV-HBV co-infected patients.

Design: Case–control observational study.

Methods: We examined liver biopsies for markers of T-cell and monocyte infiltration and activation, natural killer cells, hepatic stellate cell (HSC) activation (staining for alpha smooth muscle actin) and apoptosis [using terminal deoxyuridine triphosphate nick-end labelling (TUNEL)] in treatment-naive Asian HIV-HBV co-infected (n = 16) and HBV mono-infected patients matched for age and HBV e-antigen status (n = 16). Liver biopsies from a subset of co-infected patients (n = 15) were also compared prior to and following 48 weeks of HBV-active ART.

Results: HIV-HBV co-infected patients had a median CD4 T-cell count of 25 cells/μl and lower alanine aminotransferase levels than HBV mono-infected patients (P = 0.03). In HIV-HBV co-infected patients, hepatocyte apoptosis was increased (P = 0.04) but there were fewer intrahepatic CD4 and CD8 T cells (P < 0.001), lower activation of intrahepatic T cells, Kupffer cells and HSC (P = 0.002, 0.008 and < 0.001, respectively). Following ART, there was a significant decrease in intrahepatic HBsAg staining (P = 0.04) and Kupffer cell activation (P = 0.003).

Conclusions: We found no evidence of increased intrahepatic mononuclear and HSC activation in this cohort of HIV-HBV co-infected individuals with advanced immune suppression. An increase in intrahepatic apoptosis in HIV-HBV co-infected individuals may potentially contribute to accelerated fibrosis in this setting.

Keywords: apoptosis, hepatitis B virus, HIV, immunohistochemistry, liver

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**Introduction**

HIV-hepatitis B virus (HBV) co-infection is common with an estimated 2–4 million people worldwide infected with both viruses [1]. In the presence of HIV, HBV-related liver disease progression is accelerated and liver-related mortality is significantly increased [2,3]. Anti-retroviral therapy (ART), including agents active against both HIV and HBV (HBV-active ART) has been associated with a reduction in liver-related mortality in HIV-HBV co-infected patients in some studies [4,5] although in another study liver related mortality remains significantly elevated in HIV-HBV co-infected patients [6].

The pathogenesis of accelerated liver disease progression in HIV-HBV co-infection is poorly understood. In treatment-naive patients, HIV-HBV co-infection is associated with higher HBV DNA levels [3,7] and lower alanine aminotransferase (ALT) levels than in HBV mono-infected patients [3]. Comparative studies of liver biopsies from HIV-HBV co-infected and HBV mono-infected patients showed lower inflammatory activity but higher fibrosis in HIV-HBV co-infected patients, using the Histology Activity Index (HAI) described by Knodell [3,8,9]. Lower ALT levels and lower inflammatory activity reflect less hepatocyte destruction, possibly due to a depressed HBV-specific T-cell response [10–12]. These data suggest that factors other than the adaptive immune response to HBV may drive liver disease progression.

HIV infection is characterized by a significant increase in immune activation, with increased T-cell turnover [13], increased frequencies of activated T and B cells [14] and increased serum levels of pro-inflammatory cytokines and chemokines [15]. In addition, there are increased concentrations of lipopolysaccharide (LPS) and bacterial 16S rDNA in blood [16] secondary to massive depletion of CD4 T cells from the gut [17,18]. ART leads to a reduction in levels of LPS and immune activation but both remain persistently elevated compared with uninfected individuals [16,19,20]. In alcoholic liver disease and hepatitis C virus (HCV), LPS is increased in both the portal vein and systemic circulation and may cause liver injury through activation of hepatic Kupffer cells and HSCs [21–23].

We hypothesized that increased intrahepatic immune and HSC activation contributes to liver disease in HIV-HBV co-infected individuals and that intrahepatic immune activation persists following HBV-active ART. We therefore compared immunohistochemical staining of liver biopsies from Asian HIV-HBV co-infected individuals and HBV mono-infected individuals who were all naïve to anti-HIV and anti-HBV therapy. We also examined liver biopsies prior to and following HBV-active ART.

**Materials and methods**

**Patient recruitment**

HIV-HBV co-infected patients were recruited as a substudy of the Tenofovir in Co-infection (TICO) study. This randomized clinical trial compared the use of lamivudine (LMV) versus tenofovir (TDF) versus combination LMV/TDF in HIV-HBV co-infected individuals commencing HBV-active ART in Thailand [24]. Liver biopsy at enrolment and after 48 weeks of treatment was optional in the TICO study. All participants consenting to liver biopsy were enrolled in this substudy.

Patients were recruited through the HIV-Netherlands Australia Thailand (HIVNAT) network and were screened at the HIV outpatient clinic at the King-Chulalongkorn Memorial Hospital, Bangkok, Thailand. Inclusion criteria have been previously reported but in brief, patients were required to have an HBV DNA greater than 20,000 IU/ml; no prior anti-HBV or anti-HIV treatment; and no detectable antibodies to HCV and hepatitis D virus.

Hepatitis B virus mono-infected patients were recruited from a single clinical site, St. Vincent’s Hospital, Melbourne, Australia as a sub-study of a prospective cohort of clinic-based patients initiating anti-HBV therapy [25]. Inclusion criteria to the HBV mono-infected prospective cohort were no antibody to HIV or HCV and HBV DNA greater than 351 IU/ml. HBV e-antigen (HBeAg)-positive patients had documented elevation of serum ALT for 3–6 months prior to liver biopsy (ALT > 30 IU/ml for men, and >19 IU/ml for women [26]). No patient had evidence of decompensated liver disease or hepatocellular carcinoma (HCC) [25]. HBV mono-infected patients who were of Asian ethnicity were matched for age and HBeAg status with the HIV-HBV co-infected patients. All participants gave informed written consent and the study was approved by the relevant Human Research Ethics Committees in both Thailand and Australia.

**HIV and HBV quantification**

HIV-1 RNA was quantified using the COBAS AMPLICOR assay (Roche Diagnostics) with a lower limit of detection of 50 copies/ml. HBV DNA quantification was performed at a central laboratory (Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia) using the Versant HBV DNA 3.0 bDNA assay (Bayer Diagnostics, Tarrytown, New York, USA) and COBAS TaqMan HBV Test (Roche Diagnostics, Branchburg, New Jersey, USA), as previously described [25].

Intrahepatic HBV covalently closed circular DNA (cccDNA) and total HBV DNA were quantified by real-time polymerase chain reaction using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) following real-time polymerase chain reaction and real-time polymerase chain reaction using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany).

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Although in another study liver related mortality remains higher in HIV-HBV co-infected patients in some studies [4,5] compared with both viruses [1]. In the presence of HIV, HBV-HIV-hepatitis B virus (HBV) co-infection is common in individuals and HBV mono-infected individuals who were all HIV mono-infected. We examined liver biopsies prior to and following HBV-active ART. We noted that HSC activation persists following HBV-active ART. We found that HSC activation contributes to liver disease in HIV-HBV co-infected patients. Intrahepatic HBV covalently closed circular DNA (cccDNA) and total HBV DNA were quantified by a central laboratory (Victorian Infectious Diseases, New York, USA) and COBAS Diagnostics, Tarrytown, New York, USA) and COBAS AMPLICOR HIV-1 RNA was quantified using the COBAS AMPLIGEN instrument (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Liver biopsy
Liver biopsies obtained in Thailand were fixed in formalin and embedded in paraffin via a 12-step process including incubation in 95% ethanol, 100% ethanol and xylene. Sections were cut, mounted on slides and sent to Australia for immunohistochemistry. Liver biopsies obtained from HBV mono-infected patients in Australia were fixed in formalin and embedded in paraffin via a similar process. There were minor differences in the methods used at each site including total duration of liver processing, which was 6.5 h in Australia and 13.5 h in Thailand; incubation in 10% formalin was performed at 42°C in Australia and 37°C in Thailand; and use of 90% ethanol in Australia and 95% ethanol in Thailand. Liver biopsies were graded according to the METAVIR scoring system for activity and fibrosis [28] by two pathologists, one in Australia (J.S.) and one in Thailand (N.W.), who were blinded to clinical data. Where there was a discrepancy of more than 1 grade (n = 2), these biopsies were reviewed by a third independent pathologist and majority agreement determined the final result. Phase of HBV disease was determined on the basis of HBV DNA, ALT, HBcAg status and METAVIR score on liver biopsy as described in [29].

Immunohistochemistry
Immunohistochemistry for all liver slides was performed at St Vincents Hospital, Melbourne, Australia using the BenchMark XT automated staining system (Ventana, Tucson, Arizona, USA). A standard 30-min heat-decoaker step was used for all slides, except for CD4/CD8 double stain, in which a pascal decloaker (Dako, Carpinteria, California, USA) was used for 3 min at 125°C. Biopsies were then incubated with primary antibodies to HBV surface antigen (HBsAg, Dako); HBV core antigen (HBcAg, Dako); CD3 (Novocastra, Newcastle, UK); CD4 (Novocastra); CD8 (Novocastra); CD69 (Novocastra); CD56 (Chemicon, Billerica, Massachusetts, USA); CD68 (Dako); CD163 (Novocastra); p24 (Dako); alpha smooth muscle actin (aSMA) (Dako). Secondary antibodies used were standard for the BenchMark XT. The chromogen was diaminobenzidine (DAB) except in the case of the CD4/CD8 double stain, in which fast red was used for CD8, and DAB for CD4. Apoptosis was determined by terminal dUTP nick-end labelling (TUNEL) using Calbiochem FragEL DNA Fragmentation Detection Kit (QIA21; EMD Chemicals, Darmstadt, Germany) according to the manufacturer's instructions.

Liver sections were scored by microscopic examination by two independent investigators (J.S. and D.I.) who were blinded to patient identity and clinical data, using a semi-quantitative scale of 0–4, corresponding to positivity in 0, less than 5, 5–9, 10–29, at least 30% of cells, as previously described [25]. The distribution pattern of HBsAg staining was classified according to Chu and Liaw [30] and HBcAg staining was described according to Kim et al. [31] (Fig. 1a and b).

Statistical methods
Continuous and categorical variables were compared between groups, using the Mann–Whitney test and chi squared or Fisher’s exact test, respectively. Comparisons between baseline and week 48 were performed using the Wilcoxon signed-rank test. Spearman’s correlation was used when variables were not assumed to be normally distributed. All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, California, USA) and SPSS version 16 (SPSS Inc., Chicago, Illinois, USA).

Results
Patient demographics
HIV-HBV co-infected patients (n = 16) and HBV mono-infected patients (n = 16) were all Asian and were matched for age and HBcAg status. Mono-infected patients were mainly from Vietnam (n = 6) and Malaysia (n = 4), but also China (n = 2), Burma (n = 2), Cambodia and East Timor. The cohort was predominantly male (75%) and HBcAg-positive (63%) with a median age of 34 years (Table 1). HIV disease was advanced with median (range) peripheral CD4 cell count of 25 (5–225) cells/μl. Median (range) HIV viral load was 68 373 (9176–425 837) copies/ml. AIDS-defining illnesses were experienced by five of 16 (31%) with 10 of 16 (63%) taking cotrimoxazole, nine of 16 (56%) fluconazole, and five of 16 (31%) isoniazid and rifampicin. HBV DNA levels were not significantly different between groups at baseline (P = 0.92). However, serum qHBsAg was higher in the co-infected group (P = 0.02). ALT levels were significantly lower in the co-infected group (P = 0.03). An elevated ALT (>30 IU/ml) for men, and >19 IU/ml for women [26]) was present in 88 and 81% of the mono-infected and co-infected groups, respectively (P = 1.0). There was no significant difference in activity and fibrosis (median METAVIR score was A1F1 in each group). Genotype C HBV was predominant in the co-infected group (90%), with a mix of both genotypes B (56%) and C (44%) in the mono-infected group. There was no significant difference in total intrahepatic HBV DNA or HBV cccDNA between HIV-HBV co-infected and HBV mono-infected patients (Table 1).
Immunohistochemistry in co-infected and mono-infected patients

Given the recruitment strategies for the HIV-HBV co-infected and HBV mono-infected groups differed, we first compared the clinical phases of CHB and found that this was similar in HIV-HBV co-infected and HBV mono-infected patients \( (P=0.54; \text{Fig. 1b}) \). The majority of patients were in the immune-clearance phase \( (44\% \text{ and } 56\% \text{ for co-infected and mono-infected patients, respectively}) \), a small number in the immune-tolerant phase \( (19\% \text{ and } 6\% \text{ for co-infected and mono-infected patients, respectively}) \), no patients in the immune-control phase, and an equal number of co-infected and mono-infected patients in the immune-escape (HBeAg-negative CHB) phase \( (38\% \text{ for both co-infected and mono-infected patients; \text{Fig. 1b}}) \).

We also examined the distribution and expression of intrahepatic HBV proteins to further determine whether there was a significant difference in disease stage of the two cohorts. Previous work has demonstrated that a membranous HBsAg pattern may be associated with higher levels of HBV replication \( [30] \), that a nuclear
HBcAg pattern is associated with immune tolerance, and that cytoplasmic HBcAg can be related to the immune clearance phases of HBV [31]. We found that expression of intrahepatic HBcAg was similar in co-infection and mono-infection (P = 0.79) and staining patterns of either HBsAg or HBcAg were similar for co-infected and mono-infected patients (P = 0.13 for HBsAg and P = 0.96 for HBcAg; Fig. 1b).

Liver biopsies were then examined for markers of T-cell infiltration and activation (using anti-CD3, CD4, CD8 and CD69), monocyte infiltration and activation (anti-CD68 and CD163), natural killer (NK) cells (using anti-CD56) stellate cell activation (anti-αSMA) and intrahepatic apoptosis (TUNEL; Fig. 2). We found significantly more apoptotic hepatic cells in HIV-HBV co-infected patients than HIV mono-infected patients (TUNEL; P = 0.04). Although we did not perform double staining, based on morphology we identified in a subset of patients that the majority of the TUNEL-positive cells were hepatocytes (median [range] 75% (70–90%) of TUNEL-positive cells; n = 10). As expected given the advanced immune suppression of these patients, intrahepatic total, CD4 and CD8 T cells were significantly lower in co-infected than HBV mono-infected patients (P < 0.0001 for both; Table 1). Surprisingly, there was less T-cell activation (expression of anti-CD69; P = 0.002), fewer Kupffer cells (anti-CD68; P < 0.0001), less Kupffer cell activation (anti-CD163; P = 0.01) and less HSC activation (anti-αSMA; P < 0.001) in co-infected patients.

In HIV-HBV co-infected patients, intrahepatic HBsAg and HBcAg staining correlated with peripheral HBV DNA levels (r² = 0.34, P = 0.049 and r² = 0.51, P = 0.002, respectively) and HBsAg and HBcAg staining correlated with ALT levels (r² = 0.49, P = 0.003 and r² = 0.34, P = 0.02, respectively). There was a weak association between increased intrahepatic T cells and decreased peripheral CD4 T cell count (r² = 0.24, P = 0.08) and increased intrahepatic TUNEL staining and both lower peripheral CD4 T cell counts (r² = 0.22, P = 0.08) and higher baseline HIV RNA (r² = 0.20, P = 0.09), although none of these correlations reached statistical significance. There were no other significant correlations between HIV RNA, HBV DNA or ALT with any of the intrahepatic stains (data not shown).

To ensure there were no differences in staining avidity of liver biopsies collected and prepared in Bangkok and Melbourne, we assessed staining using an antibody to hepatitis paraffin 1 (anti- HepPar1). HepPar1 is mainly used to distinguish HCC from other types of adenocarcinoma metastatic to the liver, but strong expression is also seen in hepatocytes [32]. We found that staining...
intensity was no different in co-infected patients compared to mono-infected patients (Table 1).

Intrahepatic changes following HBV-active antiretroviral therapy in HIV-HBV co-infected patients

We then examined changes in intrahepatic inflammation in a subset of HIV-HBV co-infected patients who had matched liver biopsies prior to and following HBV-active ART (n = 15). As expected, we observed a significant decline in HBVDNA (P = 0.008) and qHBsAg (P = 0.003) following HBV-active ART. In addition, circulating CD4 T cells significantly increased (mean increase 160 cells/μl, P < 0.001) and HIV RNA was undetectable in all but one individual (P < 0.001; Table 1). When we examined changes in the liver, we found a significant decline in intrahepatic HBsAg staining (P = 0.04) but no change in HbcAg staining (P = 0.20; Table 1). Interestingly, Kupffer cell activation (CD163) significantly declined on treatment (P = 0.003) but there was no significant change in the number of CD4 and CD8 T cells, T-cell activation, αSMA or TUNEL staining (Table 1).

Discussion

The mechanism behind increased liver-related mortality in HIV-HBV co-infected individuals compared to HBV mono-infected individuals has not been determined. Here we present the first comparative study of intrahepatic immune activation and HSC activation in Asian HIV-HBV co-infected and HBV mono-infected patients. Overall, the main findings were significantly increased apoptosis but fewer intrahepatic T cells, Kupffer cells and NK cells in HIV-HBV co-infected individuals compared to HBV mono-infected individuals. Surprisingly, there was also less mononuclear and HSC activation in co-infected individuals.

Increased hepatocyte apoptosis has previously been reported in HIV-HCV co-infected patients compared to HCV mono-infected patients associated with lower CD4 cell counts [33]. Similarly, we found a trend towards increased intrahepatic apoptosis with lower baseline CD4 cell count and higher baseline HIV RNA. However, intrahepatic apoptosis did not change after 48 weeks of treatment with HBV-active ART, despite suppression of HIV RNA and recovery of CD4 T cells (P = 0.73). The majority of intrahepatic apoptotic cells were hepatocytes based on cell morphology. However, given we did not co-stain the slides for TUNEL and other cell markers, up to 30% of intrahepatic apoptotic cells may have included cells other than hepatocytes. Given the recent report that increased hepatocyte apoptosis may be associated with hepatocyte proliferation and carcinogenesis, our finding of increased intrahepatic apoptosis in HIV-HBV co-infection, may be an important factor in...
driving liver disease even following suppressive HBV-active ART [34].

It is unlikely that apoptosis of hepatocytes was caused by HBV itself, as HBV is generally considered a noncytopathic infection [35]. An alternate explanation could be direct infection of hepatocytes with HIV, as previously demonstrated both in vitro and in vivo (reviewed in [36]), which could potentially lead to hepatocyte death. Alternatively, intrahepatic accumulation of HBV proteins such as HBsAg, as we have recently demonstrated in vitro in the setting of HIV-HBV co-infection of hepatic cell lines [37], may potentially facilitate hepatocyte apoptosis via endoplasmic reticulum stress [38,39]. Compared with HBV mono-infected patients, serum qHBsAg was significantly higher in co-infected patients prior to ART and there was a trend to increased intrahepatic HBsAg ($P = 0.06$; Table 1).

The finding of reduced intrahepatic inflammatory activity in HIV-HBV co-infection was consistent with two previous studies using Knodell score to quantify intrahepatic inflammation [3,8]. Although the HAI described by Knodell is widely used [9], this score does not specifically assess activation of $T$-cell subsets, Kupffer cells or HSCs [40,41]. In these two previous studies that compared HIV-HBV co-infected to HBV mono-infected patients, the groups were not specifically matched for age and HBeAg status and patient ethnicity and HBV genotype were likely to have differed from the present study [3,8]. It is probable that the patients described in these previous studies were predominantly Caucasian with adult acquired genotype A HBV, but genotype and ethnicity were not specifically reported.

The liver has been termed a ‘graveyard for $T$ cells’ [42]. In other words, $T$ cells migrating to the liver or primed in the liver in response to intrahepatic antigen presenting cells have an increased chance of undergoing apoptosis [43]. Untreated HIV-HBV and HIV-HCV infection is characterized by high levels of chemokines such as CXCL10 as we and others have recently shown [44,45]. CXCL10 binds to CXCR3 which is expressed on both resting and activated $T$ cells which can enhance migration of both antigen-specific and nonspecific circulating $T$ cells to the liver, as previously described in HCV infection [45,46]. Therefore, it is possible that the few circulating activated CD4 and CD8 $T$ cells in this patient cohort that migrated to the liver may have been at increased risk of reduced survival and intrahepatic apoptosis.

Studies of intrahepatic NK cells, Kupffer cells and HSCs in HIV-HBV co-infection or HIV mono-infection have not been reported. In the present study, there was less intrahepatic CD56 staining (NK cells and NK T cells), CD68 (Kupffer cells) and CD163 staining (activated Kupffer cells) in HIV-HBV co-infected individuals. Reduced Kupffer cell activation could be a consequence of Kupffer cells becoming tolerant in vivo to repeated exposure to high levels of LPS leading to production of anti-inflammatory cytokines such as IL-10 in place of pro-inflammatory cytokines [47]. However, in contrast to our findings, in HIV-HCV co-infected patients, circulating monocytes demonstrated a loss of LPS tolerance and persistent production of inflammatory cytokines in response to LPS, although Kupffer cells were not specifically examined in this study [22].

The finding of reduced HSC activation in HIV-HBV co-infected individuals compared to HBV mono-infected individuals was surprising. We had expected to find increased HSC activation in the presence of high levels of HIV in untreated co-infected patients, since HIV can directly activate HSC via binding of gp120 to surface-expressed CXCR4 leading to increased production of αSMA and TGF-β in vitro [48–50]. These previous in-vitro studies may not translate to findings in vivo. Alternatively, the signalling pathways in HSCs may be altered in the setting of HIV infection.

We had initially hypothesized that hepatic mononuclear cell and HSC activation would be increased in HIV-HBV co-infection compared to HBV mono-infection. There may be several explanations for our findings. First, we recruited patients from different sites. However, all patients were of Asian ethnicity and were matched by age and HBeAg status, with likely similar duration of HBV infection, assuming childhood acquisition as is common in Asia [51]. Furthermore, there were no significant differences in HBV disease stage between the HIV-HBV co-infected and HBV mono-infected patients. Second, differences in sample processing may have affected immunohistochemical staining. However, formalin fixation protocols at the two sites were very similar (see Materials and methods section) and the staining and semi-quantitative scoring were all performed at a single site. In addition, the hepatocyte paraffin 1 (HepPar1) stain was of similar intensity in both groups, consistent with similar staining avidity in samples from both sites. Finally, our findings may have been affected by recruiting relatively young patients with mild liver disease and early fibrosis. It is important to note that the HIV-HBV co-infected patients had profound immunosuppression and therefore our findings may potentially not be applicable to patients with higher CD4 $T$-cell counts.

This is the first study to examine intrahepatic mononuclear and HSC activation in Asian HIV-HBV co-infected and HBV mono-infected individuals, matched for age and HBeAg status. We found no increase in activation of intrahepatic mononuclear cells or HSC in HIV-HBV co-infected patients. However, a significant increase in intrahepatic apoptosis may potentially explain more rapid progression of liver disease commonly reported in this setting.
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