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Similar frequencies, phenotype and activation status of intrahepatic NK cells in chronic HBV patients after long-term treatment with tenofovir disoproxil fumarate (TDF)

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ABSTRACT

Background: Currently, much effort is directed at further improving treatment for chronic hepatitis B patients by assessing the effect of immunomodulatory agents during therapy with nucleotide analogues (NUC). Although there are some reports on the effect of NUC therapy on peripheral natural killer (NK) cells, no studies investigated the long-term effects of NUC treatment on intrahepatic NK cells of chronic HBV patients. We aimed to prospectively investigate cell frequencies, phenotype, and activation status of intrahepatic NK cells of CHB patients on prolonged treatment with TDF.
Methods: Fine needle aspiration biopsies were collected from 11 chronic HBV patients at baseline, and at 12, 24, and 48 weeks of treatment with a daily 245 mg dose of TDF. Four patients underwent an additional aspiration biopsy after appoximately 6 years of treatment.
Results: Longitudinal evaluation of these patients during tenofovir therapy showed that all patients achieved a viral load reduction with undetectable DNA load after 48 weeks of therapy. Repeated sampling of the liver during therapy showed that the frequency of distinct lymphocyte populations in the liver remained unchanged despite viral load reduction. During the course of therapy, no modulation of the expression levels and frequencies of CD69, HLA-DR, NKG2A and NKG2D on liver NK cells were detected. However, evaluation of intrahepatic NK cell activation after continuous TDF therapy for 6 years demonstrated a mild increase in 3 out of 4 patients.
Conclusions: Our findings provide a unique insight in the intrahepatic NK cell compartment in chronic HBV patients during prolonged treatment. We observed that long-term NUC-induced viral suppression, accompanied by gradual decrease of HBsAg levels, had no or only a limited effect on the frequencies, phenotype, and activation status of intrahepatic NK cells.

1. Introduction

Current treatment of patients with chronic hepatitis B virus (HBV) infections consists of pegylated interferon-α and/or nucleos(t)ide analogues (NUC). There is limited data on the effects of treatment with NUC on the adaptive and innate immune response against HBV (Rehermann and Bertoletti, 2015). In peripheral blood of NUC-treated chronic HBV patients a transient improvement of T-cell function has been observed (Boni et al., 2003). Circulating NK cells obtained from chronic HBV patients exerted normal cytotoxicity comparable to healthy individuals, but they were less activated and produced less IFN-γ (Oliviero et al., 2009; Peppa et al., 2010; Tjwa et al., 2011; Sun et al., 2012). The defective ability of NK cells to produce IFN-γ may have important consequences for the control of viral replication via its direct cytotoxic effects and ability to promote Th1 responses (Mondelli et al., 2010). Since HBV replicates exclusively in hepatocytes, examination of the liver of HBV patients is crucial to better understand the role of NK cells in antiviral immunity to HBV. In addition, a better understanding of

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the local immune response against HBV during treatment may aid in the development of treatment regimens with higher (functional) cure rates. It was reported that the frequency of liver NK cells in HBV patients was lower than in healthy individuals (Zhang et al., 2011), but higher than in patients with chronic HCV infections (Bonorino et al., 2009). We and others showed partial restoration of the antiviral potential of blood NK cells following treatment of chronic hepatitis B with entecavir (Tjwa et al., 2011) or lamivudin and adefovir (Peppa et al., 2010). Restoration of NK cell activity in blood upon viral load reduction, demonstrated 6 months after start of entecavir therapy, was accompanied by an enhanced number of IFN-γ-producing $CD56^+$ NK cells, and by normalization of the expression of the activating receptor NKG2A on circulating NK cells. At present, no longitudinal studies have been conducted in chronic HBV addressing the effect of viral load reduction on the intrahepatic NK cell compartment.

In the present study, for the first time multiple longitudinal sampling of the liver was performed to determine the frequency, phenotype and activation status of liver NK cells in chronic HBV patients during the course of tenofovir disoproxil fumarate (TDF) therapy up to 6 years after start of treatment.

1.1. Patients and methods

1.1.1. Patients

Fine needle aspirate biopsy (FNAB) specimens and peripheral heparinized blood samples were obtained simultaneously from 11 patients with chronic hepatitis B prior and during treatment with the nucleotide analogue tenofovir dipivoxil fumarate (TDF; 245 mg o.i.d.). Patients who had received anti-viral therapy within 6 months prior to treatment were excluded. All patients were negative for antibodies against hepatitis C, hepatitis D and human immunodeficiency virus. Quantitative HBsAg levels were measured using the Roche Elecsys immunoassays. The study was approved by the local ethics committee, and all patients in the study gave informed consent before FNAB and blood donation.

1.1.2. Liver and peripheral blood lymphocyte cell enumeration

Liver leukocyte specimens were obtained via FNAB as previously described (Claassen et al., 2011) at baseline, week 12, 24 and 48 of antiviral therapy. Four patients underwent an additional FNAB after appoximately 6 years of TDF treatment. To determine the percentage of NK cells, cells were incubated with CD56 (MY31, BD Bioscience, Belgium), CD3 (UCHT1, Biolegend, USA) and CD45 (2D1, BD Bioscience, Belgium). Stained cells were analyzed using a multicolor flow cytometer (Canto II) and Diva software (both BD Bioscience, Belgium).

1.1.3. Expression of cell surface molecules by FACS

For *ex vivo* NK cell phenotypic analysis, PBMC were stained with antibodies against the following molecules: CD3 (UCHT1), HLA-DR (L243), CD69 (L78), CD25 (2A3) from BD Bioscience, Belgium; CD56 (N901), NKG2A (Z199), NKG2D (ON72) from Beckman Coulter, USA. As controls, cells were stained with corresponding isotype-matched control antibodies. Stained cells were analyzed as described above. Percentage positive cells were determined.

1.1.4. Statistical analysis

Data are expressed as mean percentage ± SEM, unless indicated otherwise. Data were analyzed with Prism 5.0 (GraphPad software, USA) using the Mann-Whitney *U* test to compare variables between 2 independent groups and the Wilcoxon matched pairs test between paired variables. In all analyses, a two-tailed P-value of less than 0.05 (confidence interval 95%) was considered statistically significant.

2. Results

To examine the effect of antiviral therapy on the intrahepatic NK cell compartment, we performed multiple aspirate liver biopsies from 11 chronic HBV patients before and during TDF treatment (Table 1). From the total aspirate, on average 15,000 leukocytes were acquired by flow cytometry at each time point, which allowed us to perform flow cytometric analysis but no other assays. At week 48 of treatment, serum HBV DNA was undetectable in all patients, and 7 patients had normalized ALT levels at week 48.

2.1. The frequencies of intrahepatic and circulating NK cells remain stable upon therapy

The frequency of intrahepatic NK cells, defined as $CD45^+CD3^-CD56^+$ cells (Spaan et al., 2016) was 1.5 fold higher than circulating NK cells in blood (Fig. 1). The number of intrahepatic NK cells monitored by evaluating aspirate biopsies at 4 different time-points, remained constant throughout the observation period. A weak reduction of liver NK cells was observed at week 48, but this was not significant. In agreement with our previously published findings (Tjwa et al., 2011), the number of blood NK cells did not change upon therapy. Also when baseline HBV DNA levels of patients were considered, we did not observe changes in the frequency of peripheral or intrahepatic NK cells in patients with a low or high viral load prior to start of treatment (data not shown).

2.2. The activation status of liver NK cells is distinct from blood NK cells, but is not changed by antiviral therapy

Next, the activation status of intrahepatic NK cells from CHB patients was determined during the course of NUC treatment as the percentage of liver NK cells expressing the activation markers CD69 and HLA-DR. Prior to treatment, the activation status of NK cells differed between liver and blood in patients (Fig. 2). The frequencies of NK cells expressing CD69 were higher at baseline on intrahepatic NK cells as compared to circulating NK cells, whereas the frequency of HLA-DR expressing NK cells was lower on liver NK cells as compared to their circulating counterpart. During a period of 48 weeks after start of treatment with TDF, no modulation of the frequency of $CD69^-$, and HLA-DR-expressing liver NK cells were observed. Similarly, also on blood NK cells no modulation of the expression of these activation markers on NK cells was observed during the 48 weeks of treatment.

2.3. NKG2A and NKG2D expression on liver NK cells is not dependent of viral load

At baseline, the frequency of the inhibitory receptor NKG2A and the activating receptor NKG2D expressing NK cells was comparable in the liver and blood of patients. Antiviral therapy did not affect the expression and the frequency of NKG2A and NKG2D positive NK cells in chronic HBV patients after 48 weeks of NUC therapy (Fig. 3).

2.4. The effect of continuous TDF therapy for 6 years on intrahepatic and circulating NK cells

Treatment of chronic HBV patients with TDF for 48 weeks resulted in undetectable serum HBV DNA in all patients. However, in contrast to HBV DNA, serum HBsAg levels were virtually unaltered after 48 weeks of treatment (Table 1). We hypothesized that the limited effects of NUC-induced viral load decline may be the consequence of the continuing presence of viral proteins in serum at week 48. We showed previously that prolonged NUC treatment may result in a slow but progressive HBsAg decline, especially in