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The genotoxicity of Tenofovir disoproxil fumarate in HBV-infected patients

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Abstract

Tenofovir disoproxil fumarate (TDF) is an acyclic nucleotide reverse transcriptase inhibitor used as antiviral therapeutic drug in hepatitis B virus (HBV) infection treatment. We investigated whether HBV itself or TDF could enhance genotoxicity in HBV infected patients. A total of 30 HBV-infected patients were included in this study: 10 were untreated, 10 took therapy for 6 months, 10 took therapy for 12 months and a further 15 were control group. We used the micronucleus test (MN) and mitotic index (MI) to assess the genotoxic effects of TDF. MN and MI frequency were significantly increased in all the HBV-infected patient groups when compared to control. However, significant differences were not found between different time points within the therapy groups. The current data indicate that chronic HBV infection without treatment has an effect on MN and MI. TDF administration at therapeutic doses for 12 months also did not reduce the MI and MN, suggesting its protective action against HBV induced genotoxic damage. Larger-scale and longtime studies are required for a better understanding of the genotoxicity of TDF.

Keywords: Tenofovir disoproxil fumarate, hepatitis B, micronuclei, genotoxicity

Introduction

Chronic hepatitis B infection is an inflammation of the liver that can progress to acute and chronic hepatitis, cirrhosis, fibrosis and primary hepatocellular carcinoma [1]. Hepatitis viruses are the most common cause of hepatitis. Hepatitis B virus (HBV) replicates via an RNA intermediate. Early events in the replicative cycle of HBV are the binding and entry of the virus to host hepatocytes. So the cytosolic transport of core viral particles to the nucleus, and the formation of covalently closed circular DNA (cccDNA) are occur [2]. HBV-induced cell transformation is a multistep process that requires the continued presence of viral proteins, viral genome integration into the host DNA and increased in oxidative stress [3,4].

The basic aim of treating hepatitis B patients is to stop viral replication. Antiviral therapy using tenofovir disoproxil fumarate (TDF), also known as Viread, is the mainstay of human immunodeficiency virus (HIV) and hepatitis B virus. As an acyclic nucleotide analog TDF, inhibits viral reverse transcriptase enzyme and prevents the formation of phosphodiester linkage essential for viral DNA chain elongation thus causes the premature termination of viral DNA transcription [5,6].

Evaluation of the genotoxic potential of medicinal drugs is essential to ensure that they are safe for use [7].

Nucleos(t)ide reverse transcriptase inhibitors (NRTIs) may incorporate into nDNA and mtDNA of host cell. Chronic use of NRTIs may be due to genomic damage.

Genotoxic events have been known as crucial step in the initiation of cancer. Genotoxic substances induce damage in cells through interaction with the DNA and can result, including single- and double-strand breaks, cross-links between DNA bases and proteins, and chemical additions to the DNA. The occurrence of genomic damage, if left unrepaired, may result in the formation of DNA adducts, chromosome/chromatid breaks, or aneuploidy and is associated with the formation of micronucleus (MN), sister chromatid exchange (SCE), and overall genomic instability [8]. MN is indicator of genomic instability and cytogenetic damage in dividing cells. Numerous reports based on experiments in vitro and in vivo have described the induction of MN by various NRTIs [9,10]. Only a few in vitro studies of TDF show that TDF has a weak genotoxic potential when tested in different systems [11-14]. Nevertheless, in an in vitro study, tenofovir–emtricitabine combination was found to be more cytotoxic than zidovudine–lamivudine [15]. There is no published data on the induction of genomic damage by TDF in HBV- infected patients. For this reason, we were designed this study to determine the safety and genotoxic outcome data of the antiviral drug TDF in patients with chronic HBV infection as determined by mitotic index (MI) and micronucleus tests (MN)

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Material and Methods

Patients

We studied HBV-infected adult patients (18 to 65 years old), who were treated with TDF therapy at Departments of Infectious Diseases and Gastroenterology. There is institutional ethical approval of the study protocol (2013-144). Patients with a history of alcohol and other chronic medicines consumption, smoking were not included in the study. In addition, exclusion criteria were a history of severe concomitant medical disease, co-infection, concurrent malignancy, and immune-suppression. The HBV patients were divided into three groups as no antiviral therapy group, six months therapy group and 12 months and more therapy groups. Healthy people were enrolled to the study as a control.

Clinical, biochemical, and serologic tests

Long-term persistent HBV surface antigen presence for six months, confirming chronic HBV infection. The control group was confirmed with the absence of HBV infection by serological tests. Clinical, biochemical, serologic tests and, HBV DNA quantitative level and hepatitis B early antigen (HBeAg) status were recorded at the time of HBV diagnosis and on follow up visits.

Chemicals

Cyclophosphamide, 5-Bromo-2-deoxyuridine and colchicine were purchased from Sigma Chemicals. Peripheral Blood Karyotyping Medium was purchased from Biological Industries. Methanol-acetic acid and Giemsa solution was from Merck, India.

MN assay

0.5mL heparinized bloods from patients and control group were added to the Karyotyping Medium and were further incubated at 37°C for 72 hours. The cells from the culture medium at 72nd hour was collected and were treated with hypotonic solution (0.075M KCl) at 37°C for 10 min. The cells fixed with methanol:acetic acid solution and then the dried slides were stained by the Fluorescein plus Giemsa technique. Finally, the slides were examined under a light microscope (Olympus Optical Co., Tokyo, Japan). A total of 1000 cells from each set of slides were scored.

MI assay

To measure cellular proliferation, a total 3000 cells per donor were scored for the determination of MI. For calculation of MI the following formula was used:

$$MI = \frac{100 \times \text{cells in metaphase}}{3000}$$

Statistical analysis

Data were analyzed using a statistical software program (Windows for SPSS version 21). The data are expressed as a median, minimum and maximum. The normality assumption was analyzed using Shapiro-Wilk test and continuous variables showed normal distribution. The significance of differences between the means the MN frequencies among treated cultures and their controls were determined using the Oneway Analysis of Variance test. Least Significant Difference test was applied for multiple comparisons. A value of <0.05 was accepted as significant for two tailed p-values.

Results

In this study, a total of 45 subjects were divided into four groups: group 1 included 10 patients with HBV who are not taking any therapy and had no any history of therapy; group 2 involved 10 HBV patients taking TDF therapy for 6 months; Group 3 consisted of 10 patients with HBV taking TDF therapy for at least 12 months; and group 4 was 15 were healthy control subjects. The median patient age was 44.2 years (range 18-65 years). 63.3% were male. The demographic, virological and serological parameters of all patients were shown on Table 1.

Virological and biochemical responses were similar between the patient groups over time. There was no indication that variables such as HBsAg and ALT levels in these patients treated with TDF. All the patients were matching ratio as of HBeAg-negative and anti-HBeAg positive.

Data on the mean frequency of MN for cells and MI of are presented in Table 2. MI and MN frequencies significantly increased in all the HBV patient groups. Yet, MI and MN frequencies between different time points within each therapy group did not significantly differ statistically ($p > 0.05$).

Table 1. Demographic, serologic and biochemical variables of the groups

Parameters Mean (min-max)	Naïve Patients (No treatment group) n= 10	Treatment group at 6 months n=10	Treatment group at 12 months or more n=10	Control Group n=15
Sex (male/female)	8/2	5/5	6/4	8/7
Age (years)	41.0 (18-58)	46.4 (34-55)	45.3 (29-65)	33.5 (25-43)
HBsAg	3068.3 (1563-5201)	3351.7 (2169-4894)	4210.4 (2523-5248)	-
ALT (IU/L)	32.4 (12-71)	48.3(16-117)	23.4 (10-50)	-
HBV DNA Positive/negative	3/7	1/9	0/10	-
HBe Ag Positive/negative	3/7	4/6	3/7	-
Anti HBe Positive/negative	6/4	5/5	6/4	-

Table 2. The frequency of MN and MI in HBV-infected patients and entreated with TDF for 6 and 12 months.

	MN median (min-max)		MI median (min-max)	
Control	0,100	(0,00-0,40)	2,100	(1,17-4,00)
Group I (non-treatment)	0,200*	(0,00-0,40)	4,400*	(3,33-7,17)
Group II (6 months treatment)	0,350*	(0,20-0,90)	3,450*	(1,60-5,20)
Group III (12 months treatment)	0,200*	(0,10-0,80)	4,300*	(2,70-7,27)

+ A total 1000 cells were scored for the MN assay and 3000 cells were scored for the MI

* significant difference from control

Discussion

In this study, we report the safety and genotoxic outcome data of the antiviral drug TDF in patients with chronic HBV infection. We have shown that TDF has effective virological suppression with biochemical and serological tests in HBV-infected patients.

In our study, we found that MN formation and MI were significantly increased in HBV-infected patients. MN may occur in whole chromosomes or acentric chromosomal fragments that are left behind during cellular division. The MN test can be used to determine the clastogenic and aneugenic effects of mutagens [16]. Some viruses can cause damage to host chromosomes. Some authors [17] previously reported a higher frequency of chromosomal aberrations in HCV/HBV-infected patients. Epidemiological studies have proved a strong correlation between chronic HBV infection and the development of HCC. This association has been examined via exploring some specific viral proteins and genetic instability of host genome. HBV produces HBsAg and hepatitis B virus -X (HBX) oncogenic proteins [18]. The HBX gene, which plays an important role in viral genome regulation, can frequently integrate into the host genome. HBX also effects the centrosome replication process, resulting in the rearrangement of chromosomes with MN [3,19,20]. With regards to genotoxicity the other mechanism is the direct integration of the virus DNA into the host genome. Viral DNA normally can't integrate easily into the host genome, whereas this integration has been found to be frequent (in around 80%) in HCC cases associated with HBV. The integration of HBV into the genome can affect the expression of the genes in the insertion region, altering chromosomal stability and unrepaired DNA damage results in point mutations and large deletions, and is associated with the induction of MN and overall genomic instability [21].

The goal of HBV therapy in HBV-infected patients is to suppress the replication of viral DNA. TDF is the most widely used and most potent drug for the treatment of HBV infection. Kim et al. (2015) [12] demonstrated that the incidence of HCC in patients treated with TDF was lower than expected. Idilman et al (2015) [13] reported that long-term TDF therapy effectively maintained virological and biochemical responses in HBV patients and that long-term suppression of HBV with TDF consistently reduced the risk of developing HCC. Although TDF can effectively suppress HBV replication during therapy, it is difficult to completely eradicate HBV infection because of the persistence of cccDNA in the nuclei of infected hepatocytes and therefore may need to be taken long term. However, some previous studies have demonstrated that various NRTIs become inserted into DNA of host cells, where they stop the extension of the DNA chain and cause single- and double-strand breaks, thereby inducing chromosomal instability, such as SCE, MN and chromosomal aberrations [8-11] and are able to stimulate repair mechanism [14]. Data on TDF genotoxicity are limited. There are a few studies on the genotoxicity of TDF on in vitro systems. Brüning et al.[22] reported that a combination drug of the cytidine analogue emtricitabine and the adenosine analogue TDF, because of mis-incorporation into the host DNA induces DNA damage and cell cycle arrest in human ovarian cancer cells. We did not find a study of the clastogenic potential of TDF using the MN test in HBV-infected human peripheral lymphocytes. In this study, we examined the possible genotoxic and cytotoxic effects of 6 and

12 months of TDF treatment in the lymphocytes of HBV-infected patients. In our study, MN formation and MI were increased in the TDF therapy groups compared to lymphocytes from non-infected healthy individuals. However, statistically significant differences in the frequencies MI and MN were not found between the therapy groups. These data are in agreement with other studies showing that the widespread chronic use of other antiviral drugs have generated concern that some manifestations of drug-limiting toxicity may be due to genomic damage by incorporated into nDNA and mtDNA in host cells, where they arrest DNA chain extension, and cause single- and double-strand breaks, thus inducing chromosomal instability and are able to stimulate an exonucleolytic repair mechanism [11,13]. The effect of TDF on MN frequency could be attributed to the small sample size and short duration of this study. These are the situations limiting this study. It is well known that prolonged HBV infection is a slow progressive process leading to severe liver disease. The long-term viral response to treatment with TDF shows undetectable levels of HBV DNA with 5 years of treatment, and a total of 12% of patients achieve a loss of HBsAg after 7 years of TDF treatment [23]. In addition, host factors including age, gender and family history, as well as viral factors including viral DNA, genotype profile, mutations, and hepatitis B surface antigen (HBsAg) level are associated with the development of these genotoxic complications [24]. In addition, a relative reduction in the frequency of MN in 12 months of treatment compared to 6 months may be due to the repair of damaged genetic material, elimination of cells or chromosomes, or the inactivation of the drug. NRTIs are known to lead to nucleotide pool imbalances, which weakens the functioning of DNA-repair [9, 24]. DNA-repair systems may have converted the initial DNA lesions induced by TDF into large deletions and aberrations that can be detected by genotoxicity test systems. The genotoxic potential of TDF is mainly related to the effects of adenosine nucleotide pool imbalance. A potential link between HBV and antiviral drugs with the DNA repair system has been suggested by several reports. These studies suggest that HBsAg, Hepatitis B core protein and HBX interact with DNA repair proteins and p53.

Conclusion

To our knowledge, this is the first study on the genotoxicity of TDF at therapeutic doses and duration of treatment in HBV-infected patients through the observation of MN formation. This data indicates that sub-chronic HBV infection is quite efficient in inducing genetic damage and cell growth kinetics. We therefore conclude that TDF also has mutagenic properties after 12 months treatment at a therapeutic dose. However, it is necessary to study the administration of TDF in HBV-infected patients for a longer duration of treatment and in a larger sample size to further prove our conclusions.

Competing interests

The authors declare that they have no competing interest.

Financial Disclosure

There are no financial supports

Ethical approval

There is institutional ethical approval of the study protocol (2013-144).

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