European Review for Medical and Pharmacological Sciences 2019; 23: 9093-9098

Effects of lamivudine on cell proliferation of liver cancer and expressions of HBsAg, HBeAg, and MMP-9 in patients

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Abstract. – OBJECTIVE: To explore the effects of lamivudine on cell proliferation of liver cancer and expressions of HBsAg, HBeAg, and MMP-9 in hepatoma cells.

MATERIALS AND METHODS: In the intervention group, HepG2.2.15 cells were cultured with lamivudine at 100, 200, and 300 μmol/L for 24 hours, 48 hours, and 72 hours. In the control group, HepG2.2.15 cells were cultured without lamivudine. MTT assay was used to assess the proliferative activity of cells after the intervention by lamivudine for 24 hours, 48 hours, and 72 hours. ELISA was used to measure the expression levels of HBsAg, HBeAg, and MMP-9 after the intervention by lamivudine for 48 hours and 72 hours.

RESULTS: There was no significant difference between the intervention group and the control group in the proliferation activity of cells ($p>0.05$). After 48 hours and 72 hours of intervention by lamivudine, the expressions of MMP-9, HBsAg, and HBeAg in the control group were statistically lower than those in the intervention groups with lamivudine at 100 μmol/L, 200 μmol/L, and 300 μmol/L ($p<0.05$). The expressions of MMP-9, HBsAg, and HBeAg in HepG2.2.15 gradually decreased with the increase of intervention concentration and intervention time of lamivudine ($p<0.05$).

CONCLUSIONS: Lamivudine cannot directly inhibit the proliferation of liver cancer cells, but it can reduce the expressions of MMP-9, HBsAg, and HBeAg in hepatoma cells, inhibit the replication of HBV disease in hepatoma cells, and suppress tumor growth.

Key Words:
Lamivudine, Liver cancer, HepG2.2.15, Cell proliferation, HBsAg, HBeAg, MMP-9.

Introduction

Liver cancer is one of the most common and most malignant tumors in the world. Because of the increasing number of patients with hepatitis B virus (HBV), which is recognized as a global pathogen of liver cancer, the morbidity of liver cancer is also mounting year by year. Generally, patients with liver cancer have a poor prognosis due to its high recurrence rate and metastasis rate[1-3]. Therefore, exploring an effective treatment for liver cancer has important clinical significance for liver cancer patients.

Chronic HBV infection is an important factor in the development of chronic infection into liver cancer. So, hepatitis B virus surface s antigen (HBsAg) and hepatitis B virus surface e antigen (HBeAg) are closely related to the occurrence and development of liver cancer[4-6]. HBsAg is the most expressed envelope protein in HBV replication. Studies[7,8] have shown that HBsAg can neutralize with antibody to induce immune tolerance, which helps the survival of virus and the progression of HBV infection. HBeAg, a soluble protein in the core particle of hepatitis B virus, which presents later than HBsAg but disappears earlier than HBsAg, is the second serological antigen marker in HBV infection following HBsAg. Matrix metalloprotein-9 (MMP-9) is a gelatinase that efficiently degrades IV, V collagen, and gelatin in the extracellular matrix[9,10]. Researchers[11-13] have shown that MMP-9 is closely related to the growth and metastasis of various tumors, such as liver cancer and breast cancer. Lamivudine, a representa-

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tive drug in nucleotide analogues, can inhibit the DNA strand elongation of HBV during reverse transcription of hepatocytes, and can suppress the development of liver cancer by preventing the proliferation of virus[14].

However, so far little is known about the *in vitro* experiment of lamivudine in hepatoma cells. In order to further clarify the mechanism of inhibition of lamivudine on the development of liver cancer, this study is going to explore the effects of lamivudine on cell proliferation of liver cancer and the expressions of HBsAg, HBeAg, and MMP-9 in hepatoma cells.

Materials and Methods

Experimental Reagents and Materials

Human hepatoma cell line HepG2.2.15 was purchased from Shanghai Institute for Biological Sciences and then frozen. The microplate Reader SpectraMax M5 was purchased from Molecule Devices (Shanghai, China). DMEM medium was purchased from Gibco (Rockville, MA, USA). Fetal bovine serum (FBS), trypsin, and phosphate buffer powder were purchased from Gibco (Rockville, MA, USA). MTT cell proliferation kit and cytotoxicity test kit were purchased from Sigma-Aldrich (Saint Louis, MO, USA). MMP-9 kit was purchased from Shanghai Bogoo Biological Technology Co., Ltd (Shanghai, China). HBsAg and HBeAg test kits were purchased from Beijing North Institute of Biological Technology. Lamivudine tablet was purchased from GlaxoSmith-Kline Investment Co., Ltd. (Beijing, China).

Cell recovery, Culture, Subculture, and Freezing

HepG2.2.15 cells were taken out and cultured in a medium containing 10% fetal bovine serum at 37°C in an incubator with 5% CO_2. Washing with PBS was conducted when 85% of cells were attached. After that, the cells were digested with 1 ml of 25% trypsin, and then the cells were cultured for 48 hours in the culture solution at a concentration of 10% at 37°C, with 5% CO_2, to perform the subculture. The cells in the logarithmic phase were selected for subsequent experiments.

MTT Assay for Cell Proliferation

The freezing HepG2.2.15 cells in logarithmic phase were recovered. Then, the cells were seeded in a 96-well plate with about 3×10^5 cells in each well. The cells were divided into the control group and lamivudine intervention group. In the control group, only the culture medium was added. The lamivudine intervention group was comprised of three groups with different lamivudine concentrations of 100 μmol/L, 200 μmol/L, and 300 μmol/L. After 48 hours of culture, 20 μl of MTT solution was added to the cells to perform the inoculation at 37°C for 4 hours. The supernatant was discarded before 100 μl of dimethyl sulfoxide (DMSO) solution was added. The optical density in each well was measured at a wavelength of 490 nm with a microplate reader, and cell proliferation rates after 24 hours, 48 hours, and 72 hours of intervention were calculated.

Detection of the Expressions of MMP-9, HBsAg, and HBeAg in Cancer Cells by ELISA

The cells and supernatants were collected 48 hours and 72 hours after the lamivudine intervention. The cells were digested with trypsin and centrifuged at 8000 r/min for 3 minutes, and then, the supernatant was discarded. Next, the cells were resuspended with 200 μL of PBS before the detection. The detection of MMP-9, HBsAg, and HBeAg levels was in strict accordance with the ELISA kit instructions.

Statistical Analysis

The statistical analysis of data was performed by SPSS 19.0 software (Bizinsight Information Technology Co., Ltd., Beijing). The measurement data were expressed by the mean ± standard deviation. The comparison between groups was conducted by One Way ANOVA. Repeated measures ANOVA were used for analyzing the comparison between different time points in the group. LSD/t-test was used as the back testing for pairwise comparison. The independent sample *t*-test was used for comparison between the two groups. The paired *t*-test was used for comparison between different time points. All the figures were drawn using GraphPad Prism 6 software. A statistical difference was recognized when $p<0.05$.

Results

The Effect of Lamivudine on the Proliferation of HepG2.2.15 Cells

The activity of cell proliferation of the control group after 24 hours, 48 hours, and 72 hours of drug intervention was (1.13 ± 0.14), (1.36 ± 0.13), and (1.65 ± 0.15), respectively. The activity of

Table I. The effect of lamivudine on the proliferation of HepG2.2.15 cells.

Time	Control group	100 µmol/L	200 µmol/L	300 µmol/L	F	*p*
24 hours	1.13 ± 0.14	1.18 ± 0.31	1.21 ± 0.08	1.21 ± 0.10	0.150	0.927
48 hours	1.36 ± 0.13	1.38 ± 0.14	1.38 ± 0.12	1.37 ± 0.06	0.448	0.726
72 hours	1.65 ± 0.15	1.64 ± 0.16	1.63 ± 0.18	1.62 ± 0.11	0.392	0.762
F	20.95	4.459	7.551	14.95		
p	< 0.05	< 0.05	< 0.05	< 0.05		

cell proliferation of the intervention group with lamivudine at a concentration of 100 µmol/L after 24 hours, 48 hours, and 72 hours of drug intervention was (1.18 ± 0.31), (1.38 ± 0.14), and (1.64 ± 0.16), respectively. The activity of cell proliferation of the intervention group with lamivudine at a concentration of 200 µmol/L after 24 hours, 48 hours, and 72 hours of drug intervention was (1.21 ± 0.08), (1.38 ± 0.12), and (1.63 ± 0.18), respectively. The activity of cell proliferation of the intervention group with lamivudine at a concentration of 300 µmol/L after 24 hours, 48 hours, and 72 hours of drug intervention was (1.21 ± 0.10).), (1.37 ± 0.06), and (1.62 ± 0.11), respectively. There was no significant difference in the cell proliferation activity of human hepatoma HepG2.2.15 cells between different concentrations of lamivudine at the same time point (p>0.05). Check Table I and Figure 1 for details.

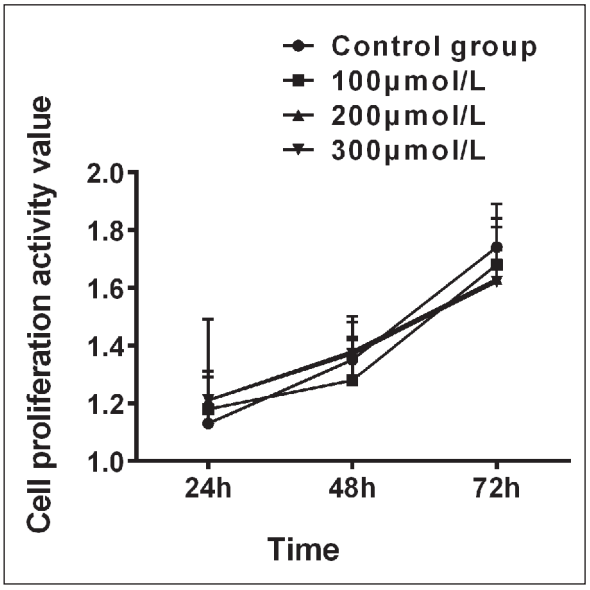

Figure 1. Effect of lamivudine on the proliferation of HepG2.2.15 cells. The cell proliferation activity was detected by MTT assay. The results showed that there was no statistical difference in the cell proliferation activity of human hepatoma HepG2.2.15 cells between different concentrations of lamivudine at the same time point (p>0.05).

Expression of MMP-9 in Each Group

After 48 hours and 72 hours of intervention by lamivudine, the expression of MMP-9 in the control group was statistically lower than that in the intervention group with lamivudine at 100 µmol/L, 200 µmol/L, and 300 µmol/L (p<0.05). The expression of MMP-9 in hepatoma cells gradually decreased with the increase of intervention concentration and intervention time of lamivudine (p<0.05). See Table II and Figure 2 for details.

Expressions of HBsAg and HBeAg in Each Group

After 48 hours of intervention, the expressions of HBsAg in the control group, the intervention group with lamivudine at 100 µmol/L, the intervention group with lamivudine at 200 µmol/L, and the intervention group with lamivudine at

Table II. Expression of MMP-9 in each group (ng/ml).

Time	Control group	100 µmol/L	200 µmol/L	300 µmol/L	F	*p*
48 hours	1.19 ± 0.08	0.98 ± 0.10*	0.81 ± 0.12*	0.69 ± 0.11*	4.413	< 0.05
72 hours	0.87 ± 0.15	0.65 ± 0.08*	0.53 ± 0.11*	0.41 ± 0.09*	4.388	< 0.05
t	3.260	4.463	2.979	3.412		
p	< 0.05	< 0.05	< 0.05	< 0.05		

Note: *p < 0.05 when compared with the control group.

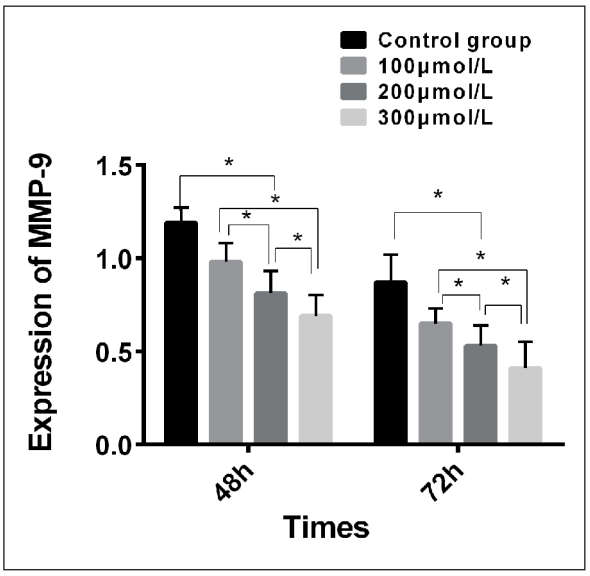

Figure 2. Expression of MMP-9 in each group. According to ELISA results, after 48 hours and 72 hours of intervention by lamivudine, the expression of MMP-9 in the control group was statistically lower than that in the intervention group with lamivudine at 100 μmol/L, 200 μmol/L, and 300 μmol/L (p<0.05). The expression of MMP-9 in hepatoma cells gradually decreased with the increase of intervention concentration and intervention time of lamivudine (p<0.05). Note: *Indicates p<0.05.

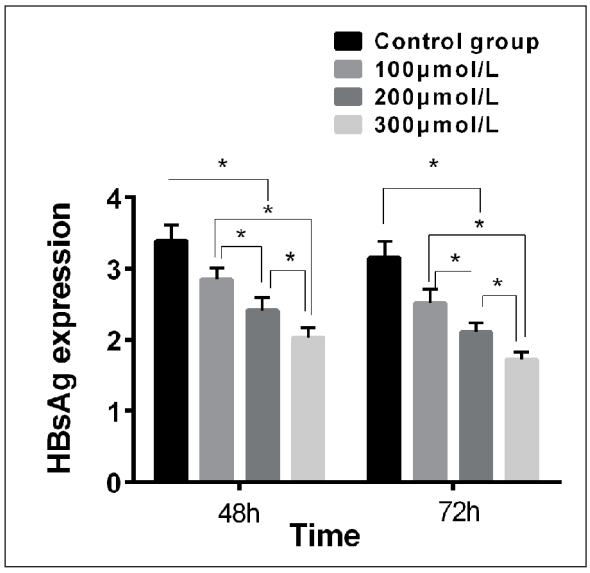

Figure 3. Expression of HBsAg in each group. According to ELISA results, after 48 hours and 72 hours of intervention by lamivudine, the expression of HBsAg in the control group was statistically lower than that in the intervention group with lamivudine at 100 μmol/L, 200 μmol/L, and 300 μmol/L (p<0.05). The expression of HBsAg in hepatoma cells gradually decreased with the increase of intervention concentration and intervention time of lamivudine (p<0.05). Note: *Indicates p<0.05.

300 μmol/L were (3.39 ± 0.22), (2.85 ± 0.16), (2.41 ± 0.18), and (2.03 ± 0.14), respectively; while the expressions of HBeAg were (3.32 ± 0.19), (2.83 ± 0.17), (2.42 ± 0.15), and (2.13 ± 0.11), respectively. After 72 hours of the control group, the expressions of HBsAg in the control group, the intervention group with lamivudine at 100 μmol/L, the intervention group with lamivudine at 200 μmol/L, and the intervention group with lamivudine at 300 μmol/L were (3.15 ± 0.23), (2.52 ± 0.19), (2.11 ± 0.13), and (1.72 ± 0.11), respectively; while the expressions of HBeAg were (3.13 ± 0.22), (2.49 ± 0.13), (2.09 ± 0.14), and (1.85 ± 0.13), respectively. The expressions of HBsAg and HBeAg in human hepatoma HepG2.2.15 cells decreased gradually with the increase of intervention concentration and intervention time of lamivudine (p<0.05). See Figure 3 and Figure 4 for details.

Discussion

Liver cancer, a malignant tumor currently incurable with mounting morbidity due to changes in the environment and living habits, poses a serious threat to human health[15,16]. As a member

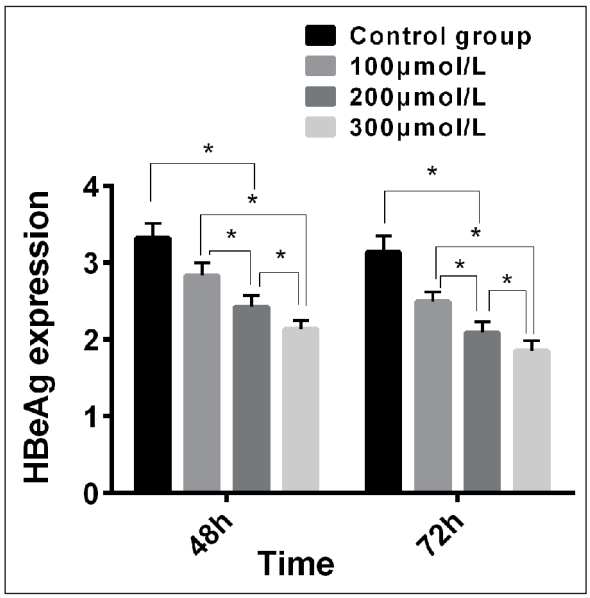

Figure 4. Expression of HBeAg in each group. According to ELISA results, after 48 hours and 72 hours of intervention by lamivudine, the expression of HBeAg in the control group was statistically lower than that in the intervention group with lamivudine at 100 μmol/L, 200 μmol/L, and 300 μmol/L (p<0.05). The expression of HBeAg in hepatoma cells gradually decreased with the increase of intervention concentration and intervention time of lamivudine (p<0.05). Note: *Indicates p<0.05.

of the matrix metalloproteinase family, MMP-9 can cause a reduction of cell adhesion when it is upregulated, thereby promoting the invasion and metastasis of tumor cells[17]. Because of the ability of MMPs family to promote the expression of vascular endothelial growth factor and the formation of new tumor blood vessels, MMPs are regarded as important targets in anti-tumor therapy[18]. Both HBsAg and HBeAg are closely related to HBV-DNA and are important indicators that reflect the replication and infection of the virus, as well as risk factors for poor prognosis of liver cancer[19]. Hann et al[20] showed that antiviral therapy has a remarkable efficacy for hepatitis-related liver cancer. They study conducted an *in vitro* experiment on the effect of lamivudine, a typical nucleoside antiviral drug, on the cell proliferation of liver cancer and the expressions of MMP-9, HBsAg, and HBeAg in hepatoma cells.

The current study selected HepG2.2.15 cell line as the experimental model to evaluate the effect of lamivudine. The results showed that there was no significant difference in the proliferation activity of HepG2.2.15 cells in different concentrations of lamivudine ($p>0.05$), which indicated that lamivudine did not directly inhibit the proliferation of liver cancer cells. Boyd et al[21] investigated the effect of lamivudine on liver cancer cells; and found that lamivudine cannot directly inhibit the proliferation of liver cancer cells, instead, it indirectly affected the cell proliferation through its inhibition of the virus. Such finding is consistent with our study. In order to investigate the effect of lamivudine on HBV-associated virus in hepatoma cells *in vitro* and its possible mechanism of action on hepatoma cells, this work measured the expression levels of MMP-9, HBsAg, and HBeAg in hepatoma cells after 24 hours, 48 hours, and 72 hours of lamivudine intervention. The expression levels of MMP-9, HBsAg, and HBeAg in the control group after 48 hours and 72 hours of lamivudine intervention were statistically lower than those in the intervention groups with lamivudine at 100 μmol/L, 200 μmol/L, and 300 μmol/L ($p<0.05$). The expression levels of MMP-9, HBsAg, and HBeAg in hepatoma cells decreased gradually with the increase of intervention concentration and intervention time of lamivudine ($p<0.05$). Such results suggest that lamivudine has a significant inhibition on HBV viral replication and MMP-9 expression in hepatoma cells. Scholars[22,23] discovered that lamivudine had a significant inhibition on HBsAg and HBeAg expressions in HepG2.2.15 cells, and gave an explanation for its action of mechanism that the phosphorylation of lamivudine in HepG2.2.15 cells enabled lamivudine to compete with deoxycytidine triphosphate (dCTP) for antiviral activity in the replication of HBV virus. This also helps explaining some of the results of this study. Researches on the effect of lamivudine on the expression of MMP-9 in hepatoma cells are rare. Zhang et al[24], believed that the upregulation of MMP-9 gene transcription may be triggered by the mutation of tumor suppressor p53. Wang et al[25] stated that MMP-9 and p53 mutations are closely related to the occurrence and development of liver cancer. So we speculated that lamivudine's inhibition on the occurrence and development of liver cancer may be achieved through its effect on the expressions of p53 and MMP-9.

Conclusions

In summary, lamivudine can not only reduce the expression levels of MMP-9, HBsAg, and HBeAg in hepatoma cells, but also inhibit the replication of HBV disease in hepatoma cells and tumor growth. However, lamivudine cannot directly suppress the proliferation of liver cancer cells. So we suggested that lamivudine controlled the expression levels of MMP-9, HBsAg, and HBeAg through an unclear mechanism and hence inhibited the invasion and metastasis of hepatoma cells. We will conduct an in-depth exploration of the mechanism of lamivudine in subsequent experiments.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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