SPECIFIC INHIBITION OF HEPATITIS B VIRUS GENE EXPRESSION BY AN ANTISENSE OLIGONUCLEOTIDE *IN VITRO*

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Summary. – It was previously shown that a number of antisense oligonucleotides against hepatitis B virus (HBV) mRNAs were highly effective in inhibition of HBV gene expression (Yao *et al.*, 1995). Here, using radioisotope techniques, we report a specific inhibition of HBV surface antigen (HBsAg) production *in vitro* by 2.2.15 cells (Hep-G2 cells transfected with HBV genome) by the antisense oligonucleotide 15-S-asON, a 15-mer phosphorothioate analogue complementary to the cap site of the SPII promoter of HBV mRNA, at a concentration of 2 – 5 μmol/l. After 24 and 48 hrs of incubation of cells with 15-S-asON, the intracellular concentration of the latter rose to 69.4 and 75.8 nmol/l, respectively, and the HBsAg level assayed by ELISA was reduced by 50.0% and 70.6%, respectively. These results were checked by use of the radioimmunoprecipitation method: 2.2.15 cells exposed to 15-S-asON and labelled with [^{35}S]-methionine for 48 hrs showed a decrease of the HBsAg level by 81.26% but almost none of the total proteins. No cytotoxicity of the 15-S-asON was observed with regard to the cell morphology and growth. These results indicate that the tested antisense oligonucleotide specifically inhibits the HBV gene expression.

Key words: hepatitis B virus; Hep-G2 cells; antisense oligonucleotide; inhibition of gene expression

Introduction

HBV infection is a major world-wide cause of acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma which have a wide range of severe clinical manifestations. At present, the vaccination is the only protective measure against HBV infection. However, approximately 300 million carriers of HBV are at high risk of chronic liver diseases. The search for a therapeutic agent that could effectively inhibit HBV infection of the millions of people who become infected every year has remained open. In the past few years, several antiviral strategies designed to terminate chronic HBV infection have been studied. Among them, α-interferon has been shown to be useful with a clinical, biochemical and serological remission in 30 – 40% of highly selected patients (Perillo *et al.*, 1990). To date, no satisfactory medical treatment of chronic HBV infection is available. Thus other therapeutic strategies need be explored. Antisense oligonucleotide strategies have been employed in a variety of systems both to understand normal gene function and to block gene expression therapeutically. This concept has been successfully applied to inhibit influenza virus (Zerial *et al.*, 1987), Rous sarcoma virus (Stephenson and Zamecnik, 1978), human immunodefficiency virus (Zamecnik *et al.*, 1986; Agrawal *et al.*, 1989), vesicular stomatitis virus (Lemaitre *et al.*, 1987), herpes simplex virus (Smith *et al.*, 1986), hepatitis B virus (Goodarzi *et al.*, 1990; Blum *et al.*, 1991; Wu and Wu, 1992), and hepatitis C virus (Wakita and Wands, 1994). Here, we report on an antisense oligodeoxyribonucleotide specifically inhibiting the HBV gene expression as evidenced with radioisotope techniques.

Abbreviations: ELISA = enzyme-linked immunosorbent assay; HBsAg = HBV surface antigen; HBV = hepatitis B virus

Materials and Methods

^{32}P-labelled antisense oligodeoxyribonucleotide. A 15-mer phosphorothioate analogue (15-S-asON), complementary to a portion of the cap site of mRNA transcribed from the SPII promoter (corresponding to nucleotides 3188-3202 of the human HBV DNA) was synthesized on an automated DNA synthesizer. The phosphorothionate analogues are known to be more resistant to nucleases and taken up by cells more efficiently than unmodified deoxyribonucleotides. To evaluate the uptake of 15-S-asON by cells, it was end-labelled with ^{32}P (Sambrook *et al.*, 1989).

A 16-mer oligodeoxyribonucleotide with random sequence was synthesized and used in control experiments.

Experimental protocol. HepG2 cells transfected with cloned HBV DNA (2.2.15 cells) were used in the experiments. An aliquot of 2 x 10^5 cells was seeded into each well of the 24-well culture plate (Nunclon). Later, the medium was changed for 10 μg (2 μmoles) of 15-S-asON in 1 ml of culture medium. The latter consisted from Eagle's Minimal Essential Medium supplemented with 10% foetal bovine serum and 380 μg/ml G418. The incubation proceeded at 37 °C in humidified atmosphere of 5% CO_2 in air. At given time intervals the culture supernatant was saved, the cells were removed from wells, washed 5 times in cold saline and used for various assays (viable cell count, 15-S-asON uptake, viral DNA). The culture supernatant was used for HBsAg assays by ELISA or radioimmunoprecipitation. All biological experiments as well as the assays were performed in triplicate and the results were expressed as means ± SE.

Uptake of 15-S-asON by cells was measured by adding ^{32}P-labelled 15-S-asON (10 μCi or 2 μmoles per well) to the culture medium and assaying the radioactivity of samples taken at various time intervals by scintillation counting.

ELISA of HBsAg in culture medium was done by use of a commercial kit (Sino-American Biotechnology Co.) following the protocol of the manufacturer.

Radioimmunoprecipitation assay of HBsAg. In the experiments on the effect of 15-S-asON on the HBsAg expression to the culture medium was added also 10 μCi per well of [^{35}S]-methionine (Chinese Medical Scientific Academic Radio-Institute, specific activity 1000 Ci/mmol, 1 mCi/ml). After 48 hrs incubation the saved culture medium was treated with a specific mouse anti-HBsAg monoclonal antibody (obtained from the Biological Institute of the Fourth Military Medical University) and precipitated with protein A-Sepharose (Sigma). The radioactivity of precipitates was measured by scintillation counting and the results were expressed as means ± SE in cpm/well.

Radioimmunoprecipitation assay of total proteins was done similarly as described for HBsAg, just 10% trichloroacetic acid was employed for precipitation.

Dot-blot hybridization of viral DNA. HBV DNA was extracted from cells according to the method of Sells *et al.* (1987). A ^{32}P-labelled HBV DNA probe was employed and the relative quantification was achieved by densitometric scanning.

Viable cell count was done microscopically after trypan blue staining.

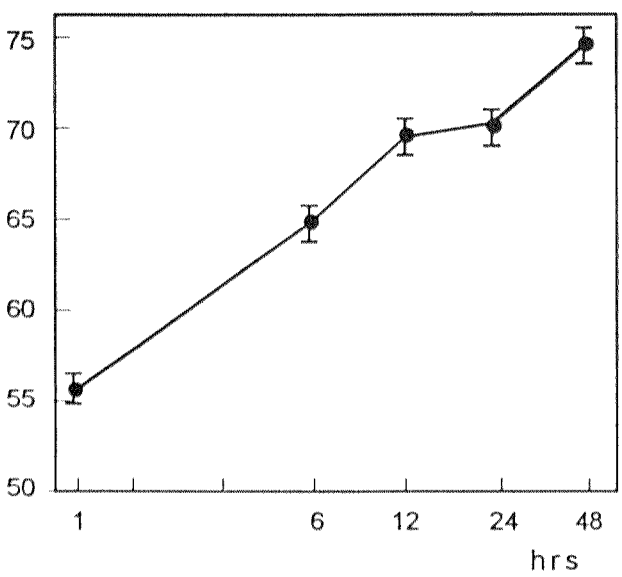

Fig. 1
Uptake of 15-S-asON by 2.2.15 cells
Ordinate: nmoles of 15-S-asON taken up by 2 x 10^5 cells. The concentration of 15-S-asON was 2 μmol/l.

Results and Discussion

Uptake of 15-S-asON by cells

The cells were exposed to ^{32}P-labelled 15-S-asON (10 μg/ml or 2 μmoles/ml) in culture medium at 37°C and after 1, 6, 12, 24 and 48 hrs the radioactivity of the cells was measured. Fig. 1 shows that the uptake of the antisense oligonucleotide by the cells increased with the exposure time and reached in average 69.4 and 75.8 nmoles per 2 x 10^5 cells at 24 and 48 hrs, respectively.

Inhibition of HBsAg expression by 15-S-asON

In the experiment depicted in Fig. 1, simultaneously the content of HBsAg in culture medium was estimated by ELISA. The results (Table 1) show that whereas control cells produced a high amount of HBsAg into medium at 24 – 48 hrs, the cells exposed to 15-S-asON produced significantly less HBsAg, namely amounts reduced by 50.0% and 70.6, respectively.

Specificity of inhibitory effect of 15-S-asON on HBsAg

To exclude the possibiliy that lower HBsAg content of the medium under the influence of the antisense oligonucleotide was not caused by a block in protein secretion from the cells, we investigated the synthesis of HBsAg by use of labelled methionine and radioimmunoprecipitation assay, in which specific anti-HBsAg monoclonal antibodies were employed. Table 2 shows that the labelled immunoprecipitable HBsAg in the medium under the influence of 15-S-asON was reduced at 48 hrs by 81.26%. On the other hand, total proteins were synthesized at a slightly reduced rate only. Altogether, these data indicate that the observed inhibition of HBsAg synthesis by the antisense oligonucleotide was specific and could not have been due to a generalized inhibition of total protein synthesis.

Table 1. Inhibition of HBsAg expression by 15-S-asON in 2.2.15 cells

Incubation (hrs)	HBsAg[a]		Inhibition rate
	15-S-asON[b]	Control	
24	4.60 ± 0.25	7.10 ± 0.21	50.0%
48	3.60 ± 0.24	7.20 ± 0.21	70.6%

[a] HBsAg in the medium was estimated by ELISA. The results are expressed as P/N values.

$$\text{Inhibition rate} = \frac{\text{control} - \text{experiment}}{\text{control} - 2.1} \times 100$$

[b] The concentration of 15-S-asON was 2 μmol/l.

Table 2. Effect of 15-S-asON on HBsAg and total protein synthesis in 2.2.15 cells after 48 hrs of incubation

	HBsAg (cpm/well)	Total protein (cpm/well)
15-S-asON[a]	12.143 ± 1.126	577.140 ± 7.749
Control	64.789 ± 2.944	618.562 ± 9.268
Inhibition rate[b]	81.26%	6.70%

[a] The concentration of 15-S-asON was 5 μmol/l.

[b] $\text{Inhibition rate} = \frac{\text{control} - \text{experiment}}{\text{control}} \times 100$

Effect of 15-S-asON on the viability of cells

The viability of 2.2.15 cells was estimated after 24 and 48 hrs of incubation with the antisense oligonucleotide (10 μg/ml) or the control one. The results show (Table 3) that the viable cell counts were similar (the differences were non-significant) in both the experimental and control series. Thus 15-S-asON in the tested concentration was not cytotoxic for 2.2.15 cells.

Table 3. Effect of 15-S-asON on the viability of 2.2.15 cells

Incubation (hrs)	Viable cell count (x 10^5/well)		p value of difference
	15-S-asON[a]	Control	
24	3.13 ± 0.03	3.50 ± 0.36	>0.05
48	3.13 ± 0.06	3.07 ± 0.23	>0.05

[a] The concentration of 15-S-asON was 2 μmol/l.

Because of the specificity of formation of DNA-mRNA hybrids implicated in the antisense DNA-mediated inhibition of translation, the antisense oligonucleotides strategy has been successfuly used in studies on normal gene expression *in vitro*. For similar reasons, antisense oligonucleotides have also been employed in anti-viral and anti-tumor therapies. E.g., Blum *et al.* (1991) have reported that a cotransfection with HBV DNA and the antisense oligonucleotide ATC-40 completely blocked the HBsAg and HBeAg syntheses as well as the HBV replication. Agrawal *et al.* (1989) following an addition of infectious human immunodefficiency virus together with an antisense oligonucleotide to cell culture medium have demonstrated a specific inhibition of virus replication. Lemaitre *et al.* (1987) have observed a substantial specific antiviral effect of an antisense oligonucleotide in a model of acute viral infection.

Our experiments presented here differ form those mentioned above in the fact that the cells used by us contained a pre-existing, stable viral genome. Our data indicate that although a stable infection was established in the cells under study, the application of an antisense oligonucleotide could dramatically inhibit viral gene expression an a specific manner. However, *in vivo*, a persistent production of HBV is usually due to the presence of integrated HBV DNA. Integration of the viral gene into that of the host is usually associated with a cessation of production of complete viral particles. Whether antisense oligonucleotides can be effective in the case of the infection generated by unintegrated viral DNA remains to be investigated.

In our experiments, the specific inhibition of HBsAg expression was found by both the ELISA and radioimmunoprecipitation method. However, the exposure of 2.2.15 cells to 15-S-asON for 24 – 48 hrs had no significant effect of HBV DNA synthsis as measured by the dot-blot hybridization method (data not shown). It is generally believed that antisense oligonucleotides anneal to their target sequences on mRNAs and so block their interactions with transcription factors and/or ribosomal RNA, or they cause degradation of the DNA/RNA hybrids by RNase H. Thus, in this way they inhibit the gene expression at the level of translation. Our results are consistent with this hypothesis. Perhaps, if 15-S-asON were applied several times at certain time intervals in our system, also the viral DNA synthesis might be inhibited through a block of formation of certain viral proteins or enzymes. Further experiments are needed to answer this question.

It should be mentioned that the oligonucleotide used in the presented work is linked by phosphorothioate bonds which are known to be more susceptible to nuclease degradation than normal phosphodiester bonds. Besides this modification, a variety of other synthetic strategies has been developed to confer nuclease resistance to antisense nucleotides. Further, to deliver these analogues specifically to liver cells, the work is in progress to encapsidate them into liposomes or L-polylysine conjugated with an asialoglycoprotein (ASGP). The latter can be specifically targeted

to liver cells via a cell surface-associated ASGP receptor unique to hepatocytes (Wu and Wu, 1992). Finally, the potential of triple-helix-forming oligonucleotides as inhibitors of HBV are explored as a further antiviral strategy.

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