INHIBITION OF HEPATITIS B VIRUS *IN VITRO* BY ANTISENSE OLIGONUCLEOTIDES

Z.Q. YAO, Y.X. ZHOU, J. GUO, Z.H. FENG, X.M. FENG, C.X. CHEN, J.Z. JIAO, S.Q. WANG*

Department of Infectious Diseases, Tangdu Hospital, The Fourth Military Medical University, Xi'an 710038; *Institute of Radiation Medicine, Peking, P.R. of China

Received November 2, 1995; revised February 13, 1996

Summary. – A series of antisense phosphorothioate oligodeoxynucleotides against hepatitis B virus (HBV) were synthesized and evaluated for their antiviral effect in Hep-G_2 cells transfected with HBV genome. The inhibitory effect of the tested antisense oligonucleotides was sequence-specific, dose- and time-dependent, and synergistic for certain combinations. In virus-inhibitory concentrations the oligonucleotides were harmless to 2.2.15 cells. The most effective antisense oligonucleotides were found directed against the HBV mRNA transcribed from the cap site of SP II promoter, the portion of polyadenylation signal and the initiation region of gene S, with an inhibition of the HBsAg and HBeAg production by 85 – 95% and 50 – 60%, respectively. To our surprise, antisense oligonucleotides directed against three key sites of HBV X gene blocked the expression of HBsAg, HBeAg and HBxAg. This fact might be related to the trans-activation of HBV X protein. Using radioisotope labelling, we demonstrated that Lipofectin promoted the cellular uptake and antiviral effect of antisense oligomers in 2.2.15 cells. These results suggest a therapeutic potential of antisense oligonucleotides in the treatment of patients chronically infected with HBV.

Key words: hepatitis B virus; antisense oligonucleotides; antiviral effects *in vitro*

Introduction

HBV infection is a major cause of acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, which have a wide range of severe clinical manifestations. It is estimated that more than 2000 million persons of the world population have been infected till now, 350 million persons are virus carriers, and 1 – 1.5 million persons die each year from the acute and chronic sequelae of HBV infection, making it one of the major cause of morbidity and mortality world-wide. However, vaccination against HBV is at present the sole protective measure available against HBV infection. The search for a therapeutic agent that could effectively inhibit HBV infection still proceeds. Recently, several antiviral strategies of termination of chronic HBV infection have been proposed and tested. Among them, interferon-alpha has been shown to be useful with a clinical, biochemical and serological remission in 30 – 40% of highly selected patients. To date, no satisfactory medical treatment of chronic HBV infection is available. Therefore, other therapeutic strategies have to be explored.

Pharmacologically, "code blockers" are ideal agents for antitumor and antiviral treatment because of their specific mode of action. Recently, 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 in inhibiting influenza virus (Zerial *et al.*, 1987), Rous sarcoma virus (Stephenson *et al.*, 1978), vesicular stomatitis virus (Degols *et al.*, 1989), herpes simplex virus (Smith *et al.*, 1986), human immunodeficiency virus (Agrawal *et al.*, 1988; Zelphati *et al.*, 1994), hepatitis B virus (Blum *et al.*, 1991; Wu and Wu, 1992) and hepatitis C virus (Wakita and Wands, 1994). We have previously shown (Yao *et al.*, 1995 a, b) that a number of antisense oligonucleotides against HBV mRNA were effective in inhibition of viral gene expression both *in vitro* in 2.2.15 cells and *in*

Abbreviations: ELISA = enzyme-linked immunosorbent assay; HBV = hepatitis B virus; ORF = open reading frame

vivo in nude mice bearing 2.2.15 tumor. Here, we report a characteristic of antisense oligonucleotides inhibiting of HBV replication and gene expression.

Materials and Methods

Phosphorothioate oligodeoxynucleotides were synthesized in an automated synthesizer (391 PCRMATE EP, ABI). We chose 10 different target sites for antisense blocking. The oligonucleotides, their target sites and complementary sequences in the HBV genome are shown in Table 1. A 21-mer sense oligonucleotide and a 16-mer oligonucleotide with random sequence were synthesized as controls. To measure the uptake of an oligonucleotide and its lipid complex, asON1 was end-labelled with ^{32}P-phosphate and then left to react spontaneously with Lipofectin Reagent to form a lipid-DNA complex. Two kinds of galactose-terminal glyco-poly-L-lysine were also synthesized as carriers of the antisense oligonucleotides.

Antiviral experimental protocol. To assay a potential inhibitory activity of an oligonucleotide on HBV gene expression, we used Hep-G$_2$ cells transfected with cloned HBV DNA (2.2.15 cells). Briefly, 2.2.15 cells (1.0 x 10^5 cells/well) were seeded onto a 24-well culture microplate and grown in Eagle's Minimal Essential Medium (MEM) supplemented with 10% foetal bovine serum (Sigma) and 380 μg/ml geneticin (Gibco) at 37 °C in a moist 5% CO_2 atmosphere for 10 days. Different oligonucleotides in various concentrations were then added to the cultures. The supernatant was taken at different times for assays.

HBsAg and HBeAg assays were done with commercial ELISA kits of the Sino-American Biotechnology Co. The results of ELISA were calculated as ratios of mean positive/negative (P/N) values. The inhibition was expressed as the inhibitory rate as follows:

$$\frac{(\text{Mean P/N of control group}) - (\text{Mean P/N of treated group})}{(\text{Mean P/N of control group}) - 2.1} \times 100\%$$

Southern blot analysis. For HBV-DNA detection, the supernatant of several wells after 10 days of incubation was pooled, HBV-DNA was extracted according to Offensperger *et al.* (1993) and identified by Southern blot analysis using digoxigenin-labelled HBV probe.

Cell count, total and viable, was estimated by standard procedures using Trypan Blue.

Results and Discussion

Sequence-specific effect

As shown in Table 1, the degree of inhibition by antisense oligonucleotides varied depending on the target site. The oligonucleotides directed against the HBV mRNA transcribed from the cap site of SP II promoter (asON1), from the portion of plyadenylation signal (asON2) and from the initiation region of gene S (asON5) showed high degree of inhibition of HBsAg (by 85 – 90%) and HBeAg (by 50 – 60%), while asON3, complementary to poly(A)-signal of HBV-DNA, showed a medium blocking activity only. In contrast, the oligonucleotide directed against the middle site of gene S (asON6) had little effect on the expression of HBsAg and HBeAg similarly to the sense control (asON10) and the non-complementary random control. These results are consistent with the previous notion that antisense oligonucleotides are, in general, most effective

Table 1. Inhibition of HBV gene expression by antisense oligonucleotides complementary to different target sites

Oligonucleotide	Target site	Complementary sequence in HBV	Inhibitory rate (%)		
			HBsAg	HBeAg	HBxAg
asON1	Cap site/SP II	3188-3202	90	60	
asON2	Poly-A signal/mRNA	1903-1923	89	61	
asON3	Poly-A signal/DNA	1903-1923	63	42	
asON4	Pre-S$_2$ region	114-126	81	50	
asON5	Initiator/gene S	149-163	86	49	
asON6	Middle site/gene S	552-556	16	18	
asON7	Key site1/gene X	1510-1530	61	53	62
asON8	Key site2/gene X	1555-1581	53	45	60
asON9	Key site3/gene X	1768-1791	62	51	68
sON10	Sense oligonucleotide (control)	1510-1530	10	14	13
ON11	Random oligonucleotide	?	8	3	

10-day-old preconfluent cultures of 2.2.15 cells were incubated with various oligonucleotides (15 μmol/l) at 37°C for 2 days. Then HBsAg, HBeAg and HBxAg were assayed by ELISA in the culture supernatants.

Table 2. Inhibition of HBV gene expresion by antisense oligonucleotides in different concentrations

Oligonucleotide	HBsAg inhibition (%)						HBcAg inhibition (%)					
	1.0	2.5	5.0	10.0	15.0	20.0	1.0	2.5	5.0	10.0	15.0	20.0
			(μmol/l)						(μmol/l)			
asON1	40	51	86	88	90	88	16	26	46	59	60	66
asON5	45	55	78	86	86	89	18	25	40	49	49	54
asON7			35	49	61	65			30	40	53	62
asON8			29	42	53	61			25	37	45	50
asON9			38	52	62	69			28	39	51	57

For legend see Table 1.

when directed against the key transcriptinal regulatory site or translational initiation site.

The HBV genome contains four open reading frames (ORF), three of which code for surface (S), core (C) and DNA polymerase (P) proteins. The fourth ORF codes for the smallest protein of 154 amino acids (X protein). It has been reported that the X gene product of HBV is a transactivitor of various cellular and viral promoters and enhancers. Further study of the X protein-responsive element has indicated that the amino acid sequences 46-52, 61-69 and 132-139 of HBV X protein, which are highly conserved among hepadnaviruses, were found to be essential for the transactivation function (Arii *et al.*, 1992). Here, we used the antisense strategy to block these three target sites. To our surprise, antisense oligomers directed against the three key sites of HBV X gene blocked the expression of HBsAg, HBeAg and HBxAg, which suggested that the X gene may play an important role in viral replication and expression.

Dose-related effect

As shown in Table 2, the inhibitory effect of antisense oligonucleotides on the HBsAg and HBeAg expression showed a dose-dependent manner between concentrations of 1.0 – 20.0 μmol/l.

Table 3. Inhibition HBV gene expression by antisense oligonucleotides after different incubation times

Oligonucleotide (15 μmol/l)	HBsAg inhibition (%)					HBcAg inhibition (%)				
	2	4	6	8	10	2	4	6	8	10
	(days)					(days)				
asON2	61	89	94	95	95	41	61	64	79	74
asON3	42	63	67	65	68	35	42	39	47	43
asON4	55	81	89	88	88	37	59	58	61	59

10-day-old preconfluent cultures of 2.2.15 cells were incubated with various oligonucleotides at 37°C for different time intervals. After 1, 4, 6, 8 and 10 days of incubation HBsAg and HBcAg were assayed by ELISA in culture supernatants. The medium with an oligonucleotide was changed for a fresh one every 2 days.

Time-dependent effect

Using the antisense oligonucleotide directed against the cap site of SPII promoter (asON1), the duration of the inhibitory effect was examined. asON1 was applied to cells in two concentrations, 5.0 μmol/l and 10.0 μmol/l. The expression of HBsAg and HBeAg was subsequently assayed at 2-day-intervals. The results showed that inhibitory effect of single treatment lasted for 5 days and then began to decline gradually. However, the inhibitory effect did not diminish to the control level at least within 15 days after the administration of asON1. This was particularly apparent at its higher concentration. A high level of inhibition could be seen with a repeated addition of antisense oligonucleotides in the same concentration of 15 μmol/l at 2-day-intervals (Table 3).

The results of Southern blot detection of HBV DNA were in accord with those of HBV antigens detection mentioned above.

Effect of complexing oligonucleotide with liposome

It was reasonable to assume that a prerequisite for an inhibitory activity of an oligonucleotide is its uptake by cells. Therefore the uptake of one of the tested compounds, asON1, by 2.2.15 cells was subjected to study. For this purpose the ^{32}P-labelled asON1 was prepared. Since it is known that the uptake of substances by cells in general can be improved by complexing them with lipids, we prepared also an ^{32}P-asON1-Lipofectin complex and followed its uptake by cells, as well as its inhibitory effect on HBsAg expression in the same cells.

The cell-bound ^{32}P was assayed in 2.2.15 cells treated with asON1 (2.0 μmol/l) and asON1-Lipofectin (the same concentration of asON1) for 1 – 48 hrs. The cell-bound radioactivity was assayed by scintillation counting. Fig. 1 shows that the average uptake of asON1 increased along

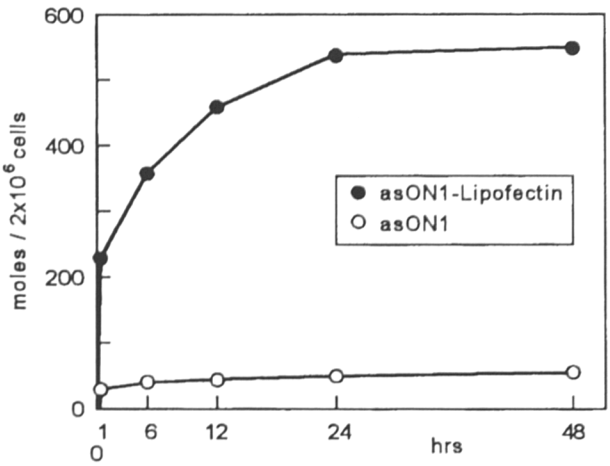

Fig. 1
Uptake of asON1 and asON1-Lipofectin by 2.2.15 cells
10-day-old monolayers of 2.2.15 cells were incubated with ^{32}P-asON1 (2.0 μmol/l) and ^{32}P-asON1-Lipofectin (2.0 μmol/l asON1) at 37°C and after 0, 1, 6, 12, 24, and 48 hrs the monolayers were washed and assayed for ^{32}P-radioactivity by scintillation counting.

with the incubation time. The concentrations of lipid-encapsulated asON1 in cells (560 and 590 nmoles at 24 and 48 hrs, respectively) were much higher than those of asON1 alone (69.4 and 75.8 nmoles at 24 and 48 hrs, respectively). In this experiment, the culture supernatants were also assayed for HBsAg by ELISA. The inhibitory effects of asON1-Lipofectin complex at 24 and 48 hrs (78% and 89%) were much higher than those of asON1 alone (50% and 70%). These results suggest that the liposome technique can improve the antiviral activity of antisense oligonucleotides and that the uptake of a substance by a cell is a prerequisite of its antiviral effect.

Two kinds of galactose-terminal glyco-poly-L-lysine that can be bound and internalized specifically by asialoglycoprotein receptors at cell surface that are unique to hepatocytes were used in this study. These glyco-poly-L-lysines can bind the tested oligonucleotides in a strong manner based on electrostatic attraction. The uptake of the two asialoglycoprotein-modified asON2, which had been fluorescein-labelled, by 2.2.15 cells was assayed by flow cytometric analysis. At the same time the inhibition of HBV gene expression was followed (data not shown). The results showed that the synthesized asialoglycoproteins could deliver well asON2 into 2.2.15 cells. Whereas asON2 (2.5 μmol/l) alone inhibited the expression of HBsAg and HBeAg by 58 – 62% and 38 – 42%, respectively, the asialoglycoprotein-asON2 inhibited the expression of HBsAg and HBeAg by 82 – 88% and 57 – 62%, respectively, which suggests that the asialoglycoprotein carrier system could improve the anti-HBV activity of asON2. In another experiment we injected this fluorescein-labelled asialoglycoprotein-asON2 complex into mice by intraperitoneal route. After 8 hrs, we observed that asON2 was specifically delivered to liver. However, the non-complexed asON2 was distributed in every organ and tissue. These results imply that the asialoglycoprotein delivered asON2 specifically to liver *in vivo*.

Synergistic effect

An increased inhibitory effect was observed when asON1 and asON5 were used together at a concentration of 1.0 μmol/l (inhibition of HBsAg by 72%, and of HBeAg by 33%) as compared with their effect when used alone (shown in Table 1). A simultaneous application of two or more antisense oligonucleotides acting synergistically represents a promising strategy for *in vivo* systems too.

Effect on cells

A relative non-toxicity of the antisense oligonucleotides of concern for cells was demonstrated in several ways. (1) By ^{35}S-methionine incorporation assay, we found that the radiolabelled immunoprecipitable HBsAg in the medium after treatment with antisense oligonucleotides was sharply reduced, while neither antisense nor random oligonucleotides had a significant effect on total newly synthesized protein in the medium (data not shown); (2) Although an antisense oligonucleotide was able to block the HBsAg and HBeAg production by 86% and 55%, respectively, the same drug in the same concentration had little effect on the expression of AFP (Yao *et al.*, 1995c); (3) We have established an animal model producing HBV markers in athymic nude mice by sc administration of 2.2.15 cells (HBV-infected nude mice bearing 2.2.15 tumor). Antisense oligonucleotides were injected by infiltration into or around the transplanted tumor in a daily dose of 20 μg per gram of body weight. Such a treatment for a total of 10 days resulted in an effective inhibition of HBV replication and gene expression *in vivo*. However, no pathologic changes were found by gross and microscopic examination of liver, spleen, kidney, lung, heart, and brain of the treated mice (data not shown); (4) The growth of 2.2.15 cells was not affected by treatment with an antisense oligonucleotide (Table 4). The cells remained viable throughout the experiment and no morphological abnormalities and

Table 4. Effect of antisense oligonucleotide asON1 on propagation of 2.2.15 cells

Incubation time (hrs)	Cell count		
	Trated cells	Control cells	P value
24	3.13 ± 0.03	3.5 ± 0.36	> 0.05
48	3.13 ± 0.06	3.1 ± 0.02	> 0.05

Cell count = (mean ± S.E.) x 10^5/well.

cytotoxicity were observed with concentrations of oligonucleotide below 20 μmol/l.

In conclusion, all these results indicate that the antisense obligodeoxynucleotides specificity targeted to HBV genome had high antiviral activity and no cytotoxicity, which suggests a therapeutic potential of these substances for the treatment of patients chronically infected with HBV.

Acknowledgements. This work was supported in part by grant No. 39200116 of the National Natural Science Foundation of China.

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