BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 217–223 (1996)
Article No. 0038

In Vivo Inhibition of Hepatitis B Virus Gene Expression by Antisense Phosphorothioate Oligonucleotides

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Received December 1, 1995

While an important goal of treatment for hepatitis B is to prevent the development of hepatocellular carcinoma, there has been no effective therapy for it. Antisense oligodeoxynucleotide treatment could in principle inhibit hepatitis B virus gene expression and suppress tumor development. We used a mouse model for hepatocellular carcinoma, which is transgenic for the hepatitis B virus HBx gene, to study antisense phosphorothioate oligodeoxynucleotides. Among 2 series of sense and antisense oligodeoxynucleotides, only antisense sequences covering the initiation codon of the HBx gene effectively inhibited the expression of the HBx gene in the liver. Intraperitoneal injection of this antisense oligodeoxynucleotide thrice a week for 8 weeks resulted in the prevention of preneoplastic lesion development in the liver without inflammation in the liver or developmental disturbance of the mice. Antisense phosphorothioate oligodeoxynucleotides can inhibit the expression of a hepatitis B virus gene and may be a promising method for the prevention of hepatocellular carcinoma in hepatitis B virus infection.

A strong relationship has been indicated between hepatitis B virus (HBV) infection and hepatocellular carcinoma (HCC) (1). Vaccination against mother-to-infant transmission has dramatically reduced the number of chronic HBV carriers (2). However, treatment of chronic hepatitis B with agents such as interferon has a very limited effect (3) and cannot be expected as a prophylactic method for development of HCC. Recently, the oncogenicity of a viral transactivator of HBV, HBx protein, has been demonstrated by *in vitro* and *in vivo* experiments (4–8), and the HBx gene is implicated in the pathogenesis of HCC in human HBV carriers (9–12). In the HBx gene transgenic mouse, a mouse model for HCC in HBV infection, expression of the HBx gene starts at birth and continues throughout the life of the mouse. Preneoplastic liver lesions develop from the age of 2 months and hepatic tumors finally appear from the age of 12 months (5). We studied antisense phosphorothioate (PS)-oligodeoxynucleotides (ODNs) (12, 13) in this transgenic mouse model in an attempt to down-regulate HBx gene expression *in vivo* and thereby suppress the development of preneoplastic liver lesions.

METHODS

Transgenic mice. Production of HBx gene transgenic mice was described previously (4). In the present study, we used male mice from the H9 strain of the HBx transgenic mouse which was homozygous for the HBx gene (5). Mice were maintained and cared for in accordance with the guidelines established by the National Institutes of Health.

Oligodeoxynucleotides. Two sets of sense and antisense PS-ODNs, S1, AS1, S2 and AS2, (Lynx Therapeutics, Inc. Hayward, CA) were designed as follows; AS1 (CGGCCCCGAGACGGGTCGTCCGCGGGA), S1 (TCCCGCGGACGACCCGTCTCGGGGCCG), AS2 (TTGGCAGCACACCCTAGCAGCCATGGA), S2 (TCCACGGCTGCTGCTAGGGTGTGCTGCCAA). AS1 and S1 ODNs cover +73 to +99 nt of the coding region of the HBx protein, and AS2 and S2 ODNs cover −3 to +24 nt. HPLC-purified ODNs as their sodium salts were suspended in phosphate-buffered saline (PBS) (pH 7.4) and adjusted to a concentration of 2 mg/ml. Approximately 25 μg/g BW of these ODNs were injected into mice intraperioneally according to the protocol described in the Results section.

RNA preparation and reverse transcription-polymerase chain reaction. RNA was extracted from liver tissues as de-

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0006-291X/96 $12.00

scribed by Chomczynski and Sacchi (14). RNA was analyzed by reverse transcription (RT) -polymerase chain reaction (PCR) as described previously (15) using 2 pairs of primers. For the detection of HBx transcript, primers X1 (GACGTCCTTTGTCTACGTCC) and X2 (AGCCTCCTAGTACAAAGACC) were used. For the detection of mouse albumin gene expression, primers Al1 (AGAGTGAGATCGCCCATCGG) and Al2 (AGTGTGAATGGACTTGTCACAG) were used. The resultant samples were separated in 2% agarose gel and subjected to Southern blotting.

Southern blotting. Southern blotting was carried out as described previously (15) using a probe X3 (CATGGAGACCACCGTGAACG) for the detection of HBx transcripts and a probe A13 (GGCCTAGTCCTGATTGCCTT) for the detection of albumin gene expression.

Western blotting. Whole tissue homogenates were resuspended in Western blotting sample buffer (5% β-mercaptoethanol, 2% sodium dodecylsulfate (SDS), 62.5 mM Tris-HCl, 1 mM EDTA, 10% glycerol). Equivalent amounts of protein were separated by 12.5% SDS/polyacrylamide electrophoresis and then electro-transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) as described previously (6). The filter was then reacted with anti-HBx rabbit serum, followed by biotinylated anti-rabbit IgG (Vector Labs, Inc., Burlingame, CA) and visualized using the avidin-biotin peroxidase method (Vectastain kit, Vector Laboratories, Burlingame, CA) and an ECL kit (Amersham Intl., Buckinghamshire, UK).

Histological studies. Tissue sections fixed in 10% neutral-buffered formalin were used for hematoxylin and eosin staining. For the evaluation of preneoplastic liver lesions, sizes of foci consisting of hepatocytes with cytoplasmic vacuolations were calculated. The maximal diameter of each focus was determined as its size, which was scored as following (1+; $\leq$ 100 μm, 2+; 100 μm $\leq$ < 500 μm, 3+; 500μm $\leq$ < 1,000 μm; 4+; 1,000 μm $\leq$ < 1,500 μm, 5+; 1,500 μm $\leq$). A total of 30 foci were evaluated in each mouse, and scores were described as mean ± SD. All slides were reviewed blindly by two independent observers; and when the results differed by more than 20%, the slides were reevaluated.

BrdU labeling. For bromo deoxyuridine (BrdU) incorporation studies, mice were given BrdU intraperitoneally. After fixation with 10% neutral-buffered formalin, samples were stained with anti-BrdU antibody and visualized as described previously (5). For the evaluation of BrdU incorporation, 30 microscopic fields were examined for the presence of positive nuclei in each mouse. The number of positive nuclei per field was expressed as mean ± SD.

Statistical analysis. The unpaired t-test was used for the evaluation of differences. The p values were considered significant when they were less than 0.05.

RESULTS

In our approach to find PS-ODNs that effectively down-regulate HBx gene expression, we prepared two sets of ODNs (Matsukura et al., 1987; Stein and Chen, 1993). One set covers the GC-rich region in the HBx gene (AS1 and S1) and the other covers the initiation codon of HBx protein (AS2 and S2).

Down-regulation of HBx gene expression. As an initial trial, we administered each of 4 different ODNs (AS1, S1, AS2 and S2) or PBS to the HBx-transgenic mice (3 month-old), respectively, for 7 consecutive days (1 mg/day). As an initial trial, we administered each of 4 different ODNs (AS1, S1, AS2 and S2) or PBS to the HBx-transgenic mice (3 month-old), respectively, for 7 consecutive days (1 mg/day). Three hours after the final administration, mice were sacrificed and gene expression in the liver was examined by means of the RT-PCR method. While no suppression of HBx gene expression was observed in the mice treated with AS1 or S1 PS-ODNs (Fig. 1, panel B), there was a marked reduction in the level of HBx gene transcripts in the AS2-treated mice compared to the PBS-control mouse (Fig. 1, panel A). In the S2-treated mice, there was a less marked decrease in the level of HBx gene expression, possibly due to interference with transcription of DNA. Alternatively, translation of mRNA might be disturbed, because there was a 78% homology including an identical stretch of 9 bases between the complementary sequence of S2-ODN and that of HBx-mRNA located immediately down-stream of RNA start site. The level of albumin gene transcripts as a control for cellular gene expression was not reduced in the AS2-treated mouse compared to that in the transgenic mice that were either PBS-injected or S2-treated (Fig. 1, panel B). No histologic damage that may be related to administration of ODNs, such as hepatocyte necrosis or inflammation was observed (17). These mice had foci of altered hepatocytes in the liver as preneoplastic lesions (5), which are typical for 3 m.o. HBx gene transgenic mice (data not shown).

Long-term treatment with antisense oligodeoxynucleotides. Successful inhibition of HBx gene expression by the administration of AS2 ODNs prompted us to design the next experiment in-

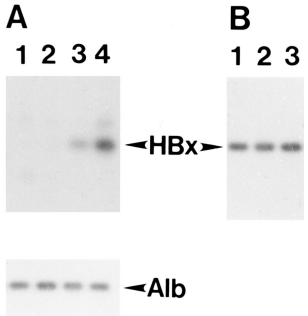

FIG. 1. Down-regulation of HBx gene expression in the transgenic mouse liver by the administration of oligodeoxynucleotides. (A) HBx gene mRNA levels were markedly reduced in the AS2-treated transgenic (lanes 1 and 2), compared to S2-treated (lane 3) or PBS-treated (lane 4) transgenic mouse. Mouse albumin gene expression was not altered by the administration of oligodeoxynucleotides (lower panel). (B) There was no change in the levels of HBx gene mRNA among AS1- (lane 1), S1- (lane 2) and PBS- (lane 3) treated transgenic mice.

volving relatively long-term treatment with AS2 ODNs. Since preneoplastic liver lesions appear from the age of 2 months in HBx transgenic mice, we decided to treat mice from 1 or 2 w.o. to 10 w.o. and examine the degree of preneoplastic lesion development. We first started treatment with the following protocol: 0.2 mg of ODNs from 1 w.o. to 2 w.o., 0.5 mg from 2 w.o. to 3 w.o. and 1.0 mg from 3 w.o., three times a week. However, 2 of 2 AS2-treated and 1 of 1 S2-treated mice showed poor growth and died before they reached 4 w.o. Since a relatively large amount of ODNs may have been toxic to young mice, we then redesigned the protocol as follows: 0.2 mg from 2 to 4 w.o., 0.5 mg from 4 to 5 w.o. and 1.0 mg from 5 to 10 w.o. Young mice tolerated this protocol well and there was no difference between their body weights and those of PBS-injected transgenic mice of the same age.

Having found an effective antisense sequence and a safe administration protocol, we applied the treatment to a large number of mice. Because preneoplastic foci appear in the transgenic mouse liver at about 2 m.o., mice were treated from 2 w.o. to 10 w.o. and sacrificed 3 hours after the final administration of ODNs. In this protocol, 13 transgenic mice were treated with AS2, 3 were treated with S2, and 5 were treated with PBS, all for 8 weeks.

At the time of sacrifice, there was no significant difference in body weight between AS2-treated, S2-treated and PBS-injected mice. Expression of the HBx gene was examined by Western blotting using anti-HBx rabbit serum. A marked reduction in the level of HBx protein was observed in 3 of 3 AS2-treated mice examined, compared to that in one S2-treated mouse (Fig. 2). No deformity of the liver or no significant difference in liver weight between AS2-treated mice and PBS-treated control transgenic mice was observed. On histologic examination, no infiltration of inflammatory cells at the portal or central areas in the liver was observed. In 10 out of the 13 AS2-treated mice, the preneoplastic altered foci consisting of vacuolated hepatocytes in the liver were found to be significantly smaller than those in PBS-treated or S2-treated transgenic mice (Fig. 3 and Table 1). Although very small vacuolated foci were observed in AS2-treated liver possibly due to HBx gene expression at a very young age in the mice, there were definite reductions of the size and severity of altered foci compared to those of the control transgenic mice.

As another indicator for preneoplastic liver lesion development in HBx transgenic mice, we then evaluated the DNA synthesis in hepatocyte nuclei induced by HBx protein (5) by examining the BrdU incorporation into AS2-treated liver. Average numbers of positive nuclei per microscopic field were significantly reduced in the AS2-treated mice than those in the S2- or PBS-treated transgenic mice (Fig. 4 and Table 2). The development of preneoplastic liver lesion was thus markedly inhibited by 8-week administration of antisense PS ODNs which effectively inhibited the expression of the HBx gene.

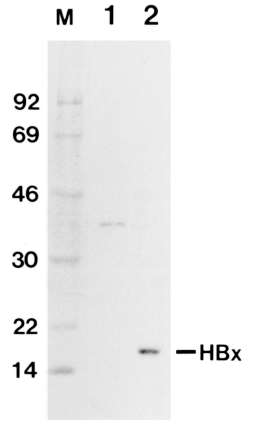

FIG. 2. Expression of HBx protein in the transgenic mouse liver after long-term administration of antisense oligodeoxynucleotides. Liver sample from an antisense oligodeoxynucleotides (AS2)-treated mouse (lane 1) or a sense of oligodeoxynucleotides (S2)-treated mouse (lane 2) was analyzed by Western blotting. M: molecular weight marker.

DISCUSSION

Antisense ODN strategies have been applied to a variety of eukaryotic systems to block gene expression therapeutically *in vitro* (12, 13). These studies include inhibition of the gene expression of human T cell leukemia virus type I (18), human immunodeficiency virus 1 (16, 19, 20), and

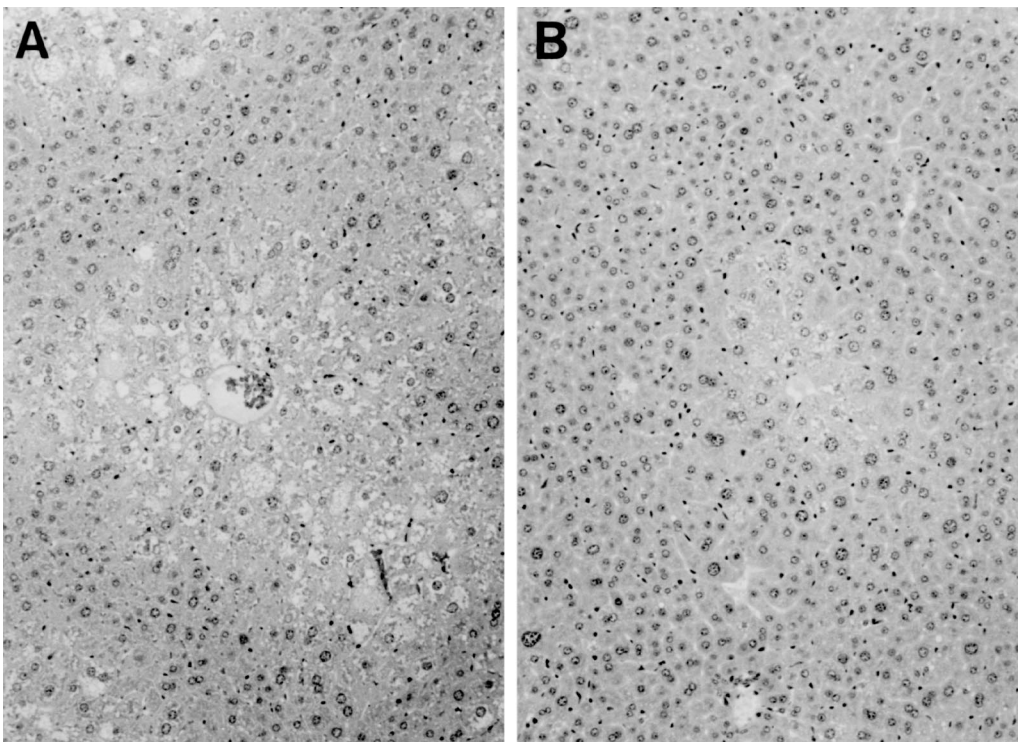

FIG. 3. Histopathology of transgenic mouse liver after oligdeoxynucleotides treatment. Eight-week treatment of HBx gene transgenic mice with AS2 antisense phosphorothioate oligodeoxynucleotides markedly inhibited the development of preneoplastic lesions consisting of hepatocytes with cytoplasmic vacuolations (B; AS2-treated mouse), compared to sense oligodeoxynucleotide (S2)-treated mouse (A). (x40, hematoxylin-eosin staining).

TABLE 1
The Size of Preneoplastic Liver Foci in AS2-, S2- or PBS-Treated Transgenic Mice

Mice	n	Sizes of foci (scores)[a]
AS-2 treated mice	13	1.29 ± 0.61
S2-treated mice	3	4.08 ± 0.85*
PBS-treated mice	5	4.43 ± 0.96*

[a] The maximal diameter of each focus was determined as its size and scored as following (1+; ≤ 100 μm, 2+; 100 μm ≤ < 500 μm, 3+; 500 μm ≤ < 1,000 μm; 4+; 1,000 μm ≤ < 1,500 μm, 5+; 1,500 μm ≤). A total of 30 foci were evaluated in each mouse, and the scores were described as mean ± SD.

* Significantly higher than AS2-treated mice ($p < 0.05$).

HBV (21, 22). In recent years, this strategy has also been applied to *in vivo* control of gene expression or inhibition of tumor growth (23–25). In contrast, little has been reported on ODNs which inhibit viral replication or gene expression *in vivo*, particularly in the case of HBV gene expression. While *in vitro* inhibition of HBV gene expression has been reported (21, 22), only one study of *in vivo* inhibition of hepadna virus replication has been reported (26). Suppression or prevention of hepatic tumor development *in vivo* has not been reported to our knowledge, probably due to the lack of appropriate animal model systems. In the present study, we employed a transgenic mouse system which carried a DNA fragment from the HBV genome (4, 5). These transgenic mice developed preneoplastic foci in the liver at 2 months of age as a consequence to HBx protein expression from birth, which leads to the development of hepatic tumors including benign adenomas and malignant HCCs (5). This experimental model for hepatocarcinogenesis allowed us to evaluate the effect of inhibition of HBx gene expression by examining the histopathological changes in liver tissues of the transgenic mice.

We found an antisense sequence that could effectively inhibit the expression of HBx protein. Marked inhibition of HBx gene expression in AS2 ODN-treated mice compared to that in the control S2 ODN-treated mice, lack of inhibition by another antisense ODN (AS1), and no change in the levels of control albumin gene expression indicate that the inhibition of HBx gene expression

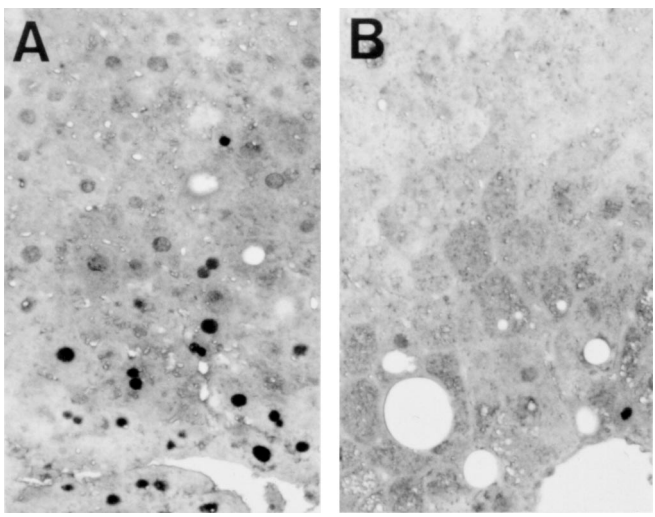

FIG. 4. Bromo deoxyuridine incorporation into nuclei of liver cells. Increased DNA synthesis in the nuclei (black dots) observed in the HBx transgenic mouse liver was suppressed by the administration of AS2 antisense phosphorothioate oligodeoxynucleotides (B; AS2-treated mouse), compared to that in sense (S2)-treated mouse (A). (X 40, counter-stained with eosin).

TABLE 2
Bromo Deoxyuridine Incorporation into Liver Cell Nuclei in AS2-, S2- or PBS-Treated Transgenic Mice

Mice	n	Number of positive nuclei per field[a]
AS2-treated mice	5	1.1 ± 1.2
S2-treated mice	3	15.2 ± 6.4*
PBS-treated mice	3	23.1 ± 5.8*

[a] Mean ± SD. In each mouse, 30 microscopic fields were examined for the presence of positive nuclei.

* Significantly higher than AS2-treated mice ($p < 0.01$).

observed in this study was specific to the selected antisense sequence. This antisense sequence exists in the region covering the initiation codon of the protein, which is one of the common sites for effective antisense ODNs (12, 16). Preneoplastic hepatic lesion development in the transgenic mice was substantially prevented with the inhibition of HBx gene expression by the administration of AS2 ODN for 8 weeks from 2 w.o to 10 w.o. It is noteworthy that this is the first example of phenotype prevention by the down-regulation of HBV gene expression *in vivo*. While genetic or epigenetic events other than HBx gene expression are assumed to be necessary for the development of HCC in human HBV infection (5), AS2 treatment might suppress the initiation of hepatocarcinogenesis through abrogation of HBx gene expression.

Oppensperger et al. reported that an antisense ODN against the 5′-region of the pre-S gene was most effective in the inhibition of duck hepatitis B virus replication (26). They could not examine the effect of the antisense ODN in the HBx region, because duck hepatitis B virus lacks the HBx gene (27). Since the HBx gene is considered to be indispensable for the establishment of woodchuck hepatitis virus infection (28, 29) and to regulate the viral replication by its transactivating function, the antisense sequence described in this study may also be effective in the inhibition of HBV replication.

In contrast to successful treatment of chronic hepatitis C with interferon (30–32), the efficacy of anti-viral therapy for chronic hepatitis B is inadequate (3). Since new infection by HBV can be prevented by means of effective vaccines (2), the most urgent clinical issue remaining to be resolved is prevention of development of HCC in HBV carriers, who are at very high risk for HCC (1). The inhibition of HBx gene expression and prevention of preneoplastic hepatic lesion development in the mouse model of hepatocarcinogenesis for human HBV infection obtained in the present study suggest that this antisense ODN may prevent the development of HCC in human HBV infection. Alternatively, the present results suggest that a conventional, small molecule might be found to be an effective drug against HCC by its inhibition of HBx protein.

ACKNOWLEDGMENTS

This work was partly supported by a grant-in-aid from the Ministry of Education, Science and Culture of Japan (Grant Nos. 04807160 and 06807169 to M.M., and 06282214 and 07274215 to K.K.). We thank Lynx Therapeutics Inc. (Hayward, CA) for the ODNs, and Gerald Zon for his useful discussion and critical reading of the manuscript.

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