p73β inhibits transcriptional activities of enhancer I and X promoter in hepatitis B virus more efficiently than p73α

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INTRODUCTION

p73 gene maps to chromosome 1p36.1, a region which was frequently deleted in several tumors, including neuroblastoma, colorectal cancer and breast cancer. Moreover, it has been found to share significant homology with the tumor suppressor gene p53 within the transactivation domain, DNA binding domain and oligomerization domain. Both p53 and p73 have redundant functions in the regulation of gene expression, because they have amino acid sequence identity reaching to 63% in the DNA binding domain, p73 can activate p53-regulated genes and suppress growth or induce apoptosis, and expression of p73 can be induced by DNA damage as p53 does.

METHODS: To construct an x-gene inactivated HBV plasmid which was cotransfected with p73α or p73β expression vectors into HepG2 cells. After transiently transfection, HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) were detected by ELISA. Viral transcripts synthesized by HBV were monitored by Northern blotting analysis. The activities of HBV regulatory elements, including enhancer I/X promoter (ENI/Xp) and enhancer II/core promoter (ENII/Cp) were measured by luciferase assays.

RESULTS: Both p73α and p73β could repress HBsAg and HBeAg expression by downregulating the ENI/Xp and ENII/Cp activities. But p73β exerted stronger inhibition on the activity of ENI/Xp than p73α, resulting in much lower level of viral transcripts and the antigens expression.

CONCLUSION: p73β as a novel member of p53 family can efficiently inhibit HBV transcription mainly through downregulating the activities of the HBV ENI/Xp regulatory elements.

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MATERIALS AND METHODS

Construction of plasmid

p3.8II, kindly provided by Prof. Wang Yuan, is an HBV plasmid which contains terminally redundant HBV genome
and can replicate in liver cell. The x-gene inactivated p3.8IIXm was constructed by changing the start condon of the X open reading frame on p3.8II. The pENI/XpLuc and pENII/CpLuc reporter plasmids were constructed by inserting nt1067-1403 and nt1430-1879 which contain enhancer I/X promoter and enhancer II/core promoter respectively in front of the pGL3 basic vector (Promega). The mammalian expression vectors pcDNA3-HA-73α and pcDNA3-HA-73β encode epitope tagged p73 proteins which were kind gifts from Dr. Lu Hua. p53 expression plasmid pRC/CMV hp53 was provided by Dr. Judith Roth. All the constructs were confirmed by restriction enzyme analysis and DNA sequencing.

**Cell culture and transfection**

HepG2 cells were cultured in DMEM supplemented with kanamycin (250 IU/ml), gentamycin (40 IU/ml) and 10 % fetal calf serum in 5 % CO2. HepG2 cells were cultured in DMEM supplemented with gentamycin (40 IU/ml) and 10 % fetal calf serum in 5 % CO2. Transfection was carried out by the calcium phosphate method[24]. Each transfection reaction contained a constant amount of 10 µg DNA per 6 cm dish.

**Preparation of cell lysate and measurement of HBV antigens**

Five days after transfection, cells were washed twice with PBS and were detached from dishes with 10 mM EDTA in PBS. After centrifugation (5 000 rpm, 1 min), cells were resuspended in 100 µl 250 mM Tris-HCl (pH7.5) and lysed by three thawing and freezing cycles. After centrifugation, the supernatant was transferred and stored at 4 °C. The protein concentration was estimated by Bradford method (Biocolor). Culture medium was collected every day after transfection. HBsAg and HBeAg were measured with ELISA kits (Sino-America). All procedures were performed according to the descriptions of the manufacturers.

**RNA analysis**

Total RNA was extracted from transfected cells using TRIzol reagent (Gibco BRL). The RNA samples were treated with RNase-free DNaseI (Pharmacia). For Northern blotting analysis, 20 µg of total cellular RNA per sample was separated on 1 % formaldehyde-agarose gel and blotted to a Hybond-N nylon membrane (Amersham). The membrane was prehybridized in Quick-Hyb buffer (Amersham) for at least 1 hour, followed by 2 hr hybridization at 65 °C with the probes of α32p-dCTP labeled 3.2 kb HBV DNA and G3PDH fragments. After hybridization, the membrane was washed in 2 %SSC, 0.1 %SDS buffer (RT, 20 min), 0.2 % SSC, 0.1 %SDS (65 °C, 30 min), then exposed to an X-ray film at -70 °C.

**Luciferase assay**

Cells were lysed and analyzed with a luciferase assay system (Promega). Corrections of the luciferase activity were made based on the protein concentrations of the lysates. The luciferase activity was measured with a Lumat LB9507 luminometer (Berthold) in 10 µl of the lysate after addition of 100 µl assay reagent.

**RESULTS**

**p73β represses HBsAg and HBeAg expression more efficiently than p73α**

Before investigating whether p73 could affect HBV transcription like p53, we established the X protein-minus HBV mutant p3.8IIXm to avoid the interaction between p73 and the HBV X protein. This HBV mutant had no ATG start codon of the X-ORF (Figure1). ELISA results revealed that the HBV antigens expression in mutant type of p3.8IIXm was just a half of that in wild type p3.8II.

**Figure 1** Partial DNA sequences of p3.8II and p3.8IIXm is indicated in the left panel (A). The point mutation A→C results in the amino acid changes in the ORF of HBV polymerase (P) and X protein(X) (B).

**Figure 2** Time course of HBV antigen expression. HepG2 cells were transiently transfected with X-minus p3.8IIXm, and cotransfected with pcDNA3, p53, p73α, or p73β individually. Culture medium were harvested at certain days, and detected by ELISA kit.

As shown in Figure2A, P3.8IIXm could secret HBsAg into the medium continuously starting from day 2. Cotransfection of p3.8IIXm with p73β expression plasmid resulted in a reduced level of HBsAg (87 % reduction on day 5 post transfection), which was similar to p53 (89 % reduction on...
day 5 post transfection). Cotransfection of p3.8IIXm with p73α expression plasmid exhibited weak repression on HBsAg synthesis (only 63 % reduction on day 5 post transfection). The time-dependent alteration in the level of HBeAg (Figure2B) displayed a similar pattern to that of HBsAg. On day 5 post transfection, the expression of HBeAg was changed at the level of 36 % reduction for p73α and 64 % reduction for p73β. Also as shown in Figure2B, p53 could inhibit HBeAg more efficiently (78 % reduction on day 5 post transfection) than p73α and p73β. It is concluded that both p73α and p73β can downregulate HBV expression including HBsAg and HBeAg, but p73β can repress the HBV antigens expression more efficiently than p73α.

p73β can repress the synthesis of viral transcripts including pregenomic/precore RNA and preS/S RNA

The roles of p73α and p73β in viral transcription were assessed at the HBV RNA levels after cotransfection of p3.8IIXm and p73 expression plasmids. Using Northern blot hybridization with 32P-labeled 3.2 kb HBV fragment as a probe, we detected the pregenomic RNA and precore RNA as well as preS/S mRNA. As Figure 3 shown, cotransfection of p73β resulted in the reduction of the viral transcript level like p53, but p73α seems to exhibit very weak repression on HBV transcription. These results are in accordance with the above ELISA data.

Figure 3 Northern blotting analysis of HBV viral transcripts, including pregenomic/ precoreRNA and preS/S RNA. HepG2 cells were transiently transfected with p3.8IIXm, and also with the expression plasmids. Lane 1 represents pcDNA3 (1), lane 2, p53 (2), lane 3, p73α (3), lane 4, pHBX and lane 5, p73β respectively. Total cellular RNA were extracted and α-32P-dCTP labeled 3.2 kb HBV DNA or G3PDH used as probe.

p73β inhibits HBV transcription mainly through downregulating the activity of HBV ENI/Xp regulatory elements

To explain the molecular mechanism of the p73β mediated repression of HBV transcription, we investigated the possibility of p73 regulating the enhancer and promoter activity. pENI/ Xp reporter plasmid containing HBV enhancer I and X promoter was cotransfected into HepG2 cells with p73α and p73β expression plasmids, and the levels of luciferase activity were determined. As shown in Figure 4A, the luciferase activity of the pENI/Xp was decreased with transfection of p73β and p73α, down to 65 % and 35 % of the control value respectively. Therefore, p73β can repress the activity of enhancer I and X promoter more efficiently than p73α. The activity of HBV enhancer II and core promoter were repressed by p73α and p73β at a similar level, but the effects of inhibition were weaker than that by p53 (Figure 4B). Therefore, it is concluded that p73β represses HBV gene expression mainly through the enhancer I and X promoter.

Figure 4 Effects of p73α and p73β on the regulatory sequences of hepatitis B virus. HepG2 cells were transfected with expression plasmids, pcDNA3(1), p53(2), p73α (3), and p73β (4). In each case, 1 µg of reporter plasmids, pENI/ XpLuc, containing the enhancer I/ X promoter (A) and pENII/ CpLuc, containing enhancer II/ core promoter (B), were individually cotransfected. 48 hours after transfection, the cells were harvested and luciferase activity was determined. The values obtained with reporters alone was arbitrarily set to 100 %, and the other values were normalized accordingly. The column heights reflect the average of at least three independent experiments.

DISCUSSION

HBV, an important risk factor of hepatitis and hepatocellular carcinoma, employs a reverse transcription step which is controlled by cellular transcription factors and some cellular signal transduction pathways, including the tumor suppressor gene p53[25,26]. Physiologically activated p53 can repress HBV transcription, and this repression can be abrogated by physiological levels of HBX protein. It is generally believed that p53 can interact with HBx, and the latter destroys p53 function, including the repression of virus transcription. To exclude the interaction between p73 and the X protein, we constructed the X-minus HBV strain p3.8IIXm which could replicate in hepatoma cells without producing X protein.

HBsAg and HBeAg are translated from preS/S mRNA and precore mRNA individually. HBV core promoter is in charge of the transcription of pregenomic RNA as well as precore mRNA. ENII and ENII are the two enhancers in the viral genome. ENI is usually able to upregulate all the HBV promoters, and ENII has a particularly significant stimulatory effect upon the core promoter. In this report, we found that p73β repressed HBsAg and HBeAg expression more efficiently, and it inhibited the viral transcripts level mainly through downregulating the enhancer I and X promoter. In the ENI/Xp construct, there were two sites with homology to
REFERENCES

HA-p73
2
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investigated.

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