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LIVER CANCER

Interferon-α response in chronic hepatitis B-transfected HepG2.2.15 cells is partially restored by lamivudine treatment

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Abstract

AIM: To characterize the IFN-response and its modulation by the antiviral compound lamivudine in HBV-transfected HepG2.2.15 cells.

METHODS: HepG2.2.15 and HepG2 cells were stimulated with various concentrations of IFN-α2a in the presence or absence of lamivudine. Then, total RNA was extracted and analysed by customised cDNA arrays and northern blot for interferon-inducible genes (ISGs). In addition, cellular proteins were extracted for EMSA and western blot. HBV replication was assessed by southern blot or ELISAs for HBsAg and HBeAg.

RESULTS: Two genes (MxA, Cig5) with completely abolished and 4 genes (IFITM1, -2, -3, and 6-16) with partially reduced IFN-responses were identified in HepG2.2.15 cells. In 2 genes (IFITM1, 6-16), the response to IFN-α could be restored by treatment with lamivudine. This effect could not be explained by a direct modulation of the Jak/Stat signalling pathway since EMSA and western blot experiments revealed no suppression of Stat1 activation and ISGF3 formation after stimulation with IFN-α in HepG2.2.15 compared to HepG2 cells.

CONCLUSION: These results are consistent with the assumption that chronic hepatitis B may specifically modulate the cellular response to IFN by a selective blockage of some ISGs. Antiviral treatment with lamivudine may partially restore ISG expression by reducing HBV gene expression and replication.

Key words: Hepatitis B; IFN-α; Gene expression; Lamivudine

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INTRODUCTION

Hepatitis B (HBV) is a hepatotropic DNA virus capable of causing both acute and chronic hepatitis in humans. It is estimated that over 350 million people are chronically infected with HBV worldwide. Currently approved therapeutic strategies for treatment of HBV include interferon-alpha (IFN-α), the nucleoside analogue lamivudine and the nucleotide analogue adefovir[1,2]. However, only a minority of patients treated with IFN-α has a long-term sustained response with 'eradication' of the virus. Patients with a high viral load, in particular, rarely respond to IFN therapy. Treatment with lamivudine, on the other hand, is complicated by a high rate of viral resistance and a high relapse rate after cessation of therapy, respectively[3]. Both the emergence of viral resistance and relapse after therapy are often associated with a hepatitis flare, which can sometimes be fatal. Thus, novel strategies are needed to improve treatment for this disease.

To develop new regimens it is necessary to gain further insights into the interactions between HBV and the main antiviral system of the host, the IFN-system. It has been shown that type I and type II interferons are able to suppress HBV-replication in livers from HBV-transgenic mice[4-6]. This could also be demonstrated *in vitro* by using immortalized hepatocyte cell lines from these animals[7] and involves elimination of pregenomic RNA-containing capsids, inhibition of DNA replication and reduction of steady-state levels of HBV transcripts. The effector mechanisms that have been associated with IFN-induced suppression of HBV-replication include MxA[8] and proteasome mediated activities[9,10]. Additional data suggest a role for GTP-binding proteins, signalling and various other molecules in the control of HBV replication[11]. HBV can

counteract these antiviral effector mechanisms by inhibiting proteasome activities in an HBX-dependent manner[12] and by suppressing MxA expression at the promoter level[13]. Furthermore, it has been shown that HBV replicated at higher levels in HBV-transgenic mice crossed with IRF-1 or PKR deficient mice while replication was unchanged in transgenic mice crossed with RNase L deficient mice[14].

Assuming that HBV may interfere with the expression of ISGs, one would predict that the ISG expression in cell lines with and without HBV may be different and this would be modulated by inhibition of HBV gene expression and replication. The present study was performed to test this hypothesis. Using customized cDNA arrays for ISGs, we could identify 2 ISGs (MxA and Cig5) that are completely abolished in HBV-transfected HepG2.2.15 cells and 4 genes (IFITM1, -2, -3 and 6-16) with partially reduced responses. This suppression could partially be restored in 2 genes (IFITM1, 6-16) by treatment with the nucleoside analogue lamivudine suggesting an additional therapeutic mechanism for this drug.

MATERIALS AND METHODS

Cell culture

HepG2.2.15 cells were kindly provided by G. Acs (Mount Sinai Medical Cancer, New York, NY) and maintained in Dulbecco's Modified Eagle's Medium, supplemented with 2 mmol/L L-glutamine 50 IU/mL of penicillin, 50 mg/L of streptomycin, 500 mg/L of G418, 5% (vol/vol) fetal bovine serum, at 37℃ in humidified incubators at 5% CO_2. The cells were seeded at a density of 8×10^5 cells and maintained in a confluent state for 2 to 3 d before being treated with antiviral compounds. At first, various concentrations from 0.04 μmol/L to 100 μmol/L of lamivudine were used to reach the suitable drug concentration, which profoundly suppressed HBV replication without cytotoxicity. At the same time, a time course of drug action also was evaluated. Over a period of 10 d lamivudine was added to the medium daily, then the cells were stimulated by addition of IFN-α for 6 h. Thereafter, the media were collected and DNA or RNA was extracted for further analysis.

Analysis of secreted HBV particles

Detection of HBsAg and HBeAg was carried out by using a commercially available kit (Dade Behring) according to the manufacturer's instructions. Medium samples collected from HepG2.2.15 cells were centrifuged at 1200 rpm for 10 min to remove cellular debris, transferred to clean tubes and stored at -20℃ until analysed. HBsAg and HBeAg amounts were evaluated from absorbance reading values (450 nm) compared to the constructed controls.

HBV DNA analysis

Extracellular virion HBV-DNA analysis: Medium of HepG2.2.15 cells was collected and centrifuged (10 min, 2000 × g), and polyethylene glycol (M_r, 8000) was added to the supernatant at a concentration of 10% (wt/vol) followed by overnight precipitation at 4℃. The virions were pelletted (30 min, 10 000 × g), and the pellet was resuspended in lysis buffer (10 mmol/L Tris-Cl, 5 mmol/L EDTA, 150 mmol/L NaCl, 1% SDS) at room temperature for 15 min. Proteinase K was added at a concentration of 500 μg/mL and the suspension incubated for 2 h at 56℃. The digest was extracted with phenol/chloroform, 1:1 (vol/vol) or chloroform, respectively, and the DNA was precipitated with 2.5 vol. of ethanol. The DNA pellet was dissolved in TE solution and then spotted onto Hybond-N+ membranes. Alternatively, the DNA was electrophoresed in 1.2% agarose gel followed by blotting onto Hybond-N+ membranes. The bolt was hybridized with a ^{32}P-labeled HBV DNA probe (digested by Nsi I from plasmids that contained the full length HBV genome sequence dimer and labelled with a RediprimeTM Ⅱ Random prime labelling system), washed with 2 × SSC/0.1% SDS at room temperature for 20 min, twice, and 0.1 × SSC/0.1% SDS at 60℃ for 45 min, and then autoradiographed. The intensity of the autoradiographic dots or bands was quantitated using the Cyclone Storage Phosphor System (Packard Instrument Company, Median, Conn.). All drug concentrations were tested in duplicate or triplicate, with antiviral effects being scored as the amount of HBV DNA present in the media relative to that in untreated controls.

Intracellular HBV replicative intermediates (RI) analysis: HepG2.2.15 cells were consecutively treated with various concentrations of lamivudine for 10 d. The cytoplasmic preparations containing HBV core particles were isolated from the treated cells. Cells were lysed with lysis buffer (50 mmol/L Tris-Cl, PH 7.4, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 0.5% NP-40) at room temperature for 5-10 min. The cytoplasmic fraction was separated from the nuclear fraction by centrifugation. Unprotected DNA was removed by adjusting cytoplasmic preparations so that they contained 10 mmol/L $MgCl_2$ and 500 μg/mL of DNase I (Roche, Germany) followed by a 1 h incubation at 37℃. To extract replicative intermediates (RI), EDTA, sodium dodecyl sulfate (SDS), NaCl and proteinase K (QIAGEN) were added separately and sequentially to final concentrations of 10 mmol/L EDTA, 1% SDS, 100 mmol/L NaCl and 500 mg/L of proteinase K. The sample was incubated for 1.5 h at 56℃ and then subjected to sequential phenol and chloroform extraction and isopropanol precipitation. Precipitated nucleic acids were resuspended in a small volume of TE solution and digested with 100 mg/L of RNase (Roche, Germany) for 1 h at 37℃. Twenty micrograms of cytoplasmic preparations containing HBV replicative intermediates DNA (RI) were then analysed by electrophoresis in 1.2% agarose gels, followed by blotting onto Hybond-N+ membranes. The bolt was hybridized with a 32P-labeled HBV DNA probe (digested by Nsi I from plasmids which contain full length HBV genome sequence dimer, and labelled with a RediprimeTM Ⅱ Random prime labelling system), washed with 2 × SSC/0.1% SDS at room temperature for 20 min, twice, and 0.1 × SSC/0.1% SDS at 60℃ for 45 min, and then autoradiographed as described above.

RNA extraction

Total RNA was isolated from cells using Trizol according

to the manufacturer's instructions. RNA quantity and quality was assessed by determination of the optical density at 260 and 280 nm using spectrophotometry and additional visualisation by agarose gel electrophoresis.

Gene expression profiling by customized cDNA macroarrays

Radiolabelled cDNA was generated from 20 μg total RNA by reverse transcription with Superscript Ⅱ (Gibco, MD) in the presence of ^{32}P-dCTP. Residual RNA was hydrolysed by alkaline treatment at 70℃ for 20 min and the cDNA was purified using G-50 columns (Amersham Pharmacia, UK). Before hybridisation to the macroarrays the labelled cDNA was mixed with 50 μg COT-DNA (Gibco) and 10 μg Poly-A DNA (Sigma), denatured at 95℃ for 5 min and hybridised for 1h to minimise non-specific binding. Preparation of the macroarrays (representing 150 known ISGs), hybridisation of the radioactive cDNAs and scanning and analysis of the macroarrays were carried out as described previously[15].

Northern blot analysis

5 μg of total RNA was electrophoresed through a 1.2% agarose gel containing formaldehyde and then transferred to Hybond-N+ membranes. The immobilized RNA was hybridized with a ^{32}P-labeled DNA probe (IMAGE clones PCR products, purified with Gel Extract kit, QIAGEN).

Electrophoretic Mobility Shift Assay

At 80% to 90% confluence, cells were stimulated with IFN-α for 6 h. Preparations of nuclear extracts were performed according to the instruction of the manufacturer (PIERCE, NE-PERTM Nuclear Extraction Reagent). Nuclear extracts/DNA binding reactions were performed in 20 μL containing 15 μg nuclear extract protein and 4 μL Gel Shift Binding 5 × Buffer (20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L Tris-Cl, PH 7.5, 0.25 mg/mL Poly (dI-dC) ·Poly (dI-dC)). ISRE/GAS consensus oligonucleotides (5'-AAG TAC TTT CAG TTT CAT ATT ACT CTA-3') from the promoter region of the IFN-α responsive genes were used. Mutant oligonucleotides (5'-AAG TAC TTT CAG TGG TCT ATT ACT CTA-3') were used as control. The probes were end-labeled with γ-^{32}P-ATP (U K, 3000 Ci/mol) at room temperature for 20 min. Complexes were separated from the probe in 4% naive poly-acrylamide gel in 0.5 × TBE buffer. The gels were subsequently dried and autoradiographed.

Western blot analysis

After interferon treatment, cells were washed once with ice-cold phosphate-buffered saline. Cells were lysed on ice for 30 min in 0.5 mL lysis buffer containing 50 mmol/L Tris, pH 8.0, 10% Glycerol, 0.5% NP40, 150 mmol/L NaCl, 1 mmol/L DTT, 1 mmol/L EDTA, 1 mmol/L Sodiumorthovanadate, 170 mg/L phenylmethylsulfonyl fluoride, 2 mg/L Aprotinin, 1 mg/L Leupeptin. Lysates were cleared by centrifugation in a microcentrifuge at high speed for 30 min at 4℃. Protein concentration of the supernatant was measured with Bradford reagent. Equal amounts (100 μg) of proteins were suspended in sodium-dodecyl sulphate (SDS)-sample buffer, boiled for 5 min and separated by electrophoresis (NuPAGE 4%-12% Bis-Tris Gel, Invitogen). The separated proteins were transferred to a polyvinylidene difluoride membrane (Hybond-P^{TM}, Amersham Biosciences). After blocking for 1 h at room temperature in 10% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) or 1% BSA for antibodies specific for phosphorylated epitopes, membranes were incubated with anti-p38, anti-pp38 (Santa Cruz), anti-Stat1, anti-Stat1(pY701) and anti-ERK1, anti-ERK1/2(pT202/pY204) (BD Biosciences) overnight at 4℃, and thereafter with horseradish peroxidase-conjugated anti-rabbit or anti-Mouse IgG (1:5000) (Amersham Biosciences) for 1 h at room temperature. The proteins were detected with enhanced chemiluminescence reagent (ECL, Amersham).

Southern blot analysis

Twenty micrograms of cytoplasmic preparations containing HBV replicative intermediates (RI) DNA were analysed by Southern blotting as above.

RESULTS

Differential expression of ISGs in HepG2.2.15 and HepG2 upon stimulation with IFN-α

Type 1 IFNs are known to induce an intracellular antiviral state against many viruses. Therefore, we developed a customized cDNA array methodology to study the expression of IFN stimulated genes (ISGs). At present, this system permits the analysis of several hundred genes of interest. A substantial spectrum of known ISGs is analysed with this macroarray (Table 1). The sensitivity of this method has also been assessed previously[15]. Conventionally, in most micro- and macroarray systems a 2-fold change in the expression level is regarded as being significant.

In the established hepatoma cell line, hepG2.2.15 with stably transfected HBV genomes[16], ISG expression was examined using the cDNA macroarrays (Table 2). While many ISGs, e.g., 2-5 OAS, IFI 17, and RING4, were normally stimulated by IFN-α, several other ISGs were expressed at a lower level compared with the ISG expression in HepG2 cells. The induction of 2 ISGs, MxA and Cig5, was completely inhibited in HepG2.2.15 cells, while a partial inhibition was observed for 4 ISGs, IFITM1, IFITM2, IFITM3, and 6-16 (Table 1, Figure 1). Thus, only a subgroup of ISGs was down regulated in HepG2.2.15.

Analysis of the IFN response in HepG2.2.15 and HepG2 cells

The results above suggested that the IFN-signalling pathway is only partially inhibited in HepG2.2.15. Western blotting and EMSA and were carried out to analyse Stat1 activation and ISGF3 formation in HepG2 and HepG2.2.15 cells. The phosphorylated form of Stat1 was detected by western blot in IFN-α treated cells (Figure 2). The phosphorylation of Stat1 was enhanced in HepG2.2.15, compared with HepG2. Furthermore, Figure 3 showed that the formation of ISGF3 in HepG2.2.15 cells occurred after IFN-α stimulation, as occured in HepG2 cells.

Table 1 Complete list of genes investigated in this study

Gene Name	Acc. No.	Gene Name	Acc. No.	Gene Name	Acc. No.
101F6	AA544950	IFI 16	M63838	Mdm2	Z12020
2-5 OAS	X02875	IFI 41	L22342	MEN1	U93237
2-5 OAS	D00068	IFI 44	D28915	Met	AA410591
5' nucleotidase	X55740	IFI 6-16	BC015603	Mig	X72755
60S Ribosomal protein L11	U43522	IFI27	X67325	MIP-1b/CCL4	NM_002984
72 kDa type IV collagenase	J03210	IFI4	X79448	MLK 2	X90846
9-27	J04164	IFIT 1	M24594	MMP-1	M13509
ADAM-10	AF009615	IFIT4	U72882	MxA	M33882
ADAM-17	U69611	IFIT4	AF083470	MxA	M33882
akt-1	NM_005163	IFITM2	X57351	MxB	M30818
akt-2	M77198	IFITM3	X57352	MxB	M30818
Alpha-1-antiproteinase	K01396	IFN omega 1	X58822	NCAM	M74387
Alpha-crystallin	U05569	IFN-AR1	J03171	NF-IL-6	X52560
ATF-2	X15875	IFN-AR2	L42243	NFkB	M58603
Auto Ag SS-A/Ro	NM_003141	IFN-g	M29383	NKC-4	M59807
bad	U66879	IFN-GR1	J03143	n-myc	Y00664
BAK1	X84213	IFN-GR2	U05875	p19	U40343
BAX	U19599	IFI 17	J04164	p48/ISGF3g	M87503
Bax	L22474	IFP 35	U72882	p53	M14694
bcl-2	M14745	IFP-53	X62570	p57Kip2	U22398
BRCA1	U14680	IFRG28	AJ251832	p70 S6 kinase	M60724
BST2	D28137	ikBa	M69043	PAI-1	M16006
BTG1	X61123	IL-1 α	M28983	PCBP	M80563
Calcyclin	J02763	IL-10	M57627	PDGF-alpha	X06374
Calretiulin	M84739	IL-10 R α	U00672	PDK1	Y15056
CASP	AJ006470	IL-10 R β	Z17227	PDK2	NM_002611
Caspase 7	U67319	IL-12R β	U64198	Phosph. Scram. 1	AF098642
Caspase 8	X98172	IL13RA	U81379	Phosph.glycerate kin.	V00572
Caspase-1	M87507	IL13RA 2	U70981	Pi3-kinase	NM_006219
Caspase-9	U60521	IL-15	U14407	PIAS x-beta	AF077954
Cat. o-methyltransferase	M58525	IL-15RA	U31628	pig7	AF010312
CBFA	NM_004349	IL-18	D49950	pim-1	M16750
CBP	U85962	IL-18 bprot	AB019504	PK R	AF072860
CCR1	L09230	IL2	U25676	PKR	U50648
CCR5	U54994	IL-2R α	K03122	plectin (PLEC1)	U53204
CD5	X04391	IL2RG	D11086	PLOD2	U84573
cdk inhibitor p27KIP1	U10909	IL6	X04602	PML-1	M79462
C-fox	NM_005252	IL-8	M28130	PPP3CA	L14778
CG12-1	AF070675	IL8RB	L19593	Pro. 4-hydroxyl.	M24486
C-jun	J04111	iNOS	L09210	Prot.-ATPase-like pr.	D89052
C-myc	L00058	Int-6	U62962	PTEN	U96180
C-myc	V00568	Integrin β 7	M62880	pyridoxal kinase	U89606
Collagen α1 (I)	Z74615	integrin-β-6	NM_000888	raf (c-raf-1)	X03484
Collagen α2 (I)	J03464	IP-10	X02530	RAP46/Bag-1	Z35491
Collagen, type XVI, alpha 1	M92642	IP-30	J03909	RbAp48	X74262
Complement compound C1r	J04080	IRF 1	X14454	Reticulocalbin	D42073
COX17	L77701	IRF 4	U52682	RGS2	NM_002923
Cpp32	NM_004346	IRF 5	U51127	RHO	NM_000539
CREB	NM_004379	IRF-1	L05072	RHO GDP-dis.inh. 2	L20688
CTRL-1	X71877	IRF-2	X15949	RING 10	NM_004159
CXCR4	AF005058	Irf-7	U73036	RING4	X57522
Cyclin D1	M64349	ISG15	AA406020	Smad1	U59423
Cyp19 (aromata)	M28420	ISG15	M13755	Smad2	AF027964
Cys-X-Cys,member 11	AF030514	ISG-56K	M24594	Smad4	U44378
DEAD box binding protein 1	AF077951	KIAA0129	D50919	Smad5	U73825
DEAD-box protein p72	U59321	KIAA0235	D87078	Smad7	AF015261
Destrin	S65738	KIAA0284	AB006622	SnoN	X15219
DP (β1)	M83664	LIPA	U04285	SOCS 3/ssi-3	AB004904
DR-α	J00194	LMP-2	X66401	SOCS 4/CIS 4	AB006968
E2F-1	U47677	L-selectin	M25280	SOCS1	N91935

Gene Name	Acc. No.	Gene Name	Acc. No.	Gene Name	Acc. No.
egr-1	X52541	Mad 4	X03541	SOCS-1	NM_003745
Elastase 2	M34379	MAP2K1	NM_002755	SOCS2	AF020590
ERM	X76184	MAP2K1IP1	NM_021970	SOCS-3	NM_003955
F-actin capping protein	U56637	MAP2K2	L11285	Stannin	NM_003498
Farn. pyro. syn.	J05262	MAP2K3	NM_002756	STAT 6	U16031
FAS/Apo-1	M67454	MAP2K4	L36870	STAT1 (91kDa)	M97935
fas-ligand	U08137	MAP2K5	NM_002757	STAT1 (91kDa)	M97935
Fibronectin-1	X02761	MAP2K6	U39657	STAT2	M97934
FK506 binding protein 6	AF038847	MAP2K7	AF022805	STAT4	L78440
FKHRL1	AF041336	MAP3K1	AF042838	STAT5A	L41142
Folate receptor	X62753	MAP3K11	NM_002419	STAT5B	U47686
gadd45	M60974	MAP3K14	NM_003954	Succinyl CoA Ligase	AF058953
Galectin-1	J04456	MAP3K2	NM_006609	TAP1 (Ring4)	L21204
Gamma actin	X04098	MAP3K3	U78876	TFE3	X96717
Gamma2-adaptin (G2AD)	AF068706	MAP3K4	NM_005922	TGF-bR1	L11695
GAPDH	X01677	MAP3K5	NM_005923	TGF-bR2	D50683
GATA 3	X58072	MAP3K7	NM_003188	TGF-bR3	L07594
GBP-1	M55542	MAP4K1	NM_007181	TGIF	X89750
GBP-2	M55543	MAP4K3	NM_003618	TIMP-1	M59906
Granzyme B	M17016	MAPK10	NM_002753	TIMP-2	J05593
GSK3	NM_002093	MAPK11	NM_002751	TIMP-3	U14394
HCV-ass. p44	D28915	MAPK12	NM_002969	TIMP-4	U76456
HLA-A (MHCI Ag B27)	NM_002116	MAPK13	AF004709	TNF-alpha	X01394
HLA-E	X56841	MAPK14	NM_001315	TRAF6	U78798
Homo sapins STAT	M97936	MAPK3	X60188	Transferrin	M12530
Hou	U32849	MAPK6	NM_002748	Transthyretin	D00096
HPAST protein	AF001434	MAPK7	NM_002749	TRIP14	L40387
hsf1 (tcf5)	M64673	MAPK8	NM_002750	trk oncogene	X03541
hsp90 (CDw52)	X15183	MAPK8IP2	NM_012324	TTF-2	AF073771
Hypoxia-ind. Factor-1	U22431	MAPK9	U35003	UBE2L6	AA292074
ICAM-1	M24283	MAPKAPK2	NM_004759	VCAM -1	M30257
ICSB 1	M91196	MAPKAPK3	NM_004635	VEGF-C	U43142
IDO	M34455	MCP-1/CCL2	X14768	Virpirin (Cig5)	AF026941

Genes of interest were selected from the UniGene database. These genes comprise known ISGs and genes of intrinsic interest which might or might not be induced by IFNs in different cell systems. They include genes involved in cell proliferation, immune responses and the responses to a variety of cytokines. 5′ IMAGE clones with 0.5-0.8 kb length were chosen and obtained from RZPD, Berlin, Germany.

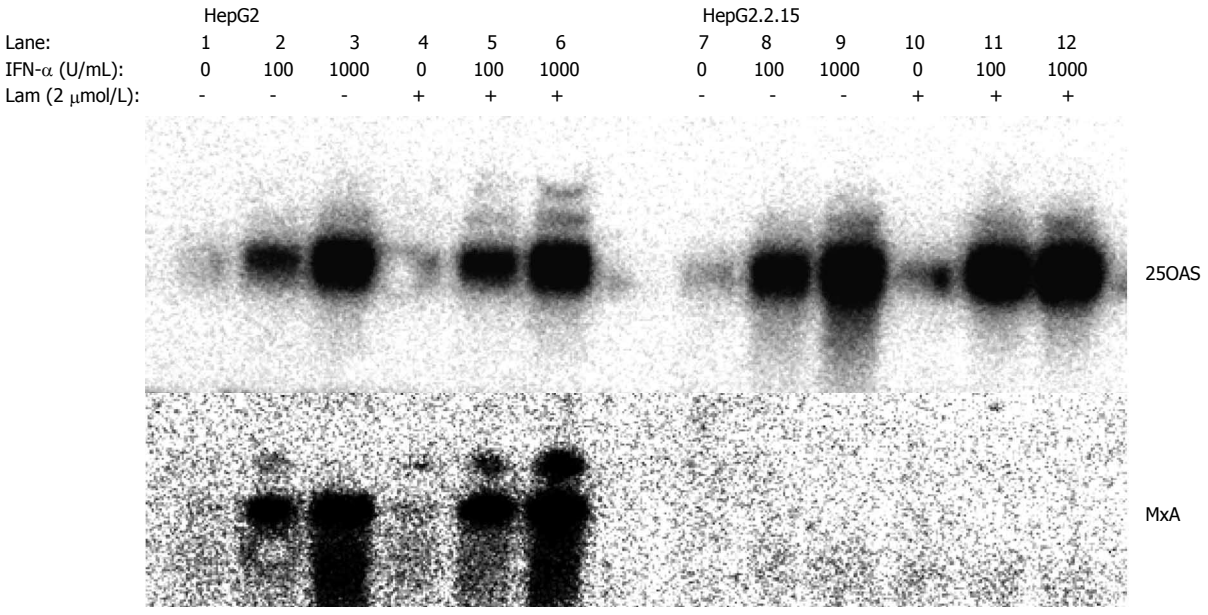

Figure 1 Northern blot analysis of ISG expression and its modulation by lamivudine in HepG2 and HepG2.2.15 cells. HepG2 and HepG2.2.15 cells were cultured in the absence or presence of 2 μmol/L of lamivudine for 10 d. Then, the cells were stimulated with 100 or 1000 IU/mL of IFN-α for 6 h. Total cellular RNAs were isolated for Northern blotting hybridization. Lam: lamivudine.

Table 2 Suppression of ISG induction in HepG2.2.15 cells, effect of lamivudine treatment

Gene	Acc. No.	HepG2	2.2.15	HepG2 /lam	2.2.15 /lam
Ⅰ complete inhibition					
MxA	M33882	5.9	0.6	4.0	0.8
cig5	AF026941	2.2	0.9	2.1	1.2
Ⅱ partial inhibition					
IFITM3	X57352	3.4	1.6	3.4	1.8
IFITM2	X57351	2.7	1.6	2.2	1.8
Ⅲ reversible inhibition					
IFI 6-16	BC015603	7.9	4.4	7.2	6.5
IFITM1	M24594	4.9	2.5	4.8	4.6
Ⅳ no inhibition					
2-5OAS	D00068	3.8	4.0	4.4	5.8
MxB	M30818	1.4	2.0	1.4	1.9
Caspase 7	U67319	2.3	2.4	2.2	2.1
IFI 17	J04164	3.3	2.9	2.8	2.7
IFI 27	X67325	1.9	2.1	1.8	2.2
IFI T4	U72882	2.7	1.9	2.6	1.8
RING4	X57522	2.4	2.3	2.5	3.5

Cells were stimulated with 100 U/mL IFN-α for 6 h with or without pretreatment with 2 μmol/L lamivudine for 10 d. Then, RNA was isolated and assayed by cDNA macroarray. Data are shown as fold induction compared to the untreated control. Lam: Lamivudine.

These data clearly show that the IFN-signalling pathway is generally not blocked in HepG2.2.15 cells. The results consistently show that both steps were evenly enhanced in HepG2.2.15. In addition, activation of ERK and p38 MAPKinase was not altered in HepG2.2.15 cells (data not shown).

Reduction of the production of HBV proteins and the HBV replication by lamivudine treatment

The difference in ISG expression in HepG2 and HepG2.2.15 cell lines may be partly due to the presence of HBV replication in the later one. Consequently, the ISG expression in HepG2.2.15 would change if the HBV gene expression or replication is suppressed. To test this hypothesis, we determined the optimal condition to reduce HBV gene expression and replication using the nucleoside analogue lamivudine. HepG2.2.15 cells were treated with lamivudine at various concentrations from 0.04 μmol/L to 100 μmol/L. The antiviral activity was determined by quantitation of secreted HBsAg and HBeAg particles, extracellular virions and intracellular HBV replicative intermediates (RI). Figure 4A shows that treatment with lamivudine led to a significant reduction of secreted HBsAg and HBeAg in the supernatant of HepG2.2.15 cells. Parallel to the reduction of HBsAg and HBeAg production, the extracellular virion DNA in the culture supernatant of HepG2.2.15 cells and intracellular HBV replicative intermediates (RI) decreased after treatment with 2 μmol/L or 20 μmol/L of lamivudine for 10 d (Figure 4B and C). Maximal levels of suppression of HBV were observed after 10 d of lamivudine treatment. At that time, levels of RI were not more than 1.5% of controls in cultures of the 2 μmol/L treatment group. Based on these results, we chose

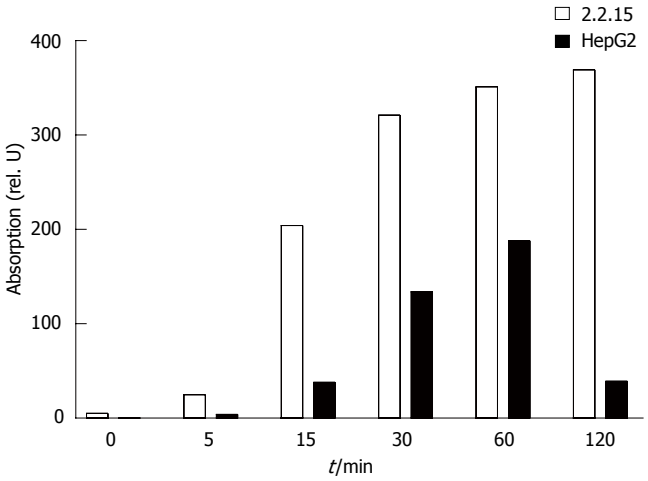

Figure 2 Analysis of Stat1 phosphorylation after IFN-α stimulation in HepG2 and HepG2.2.15 cells. Cells were stimulated with 100 U/mL of IFN-α for the indicated time points. Then, nuclear proteins were extracted and analysed by western blot. Data were quantified using Imagequant and are shown as relative units.

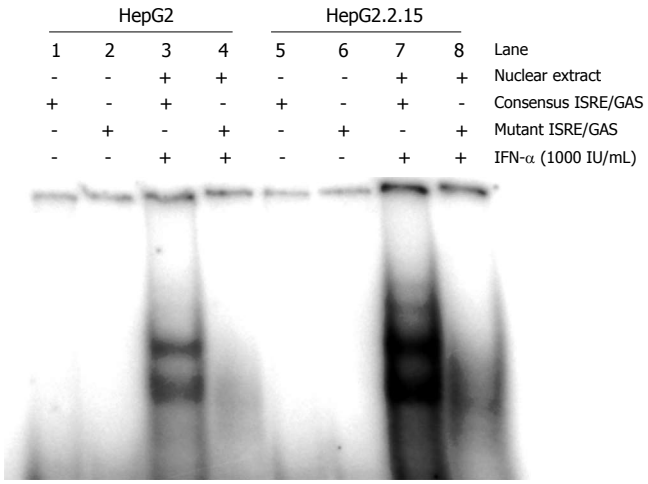

Figure 3 Analysis of ISGF3 formation after IFN-α stimulation in HepG2 and HepG2.2.15 cells. HepG2 and HepG2.2.15 cells were stimulated with 1000 IU/mL of IFN-α for 6 h followed by isolation of nuclear extracts (NE-PER™ reagent kits) for EMSA analysis.

a concentration of 2 μmol/L and duration of 10 d to suppress HBV replication in our system to study the modulatory effects of lamivudine on the IFN-response.

The IFN response in HepG2.2.15 and HepG2 cells after lamivudine treatment

The effect of lamivudine on ISG expression in HepG2.2.15 and HepG2 was investigated by using gene macroarrays. No effect was observed for the stimulation of MxA and Cig5 expression by lamivudine treatment (Table 2). Both genes did not respond with an increased expression upon IFN-α stimulation. An increase of the IFN-α concentration to 1000 units per mL or a prolonged incubation with IFN-α did not change the expression of MxA and cig5. The reduced induction of IFITM 2 and IFITM 3 expression could not be enhanced by lamivudine treatment. In contrast, IFITM1 and 6-16 expression could

be restored by lamivudine treatment of HepG2.2.15 cells (Table 1, Figure 1). This indicates that lamivudine can only partially normalize the IFN-response in HBV-transfected HepG2.2.15 cells at concentrations that profoundly inhibit viral replication and secretion of viral particles. Lamivudine had no effect on ISG expression in HepG2 cells and did not enhance the induction of many other ISGs, such as 2.5 OAS and MxB.

DISCUSSION

In the present work, we found that HepG2.2.15 and HepG2 respond differently to IFN-α. Several ISGs were not induced in HepG2.2.15 while they were expressed in HepG2 cells after IFN-α. There may be multiple reasons for the different ISG expression profiles in these cell lines, though HepG2.2.15 was derived from HepG2[16]. Previous data indicated that the expression of the IFN-inducible gene MxA was specifically inhibited by HBV proteins in HBV-transfected HepG2 or HuH7 cells[13], and this was accompanied by diminished antiviral activity of IFN[17]. In our study, we confirmed this finding with MxA expression being completely diminished in HBV-transfected HepG2.2.15 cells. In addition, we showed that additional ISG (Cig5, IFITM1, -2, -3 and 6-16) expression was completely abolished or partially reduced by HBV. The majority of ISGs, however, are expressed and inducible in both HepG2 and HepG2.2.15 cells, indicating that the HBV gene expression and replication had no effect on these ISGs. Consistently, Rosmorduc *et al*[17] demonstrated that 2´5OAS expression is not affected by HBV. Our results support the view that the HBV-mediated inhibition of the IFN-response, if any, represents a specific rather than global effect. The Stat1 activation or ISGF3 formation in HepG2.2.15 cells appeared to be normal, indicating that the Jak/Stat signalling pathway is intact and functional. These findings are corroborated by the data from Fernandez *et al*[13] who demonstrated that the inhibition of MxA induction in HepG2 cells occurs at the promoter level.

We then asked the question whether the HBV-mediated suppressive effect on the IFN-response could be reverted by treatment with the nucleoside analogue lamivudine, which is an effective inhibitor of HBV replication *in vitro*[18] and *in vivo*[19,20]. Lamivudine is phosphorylated within the cell and then incorporated into nascent viral DNA by the HBV polymerase during replication[21] resulting in the termination of HBV DNA elongation. Lamivudine also inhibits reverse transcriptase activity directly through competitive inhibition. Although some reports indicate that lamivudine exerts synergistic effects with IFN, the underlying mechanisms are not clear[22,23]. To answer this question we first established the optimal conditions for *in vitro* treatment of HepG2.2.15 cells with lamivudine. The results indicated that lamivudine exerted potent antiviral activities in our system as it strongly suppressed the formation of HBV replicative intermediates and extracellular HBV DNA at concentrations that correspond well to plasma levels found in patients that are treated with this drug. However, HBsAg and HBeAg secretion was only down regulated and not completely blocked. After treatment with lamivudine for 10 d, the induction of IFITM1 and 6-16 expression could be enhanced while MxA, Cig5, IFITM2 and IFITM3 induction remained unchanged. This indicates that lamivudine can at least partially improve the impaired IFN response in HBV-transfected cells. IFITM 1 to 3 and 6-16 belong to a family called small ISGs[24]. IFITM 1 to 3 are classified as members of the 1-8 group while 6-16 is a member of the ISG12 group. These genes were under the control of multiple elements responding to IFN-α stimulation including ISGF3 and interferon. It is likely that the lamivudine treatment partially reduces HBV gene expression and therefore contributes to the improved

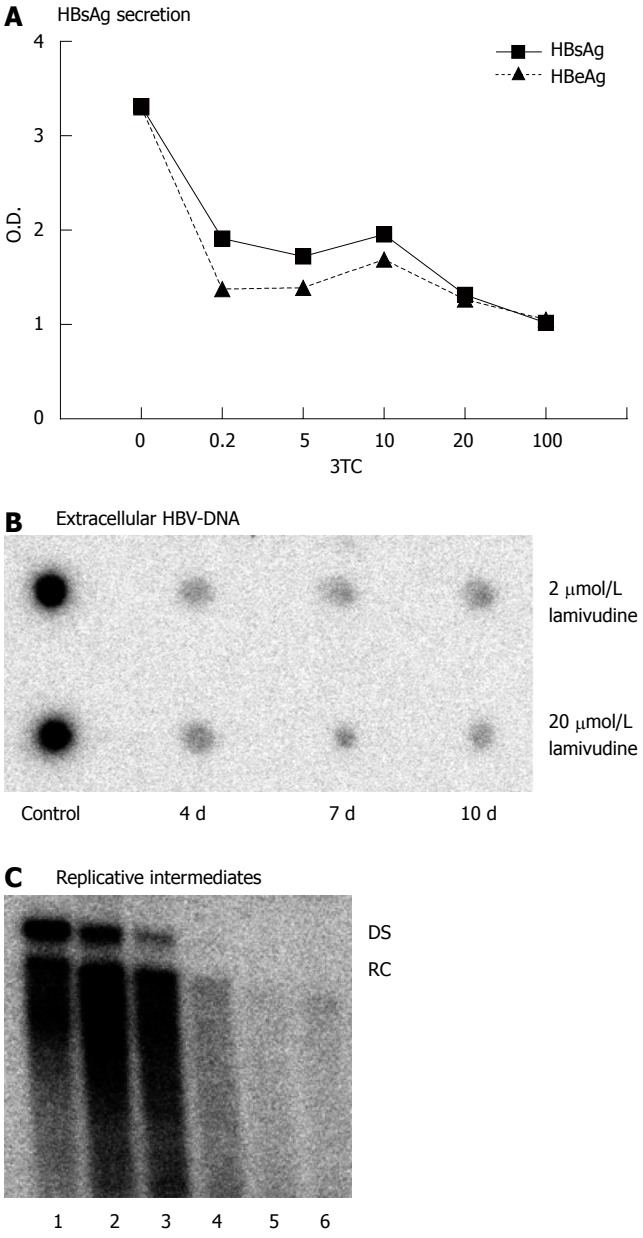

Figure 4 Antiviral effects of lamivudine in HepG2.2.15 cells. **A**: HepG2.2.15 cells were cultivated with various concentrations (0 to 100 μmol/L) of 3 TC for 10 d. Then, supernatants were harvested and assayed for the presence of HBsAg and HBeAg by ELISA; **B**: HepG2.2.15 cells were treated with 2 or 20 μmol/L of lamivudine for 4, 7 and 10 d, respectively. Then, supernatants were collected and extracellular HBV-DNA was analyzed by dot blot hybridization; **C**: HepG2.2.15 cells were treated with various concentrations of lamivudine for 10 d. Then, intracellular HBV replicative intermediates were isolated for southern blotting. Lane 1: control, lane 2: 0.04 μmol/L, lane 3: 0.2 μmol/L, lane 4: 5 μmol/L, lane 5: 25 μmol/L, lane 6: 125 μmol/L, RC, relaxed circular HBV-DNA; DS, double stranded linear HBV-DNA.

ISG expression. On the other hand, the continuing HBV protein production may still dominantly interfere with the expression of many ISGs, such as cig5 and IFITM3.

These findings are corroborated by our study that shows an improved IFN response of PBMC from HBV patients after treatment with adefovir. Some reports have also suggested a restoration of weak T helper cell and CTL responses after initiation of lamivudine therapy[25,26]. Although it is certainly a possibility, it still remains to be determined whether this effect can be explained by an enhanced responsiveness to IFNs.

In conclusion, our results suggest that HBV specifically modulates the IFN response in HepG2 cells by a selective suppression of certain ISGs. This suppression is at least partially reversible by antiviral treatment with the nucleoside analogue lamivudine.

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