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APOBEC and iNOS are not the main intracellular effectors of IFN-γ-mediated inactivation of Hepatitis B virus replication

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Abstract

Background/Aim: Interferon-gamma (IFN-γ) produced by activated T-cells is the principle mediator of non-cytolytic Hepatitis B virus (HBV) inactivation; however the intracellular pathways responsible are poorly defined. We investigated the role of IFN-γ–inducible nitric oxide synthase (iNOS) and APOBEC3 (A3) enzyme family in the inhibition of HBV replication by IFN-γ.
Methods: Hepatoma-cell lines transfected with HBV DNA were treated with IFN-γ. Viral replication, iNOS and A3 mRNAs were quantitated by TaqMan®PCR and the direct nitric oxide (NO) effect on HBV replication was investigated using an NO-donor. A3G antiviral activity was verified by co-transfection with its inhibitor, human immunodeficiency virus (HIV)-associated virion infectivity factor (Vif).
Results: IFN-γ caused a dose-dependent reduction (>50%) of HBV DNA in the absence of cytotoxicity. Although iNOS mRNA increased 45-fold in IFN-γ treated cells, NO_2^- was not detectable in supernatants and the use of an NO-donor did not inhibit HBV replication. A3 enzyme mRNAs varied between cells and were >10-fold higher in lymphocytes than in liver tissue. IFN-γ up-regulated A3G mRNA by three-fold, associated with significant HBV DNA decrease. However, A3G degradation by Vif did not abolish the antiviral effect of IFN-γ against HBV.
Conclusions: IFN-γ inhibits HBV replication and up-regulates both iNOS and A3G. However, other pathways appear to have a greater role in IFN-γ-induced HBV inactivation in the liver.

Keywords: Interferon-γ; Hepatitis B virus; Inducible nitric oxide synthase; Apobec3 enzymes

1. Introduction

Hepatitis B virus (HBV) is a non-cytopathic virus and the outcome of HBV infection, as well as the diversity of clinical manifestations, depends primarily on the host immune response (Rehermann and Nascimbeni, 2005). Cytokines secreted by virus-specific cytotoxic T-lymphocytes (CTL) or by antigen-nonspecific macrophages and T-cells have a key role in intracellular inactivation of HBV (Guidotti and Chisari, 2001). The importance of these cytokine-mediated, non-cytolytic antiviral mechanisms was clearly demonstrated in an experimental HBV infection of chimpanzees, and in transgenic mice (Guidotti et al., 1996, 1999; Thimme et al., 2003). Interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) were found to activate two virucidal pathways – one eliminating the HBV nucleocapsid particles with their cargo of viral genomes, the second causing degradation of viral RNA (Guidotti et al., 1996; Biermer et al., 2003; Wieland et al., 2000, 2005). Cytokine-mediated inhibition of HBV replication has also been demonstrated in primary human hepatocytes and in human hepatoma-derived cell lines following transient or stable transfection with cloned HBV DNA (Biermer et al., 2003; Pasquetto et al., 2002; Rang et al., 1999, 2001). However, the cellular effectors that mediate IFN-γ or TNF-α-induced inhibition of HBV replication and their relative contribution to clearance of the virus from infected hepatocytes are poorly defined (Guidotti et al., 2002).

Studies in transgenic mice suggested that the antiviral effects of IFN-γ might be mediated by nitric oxide (NO), a gaseous signalling molecule produced in many cell types includ-

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ing macrophages and hepatocytes following the induction of inducible nitric oxide synthase (iNOS) (Guidotti et al., 2000). Inducible synthesis of NO represents an important mechanism in the control of pathogens and has previously been shown to inhibit replication of animal viruses both *in vitro* and *in vivo* (Akerström et al., 2005; Karupiah et al., 1993; López-Guerrero and Carrasco, 1998; Rimmelzwaan et al., 1999; Zaragoza et al., 1997). At present, it is unclear whether the antiviral effects of NO extend to control of HBV in human cells or how elevated NO levels should be for interfering with virus replication.

Recently, the replication of HBV in transfected hepatoma cells was shown to be inhibited by APOBEC3G (A3G) and related members of the APOBEC3 (A3) family of cytidine deaminase enzymes, previously identified as cellular proteins capable of limiting replication of human immunodeficiency virus (HIV) in non-permissive cells (Rösler et al., 2005; Turelli et al., 2004; Turelli and Trono, 2005; Harris and Liddament, 2004; Sheehy et al., 2002). Moreover, a subsequent study has demonstrated the expression of A3A-G in human liver and revealed that expression of mRNAs encoding APOBEC proteins was induced by IFN-α in primary human hepatocytes, thus suggesting a potential role for APOBEC family members in cytokine-mediated clearance of HBV (Bonvin et al., 2006). However, the induction of the members of APOBEC family by IFN-γ and their potential involvement as antiviral mechanisms against HBV is unknown.

In this study we used transiently and stably transfected cell lines to examine the effects of IFN-γ on iNOS and APOBEC expression and their role as possible pathways for HBV inactivation. Our results demonstrate that while viral replication is inhibited by INF-γ, and iNOS mRNA and several members of the APOBEC family are induced in liver cells by IFN-γ, its antiviral effect is due to neither increased NO production nor the activity of APOBEC proteins, suggesting that other pathways have a greater role in immune-mediated HBV control.

2. Methods and materials

2.1. Cell culture and transfection

The human hepatoma-cell lines Huh-7, HepG2 and HepG2.2.15 (stably transfected with HBV DNA with full virion production) (Sells et al., 1988) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Huh-7 and HepG2 were plated at 2×10^5/well in 12-well plates or at 3×10^6/well in a 10 cm dish, 18 h before transfection. Cells were then transfected using FUGENE 6 reagent (Roche, Welwyn Garden City, UK), according to the manufacturer's protocol and treated for 48 h with cytokines or NO-donors, as indicated below.

Plasmid, pHBV-dimer (pSM2) (Rang et al., 1999) was a gift from Dr Andreas Rang, Munich Germany. A3G DNA and virion infectivity factor (Vif) DNA were cloned into a pcDNA5/FRT/V5-His-TOPO vector (Invitrogen, Paisley, UK), as described previously (Suspene et al., 2004; Jarmuz et al., 2002).

2.2. Specimen and samples

Paired samples of peripheral venous blood and a spare portion of diagnostic liver biopsy specimens were obtained from four patients with chronic Hepatitis B, with informed consent. One patient was HBeAg positive, while three were anti-HBe positive with serum HBV DNA ranging from 6 to 7 $\log_{10}$ IU/ml and all with raised ALT. The use of clinical material for these experiments was approved by the UCLH research Ethics committee. Peripheral blood mononuclear cells (PBMCs) were isolated by standard lymphoprep density centrifugation from the collected fresh blood.

2.3. Cytokines and reagents

Recombinant human IFN-γ was purchased from R&D Systems (Abingdon, UK); NO-donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) – from Sigma–Aldrich (Dorset, UK); DNAseI and Proteinase K – from Roche (Welwyn Garden City, UK). Lactate dehydrogenase (LDH) was tested in cell culture supernatants as a measure of cell lysis using a commercially available enzyme-immunoassay (Roche, Welwyn Garden City, UK).

2.4. Purification and quantitation of HBV DNA from intracellular core particles

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed by adding 1 ml lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40) per well at 4 °C for 30 min. The lysate was centrifuged for 2 min at 5000 rpm to remove debris and nuclei. The supernatant was adjusted to 10 mM $MgCl_2$ and treated with 100 μg/ml DNaseI for 30 min at 37 °C. The reaction was stopped by adding EDTA and sodium dodecyl sulfate (SDS) to final concentrations of 25 mM and 1%, respectively, and proteins were digested with 0.5 mg/ml Proteinase K for 2 h at 37 °C. DNA was further purified by phenol/chloroform (1:1) extraction and ethanol precipitation. HBV DNA was quantified by real-time TaqMan PCR (ABI prism 7700, Applera, Warrington, UK), as previously described with minor modifications (Garson et al., 2005). The amplification was performed in 25 μl reactions (in duplicate for all samples) containing 12.5 μl of 2× TaqMan Universal PCR mix, 0.6 μM forward and reverse primers, 0.15 μM probe and 5 μl of template. Dilutions of pHBV-dimer were used to derive a standard curve ranging from 10^9 to 10^4 copies/ml in each experiment and the standard curve was validated using the WHO HBV international standard 97/746 (National Institute for Biological Reference Standards, Potters Bar, UK). HBV DNA quantitation was normalized using human β-actin, which was quantitated with a commercial human β-actin kit (Eurogentec Ltd., Hampshire, UK).

2.5. RNA extraction and quantification by real-time PCR

Total RNA was extracted from the cell lines, human liver biopsies and PBMC using RNeasy kit (Qiagen, West Sussex,

UK,) and subsequently was reverse transcribed with random hexamers and M-MLV reverse transcriptase (Ambion, Spitfire Close, UK). The cDNAs were quantitated by real-time PCR using primers and probe specific for iNOS and A3 and the results were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantification was performed using the Ct comparison ($\Delta\Delta C_T$) between iNOS and GAPDH after validation of the method. For A3 enzymes, standards curves with known number of copies of A3 enzymes and GAPDH were used (Suspene et al., 2004). The identification numbers of the predesigned and pre-optimized primers and probes sets (Applera, Warrington, UK) were as follows: iNOS: Hs00167257_ m1; APOBEC 3A (A3A): Hs00377444_ m1; APOBEC 3B (A3B): Hs00358981_ m1; APOBEC 3D (A3D): Hs00537163_ m1; APOBEC 3F (A3F): Hs00736570_ m1; APOBEC 3G (A3G): Hs00222415_ m1.

2.6. Measurement of NO_2^-

Nitrite levels were determined with the Griess colorimetric assay using sodium nitrite standards. 100 μl of Huh-7, 2.2.15 supernatants or sodium nitrite was mixed with 100 μl of Griess reagent (1% sulfanilamide, 0.1% napthyl-ethylenediamine) and after 10 min of incubation, the absorbance was measured at 540 nm.

2.7. Western blot analysis

Cytoplasmic extracts were precipitated with 10% trichloroacetic acid and proteins solubilized in SDS buffer. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes after electrophoresis on SDS polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were blocked in 10% fat-free milk powder. Detection of A3G was performed with mouse anti-human A3G antibody and followed by HRP-labelled rabbit anti mouse IgG (Dako, Cambridgeshire, UK). Bands were detected by enhanced chemiluminescence (Perbio, Northumberland, UK).

2.8. Statistical methods

Statistical analysis was performed using SPSS 12.0 software package. Statistical differences between the control and treatment groups were calculated by paired sample *t*-test and considered significant at $p < 0.05$.

3. Results

3.1. Effect of IFN-γ on HBV replication

Stably HBV-transfected 2.2.15 cells were cultured over a period of 24 and 48 h with 300 IU/ml and 3000 IU/ml of IFN-γ. The encapsidated viral genomes measured in the cytoplasmic pool were reduced by 45% ± 14 ($p < .05$) after 24 h treatment with 3000 IU/ml of IFN-γ. After 48 h, 300 and 3000 IU/ml IFN-γ induced a reduction of 43 ± 23% ($p < .05$), 47 ± 7% ($p < .01$) (Fig. 1A). In transiently transfected Huh-7 cells treated for 48 h with 1000 and 5000 IU/ml IFN-γ cytoplasmic HBV DNA was reduced by 45 ± 6.48% ($p < .001$) and 69 ± 10.59% ($p < .001$), respectively, in comparison to non-treated cells (Fig. 1C). This antiviral activity in the cell lines was not due to cytotoxic effects of the cytokine, as the release of LDH in presence of IFN-γ was not increased over control cells (Fig. 1B and D).

3.2. IFN-γ stimulation of inducible nitric oxide synthase (iNOS)

Huh-7 cells transiently transfected with HBV and treated with 1000 and 5000 IU/ml IFN-γ resulted in a marked increase of iNOS mRNA, 30-fold ($p < .05$) and 43-fold ($p < .05$), respectively, over the iNOS level in non-treated Huh-7 cells (Fig. 2). To evaluate the NO levels directly, we measured the production of nitrite (NO^-_2), an end product of NO oxidation. However, NO production was undetectable, i.e. below 2 μmol/l NO^-_2, which was the lower limit of detection of the assay both in control and IFN-γ treated cells. To determine whether increased NO levels could inhibit HBV replication, the cells were incubated with the NO-donor, SNAP which was added directly to 2.2.15 (Fig. 3A) and to Huh-7, 5 h after transfection (Fig. 3B). In both cell lines, SNAP induced a continuous dose-dependent release of significant amounts of NO which we verified by quantitation of NO_2^- levels in comparison to the non-treated cells ($p < .01$). In parallel, measurement of the cytoplasmic HBV DNA in both cell lines did not show any significant antiviral effects with 100 or 200 μM of SNAP relative to controls (Fig. 3C and D).

3.3. Apobec3 gene expression in hepatoma-derived cell lines, liver tissue and PBMC

We also measured the effect of IFN-γ on the expression of genes encoding members of the A3 family of cytidine deaminases. IFN-γ treatment (5000 IU/ml) resulted in a 2.5-fold ($p < .05$) increase in the expression of A3G in Huh-7 cells, as measured by TaqMan real-time PCR (Fig. 4A). A greater induction was observed in HepG2 cells also treated with 5000 IU/ml, where A3B, A3F and A3G expression increased 2.5-, 3.8- and 5.0-fold, respectively ($p < .05$ Fig. 4B). In addition, we compared the expression of A3A, A3B, A3F and A3G in paired PBMC and liver biopsy specimens isolated from four patients with chronic HBV infection (Fig. 4C). These results revealed quantitative and qualitative differences in enzyme expression in liver and mononuclear cells. Overall, A3G was 10-fold higher ($p < .05$) in PBMC than the corresponding level in liver; similarly, A3A was 30-fold higher ($p < .001$) in PBMCs, while the levels of A3B and A3F were similar in these two cell types.

3.4. Effect of Vif protein on A3G inhibition of HBV replication

Co-transfection of pHBV-dimer with A3G plasmid in Huh-7 cells resulted in a 75% ($p < .05$) reduction of encapsidated HBV DNA when the ratio of plasmid DNA encoding A3G and HBV during transfection was 3:1. When this ratio was increased to 4:1, the HBV DNA reduction was even greater – 100% ($p < .001$)

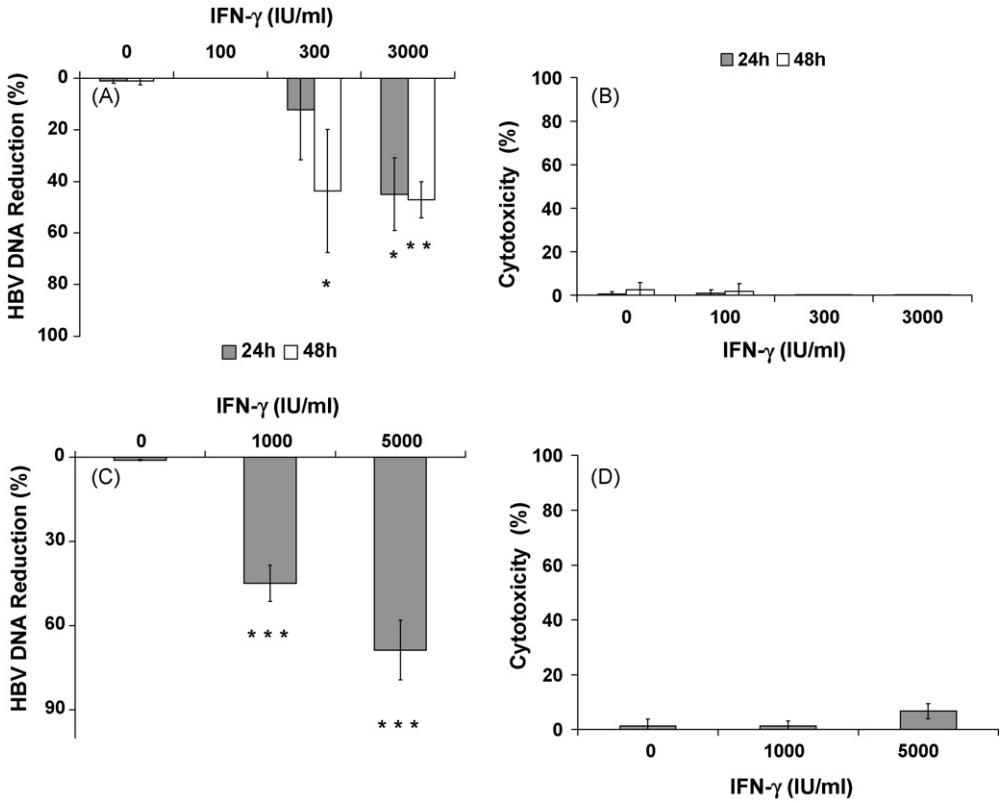

Fig. 1. Effect of IFN-γ on HBV replication in stably and transiently transfected cell lines. (A) 2.2.15 cells were treated with 100, 300 and 3000 IU/ml IFN-γ for 24 and 48 h. Cytoplasmic HBV DNA was quantitated by real time PCR using HBV pre-S specific primers and probe and normalized for β-actin. Values are expressed as mean ± S.D. of percentage of HBV DNA produced in absence of IFN-γ which was given the value of 100. (B) 2.2.15 cytotoxicity was measured by LDH quantitation. Values are expressed as percentage. (C) Huh-7 cells were transiently transfected with HBV DNA plasmid and cultured with IFN-γ (1000 and 5000 IU/ml) for 48 h and cytoplasmic HBV DNA was quantitated as described in (A). (D) Huh-7 cytotoxicity was measured as described in (B). The data are representative of three independent experiments, each done in triplicate. *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$) indicates statistically significant differences between the indicated group versus control which correspond to the non treated cell lines.

compared with control cells (Fig. 5A). To investigate whether the expression of APOBEC proteins stimulated by IFN-γ could act as an intracellular effector of cytokine-mediated inhibition of HBV replication, we employed the unique effect of Vif, an HIV protein, known to selectively inhibit A3G in cells (Mariani et al., 2003; Sheehy et al., 2002). Recent data have established that Vif targets A3G for proteosomal degradation when co-expressed in the same cell (Conticello et al., 2003; Marin et al., 2003; Mehle et al., 2004). The effect of Vif on A3G expression was examined by co-transfection of A3G and Vif plasmids in Huh-7 and subsequent analysis of the effect on A3G protein expression and HBV DNA levels. When A3G plasmid was transfected alone, the A3G protein was readily detectable by western blot (Fig. 5B, lane 2), while in the presence of Vif the 46 kDa protein was undetectable, confirming that Vif reduces A3G in the cells (lane 3). Moreover, endogenous level of A3G was not detectable by Western blot in Huh-7 cells treated with 5000 IU/ml IFN-γ (lane 4), suggesting a low level of endogenous A3G in the cells. Co-transfection of HBV and A3G in Huh-7 resulted in a 70 ± 4.8% ($p < .05$) reduction of HBV DNA; the inhibition of A3G protein by Vif induced a two-fold ($p < 0.05$) increase of HBV DNA levels, compared to those observed in the absence of A3G (Fig. 5C), showing that Vif can partially reduce A3G antiviral activity against HBV. To investigate a potential role of endogenous A3G in the reduction of HBV replication mediated by IFN-γ, we co-transfected cells with pHBV-dimer and Vif plasmid, followed by 48 h treatment with 5000 IU/ml of IFN-γ. The inhibition of A3G by Vif did not affect the INF-γ-induced inhibition of HBV replication, as there was a 50% ($p < .05$) decrease of cytoplasmic HBV DNA even

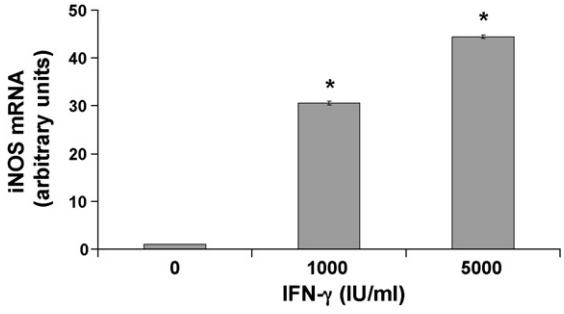

Fig. 2. Effect of IFN-γ on iNOS mRNA . Huh-7 cells were transiently transfected with HBV DNA and cultured with IFN-γ (1000 and 5000 IU/ml) for 48 h. iNOS cDNA was quantitated by real time PCR using iNOS mRNA specific primers and probe and normalized for GAPDH. Values are expressed as relative to iNOs mRNA produced in absence of IFN-γ which was given the arbitrary value 1. The data are represented as mean ± S.D. of duplicates. The data are representative of three independent experiments, each done in duplicate. * Indicates statistically significant differences ($p < 0.05$) between the treated and non-treated groups.

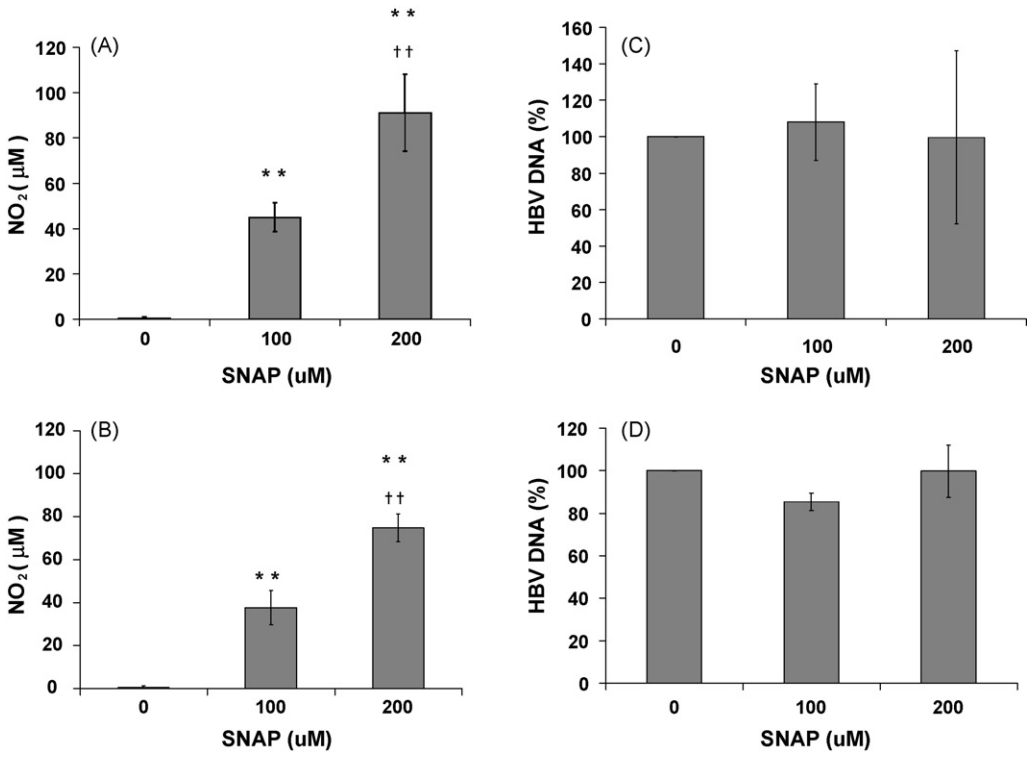

Fig. 3. Effect of NO on HBV replication. 2.2.15 cells and Huh-7 cells transiently transfected with HBV DNA plasmid were treated with 100 and 200 μM of SNAP (NO inducer) for 48 h. The NO2 levels in (A) 2.2.15 and (B) Huh-7 supernatants were quantitated by the Griess assay. Cytoplasmic HBV DNA in (C) 2.2.15 and (D) Huh-7 was quantitated by real-time PCR using HBV pre-S specific primers and probe and normalized for β-actin. Values are expressed as mean ± S.D. of percentage of HBV DNA produced in absence of SNAP which was given the value of 100. The data are representative of three independent experiments, each done in duplicate. ** and †† indicate statistically significant differences ($p < 0.01$) between the indicated group (** versus control, †† versus 100 μM SNAP).

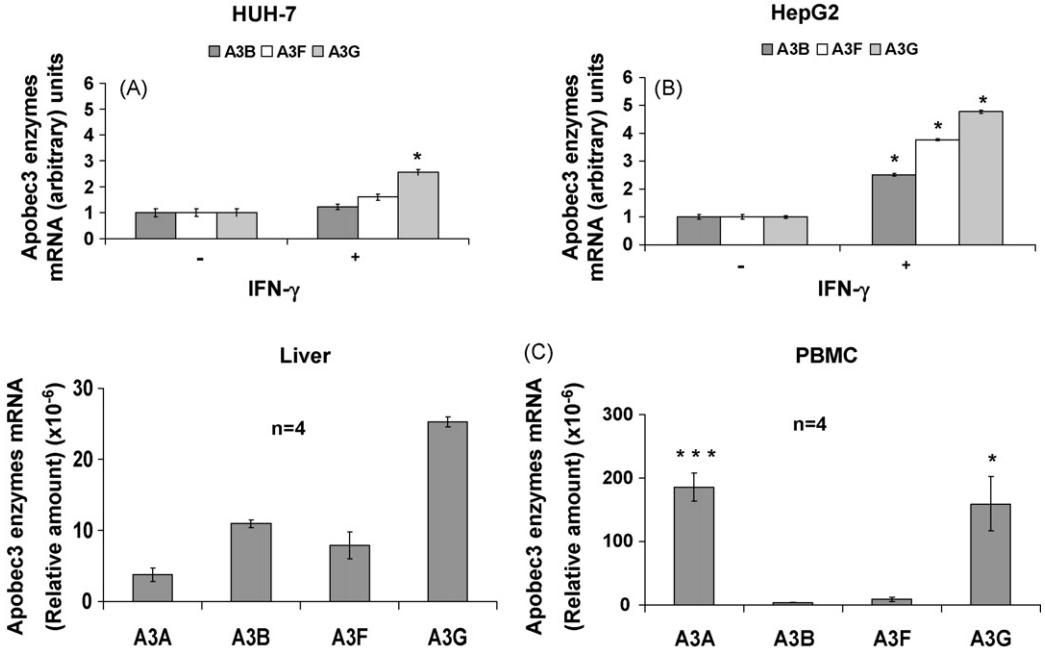

Fig. 4. Effect of IFN-γ on A3 enzymes mRNA in cell lines. (A) Huh-7 and (B) HepG2 cells were treated with 5000 IU/ml IFN-γ for 48 h. Apobec enzymes cDNA were quantitated by real-time PCR using A3 enzymes specific primers and probe. Values were normalized to GAPDH and are expressed as relative to A3 mRNA produced in absence of IFN-γ which was given the arbitrary value1. The data were represented as mean ± S.D. of duplicates and are representative of three independent experiments. * ($p < 0.05$) indicates statistically significant differences between the controls and the treated groups. (C) Level of expression of A3 enzymes in liver specimen and lymphocytes from four chronic HBV patients. A3 enzymes cDNA were quantitated as stated above. Values are expressed as relative to GAPDH expression and represented as mean ± S.D. of duplicates. *** ($p < 0.001$) and * ($p < 0.05$) indicate statistically significant differences of A3 enzyme expression between paired PBMC and liver specimen.

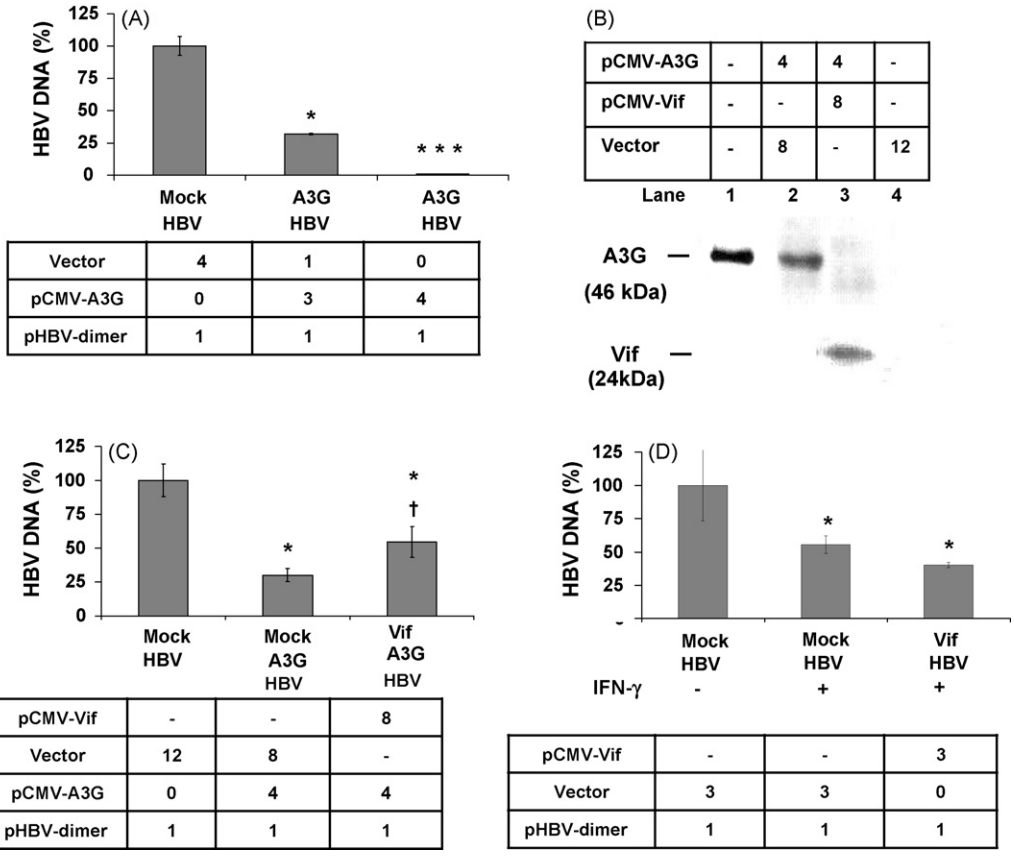

Fig. 5. Direct effect of A3G on cytoplasmic HBV DNA and effect of A3G inhibition on IFN-γ inhibitory activity. (A) Huh-7 was transfected with A3G and HBV DNA plasmids at different ratios as stated in the table. Cytoplasmic HBV DNA was quantitated by real time PCR using HBV pre-S specific primers and probe and normalized for β-actin. (B) Western blot analysis of A3G and Vif in cytoplasmic extracts from Huh-7 cells. Recombinant A3G was used as positive control (lane 1). Huh-7 cells were transfected with A3G (lane 2), co-transfected with A3G and Vif (lane 3) or not transfected but treated with 5000 IU/ml IFN-γ (lane 4). Huh-7 was (C) co-transfected with A3G, HBV DNA and Vif (A3G inhibitor) and (D) co-transfected with HBV and Vif and subsequently treated with 5000 IU/ml IFN-γ. The amount of plasmids used is summarized in the table. Cytoplasmic HBV DNA was quantitated as described in (A). Values are expressed as mean ± S.D. of three independent experiments. The control group, Huh-7 cells mono-transfected with HBV DNA plasmid was given the value of 100. *** ($p < 0.001$), * and † ($p < 0.05$) indicate statistically significant differences between the indicated groups (*versus control, † † versus HBV, A3G and Vif co-transfection).

after blocking A3G (Fig. 5D). These results suggest that A3G does not play a significant role in cytokine-mediated suppression of virus replication.

4. Discussion

Previous reports have demonstrated the importance of inflammatory cytokines, in particular IFN-γ and TNF-α, in inhibiting the replication of HBV and many other viruses without damaging the infected cells (McClary et al., 2000; Guidotti et al., 1999; Cavanaugh et al., 1998). Resolution of HBV infection was shown to require non-cytolytic antiviral mechanisms involving TNF-α and mostly IFN-γ producing cytotoxic T-lymphocytes (Guidotti et al., 1996, 1999; Thimme et al., 2003). However, the cellular mechanisms involved in IFN- γ antiviral activities against HBV are still not well defined (Gordien et al., 2001; Rang et al., 2002; Guidotti and Chisari, 2001; Robek et al., 2004). In the present study, we analysed the potential role of two pathways, iNOS and A3 enzymes, which were separately shown to have antiviral activities against HBV and other viruses.

Our results demonstrate that HBV replication in 2.2.15 and Huh-7 cells is inhibited by IFN-γ in a dose- and time-dependent manner, showing that the two systems can be used to study cytokine-induced antiviral effect. In our study, transiently transfected Huh-7 cells were more susceptible to IFN-γ antiviral activity than 2.2.15 cells. This could be explained by the fact that 30–40% of Huh-7 cells were transiently transfected with HBV plasmid and produced virions whereas 2.2.15 cells are stably transfected with every cell producing high levels of virions. Therefore, the magnitude of the antiviral effect of IFN-γ in Huh-7 and 2.2.15 cells was affected by the level of virions produced in the two cell lines.

In chronically WHV-infected woodchucks it was shown that wIFN-γ is unable to significantly inhibit WHV and it is believed that the infected animals developed a ‘tolerance’ to IFN-γ because of continuous exposure to high levels of the cytokine during chronic infection (Fiedler et al., 2004; Lu et al., 2002). However, IFN-γ inhibition of HBV in our study is consistent with the observed antiviral effects in immortalized murine hepatocytes from HBV transgenic mice (Pasquetto et al., 2002), primary hepatocytes from DHBV-infected ducks (Schultz and

Chisari, 1999) or in naturally infected human hepatocytes, albeit restricted to cells from patients with low viral titers (Suri et al., 2001). The ability of IFN-γ to reduce HBV titers in transgenic mice was previously linked to the production of NO (Guidotti et al., 2000), which was also shown to have antiviral properties against various viruses (Akerström et al., 2005; Karupiah et al., 1993; López-Guerrero and Carrasco, 1998; Rimmelzwaan et al., 1999; Zaragoza et al., 1997). Therefore, we examined whether IFN-γ-induced inhibition of HBV replication in transfected Huh-7 cells was mediated by NO. Although IFN-γ was able to increase iNOS mRNA, NO_2^- was not detected in the two cell lines. As NO levels below the limit of detection of the Griess assay may still inhibit HBV replication, we also studied the direct effect of NO by using the NO-donor SNAP. When the level of HBV DNA was measured in cells incubated with different concentrations of SNAP, no reduction in the yield was observed despite a large increase in NO concentration. These experiments demonstrate that NO production alone is not responsible for the reduction in viral replication caused by IFN-γ in 2.2.15 and Huh-7 cells. This observation is further supported by a study using a mouse model of acute Hepatitis B (Chang et al., 2003). In this latter study, the iNOS expression was associated with a decrease of HBV titers. However, the absence of NO was associated with a reduced infiltration of T-cells and NKT cells into the liver, possibly suggesting that the reduced clearance of HBV in these mice was not due solely to a direct antiviral effect of NO. Interestingly, HBV transgenic mice that were deficient in interferon-regulatory factor (IRF-1), an IFN-γ-inducible transcription factor necessary for transcription of iNOS, exhibited increased levels of virus replication under basal conditions but maintained a functional antiviral response in the presence of IFN-γ, suggesting that a cytokine-mediated effector distinct from iNOS remained operative in these animals (Guidotti et al., 2000). Similarly, no viral reduction was observed in HCV replicons treated with an NO-donor (Frese et al., 2002), suggesting a possible resistance of HCV to NO. Thus, our data support the notion that NO is not an essential cellular mechanism for the antiviral activity of IFN-γ against HBV.

Recent studies have revealed that members of the APOBEC3 family of cytidine deaminases can inhibit the replication of HBV *in vitro* (Rösler et al., 2005; Turelli et al., 2004). Although G-to-A hypermutated HBV DNA can be detected in HBV-infected patients (Noguchi et al., 2005), a non-editing mechanism involving the inhibition of pregenomic HBV RNA packaging has been proposed to account for the inhibition of HBV replication observed *in vitro* (Turelli et al., 2004; Rösler et al., 2005). Bonvin et al. (2006) recently demonstrated that several APOBEC family members are expressed in human liver and showed that the expression of these enzymes in primary human hepatocytes, and hepatoma-derived cell lines is stimulated by IFN-α. Our results extend these findings and demonstrate that A3 enzymes are induced also by IFN-γ in Huh-7 and HepG2 cells. Importantly, we found that the level of induction varies between cell types, as well as between different enzymes, with A3G being stimulated to a greater level than A3F and A3B and with higher levels in HepG2 compared with Huh-7 cells. The levels of APOBEC induction by IFN-γ in our study are comparable with the induction reported by IFN-α (Bonvin et al., 2006), with the exception of the marked stimulation (up to 60-fold) of A3G in Huh-7 cells by IFN-α which was not observed with IFN-γ. Recently, A3G upregulation by IFN-γ was correlated with inhibition of HIV in CD4+ T-cells (Peng et al., 2006). By comparing the levels of A3 enzyme expression in paired liver specimens and PBMCs from chronic HBV patients, we found marked quantitative and qualitative differences between liver cells and lymphocytes. The variations in A3 enzymes expression between the different cell types studied here could be the result of not yet discovered activities, as A3 enzymes are known to have a broad profile of expression (Harris and Liddament, 2004). A3G with A3A are expressed the highest in PBMCs. In these cells, A3G levels could be explained by it's antiviral activity against vif-deficient HIV-1 which infects CD4 T cells (Harris and Liddament, 2004; Sheehy et al., 2002). However, A3A so far was only discovered in keratinocytes and we show for the first time an expression in PBMCs which suggests an unknown function of this enzyme. Moreover, we could not detect expression of A3G in hepatoma-derived cell lines – Huh-7 and HepG2 cells treated with IFN-γ by Western blot analysis, which emphasizes the low A3 enzyme levels in the liver. Furthermore, to determine whether A3G might be responsible for the effects of IFN-γ on HBV assembly in Huh-7 cells, we co-transfected cells with a plasmid encoding Vif, thus employing the specific effect of the HIV-associated virion infectivity factor to abrogate the antiviral effect by targeting A3G for degradation (Conticello et al., 2003; Marin et al., 2003; Mehle et al., 2004). As expected, we found that Vif effectively induces the degradation of A3G protein; however, the A3G inhibition by Vif did not reduce or abrogate the antiviral effect of IFN-γ, suggesting that A3G is unlikely to be a key intracellular mediator of IFN-γ despite a modest upregulation of its expression in response to this cytokine

In conclusion, the present study demonstrates that the inhibitory effects of IFN-γ on HBV replication in transfected cells are mediated by neither NO nor APOBEC3 proteins, despite the induction of the relevant cellular genes, and suggests that additional intracellular factors appear to have a greater role in IFN-γ-induced inhibition of HBV replication in the liver.

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