

The Effect and Possible Mechanism of Double-Stranded DNA on Replication of Hepatitis B Virus

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Abstract

Liver is one of the target organs for double-stranded DNA (dsDNA)-vector mediated gene delivery due to the highly efficient uptake of gene therapy vectors. Recently dsDNA was described as a pathogen associated molecular pattern that could be recognized by intracellular DNA sensors. Herein, we explored the possibility that dsDNA may change the intracellular innate immune responses of hepatocyte-derived cell and therefore regulate the replication of hepatitis B virus (HBV). A hepatoma cell line HepG2.2.15 which derived from HepG2 with integrated HBV genome, were treated with poly (dA-dT), a synthetic double-stranded DNA molecule. Unexpectedly, HBV replication was up-regulated after poly (dA-dT) transfection in HepG2.2.15 despite the delayed activation of ISGs. There was no nuclear-plasma translocation of IRF3 or NF- κ B observed at an early stage. Treatment of HepG2.2.15 cells with supernatant harvested from the cells transfected with poly (dA-dT) indicating that poly (dA-dT) -enhanced HBV replication was predominantly mediated by not secreted cytokines, but intracellular factors. By blocking the cellular signal pathways with inhibitors, we found that U0126, an inhibitor of ERK1/2, could abolish the poly (dA-dT) enhanced HBV replication. Pathway-scan results also indicated that phosphorylated MEK1/2 was enhanced after poly (dA-dT) transfection. Whether this HBV replicating enhancement is good for HBV infection disease outcome needs to be further investigated.

Keywords: Hepatitis B virus; Double-stranded DNA; MAPK/ERK

Introduction

Although host nucleic acids are normally sequestered from potential signaling receptors, a growing body of evidence has suggested that the host DNA can be recognized by and modulate innate immune response [1,2]. Genomic DNA derived from pathogens, such as bacteria or viruses, and certain CpG oligodeoxynucleotides (ODNs) also stimulate plasmacytoid dendritic cells to produce large amounts of interferon- α [3-6]. Accumulating evidence over the past few years has also suggested that double-stranded, but not single-stranded DNA, possesses strong immunological activity without the need for specific sequences like CpG motifs. This effect was observed when DNA was forced into cells by any means of transfection [7]. In fact, dsDNA induces production of type-1 IFNs in various cell types including immune cells such as dendritic cells, macrophages and B cells, and non-immune cells such as epithelial cells, fibroblasts, and thyroid cells through recognition and signaling pathways independently of TLR9 [8].

Chronic hepatitis B remains a substantial public health burden affecting approximately 350 million individuals worldwide. It is presently much more prevalent in China where the hepatitis B virus (HBV) carriers are almost 10% in the general population. These patients have an elevated risk of liver cirrhosis, hepatocellular carcinoma (HCC), and other severe clinical sequelae [9-11]. It is therefore a global health priority to cure chronic HBV infection and prevent its dire consequences. Currently approved drugs for treatment of chronic hepatitis B, including alpha interferon and nucleos(t)ide analogue inhibitors, despite get the good therapeutic effect in clinic, but still exhibit limited response, adverse effects, and emergence of drug resistance, and are rarely curative [12,13]. Therefore, scientists have not stopped their efforts for looking for better drugs to cure this virus infectious disease. Many groups focus on the development of therapy measures using an immunotherapeutic approach such as therapeutic HBV vaccine [14-16]. The gene delivery vectors for specifically target

hepatitis B virus or cellular molecules are often utilized. However, whether the vector backbones have any influences on HBV treatment is less well defined.

Given the background described above, we have explored here the possibility that dsDNA may regulate the replication of HBV in hepatoma cell line HepG2.2.15 in which HBV replicates stably. We found that poly (dA-dT), a synthetic double-stranded DNA, could enhance HBV replication in HepG2.2.15 despite the up-regulation of some ISGs. Moreover, the phosphorylated MEK1/2 was up-regulated after poly (dA-dT) transfection. And U0126, an inhibitor of ERK1/2, could block the poly (dA-dT) enhanced HBV replication. Our findings give a new insight of MAPK/ERK into HBV life cycle, namely, MAPK/ERK acts on enhancing HBV replication in HBV persistence state. Whether this dsDNA-enhanced HBV replication is good for disease outcome needs to be further investigated.

Materials and Methods

Reagents

Poly(deoxyadenylic-thymidylic) acid sodium salt (Catlog No. P0883), abbreviated as poly (dA-dT), was purchased from Sigma. NF κ B p65 antibody (sc-8008), IRF3 antibody (sc-9082), goat anti-

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rabbit and goat anti-mouse immunoglobulins/HRP were products from Santa Cruz Biotechnology. RPMI 1640 medium, Non Essential Amino Acid, L-glutamine, G418 Sulphate solution were purchased from PAA Laboratories GmbH. Fetal bovine serum superior was from Biochrom AG. Trypsin-EDTA cell digestion solution was from Gibco. Lipofectamine2000, opti-MEM serum-free medium, and total RNA extraction reagent TRIzol were from Invitrogen. RNase-free DNase set, RN easy Mini kit, Quanti Fast SYBR Green RT-PCR Kit, QIAamp DNA Mini and Blood Mini kit, Gel extraction kit, Proteinase K were purchased from Qiagen. NE-PER nuclear and cytoplasmic extraction reagents, SuperSignal® West PicoChemiluminescent Substrate were from Thermo Pierce. Amersham Rediprime II Random Prime Labelling system, Microspin S-200 HR Columns for probe purification, and QuikHyb Hybridization Solution were purchased from GE Healthcare life sciences. Redivue [α - 32 P] dCTP was from Amersham Biosciences. LightCycler® FastStart DNA Master SYBR Green I kit was from Roche.

Cell culture

Cryopreserved human hepatoma cell line, HepG2.2.15, were thawed and suspended in RPMI 1640 plus 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin, 500 μ g/ml G418, 5 mmol/L L-glutamine and 1 \times non-essential amino acid.

Cell transfection

HepG2.2.15 cells were seeded into 35mm diameter 6-well plate at a density of 7.5×10^5 cells per well. After 24 hours, each well was transfected with poly (dA-dT) and/or HBV1.3 plasmid by following the Lipofectamine 2000 (Invitrogen) manufacturer's directions. Transfected cells were harvested at the indicated time.

Quantitative real-time RT-PCR

Total RNA was extracted by TRIzol Reagents (Invitrogen) and purified by RNeasy Mini kit (Qiagen) according to the manufacturer's direction. One-step real-time RT-PCR was performed with 100 ng of total RNA using QuantiFast SYBR Green RT-PCR Kit (Qiagen) on a Light Cycler™ instrument (Roche) with 50°C for 10 min for reverse transcription followed by 40 cycles of PCR: 95°C for 10 sec, 60°C for 30 sec. The three individual genes were: IFIT1 (nucleotide [nt] 765 to 879; GenBank accession no. NM_080657), IFN- β (nt 1811 to 1924; NM_002463), and TNF- α (nt 327 to 446; NM_005101). The primers used were purchased from Qiagen. The relative mRNA copies were determined with a standard curve constructed using corresponding cDNA fragments and this value is normalized within each sample to that of a housekeeping gene (β -actin).

Quantitation of HBV DNA

HBV progeny DNA in the culture supernatants was extracted using QIAamp DNA blood mini kit (Qiagen) and quantified as genome equivalents (GE) per ml by real-time PCR with LightCycler DNA master SYBR green kit (Roche). PCR was performed on Light Cycler instrument with 95°C for 5 sec, 60°C for 5 sec and 72°C for 10 sec with the primers HBV-s1 and HBV-s2 which sequences are as following: HBVS1: 5'-TGCCTCATCTTCTTRTTGGTTCT-3', HBVS2: 5'-CCCCAAWACCAKATCATCCATATA-3'. (R: G and A; W: A and T; K: G and T). A plasmid containing a full-length HBV genome was diluted and served as standard. The detection limit of this assay was 10^3 HBV GE/ μ l.

Chemiluminescent microparticle immunoassay (CMIA)

Levels of HBsAg and HBeAg in cell supernatants were determined

by using the Architect system and HBsAg and HBeAg CMIA kits (Abbott Laboratories, Wiesbaden-Delkenheim, DE) according to the manufacturer's instructions. The assays were performed in duplicate at least three times, and the mean and standard error were computed.

Southern blot

HBV replicative intermediates were purified from intracellular core particles according to the protocol described previously [17]. The isolated HBV DNA was subjected to agarose gel electrophoresis, followed by denaturation and neutralization. Afterwards, HBV DNA was transferred to Hybond-N+ nylon membrane (GE Healthcare) and were detected by hybridization with a 32 P-labeled full length HBV probe. The image capture of the Southern blots was performed by using a Phospho-Imager (Cyclon, Parkard, Meriden, CT, USA). The total HBV replicative intermediates shown as a smear in the Southern blots including the three major forms: RC (relaxed circular), DL (duplex linear), and SS (single-stranded) DNA were quantified.

MTT assay

HepG2.2.15 cells were plated in triplicate in 96-well plates at a density of 3×10^4 cells/well and transfected with 2 μ g/ μ l of poly (dA-dT). At day0, day1, day 2, day3, day 4, day5, and day6 after transfection, cell proliferation was analyzed by methyl thiazolyl tetrazolium (MTT)-based assay. For each MTT assay, the medium in each well was replaced with 200 μ l of medium containing MTT at 0.5 μ g/ μ l, and plates were incubated at standard culture conditions. After 4h of incubation, the MTT-containing medium was removed, 200 μ l of DMSO was added to each well, and the plate was agitated for 10 minutes in the dark to dissolve the MTT-formazan crystals. Sample absorbance was recorded at 570 nm.

Western blot

Nuclear and cytosolic proteins were extracted by NE-PER nuclear and cytoplasmic extraction reagents (Thermo, Pierce) with adding protease inhibitor cocktail tablets (Roche). Protein was quantified by using a BCA assay (Pierce), and equal amounts were added to loading buffer (125 mM Tris-Cl [pH 6.8], 30% glycerol, 0.1% bromophenol blue). Samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad Laboratories). After transfer, membranes were incubated in a blocking buffer (PBS containing 5% milk and 0.1% Tween 20), followed by incubation of the primary antibody diluted in PBS containing 5% milk and 0.1% Tween 20. The primary antibodies used here were Rabbit anti-IRF3 Ab, or Mouse anti-NF- κ B Ab. Blots were washed with PBS containing 0.1% Tween 20 and incubated with a horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Piscataway, NJ) diluted in PBS containing 5% milk and 0.1% Tween 20. After washing, the result was detected using ECL Western blotting detection Kit (catalog RPN2135, GE Healthcare) according to the manufacturer's instruction.

Sandwich ELISA

CST's PathScan® Signaling Nodes Multi-Target Sandwich ELISA Kit (Cell Signaling Technology) was used to detect endogenous levels of six key signaling proteins: Akt1, phospho-Akt1(Ser473), phospho-MEK1(Ser217/221), phospho-p38 MAPK(Thr180/Tyr182), phospho-Stat3(Tyr705) and phospho-NF- κ B p65 (Ser536). Procedure according to instructions, briefly, HepG2.2.15 cells were transfected with poly(dA-dT). After 72h, cells were harvested and lysis under nondenaturing conditions. The cell lysates were applied to the microwell where a

capture antibody has been coated. After incubation with cell lysates, the target protein was captured by the coated antibody. Following extensive washing, a detection antibody was added to detect the captured target protein. An HRP-linked secondary antibody was then used to recognize the bound detection antibody. HRP substrate, TMB, was added to develop color. The magnitude of absorbance at 450nm is proportional to the quantity of bound target protein.

Statistical analysis

SPSS 12.0 statistical software was used for data analysis. Results were showed as mean values with standard deviation (SD). Statistical analysis was completed using one-way ANOVA. Tukey's test was used to determine the difference among groups when the ANOVA test indicated a significant effect. P values <0.05 were considered statistically significant.

Results

Poly (dA-dT) enhanced HBV replication in HepG2.2.15 cells

To observe the possible effects of double-stranded DNA on HBV replication, a synthetic dsDNA, poly (dA-dT), was transfected into HepG2.2.15. At day 2, day 4 and day 6 after transfection, HBV core particles were extracted and HBV replicative intermediates were detected by southern blot. The results showed HBV replication was

up-regulated in HepG2.2.15 after poly (dA-dT) transfection, especially under 2 $\mu\text{g}/\text{ml}$ of poly (dA-dT) treatment. HBV surface and envelope antigens, HBV virions in the supernatant were assayed by ELISA and real-time PCR, respectively. There were no significant differences of viral antigen expression and virion production among poly (dA-dT) treatment groups and mock group ($P>0.05$). MTT assay demonstrated that there was no obvious cytotoxicity of poly (dA-dT) on HepG2.2.15 cells. As shown in Figure 1.

Poly (dA-dT) enhanced HBV replication was not mediated by the secreted factors in the supernatant

To explore the possible secreted factors, which enhance HBV replication, the supernatants of HepG2.2.15 which were transfected with 1 $\mu\text{g}/\text{ml}$ or 2 $\mu\text{g}/\text{ml}$ of poly (dA-dT) were collected at different time points and used as inocula for naïve HepG2.2.15 cells. At day4, HBV core particles were extracted and HBV replicative intermediates were tested by southern blot. The results showed HBV replication was not significantly different by treatment of HepG2.2.15 cells with culture media harvested from cells transfected with different doses of poly(dA-dT). This indicated that poly (dA-dT) enhanced HBV replication was predominantly mediated not by secreted cytokines, but possibly by intracellular factors (Figure 2).

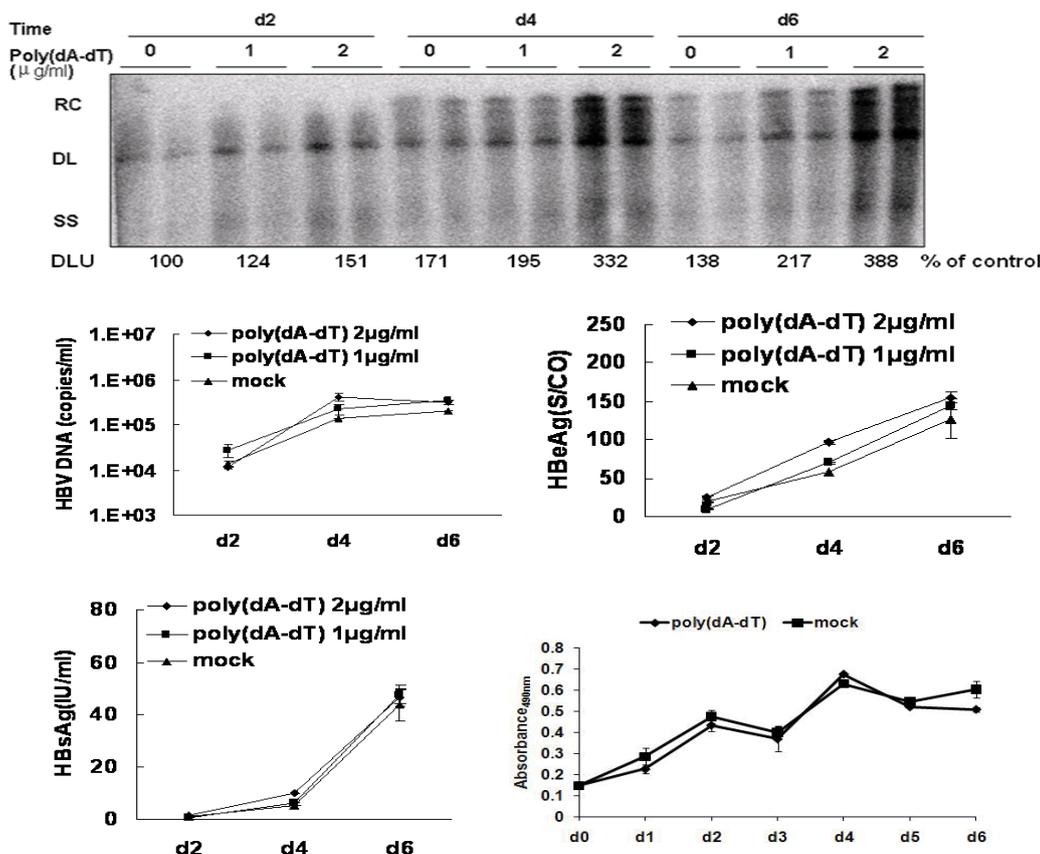


Figure 1: Poly (dA-dT) enhances HBV replication in HepG2.2.15 cells. HepG2.2.15 cells were transfected with 1 or 2 $\mu\text{g}/\text{ml}$ of poly (dA-dT) On day2, day4, and day6 after transfection, HBV core particles were extracted and the replicative intermediates were detected by southern blot (a). HBV virion secreted in the supernatant was extracted and HBV copies were quantified by real time PCR (b). HBV surface protein (c) and envelop protein (d) in the supernatant were tested by ELISA. Cell viability (e) was analyzed by MTT assay. Each sample was analyzed by duplicates. Error bars correspond to the averages \pm S.D.

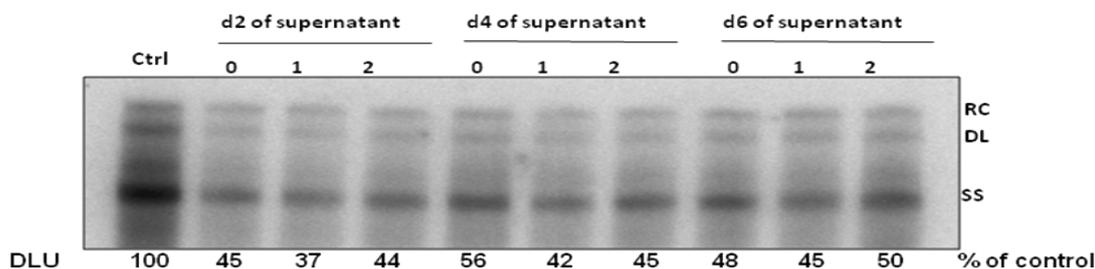


Figure 2: dsDNA-enhanced HBV replication was not mediated by secreted factors in culture supernatants. HepG2.2.15 cells were transfected with 1 µg/ml or 2 µg/ml of poly (dA-dT). At day2, day4, and day6 time points, the culture supernatants were collected and incubated with naive HepG2.2.15 cells. At day4 after incubation, HBV core particles were extracted and HBV replicative intermediates were tested by southern blot.

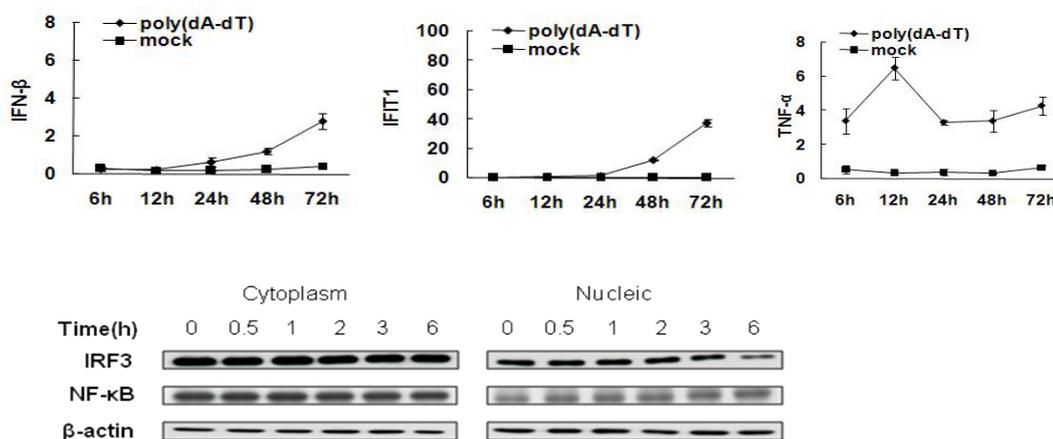


Figure 3: IFN/ISG expression in HepG2.2.15 cells after ds-DNA stimulation. HepG2.2.15 cells were transfected with 2 µg/ml poly (dA-dT). Total RNA were extracted at 6h, 12h, 24h, 48h, and 72h. IFN-β (a), IFIT1 (b), and TNF-α (c) were detected by real time RT-PCR. Each samples was analyzed in triplicates and normalized to β-actin expression. Error bars correspond to the averages ± S.D. Total proteins; cytoplasm and nucleic proteins were extracted from HepG2.2.15 cells after poly (dA-dT) transfection. IRF3 and NF-κB translocation were investigated at 0h, 0.5h, 1h, 2h, 3h, and 6h time points (d).

No IRF3 or NF-κB translocation was observed in HepG2.2.15 after poly (dA-dT) transfection

Hepatocytes are a major source of chemokines and cytokines in various viral or bacterial infections [18,19]. Poly (dA-dT) stimulation could trigger type I interferon expression [7]. Since the HBV replication was enhanced, so we want to know whether there is interferon type I induced but not play their roles in HepG2.2.15 cells after poly (dA-dT) transfection. Using real-time RT-PCR, we observed that IFN-β and interferon-stimulated genes (IFIT1) expressions were induced after poly (dA-dT) treatment with peak at 72 h. While TNF-α expression was readily detectable at indicated time points, and slightly increasing on 12 h after transfection. We also detected the cytoplasm-nucleic translocation of signaling molecules IRF3 or NF-κB p65 subunit. It demonstrated that neither IRF3 nor NF-κB p65 subunit translocation within 6 hours could be observed after poly (dA-dT) transfection. In 2011, Dr. Kumar's group reported that the regulatory HBx protein of HBV inhibited dsDNA-mediated IFN-β activation [20]. His work explains in part our observation (Figure 3).

Phosphorylated MEK1 was enhanced in HepG2.2.15 after Poly (dA-dT) transfection

Next, we addressed the question which signaling pathways were activated by poly (dA-dT) and involved in enhancement of HBV

replication. The PI3K/Akt, MAPK and NF-κB pathways are known to be activated in response to poly (dA-dT) in both macrophage and non-myeloid cell types, while IRF3 pathway activation occurs at a later phase [21]. Total cell lysates of HepG2.2.15 were prepared at 72 h after poly (dA-dT) transfection and subjected to sandwich ELISA analysis for phosphorylated Akt1, MEK1, p38 MAPK, Stat3, and NF-κB. As indicated in Figure 4, poly (dA-dT)-stimulated HepG2.2.15 exhibited an increased phosphorylated MEK1.

U0126, an inhibitor of MEK-ERK1/2, could block Poly (dA-dT) enhanced HBV replication

Next, if the pathways were blocked, the poly (dA-dT)-enhanced HBV replication would be abolished? To test this, the inhibitors of NF-κB, IRF3, PI3K, MAPK/ERK, MAPK/JNKs, and MAPK/p38 were used. HepG2.2.15 cells were pretreated with the inhibitors of Bay11-7082 (inhibitor of NF-κB), or AG490 (inhibitor of IRF3), or Rapamycin (inhibitor of PI3K), or U0146 (inhibitor of MEK/ERK), or SP600125 (inhibitor of MAPK/JNKs), or SB203680 (inhibitor of MAPK/p38). After 2 hours, the inhibitors were withdrawn and then poly (dA-dT) was transfected. The next day after transfection, the inhibitors were recovered into cell culture and southern blot was carried out at the fourth day to test the HBV replicative intermediates. The results indicated that MEK1/2 phosphorylation induced by poly (dA-dT) stimulation can be blocked specifically by pretreatment with

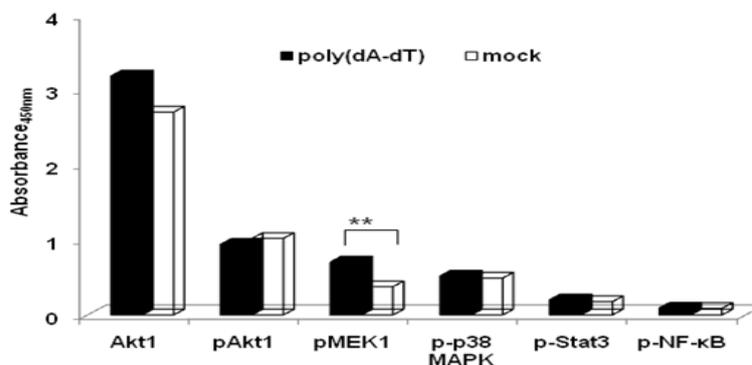


Figure 4: MEK1 phosphorylation is up-regulated in HepG2.2.15 cells after poly (dA-dT) transfection. HepG2.2.15 cells were transfected with 2 µg/ml of poly (dA-dT). After 72h, Cells were harvested and treated with lysis buffer under nondenaturing conditions. The cell lysate were investigated by Sandwich ELISA for Akt1, pAkt1, pMEK1, p-p38 MAPK, p-Stat3, and p-NF-κB. (**P<0.05)

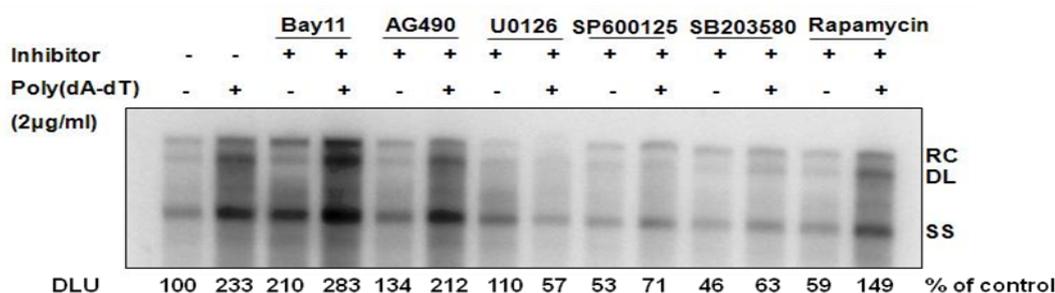


Figure 5: The involvement of MAPK/ERK pathways in the poly (dA-dT) directed-upregulation of HBV replication. HepG2.2.15 cells were pretreated with inhibitors of NF-κB (Bay11-7082, 5 µM), IRF3 (AG490, 50 µM), PI3K (Rapamycin, 1 µM), MAPK/ERK (U0126, 10 µM), MAPK/JNKs (SP600125, 10 µM), MAPK/p38(SB203580, 10 µM) or DMSO as a control for 2 hours, then inhibitors were withdrew and cells were transfected with 2 µg/ml of poly (dA-dT). 24 hours after transfection, inhibitors were included into the culture continuously. On day 4, HBV replicative intermediates were analyzed by southern blot hybridization.

MEK-ERK pathway inhibitor U0126, not by the other inhibitors (Figure 5).

Discussion

In the present study, we demonstrated that poly (dA-dT), a synthetic double-stranded DNA, is able to enhance HBV replication in HepG2.2.15 cells. Treatment of HepG2.2.15 with 1 µg/ml or 2 µg/ml of poly (dA-dT) resulted in up-regulated HBV replication despite induction of IFN production and subsequent stimulation of ISGs at late phase. In the first six hours after poly (dA-dT) transfection, there is neither IRF3 nor NF-κB activation in HepG2.2.15 cells. These findings are different from the data published by Zhu et al. [22] that dsDNA can activate IRF3 and NF-κB to trigger the innate immunity, and finally to resist the virus infection. In our issue, the most obvious difference is that the hepatitis B virus is integrated into cellular genome and under ongoing replication, which is more similar to that of the clinical HBV infectious patients. The persistence of the virus may change the innate response of cells to poly (dA-dT). Thus, we observed a surprising phenomenon that poly (dA-dT) stimulation enhanced HBV replication. The cultural supernatant collected after poly (dA-dT) transfection has no ability to inhibit HBV replication indicates that the stimulators are not the secreted factors but intracellular ones.

To explore the possible intracellular HBV stimulators, we detected the signaling nodes of Akt1, pAkt1, pMEK1, p-p38 MAPK, p-Stat3, and p-NF-κB after poly (dA-dT) transfection by using multi-target

sandwich ELISA. These molecules represent convergence points and key regulator proteins in signaling pathways controlling cellular events such as growth and differentiation and the response to stress and inflammation. Upon poly (dA-dT) stimulation, the expressional level of phosphorylated MEK1 in HepG2.2.15 cells was up-regulated. Is that resulted in enhanced HBV replication? As we know, MEK is the upstream signaling molecule of MAPK/ERK pathway. Chin et al. [23,24] reported that replication of HBV in a cell-culture system was associated with the up-regulation of phosphorylated ERK, Akt, c-myc together with increased p53, cyclin B1 and p21(cip1) expression and cell cycle progression to G2 phase. These results demonstrated simultaneous activation of the MAP Kinase and Akt pathways in HBV-replicating hepatocytes that resulted in dys-regulation in the control of cell cycle progression. This dys-regulation helps explain the early pathogenic mechanisms that underlie malignant transformation associated with chronic hepatitis B infection. We also detected the expression of the molecules about Akt1, pAkt1, pMEK1, p-Stat3, p-p38 MAPK and p-NF-κB in both HepG2.2.15 and HepG2 cells after poly (dA-dT) transfection. Comparing with HepG2, in HepG2.2.15, the level of Akt1, pAkt1, and pMEK1 are higher, p-Stat3 is lower. The level of p-p38 MAPK and p-NF-κB has no difference in both cell lines. (data unpublished) These results demonstrated that ongoing replication of HBV in HepG2.2.15 was associated with the up-regulation of pERK and pAkt. Since HBV integration into HepG2.2.15 cell genome, intercellular pERK levels tend to equilibrium. Whether poly (dA-dT) transfection up-regulate pERK expression, then break the equilibrium,

thus increase HBV replication? To answer this question, we used U0126, an inhibitor of ERK1/2, to block the MAPK/ERK pathway. The results indicated U0126 could block the poly (dA-dT) enhanced HBV replication. This means blocking of pERK up-regulation abolished enhancement of HBV replication resulted from poly (dA-dT) transfection. These results imply the MAPK/ERK pathway may play a complex role in regulation of HBV life cycle and HBV-induced hepatocyte malignant transformation.

Our data here reported an enhanced HBV replication in HepG2.2.15 cell line after poly (dA-dT) transfection, which resulted in pphospho-ERK up-regulation. Whether this HBV replicating enhancement is good for HBV infection disease outcome or not, needs to be further investigated. Next, we plan to study on the roles of pERK on both HBV replication and liver cells transformation. Additionally, the global effects of double-stranded DNA or DNA vector used for therapeutic vaccine in HBV infection related disease need to be re-evaluated.

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