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Doxorubicin Activates Hepatitis B Virus (HBV) Replication in HBV-harboring Hepatoblastoma Cells. A Possible Novel Mechanism of HBV Reactivation in HBV Carriers Receiving Systemic Chemotherapy

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Abstract. *Background: Reactivation of HBV replication is a clinically significant complication in HBV(+) patients receiving chemotherapy. We recently found that nearly half of the HBV reactivation in lymphoma patients occurred within 2 weeks of the first dose of chemotherapy. We hypothesized that mechanisms other than immunosuppression, such as direct stimulation of HBV replication by anticancer drugs, might be involved in this type of HBV reactivation. Materials and Methods: 2.2.15 cells, which secrete HBV particles constitutively, were used in the experiments. Real-time quantitative polymerase chain reaction was used to quantitate HBV DNA, and microparticle enzyme immunoassay to measure HBV surface antigen (HBsAg). Results: HBV DNA secretion in culture medium was dose- dependently increased by doxorubicin, one of the most commonly used anticancer drugs for lymphoma. One-hour exposure of cells to 1 μM doxorubicin induced a 15.4±5.9-fold and a 3.05±0.09-fold increase of HBV DNA and HBsAg on the 4th culture day, respectively. Lamivudine suppressed the doxorubicin-induced increase of HBV DNA. Conclusion: Our data suggest that cytotoxic agents may stimulate the replication of HBV and thereby contribute to the reactivation of HBV during systemic chemotherapy. Importantly, this adverse effect of cytotoxic agents may be preventable by co-administration of lamivudine.*

Reactivation of hepatitis B virus (HBV) is a frequent and clinically significant complication in patients with chronic HBV infection who receive cytotoxic therapy (1-15). This complication is especially important in HBV endemic areas such as Taiwan, where the HBV carrier rate is as high as 15～20%. Reactivation of HBV may result in clinical hepatitis, which often jeopardizes the chemotherapy schedule, and sometimes leads to hepatic failure and death.

In a recent prospective study examining HBV reactivation in HBsAg(+) lymphoma patients, we demonstrated an overall incidence of HBV reactivation of 54% (13). Unexpectedly, nearly half (13 out of 27) of the HBV reactivation occurred within the first 2 weeks of the first dose of chemotherapy (13). It was very likely that HBV reactivation of these cases might occur before the development of significant neutropenia or immunosuppression. The finding of early reactivation of HBV in patients receiving chemotherapy suggests that mechanisms other than perturbation of the balance between host immunity and HBV replication may be responsible for some of the reactivation of HBV.

One of the possibilities is that anticancer drugs may directly stimulate HBV replication. The current study addressed this hypothesis by using an *in vitro* cell culture model. The results suggest that doxorubicin, one of the most commonly used anticancer drugs for lymphoma, may stimulate the proliferation of HBV, and that this stimulatory effect may be preventable by co-administration of lamivudine.

Materials and Methods

Chemicals and drugs. Doxorubicin (Pharmacia & Upjohn S.P.A., Italy), as reconstituted according to the manufacturer's instructions, was aliquoted and stored at -70˚C. Lamivudine, provided by Prof. Yung-Chi Cheng, Department of Pharmacology, Yale University School of Medicine, USA, was prepared as 10 mM stock, aliquoted and stored at -20˚C.

Cell culture. 2.2.15 cell was derived from HepG2 cells, a human hepatoblastoma cell line, by stable transfection by a head to tail

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Key Words: Hepatitis B virus (HBV), viral reactivation, doxorubicin, lamivudine.

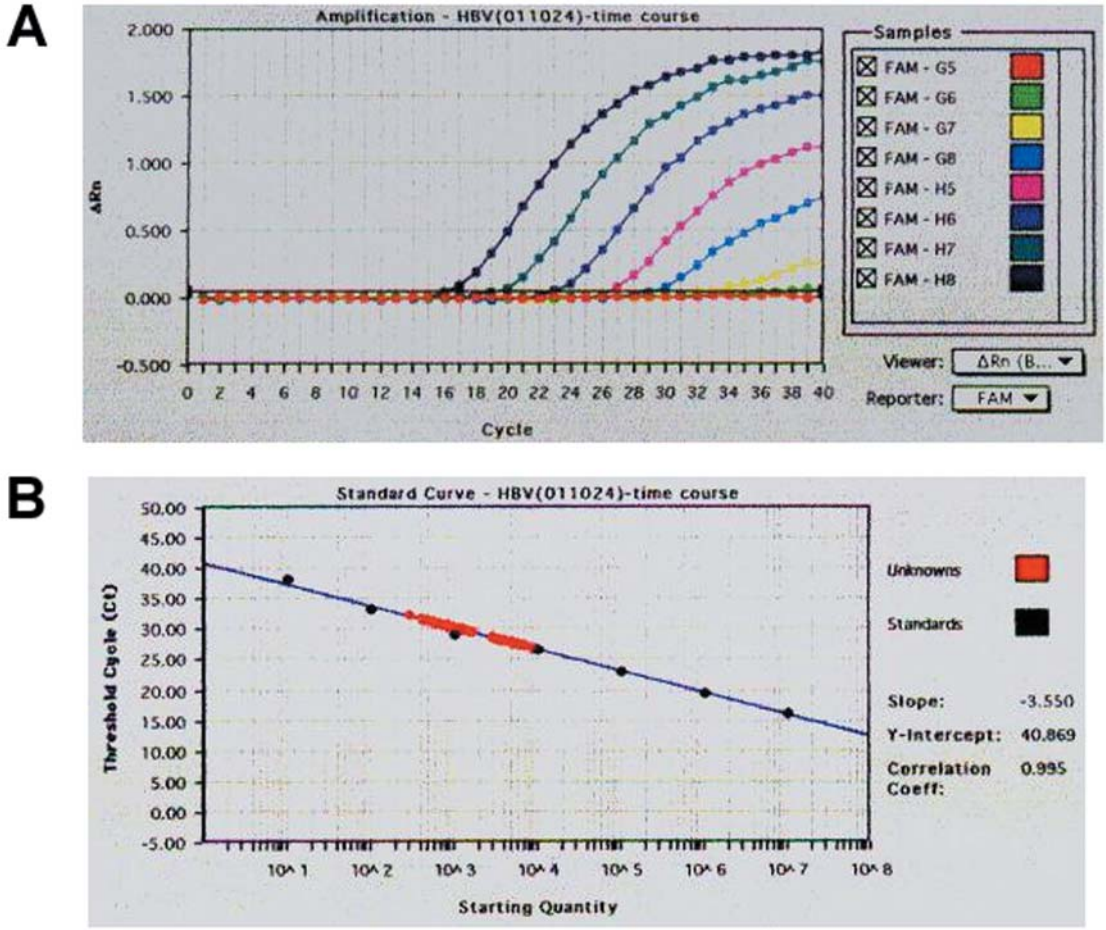

Figure 1. *Real-time quantitative PCR of HBV DNA: Real-time quantitative PCR was performed using the fluorescent TaqMan chemistry and the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A panel of serial dilutions of HBV DNA-containing plasmids, representing 10 to 10^7 copies of HBV DNA, served as the standard curve for the quantitation of HBV. The amplification plot of this serial dilution panel of HBV DNA is shown in (A), in which smaller cycle numbers for the fluorescence to rise above the baseline (the so-called threshold cycle; C_T) indicated higher amounts of initial HBV DNA. The standard curve was plotted as C_T numbers against the initial HBV DNA copy number of the standard curve panel (B). As shown in (B), the standard curve gave a correlation coefficient ≥ 0.995.*

HBV dimer (16). 2.2.15 cells constitutively release HBV in the culture medium. Cells were maintained in minimal essential medium (MEM), supplemented with 10% fetal calf serum (FCS), 2 mM of glycine and 100 U of penicillin/ streptomycin, at 5% CO_2, 37˚C.

Real-time quantitative PCR for HBV. In order to collect viral particles instead of free viral DNA in the medium, the supernatant was subjected to PEG-8000 precipitation (PEG-8000 10% and 0.5 M NaCl) overnight (17). The precipitates were then treated with lysis buffer, composed of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Tween 20 and 500 μg/ml of proteinase K, at 55˚C for 3 hours. After inactivation of proteinase K at 95˚C for 10 minutes, the samples were ready for PCR analysis. For detection of HBV DNA in 2.2.15 cells, total cellular DNA was extracted using the Qiagen DNA extraction kit (Qiagen, CA, USA) according to the manufacturer's instructions.

Real-time quantitative polymerase chain reaction (qPCR) was performed using fluorescent TaqMan chemistry and the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) (18-20). The quantitation of the target sequence was measured by the number of amplification cycles needed for the PCR product to be first detectable, the so-called threshold cycle (CT).

The PCR primers and TaqMan probe for HBV were designed specifically to the region of major S protein according to Genebank accession number AF151735 (21): forward primer (qHBV1):5'-GGACCCCTGCTCGTGTTACA-3'; reverse primer (qHBV2): 5'-GAGAGAAGTCCACCACGAGTCTAGA-3'; and probe (qHBV): 6FAM-5'-TGTTGACAAGAATCCTCACAATACCACAGA-3'-TRAMA. For an internal control of the amount of cellular DNA, a real-time qPCR for β-globin was also designed according to the Genebank accession number NG000007 (22). The forward primer (qbG1), reverse primer (qbG2) and TaqMan probe (qbG) for β-globin were: 5'-GGGCATGTGGAGACAGAGAAG -3', 5'-ACCT CTGGGTCCAAGGGTAGA -3', and 6FAM-5'-TGA TAGGCAC TGACTCTCTCTGCCTATTGGTCT -3'- TRAMA, respectively.

The amplification mixtures contained 2 ~ 5 μl of sample cDNA, 25 μl of Master Mix (Applied Biosystems), 0.4 μM each primer and

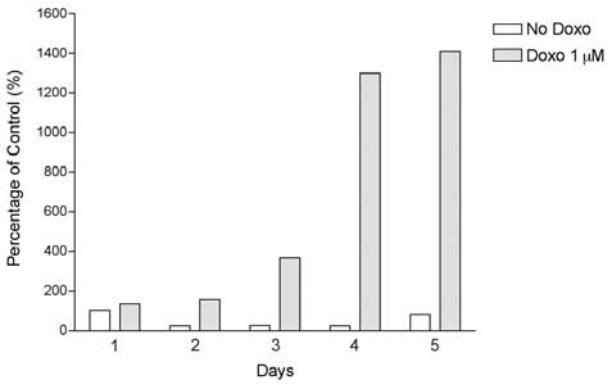

Figure 2. *Kinetics of HBV DNA excretion in 2.2.15 cells treated with doxorubicin. 2. 2.15 cells, derived from HepG2 cells by stable transfection with HBV DNA, were maintained in MEM supplemented with 10% fetal calf serum (FCS). Cells were treated with 1 μM of doxorubicin for 1 hour, and then refreshed with drug-free culture medium. The supernatant was collected at 24-hour intervals for 5 consecutive days after drug treatment. Meanwhile, the cell numbers at the end of each 24-hour interval were also determined by trypan blue exclusion. The culture supernatants were treated with PEG-8000 overnight to precipitate viral particles. The precipitates were treated with lysis buffer, and then subjected to real-time quantitative PCR analysis. The data are means of 2 different experiments, shown as excreted HBV DNA copy number/viable cell for each 24-hour interval.*

200 nM TaqMan probe. Thermal cycling consisted of an initial conditioning for 2 minutes at 50˚C and 10 minutes at 95˚C, followed by 45 cycles of 95˚C for 15 seconds and 60˚C for 1 minute. The amplification and detection were performed in an ABI Prism 7700 Sequence Detection System. The data were analyzed by Sequence Detection System software, using threshold cycle analysis.

pGem3Z/HBV, which contains 1 copy of HBV genome, was used for setting up a standard curve for real-time quantitative PCR of HBV DNA. By serial dilutions of the plasmid DNA, a standard curve for HBV DNA in the range of 10 to 10^7 copies, with a linear coefficient more than 0.995, was established (Figure 1).

Enzyme immunoassay for HBV surface antigen (HBsAg). HBsAg was determined by a microparticle enzyme immunoassay according to manufacturer's instructions (AxSYM HBsAg (V2) kit, Abbott Lab., IL, USA).

Results

Kinetics of HBV DNA excretion in 2.2.15 cells exposed to doxorubicin. The excretion of HBV DNA in the culture medium of 2.2.15 cells was evaluated daily for a total of 5 days. As shown in Figure 2, for cells without doxorubicin treatment, the excretion of HBV in medium remained minimal until day 5. However, in the cells treated with 1 μM of doxorubicin for 1 hour, the HBV DNA excreted in medium increased from day 2 and was significantly increased from day 4.

Dose-dependent increase of HBV DNA and HBsAg excretion in 2.2.15 cells treated with doxorubicin. The effect of doxorubicin

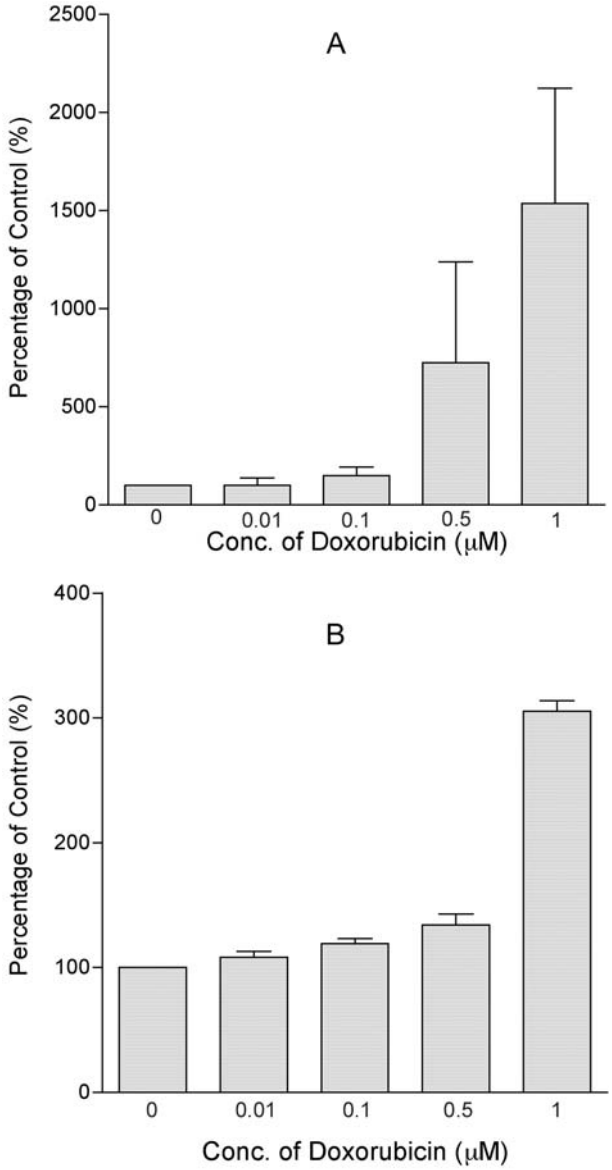

Figure 3. *Dose-dependent induction of HBV DNA (A) and HBsAg (B) in culture medium of 2.2.15 cells treated with doxorubicin. 2.2.15 cells were treated with 0.01 to 1 μM of doxorubicin for 1 hour, followed by 96 hours of incubation. The mediums were collected during the period between 72 and 96 hours after drug treatment, and subjected to analysis of HBV-DNA copy numbers by real-time quantitative PCR and to an enzyme immunoassay for HBsAg (AxSYM HBsAg (V2) kit, Abbott Lab., IL, USA). Data are expressed as mean with standard deviation of at least 3 different experiments.*

treatment on the secretion of HBV DNA and HBsAg in 2.2.15 cells was evaluated. A dose-dependent increase of HBV DNA and HBsAg secreted in culture medium was demonstrated with doses of doxorubicin ranging from 0.01 to 1 μM (Figure 3). One-hour exposure to 1 μM of doxorubicin induced a 15.4±5.9-fold and a 3.05±0.09-fold increase of HBV DNA and HBsAg, respectively.

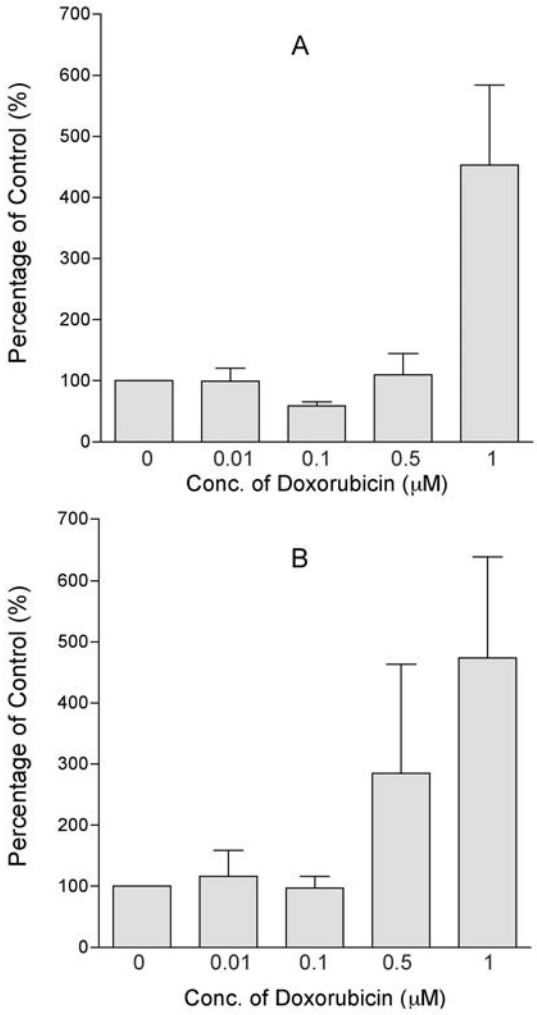

Figure 4. *HBV DNA in 2.2.15 cells treated with doxorubicin. 2.2.15 cells were treated with 0.01 to 1 μM of doxorubicin for 1 hour. Following 96 hours of incubation, cells were harvested and total cellular DNA was extracted by the Qiagen DNA isolation kit (Qiagen, CA, USA) and quantitated. The DNA was then evaluated by real-time quantitative PCR for HBV DNA and β-globin, which served as a reference of total cellular DNA. Data are expressed as mean with standard deviation of at least 3 different experiments, showing the HBV DNA normalized to the total cellular DNA in μg (A), and the HBV DNA normalized to the β-globin determined by real-time quantitative PCR (B).*

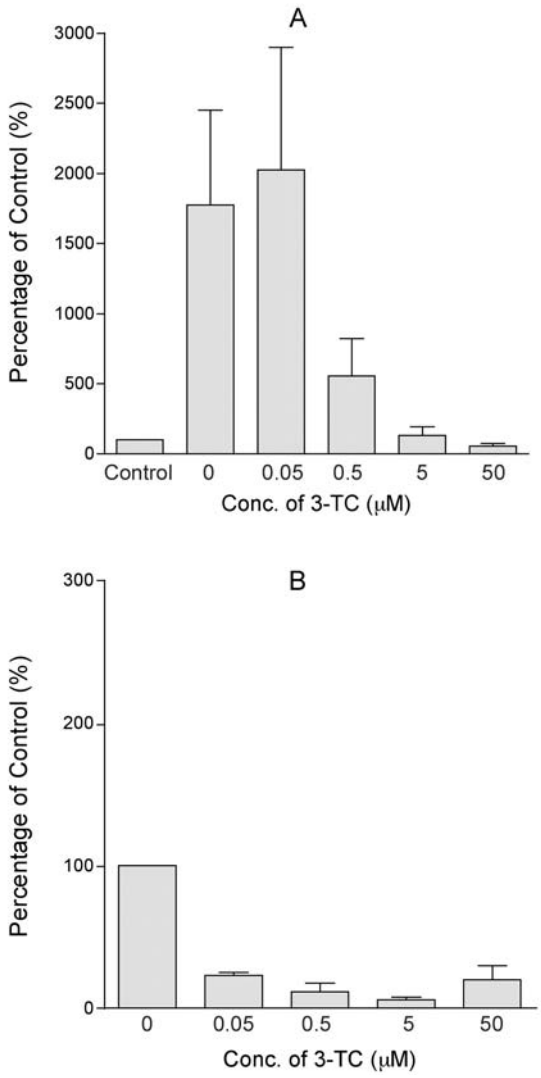

Figure 5. *Lamivudine inhibits the increase of secreted HBV DNA of 2.2.15 cells treated with doxorubicin. 2.2.15 cells were treated with 1 μM of doxorubicin (A) or medium alone (B) for 1 hour, followed by incubation with 3-TC (lamivudine) in doses ranging from 0.05 to 50 μM, for 96 hours. Medium from the period 72 hours to 96 hours after drug treatment was collected for analysis of HBV DNA by real-time quantitative PCR. The data, derived from two different experiments, are expressed as mean with standard deviation. Doxorubicin- induced HBV DNA in 2.2.15 cells treated with different doses of 3-TC is shown in (A), expressed as percentage of control, i.e. no-doxorubicin/no-3-TC 2.2.15 cells. The inhibitory effect of 3-TC on secreted HBV of routinely cultured 2.2.15 cells is shown in (B).*

Dose-dependent increase of intracellular HBV DNA of 2.2.15 cells treated with doxorubicin. We next evaluated the effect of doxorubicin on HBV DNA inside 2.2.15 cells (Figure 4). After normalization by either amount of total DNA or copy numbers of a controlled gene (β-globin), doxorubicin showed a dose-dependent increase of HBV DNA in the cells. At the concentration of 1 μM, the increments induced by doxorubicin were 4.52±2.26 (normalized by total DNA amount) and 4.43±2.83 (normalized by β-globin DNA amount)-fold, respectively.

Lamivudine inhibits the increase of HBV DNA secretion induced by doxorubicin. The finding that HBV DNA inside 2.2.15 cells and HBV DNA secreted in culture medium were increased by doxorubicin raised the possibility that replication of HBV was activated by the treatment. We then tested whether inhibition of HBV replication by lamivudine, a HBV-reverse transcriptase inhibitor, would affect doxorubicin-induced

HBV DNA secretion. Lamivudine dose-dependently inhibited the increment of HBV DNA secretion induced by 1 μM of doxorubicin (Figure 5). The 50%-inhibitory concentration (IC_{50}) for lamivudine to inhibit doxorubicin-induced HBV DNA secretion was around 35 μM, which was higher than the IC_{50} for lamivudine to inhibit the secretion of HBV DNA in untreated 2.2.15 cells (IC_{50}< 5 μM).

Discussion

The study showed that HBV DNA production by 2.2.15 cells was increased by doxorubicin. This observation supports our hypothesis that systemic chemotherapy may induce HBV reactivation by a direct effect on the virus, in addition to its indirect effect *via* immunosuppression. The possible existence of these two mechanisms of HBV reactivation in HBV carriers was suggested based on our previous prospective study, which indicated that some HBV reactivation occurred immediately after systemic chemotherapy (13). Further, the present *in vitro* study indicates that the direct activation of HBV by doxorubicin can be suppressed by lamivudine, an inhibitor of HBV replication, suggesting the possibility of prophylaxis against HBV reactivation in HBsAg(+) patients receiving doxorubicin- based chemotherapy.

Real-time quantitative PCR was used to measure viral DNA in this study. This approach has emerged as a tool to evaluate human viral diseases and several virus-associated human malignancies (21, 23-25). We confirmed that this method reliably detects HBV DNA over a wide range (21). However, this method cannot distinguish viral particles from free viral DNA. To cope with this limitation, HBV viral particles were precipitated before the assay (17). However, with the very high sensitivity of the PCR assay, it remains possible that some trace amounts of free HBV were amplified. This may partly explain the discrepancy between the degree of induction of secreted HBV DNA and secreted HBsAg in the current report.

This study used a different time schedule to evaluate HBV DNA in 2.2.15 cells compared to most previous studies which measured HBV DNA by Southern blotting (17, 26). These previous studies were often done after 10 to 14 days of culture when cells had reached confluency and the secretion of HBV became steady. One of the major reasons for using these traditional approaches is that the sensitivity of Southern blotting is relatively low and requires a higher amount of HBV DNA from confluent cells. By contrast, the high sensitivity of PCR methods allows detection of significant differences even at low DNA amounts.

Cytotoxic stress- induced activation of virus replication has been reported in other virus-harboring cells. For example, we and other investigators have observed activation of genes responsible for the lytic cycle of Epstein-Barr virus (EBV) in EBV- containing lymphoid or epithelial cells by irradiation and cytotoxic agents (27-31). It appears reasonable to speculate that, during the long history of evolution, viruses might have developed strategies which can sense and respond to cytotoxic stresses imposed on their host cells.

The possible links between cytotoxic stresses and activation of HBV replication have yet to be explored. One possibility is that perturbation of cell cycle propagation by cytotoxic drugs may affect HBV replication. In a previous study, replication of HBV increased when the cells were blocked at certain phases of the cell cycle, including G2/M (32). Doxorubicin blocks cells at the G2/M-phase. Another possibility is that alteration of signaling transduction pathways of host cells by cytotoxic drugs may affect HBV replication. Many signaling pathways have been identified as part of cellular responses to cytotoxic stresses (33-35). Alteration of some of these signaling pathways has been linked to HBV replication (36, 37). Further studies are needed to confirm these possibilities.

Acknowledgements

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