Downregulation of DNA excision repair by the hepatitis B virus-x protein occurs in p53-proficient and p53-deficient cells

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Introduction

Genes mediating DNA repair play an important role in the maintenance of gene integrity and stability in situations of genomic stress. Activities of these genes are intimately linked to cell cycle checkpoint mechanisms, which coordinate the regulation of DNA repair by HBx. The most characterized model in which a synergistic association between viral infection and chemical carcinogens seems to play an important role in cancer development is hepatocellular carcinoma (HCC). HCC has a high incidence in specific geographic areas such as southern China and central Africa (15). The concomitant exposure to liver carcinogens such as aflatoxins and HBV has been associated with a high incidence of HCC in endemic regions (16), and in laboratory animal models (17,18). One of the most documented mechanisms by which HBV contributes to HCC involves the hepatitis B-x protein (HBx) (8–12) inactivate p53. The liver carcinogen aflatoxin B1 induces a G→T transversion in codon 249 of the p53 gene (13), whereas benzo[a]pyrene induces transversions and substitutions in the p53 gene (14).

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Synergism between exposure to chemical carcinogens and infection with the hepatitis B virus (HBV) has been implicated in the high incidence of hepatocellular carcinoma. In this study we report that the HBV protein HBx, inhibits cellular DNA repair capacity in a p53-independent manner. Two alternative assays were used: the host cell reactivation assay, which measures the cell's capacity to repair DNA damage in a reporter plasmid, and unscheduled DNA synthesis, which measures the overall DNA repair capacity in damaged cells. Two p53-proficient cell lines, the hepatocellular carcinoma cell line HepG2 and liver epithelial cell line CCL13, were co-transfected with the pCMV–HBx reporter plasmid and the pCMV–CAT plasmid damaged with UVC radiation. Compared with cells transfected with control plasmid, the presence of HBx resulted in ~50% inhibition of the cell's capacity to reactivate CAT activity of UVC-damaged plasmid, and ~25% inhibition of unscheduled DNA synthesis in cells treated with either aflatoxin B1 epoxide or UVC radiation. Using the p53-deficient cell line Saos-2, we demonstrated that expression of HBx also resulted in diminished overall cellular DNA repair of damage induced by both aflatoxin B1 epoxide and UVC radiation, using both the host cell reactivation and unscheduled DNA synthesis assays. In summary, this study provides evidence for p53-independent regulation of DNA repair by HBx.

Materials and methods

Plasmid constructs

The HBx protein expression vector pCMV–HBx consists of the HBx gene ligated into the HindIII site of the pRc–CMV vector (Invitrogen, La Jolla, CA) (28). The pRc–CMV plasmid was used as a negative control. The pCMV–CAT chloramphenicol reporter construct used for the host cell reactivation assay was obtained by subcloning the CAT gene into the HindII and EcoRI sites of the pRc–CMV plasmid. The pCMV–CAT reporter plasmid (50 µg/ml), which was irradiated with 1000 J/m² from a UVC lamp at 1 J/s/m², was used in the host cell reactivation assay.

Abbreviations: CAT, chloramphenicol acetyl transferase; CMV, cytomegalovirus; HBV, hepatitis B virus; HBx, hepatitis B virus-x protein; HCC, hepatocellular carcinoma; HCR, host cell reactivation; NER, nucleotide excision repair; UDS, unscheduled DNA synthesis.
Cells were incubated with DNA–Lipofectamine complexes for 8 h at 37°C, added to equalize the amount of DNA transfected in each well when necessary. Biochemical, Cleveland, OH). HepG2 cells were grown α by DNA sequence analysis using Sequenase version 2.0 (United States

HepG2 is a hepatoblastoma cell line expressing wild-type p53, as determined

Cell lines

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Cells were washed twice in cold phosphate-buffered saline (PBS) and then lysed directly using lysis buffer (1% Triton X-100, 10 mM Tris–HCl, pH 8.0, 60 mM KCl, 1 mM EDTA, 1 mM DTT, 0.5% NP-40, 0.5 mM phenylmethyl-sulfonylfluoride, 0.01 g/ml leupeptin, 0.01 g/ml pepstatin, 0.01 µg/ml aprotinin, 5 mM sodium orthovanadate and 10 mM sodium Ppi). Total cell extracts from cells transfected with pCMV–HBx and pCR–CMV were used to examine the expression of HBx by western blot analysis. Polyacrylamide gels were prepared and stained with Coomassie blue or used for western blot analysis. Protein extracts were run at 50 V for 16 h and transferred onto nitrocellulose membrane (Costar, Cambridge, MA). The membranes were blocked overnight at 4°C with 10% low fat milk in PBS and incubated overnight with the corresponding antibody. HBx protein was detected using the monoclonal antibody 16F1 (28) and an enhanced-chemiluminescence (ECL) reagent kit (Amersham, Oakville, Ontario).

Host cell reactivation assay (HCR)

Cells were seeded at 2.7–3.0 ×10^5 cells per well in six-well plates, and grown overnight in the appropriate medium. The following day, the cells were transiently transfected using Lipofectamine (Gibco BRL, Burlington, Ontario) with the corresponding plasmids as described in the figure legends. Lipofectamine was used at a concentration of 3 µg/µl DNA. Salmon sperm DNA was added to equalize the amount of DNA transfected in each well when necessary. Cells were incubated with DNA–Lipofectamine complexes for 8 h at 37°C, 5% CO2 and harvested 12 h after transfection. Protein extracts were used to determine chromosomal acetylated transerase (CAT) activity essentially as described (29). The quantification of the reaction products in the CAT assay was performed using a Bio-Rad Gelscan Phosphomager and a Molecular Analyst (Bio-Rad, Richmond, CA) software program. The results were expressed as the percentage of chromosomal conversion to its acetylated metabolites.

Unscheduled DNA repair synthesis

Cells were seeded at a density of 5 × 10^5/well in six-well plates and cultured in complete medium until cells reached full confluence. Cells were then transfected with 5 µg pCMV–HBx or pCR–CMV DNA, using 15 µg Lipofectamine. Control samples were transfected with 5 µg of salmon sperm DNA per well. After 16 h incubation at 37°C, 5% CO2, cells were incubated for an additional 24 h in arginine-free medium (MEM Select-Amine; Gibco BRL) containing 1% dialyzed FBS, at 37°C, 5% CO2. Under these conditions, cell viability was >90% as determined by Trypan Blue staining. Cells were then treated with 50 J/m2 UV (254 nm using a 60 Hz, 0.16 A UV lamp at a distance of 19 cm), or with 100 ng/ml of aflatoxin active metabolite, aflatoxin B1 epoxide, for 2 h. Aflatoxin B1 and aflatoxin B1 epoxide were synthesized as previously described (30) and its structure was specifically identified by western blot analysis. Protein extracts were run at 50 V for 16 h and transferred onto nitrocellulose membrane. The membranes were blocked overnight at 4°C with 1% low fat milk in PBS and incubated overnight with the corresponding antibody. HBx protein was detected using the monoclonal antibody 16F1 (28) and an enhanced-chemiluminescence (ECL) reagent kit (Amersham, Oakville, Ontario).

Fig. 1. Expression of HBx in transfected cells. Cells at 70% confluence grown in 60 mm plates were transfected with 20 µg of pCMV–HBx or pCR–CMV vectors. Cells were harvested 12 h after transfection and protein extracts were then used for western blot analysis as described in Materials and methods.

To determine whether HBx modifies the ability of the cell to repair DNA damage, we analyzed the cellular DNA repair capacity by UDS (Figures 2 and 4), following HBx expression and treatment with the DNA-damaging agents aflatoxin B1 epoxide or UVC radiation. The assay detects [3H]dThd incorporation into DNA whereas repair takes place following DNA damage. Since the three cell lines investigated do not possess the adequate enzymatic machinery (cytochrome P450s) required to activate aflatoxin B1 (data not shown), we used the aflatoxin B1 active metabolite, aflatoxin B1 epoxide. Cell viability was examined by Trypan Blue staining, after transfection and carcinogen treatments, and was >90% in cells transfected with HBx or the negative controls (pCR–CMV or salmon sperm) following 24 h incubation of transfected cells in arginine-free medium as well as 4 h after treatment with UV radiation or aflatoxin B1 epoxide. Results of UDS show that HepG2 and CCL13 cells transfected with pCMV–HBx demonstrate 17–25% inhibition of repair of DNA damage induced by aflatoxin B1 epoxide or UVC radiation (Figure 2). The expression of pCR–CMV, which serves as a negative control for HBx, had no significant effect on DNA repair capacity.

To confirm and further support the results obtained by UDS, we performed the HCR assay. This procedure measures the cell’s capacity to repair a UV damaged reporter plasmid following the expression of the HBx protein. The pCMV– HBx expression or the negative control (pCR–CMV) vector was co-transfected with the pCMV–CAT reporter plasmid or the same plasmid irradiated with UVC light (1000 J/m2) into HepG2 and CCL13 cell lines. Cell extracts were harvested 12 h after transfection and CAT assays were performed (Figure 3). As compared with the non-irradiated plasmid, a decreased CAT activity was observed following transfection of the UV-treated plasmid as a consequence of DNA damage. Co-transfection of the pCMV–HBx plasmid was shown to inhibit the recovery of CAT activity from the irradiated plasmid whereas no effect of HBx was observed on the non-irradiated plasmid. Cell viability >90% was observed by Trypan Blue staining at the time of collecting the cells for CAT assay in HBx transfected cells as well in the control. HBx expression decreased cellular DNA repair capacity while promoting transactivation of Ap1 and NF-xB responsive elements (unpublished data).

To determine whether DNA repair inhibition was p53-dependent, we tested UDS on the Saos-2 cell line, which lacks endogenous p53 due to gene deletion. These cells showed the same extent of DNA repair inhibition by HBx as the p53-proficient cell lines HepG2 and CCL13 (Figure 4a). We
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Fig. 2. Unscheduled DNA synthesis assay. Confluent cells, in which semi-conservative DNA synthesis was completely inhibited, were transfected with pCMV–HBx or pRc–CMV. Cells were then treated with (a) 100 ng/ml aflatoxin B1 epoxide or (b) 50 J/m² UVC, and \(^{3}H\)dTdH was added in medium containing 2% serum. DNA was then extracted and the incorporation of \(^{3}H\)dTdH was determined. UDS was determined as described in Materials and methods. Results are expressed as percentage inhibition compared with untreated cells. Each value corresponds to the mean ± SE of three independent experiments, each in triplicate. The background value (no treatment) was 87 ± 8.9 and 53 ± 4.7 of DNA for HepG2 and CCL13, respectively.

*Significantly different from control pRc–CMV transfected cells at \(P<0.01\), using Student’s \(t\)-test.

Fig. 3. Host cell reactivation assay. HepG2 (a) and CCL13 (b) cell lines were transfected with pCMV–CAT or pCMV–CAT UV treated plasmids. HCR assay was carried out as described in Materials and methods. (a) CAT activity from non-irradiated and irradiated plasmid co-transfected with salmon sperm (control), the expression vector for HBx (pCMV–HBx) or its negative control. (b) CCL13 cells where only the results using the UV-treated reporter plasmid are shown. Results are expressed as percentage of acetylation, and the means of at least three independent experiments are shown. *Significantly different from control pRc–CMV transfected cells at \(P<0.01\), using Student’s \(t\)-test.

also subjected Saos-2 cells to the HCR assay following the same conditions used for HepG2 and CCL13. Figure 4b shows decreased CAT activity from pCMV–CAT that was UV-treated following HBx expression when compared with the negative control.

Discussion

There is mounting evidence that individual susceptibility to carcinogenesis is affected by the interaction of several factors
including genetic predisposition, exposure to genotoxic agents as well as acquired factors such as infection with viruses. Earlier epidemiological studies reported a synergistic association between exposure to environmental pollutants, such as aflatoxin B1 and chronic infection with viruses such as the hepatitis B virus (HBV), in the high incidence of hepatocellular carcinoma in endemic areas where both chronic HBV infection and exposure to aflatoxins prevail (31,32). This was also demonstrated in cell lines and transgenic mice expressing HBV products (17,18).

One of the most documented mechanisms by which HBV enhances carcinogenesis involves the HBx protein. The HBx protein is expressed in chronic hepatitis, cirrhotic liver and HCC from individuals infected with HBV (15). It is localized in both the cytoplasm and nucleus (33), and can therefore interact with cell signal transduction pathways and transcription machinery (33–35). HBx has been reported to transactivate a variety of cellular genes (36–39). Furthermore, HBx associates with the p53 tumor suppressor protein in vitro and in vivo (10,12,15), leading to p53 inhibition of its functions. Moreover, p53 inactivation by HBx has been implicated in liver carcinogenesis (12,20).

The p53 protein has been implicated in several functions including the regulation of DNA repair and the associated cell cycle checkpoint mechanisms (40). p53 associates with XPB, XPD and p62 subunits of the TFIIH complex, which is involved in both nucleotide excision repair and transcription-coupled repair mechanisms (7,25). The p53 protein also interacts with RPA, human Rad51 and BRCA1, which has been implicated in DNA repair (21,41,42). A recent study demonstrates that p53 is phosphorylated in vitro by the TFIIH-associated kinase (CDK7–cyclin H–p36 trimeric complex) enhancing its ability to bind sequence-specific p53-responsive elements (43). All of these interactions support a pivotal role of p53 in many cellular functions such as DNA repair, cell cycle checkpoint controls and/or apoptosis.

DNA repair is an important mechanism by which cells cope with DNA damage. In this study we examined repair of DNA damage induced by two carcinogens that trigger the nucleotide excision repair (NER) pathway: aflatoxin B1 and UVC light. UVC radiation induces pyrimidine dimers. Aflatoxin B1 is metabolized to aflatoxin-8,9-epoxide, the ultimate genotoxic metabolite that binds to DNA, predominantly at guanine residues, to form the trans-8,9-dihydro-(N²-guanyl)-9-hydroxy-aflatoxin B1 adduct (30). The incidence of carcinogen-induced mutations is dependent on the balance between the level of DNA damage and DNA repair capacity. Our results indicate that HBx expression was associated with inhibition of the overall DNA repair capacity in p53-proficient cells, which is in agreement with a previous study (22). Furthermore, we have found the same extent of DNA repair inhibition in the p53-deficient cell line Saos-2, which supports the idea that HBx affects the regulation of DNA repair through a p53-independent pathway. Transactivation of multiple cellular genes, another mechanism where HBx is involved, was also reported to be independent of the p53-inhibiting functions by HBx (44). The evidence supporting a role for p53 in DNA repair include: (i) the association of p53 with several DNA repair proteins (7,21,41,42); (ii) p53 can recognize and bind to both irradiated DNA and mismatch DNA (45,46); and (iii) disruption of wild-type p53 results in selective loss of global genomic nucleotide excision repair (47). However, there are some discrepancies because it was also reported that Li-Fraumeni cells exhibit defective global DNA repair but are normal for transcription-coupled repair; p53−/− mouse fibroblasts display normal rates of repair; and p53 does not influence DNA repair capacity in vitro (48–50). Our study does not rule out a p53-dependent mechanism because the assays used estimate the overall DNA repair capacity but not other DNA repair mechanisms such as transcription-coupled repair. The XBP and XPD NER proteins of the TFIIH complex are also involved in transcription-coupled repair (51), and there is evidence that p53 is involved in the regulation of this process (40). Further studies are required to understand the biological significance of these multiple interactions in relation to DNA repair and the associated cell cycle checkpoint mechanisms.

Whereas the mechanisms by which HBx interferes with DNA repair are unknown, studies by other groups have demonstrated a direct interaction of HBx with XAP-1/UVDDB, XBP and XPD proteins as well as binding of HBx to damaged DNA (22,51–53). Although the in vivo relevance of these interactions is still not known, they may account for the impaired DNA repair activity observed in our study. In addition, HBx has been reported to inhibit cell cycle checkpoint mechanisms required for DNA repair (24). However, differences in cell cycle checkpoints cannot fully explain our results. The DNA repair inhibition observed in the UDS assay was carried out on cells arrested at G0/G1 and where DNA semi-conservative synthesis was negligible, suggesting a cell cycle-independent interaction of HBx with NER pathway.

In summary, we report the first evidence that HBx-induced DNA repair inhibition occurs through a p53-independent regulatory pathway and suggests that inhibition of DNA repair mechanisms by HBV products may contribute to the observed synergistic interaction between chronic infection with HBV and exposure to liver carcinogens. Further studies are required to determine the proteins involved and the in vivo implications of these findings.

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References

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