Hepatitis B Virus X Protein Sensitizes Hepatocellular Carcinoma Cells to Cytolysis Induced by E1B-deleted Adenovirus through the Disruption of p53 Function

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ABSTRACT

Replication-selective adenovirus has been reported to kill tumor cells and hold promise for cancer therapy. In this study, we constructed an E1B Mφ 55,000-deleted adenovirus, designated Ad5WS1, and examined its cytolytic effect on human hepatocellular carcinoma (HCC) cell lines with various p53 status. The results show that Ad5WS1 lysed HCC cells lacking p53 transcription activity. However, this effect was not observed in cells harboring functional p53. Because loss of p53 transcription activity can be induced by binding to hepatitis B virus X protein (HBx), we generated HBx stable transfectants from Chang liver cells and examined their susceptibility to Ad5WS1-induced cytolysis. Expression of HBx in Chang liver cells changed the location of p53 from the nucleus to the cytoplasm, which mostly coincided with the location of HBx in the cytoplasm. Disruption of p53 transcription activity by HBx in Chang liver cells rendered them susceptible to infection with Ad5WS1. Furthermore, Ad5WS1 exerted antitumor effect, especially when combined with chemotherapeutic agent cisplatin, in BALB/c mice bearing HBx-expressing HCC. Our results suggest that E1B Mφ 55,000-deleted adenovirus may have therapeutic potential for the treatment of HCC with loss of p53 transcription activity or with HBx expression.

INTRODUCTION

The E1B Mφ 55,000 protein of adenovirus is able to bind and inactivate p53, and thus allows adenovirus to replicate in cells without the restrain of p53 growth control. A mutant adenovirus, ONYX-015, deleted in E1B Mφ 55,000 protein has been shown to replicate selectively and kill human tumor cells with defective p53 function. Significant cytolytic effects of the virus have been demonstrated on cervical carcinoma, colon carcinoma, and pancreatic adenoma cells with defective p53 function (1). However, some reports indicated that there is no correlation between the replication of E1B-deleted adenovirus and the genetic status of p53 in cancer cells (2, 3). Although the mechanism underlying oncolytic activity of E1B-deleted adenovirus is still unclear, clinical trials of ONYX-015 for various cancers have been encouraging, especially combined with chemotherapy (4–7).

The p53 protein has pleiotropic functions in the modulation of genomic stability of cells. Disruption of p53 activity is commonly found in human cancers. The HBx3 is one of the factors contributing to HBV-induced HCC. HBx is able to disrupt p53 activity through transcriptional repression (8) or direct association with p53 protein (9). The interference of HBx with p53 function may thus contribute, in part, to the development of HCC. Because most human HCCs have p53 mutations or deletions, the E1B Mφ 55,000-deleted adenovirus may have therapeutic potential for the treatment of such patients.

In this study, we tested the cytolytic effect of E1B-deleted adenovirus, Ad5WS1, on human HCC cell lines with various p53 status and HBx expression. Our results show that Ad5WS1-induced cytolysis was dependent on the transcription activity of p53 in HCC cells. We demonstrate that introduction of HBx gene into Chang liver cells could sensitize cells to Ad5WS1-induced cytolysis through the disruption of p53 transcription activity. Finally, the therapeutic efficacy of Ad5WS1 was tested in BALB/c mice bearing HBx-expressing HCC. Our results suggest that E1B Mφ 55,000-deleted adenovirus may have therapeutic potential for the treatment of HCC with loss of p53 transcription activity or with HBx expression.

MATERIALS AND METHODS

Cell Lines and Mice. The ML-1 mouse liver cell line was originally established from hepatocytes of BALB/c mice by HBx DNA transfection (10), and a highly tumorigenic subline of ML-1 cell line obtained from orthotopic tumors was used in this study. HepG2, Huh7, and Hep3B human HCC cell lines, as well as Chang liver (transformed human hepatocyte cell line), 293, and ML-1 cells were cultured in DMEM containing 10% fetal bovine serum, 1% glutamine, and 50 μg/ml gentamicin at 37°C in 5% CO2. BALB/c mice were obtained and maintained under 5% CO2. BALB/c mice were obtained and maintained under 5% CO2.

Received 3/11/02; revised 8/7/02; accepted 8/13/02.

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1 Supported by Grants (NSC90-2318-B-006-M51 to A-L. S. and NSC-2318-B-006-003-M51 to C-L. W.) from the National Science Council, Taiwan.

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The abbreviations used are: HBx, hepatitis B virus X protein; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; CPE, cytopathic effect; MOI, multiplicity of infection; TCID50, 50% tissue culture infectious dose; DAPI, 4',6-diamidino-2-phenylindole.
isothermal conditions with regular photoperiods in the Laboratory Animal Center, National Cheng Kung University Medical College.

Construction of Recombinant Adenoviruses. The wild-type E1 region from bases 343 to 2270 of adenovirus type 5 was generated by PCR using genomic DNA from 293 cells as the template. The resulting 1928-bp fragment contained a BamHI site at the 5′ end and a BglII site at the 3′ end, as well as two point mutations at bases 2253 (C→ATG) and 2262 (G→T) that generated premature translation stop codons in the E1B M region. The coding region of HBx was amplified from pHBV130 (13). Detection of Transgene Expression in the Transfectants. β-galactosidase was also used.

Grown in six-well plates were cotransfected with 2 μg of pAd5WS1, the E1B M, 55,000 reading frame at codons 79 and 82, respectively. The resulting PCR fragment was digested with BamHI and BglII, and cloned into BglII-restricted pSP72 (Promega, Madison, WI), yielding pSP72-E1AdE1B. The E1 region encompassing bases 3328–5788 was excised from pJM17, which contained the whole Ad5 genome (11), by XhoI and BglII digestion and inserted into pSP72-E1AdE1B at the XhoI/BamHI site to generate pAd5WS1. The E1B M, 55,000-deleted adenovirus, designated Ad5WS1, was rescued, propagated, and quantified by plaque assay using 293 cells as described previously (12). The E1-deleted replication-defective adenovirus Ad5lacZ encoding β-galactosidase was also used.

Generation of HBx-expressing Chang Liver Cells and Detection of Transgene Expression in the Transfectants. The coding region of HBx was amplified from pHBV130 (13) by PCR with sense primer 5′-ATG GCT GCT AGG CTG TG and antisense primer 5′-CCC AAA GCC ACC CAA GGC. The resulting 526-bp PCR product was cloned into pGem-T-easy vector (Promega), excised by EcoRI digestion, and subsequently cloned into the EcoRI site of pTCl (14), yielding pTClHBx, which directed the expression of HBx controlled by the rat β-actin promoter. Chang liver cells in six-well plates were transfected with 2 μg of pTClHBx, and clonal derivatives were isolated by G418 (400 μg/ml) selection and expanded to independent clones. Likewise, vector control clones were isolated and used for parallel studies.

Assays of CPE, Cell Viability, and Viral Replication. Confluent cells in six-well plates were mock infected or infected with Ad5WS1 at an MOI of 10 and stained with 0.05% crystal violet/10% formalin 7 days after infection. A colorimetric assay using WST-8 was also used to assess cell viability. Confluent cells in 96-well plates were infected with Ad5WS1 at an MOI of 10. After 72 h, the medium was aspirated, and 10 μl of WST-8 (Dojindo Labs, Tokyo, Japan) was added to each well and incubated at 37°C for 1 h. The absorbance at 450 nm that stands for cell growth was measured with the reference wavelength at 595 nm. For viral replication assay, confluent cells in six-well plates were infected with Ad5WS1 at an MOI of 10 for 2 h followed by removal of the unabsorbed virus by washing with PBS. Thirty h after infection, the supernatant and cells were harvested, and exposed to three cycles of freezing and thawing to release virions. Serial dilutions of the lysates were subsequently titered on 293 cells for TCID50 calculated by the Reed-Muench method. The viable cell numbers were also determined in cells seeded in 24-well plates after 4 days of Ad5WS1 infection by trypan blue exclusion.

Analysis of p53 Transcription Activity. HCC cells, HBx-transfected, or control vector-transfected Chang Liver cells grown in six-well plates were cotransfected with 2 μg of p53-luc containing a luciferase expression cassette with 15 copies of a p53 response element and 1 μg of pTRELacZ, a β-galactosidase reporter vector derived from pTREd2EGFP (Clontech, Palo Alto, CA) by lipofectin (Life Technologies, Inc., Rockville, MD). Cell lysates were harvested 44 h after transfection, and the luciferase activities were determined by a dual-light luciferase and β-galactosidase reporter gene assay system (Tropix, Bedford, MA) using a luminometer (Minilumate LB9506, Bad Wildbad, Germany).

Immunoblot Analysis. Proteins from total cell extracts were fractionated on SDS-PAGE, transferred onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham, Uppsala, Sweden), and probed with rabbit antihuman p53 monoclonal antibody CM1 (Novocastra Laboratories, Newcastle upon Tyne, United Kingdom), mouse antihuman p21WAF1/CIP1 monoclonal antibody (F-5; Santa Cruz Biotechnology, Santa Cruz, CA; or Clone 70; BD Transduction Laboratories, San Diego, CA), or mouse antihuman β-actin monoclonal antibody N350 (Amersham). Horseradish peroxidase-conjugated goat antirabbit IgG (KPL, Gaithersburg, MD) and goat antianti IgG + IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies where appropriate, and protein-antibody complexes were visualized by the enhanced chemiluminescence system (Amersham).

Immunofluorescence Double Staining. Cells grown on coverslips were fixed in 10% formalin, permeabilized with cold methanol/acetone (30:70 v/v), incubated with rabbit anti-HBx antibody 36985 (15) and mouse anti-p53 monoclonal antibody Ab-5 (Oncogene Science, Boston, MA) at 4°C overnight, and subsequently incubated with fluorescein-conjugated goat anti-rabbit IgG (KPL) and Texas Red-conjugated goat antimouse IgG (KPL) at room temperature for 1 h. Nuclei were stained with 50 μg/ml of DAPI. Expression and localization of the proteins were observed under fluorescence microscope at a magnification of ×400.

Animal Studies. ML-1 cells (1 × 10⁶) were inoculated s.c. into the right flank of 7-week-old female BALB/c mice at day 0. Groups of 6 mice were treated with 2 × 10⁷ plaque-forming units of Ad5WS1 intratumorally at days 15, 16, and 17, and/or cisplatin (4 mg/kg) i.p. at days 19, 21, and 23. All of the mice were monitored for tumor growth and survival. Tumor measurements were performed in two perpendicular axes with calipers twice weekly and volumes calculated as: length × width² × 0.45. The mean tumor volumes were calculated only when mice within the same treatment group were all alive.

Statistical Analyses. The statistical difference in tumor volumes between the different groups was assessed by Student’s t test. The survival analysis was performed using the Kaplan-Meier survival curve and the log-rank test. Any Ps < 0.05 were considered statistically significant.

RESULTS

CPEs of Ad5WS1 on HCC Cell Lines. We examined the CPE effect of Ad5WS1 on three human HCC cell lines and normal human liver cells with different genetic status of p53. HepG2 cells carry wild-type p53, whereas Hep3B and Huh7 cells have null and point mutations at p53 codon 220 resulting in substitution from tyrosine to cysteine, respectively (15). No-
tably, Hep3B was the only cell line tested here to express HBx. Chang liver cells, which carry wild-type p53, were used to represent normal human hepatocytes. Cell viability was determined 72 h after Ad5WS1 infection at an MOI of 10 by WST-8 assay. As shown in Fig. 1A, Chang liver cells were resistant to Ad5WS1 infection, whereas Hep3B cells were most susceptible to Ad5WS1-induced cytolysis among four cell lines. Ad5WS1 lysed >90% of Hep3B cells, whereas it lysed <30% and 10% of HepG2 and Huh7 cells, respectively. In contrast, these cells infected with Ad/lacZ at an MOI of 50 showed a similar degree of CPE regardless of the genetic status of p53 (Fig. 1B). Although E1-deleted Ad/lacZ vectors presumably replicate only in cells providing E1 gene products in trans, it has been reported that high doses of replication-defective adenoviral vectors may induce apoptosis of infected cells (17), which may have occurred here. Taken together, the results suggest that HCC cells lacking p53 and/or expressing HBx may be more susceptible to Ad5WS1-induced cytolysis. Nevertheless, HCC cells harboring p53 gene mutation are not necessarily sensitive to Ad5WS1 infection, as observed in Huh7 cells.

Transcription Activities of p53 in HCC Cell Lines. Because p53 gene mutation does not necessarily abrogate p53 transcription activity, it is of interest to investigate the functional status of p53 in HCC cell lines and its correlation to the sensitivity of the cells to Ad5WS1. We first examined the endogenous expression of p53 by immunoblot analysis (Fig. 2A). As expected, moderate levels of p53 were detectable in Chang liver and HepG2 cells that contain wild-type p53, but not in p53-null Hep3B cells. On the contrary, a high level of p53 was detected in Huh7 cells. Because p21 is a downstream target of p53, we next examined the p21 expression in HCC cell lines. As shown in Fig. 2B, Chang liver and HepG2 cells expressed high levels of p21. Furthermore, Huh7 cells also expressed p21, albeit at a lower level, whereas Hep3B cells did not express p21. The endogenous transcription activity of p53 in HCC cell lines was also determined by using a p53-responsive reporter gene assay. The transcription activity of p53 in Huh7 cells was comparable with Chang liver cells (Fig. 2C). Huh7 cells, although harboring mutant p53 gene, still maintained p53 transcription activity, which may contribute to their relative resistance to Ad5WS1 infection. The results from Figs. 1 and 2 show a correlation between the susceptibility of HCC cells to Ad5WS1 and the functional status of p53. The Relocalization of p53 Protein in HBx-transfected Chang Liver Cells. Three stable HBx-transfected clones derived from Chang liver cells, designated CLX1, CLX2, and CLX3, as well as three control vector-transfected clones, designated CLTCY1, CLTCY2, and CLTCY3, were chosen for comparative studies. Both mRNA specifying for HBx gene and
HBx protein were detectable in all of the three HBx-transfected clones but not in three control clones, as determined by reverse transcription-PCR and immunohistochemistry, respectively (data not shown). It is known that nuclear localization is critical for p53 to function as a transcription factor. High levels of HBx expression have been demonstrated to accumulate in the cytoplasm (18, 19). Because HBx can associate with p53, we next examined the subcellular localization of p53 in HBx-expressing Chang liver cells by immunofluorescent double staining with anti-HBx by fluorescein and anti-p53 by Texas Red (Fig. 3). Intense staining of HBx was mainly observed at the nuclear periphery and in the cytoplasm of HBx-transfected cells. As expected, p53 was found mainly in the nucleus of CLTCY1 cells. In contrast, p53 was mostly observed at the nuclear periphery and in the cytoplasm, and only some were found in the nucleus in HBx transfectants. Taken together, these findings demonstrate that p53 protein was sequestered in the cytoplasm by HBx in the stable HBx transfectants.

The Transcription Activity of p53 and Expression of p21 Were Decreased in HBx-transfected Chang Liver Cells. Retention of p53 protein in the cytoplasm by HBx led us to investigate additionally whether p53-mediated transcription activity was abrogated in HBx transfectants. We examined the transcription activity of p53 as assessed by a p53-responsive reporter gene assay, and the protein levels of p21 in HBx-transfected and control clones. Fig. 4A shows that the transcription activity of p53 decreased dramatically in HBx transfectants compared with control vector-transfected or parental cells, whereas no significant difference was found between control vector-transfected and parental cells. The expression of the cell cycle inhibitor p21, a downstream target of p53, was also reduced in the three HBx-transfected clones compared with three control clones (Fig. 4B).

Expression of HBx in Chang Liver Cells Increased Their Susceptibility to Ad5WS1 Infection. Additional evidence to support the notion that loss of functional p53 in cells renders them susceptible to Ad5WS1-induced cytolysis is presented in Fig. 5. Complete cytolyis was found in HBx transfectants 7 days after infection with Ad5WS1 at an MOI of 10, whereas no CPE was observed in the control clones (Fig. 5A). Cell viability was also measured by trypan blue exclusion 4 days after Ad5WS1 infection at an MOI of 10. The number of viable cells in HBx transfectants decreased dramatically, whereas the viability of the control clones was still >85%, as compared with their mock-infected counterparts (Fig. 5B). Furthermore, the ability of Ad5WS1 to replicate in these clones was assessed 30 h after infection with Ad5WS1 by TCID₅₀ assay. Ad5WS1 replicated to much higher levels in three HBx-transfected clones than in three control clones (Fig. 5C). There was as much as 3 orders of magnitude difference in the viral titer of Ad5WS1 on HBx-expressing clones compared with control clones. In summary, these results indicate that HBx expression in liver cells increased their sensitivity to Ad5WS1-induced cytolysis.

Ad5WS1 Exerted Antitumor Effect on HBx-expressing Tumors in Vivo. The in vivo antitumor effects of AdWS1 alone or combined with cisplatin were evaluated in terms of tumor growth and survival in groups of 6 BALB/c mice bearing HBx gene-transfected ML-1 tumors. Mice treated with either Ad5WS1 (P = 0.0004) or cisplatin (P = 0.0006) alone resulted in significant retardation of tumor growth compared with PBS-treated mice (Fig. 6, A, B, and D). Increased survival was also observed when the cisplatin-treated group was compared with
the PBS-treated group \((P = 0.048)\). As shown in Fig. 6, B–D, Ad5WS1 treatment followed by cisplatin also significantly inhibited tumor growth compared with cisplatin-treated mice \((P = 0.048)\) or PBS-treated mice \((P = 0.0002)\). However, treatment of Ad5WS1 plus cisplatin did not significantly reduce tumor size compared with Ad5WS1 treatment. At day 63 when all of the mice were still alive, the mean tumor volumes of mice with Ad5WS1 plus cisplatin, Ad5WS1, cisplatin, and PBS treatments were 329.1 ± 287.74 mm³, 504.25 ± 422.15 mm³, 817.1 ± 447.26 mm³, and 817.1 ± 1458.30 mm³, respectively. Of 6 Ad5WS1-treated mice, 1 showed a complete tumor regression and 1 showed a partial response (Fig. 6A). In contrast, no tumor regression was observed in cisplatin-treated mice (Fig. 6B). Two of 6 tumors in the combined treatment group underwent complete regression (Fig. 6C). Nevertheless, in PBS-treated group, partial tumor regression was observed in only 1 of 6 mice, and tumors ultimately grew progressively (Fig. 6D). Completely regressed tumors have been followed for >6 months without evidence of regrowth. Notably, survival of the mice injected with Ad5WS1 plus cisplatin was significantly increased versus those injected with PBS \((P = 0.002)\), Ad5WS1 \((P = 0.033)\), or cisplatin \((P = 0.021)\) alone (Fig. 6E).

DISCUSSION

In this study, our results show that Ad5WS1 extensively lysed p53-null, HBx-expressing Hep3B cells but not p53-wt HepG2 cells, p53-mut Huh7 cells, or Chang liver cells. In Huh7 cells, p53 protein has been shown to be expressed at increased levels and have a prolonged half-life leading to its accumulation in the nuclei (16). We also showed that Huh7 cells maintained p53 transcription activity despite gene mutation. Therefore, it is plausible that the susceptibility of HCC cells to Ad5WS1-induced cytolysis might be more related to p53 functional status than to p53 genotype. Our results agree with previous reports showing that the susceptibility of HCC cells to cytolytic effect induced by E1B-deleted adenovirus is related to both the genotype and functional status of p53 (20, 21). Nevertheless, Vollmer et al. (22) reported that selective replication of E1B-deleted adenovirus in HCC cells with p53 mutation or deletion occurs only at a low viral dose. Apart from HCC, a variety of tumor cells have been examined for their susceptibility to E1B-deleted Adenovirus in HBx-expressing HCC.
The intracellular distribution of HBx seems to be dependent on...the overall expression levels. In HBx-transfected hepatoma...cells, HBx is primarily localized in the nucleus in weakly expressing cells, whereas at elevated levels HBx predominately accumulated in the cytoplasm (18). Furthermore, HBx is detected, predominantly in the cytoplasm, in ~70% of the liver biopsies from patients with chronic HBV infection (19). Previous work has shown that overexpression of HBx induces a change of p53 distribution from the nucleus to the cytoplasm, and thus inhibits p53 function (25). Consistent with a previous study (24), our results confirmed that cytoplasmic retention of p53 was because of its association with HBx in Chang liver cells overexpressing HBx. Furthermore, functional abrogation of p53 by HBx was elucidated by the reduction of the p53-mediated transcription activity and inhibition of p21 expression. DNA microarray analysis has also revealed that gene expressions of p53 and p21 were down-regulated in normal human hepatocytes transfected with HBx and in HBV-infected liver tissues (26).

Our results show that disruption of p53 transcription activity by HBx rendered Chang liver cells sensitive to Ad5WS1-induced cytolysis. Staining of cells with crystal violet after infection with Ad5WS1 revealed little evidence of CPE in control cells, whereas HBx-transfected cells were completely lysed by Ad5WS1. Evaluation of cell viability by direct microscopic counting also showed similar results. In the viral replication assay, which is more sensitive than the CPE assay, the observed CPE of Ad5WS1 on HBx-transfected Chang liver clones corresponded to as much as 3 orders of magnitude difference in the viral yield compared with the control clones. However, control vector-transfected cells still produced infectious viruses, albeit to a small extent, to generate CPE on 293 cells. In the original report describing this oncolytic adenovirus (1), E1B-deleted adenovirus replicated in and lysed tumor cells deficient in p53, but replicated 100 times less efficiently in cells expressing functional p53. Using genetically matched ovarian cancer cell lines differing only in the p53 status, Ganly et al. (27) showed that mutant p53 transfectants were ~360-fold more permissive for E1B-deleted adenoviral replication than parental cells carrying wild-type p53. Although these cancer cells with wild-type p53, when infected with E1B-deleted adenovirus, exhibited no CPE, they still produced infectious virions (1, 27). These results were in line with our finding, suggesting that infectious E1B-deleted adenoviruses may still be produced to a small extent in cells harboring wild-type p53, despite the absence of an obvious CPE. Because most related reports do not include quantitative evidence of viral replication and the production of new virus in the cancer cell lines studied, the relationship among p53 status, viral replication, and CPE still awaits clarification. Despite this, intratumoral injection of ONYX-015 has shown promising results in clinical trials, and importantly, no virus was detected in the normal tissue examined (5).

In this study, we used the ML-1 HCC tumor model in syngeneic BALB/c mice to investigate the antitumor effect of Ad5WS1 in vivo. Because of the deletion of integrated HBV genome (28) in ML-1 cells, we transfected HBx gene into a ML-1 subline to generate HBx-expressing HCC cells. As immunocompetent animal tumor models are indispensable for addressing the balance among viral replication, immune recognition of tumor cells alone, and tumor cells harboring E1B-deleted adenovirus, our animal model is more relevant to clinical appli-

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Fig. 6 Antitumor effects of Ad5WS1 and/or cisplatin on BALB/c mice bearing HBx-expressing ML-1 tumors. Groups of 6 BALB/c mice that had been injected s.c. with ML-1 cells (1 × 10⁶) at day 0 were treated with 2 × 10⁷ plaque-forming units of Ad5WS1 at days 15, 16, and 17 intratumorally, with cisplatin (4 mg/kg) i.p. at days 19, 21, and 23, or both. Control mice received PBS. All individual tumor volumes in mice treated with (A) Ad5WS1, (B) cisplatin, (C) Ad5WS1 plus cisplatin, and (D) PBS are shown. Each line represents one individual tumor. E, Kaplan-Meier survival curves at day 182 are shown.
cations compared with HCC xenograft models in immunodeficient mice (21, 29). In the present study, significant inhibition of tumor growth was observed in HCC-bearing mice treated with either Ad5WS1 or cisplatin alone, or in combination. Inhibition of tumor growth and increased survival were observed when the combined treatment group was compared with the cisplatin-treated group. Nevertheless, a significant difference was found in survival but not in tumor size between the combined treatment group and Ad5WS1-treated group. It is of note that tumor volume and survival are not always well correlated, as some mice may die because of metastasis despite smaller tumor size. Our results are consistent with the in vitro data that Ad5WS1 caused severe CPE in HBx-expressing cells. In addition, Ad5WS1 and cisplatin have an additive antitumor effect on HBx-expressing ML-1 tumors, suggesting a role for p53 inactivation in enhancing chemosensitivity. This notion is supported by the findings that many forms of mutant p53 protein confer sensitivity to cisplatin in bladder cancer cells (30). The other possible mechanism contributing to this association is the E1A gene, of which the expression resulting from adenoviral replication may sensitize tumor cells to DNA-damaging agents through a p53-independent pathway (31). Combined treatment with E1B-deleted adenovirus followed by cisplatin has been demonstrated to enhance antitumor effects compared with treatment with either agent alone in SCID mice bearing Hep3B xenografts (22). Heise et al. (32) also showed that combination therapy of E1B-deleted adenovirus with cisplatin is superior over either agent alone. Furthermore, this effect is highly dependent on the sequencing of the agents; treatment with E1B-deleted adenovirus before or simultaneously with chemotherapy is significantly superior to chemotherapy followed by adenovirus. Our in vivo data are in accordance with these findings. Additionally, evidence for a potentially synergistic interaction between E1B-deleted adenovirus and chemotherapy has been obtained on several clinical trials recently (7).

Our results demonstrate that E1B-deleted adenovirus was effective in lysing HCC cells with HBx expression. Disruption of p53 activity by viral oncoprotein is found frequently during viral carcinogenesis and thought to be equivalent to mutation of p53 in cells. Normal HBx has a counteracting effect on the p53 suppressor function. HBx is highly expressed in patients with chronic hepatitis with cirrhosis compared with that in patients with chronic hepatitis without cirrhosis (33). Moreover, HBx-expressing cells are preferentially localized in the perportal region accompanying high necroinflammatory activity. It can be assumed that overexpression of HBx is highly associated with inflammatory reaction followed by cell destruction and, thus, provides an opportunity for the accumulation of genetic mutation. Therefore, to prevent cells harboring HBx gene from carcinogenesis, Ad5WS1 may be explored to target selectively and kill hepatocytes that express HBx.

In summary, our results indicate that E1B-deleted adenovirus can selectively replicate in and kill HBx-expressing liver cells. The susceptibility of HCC to E1B M, 55,000-deleted adenovirus is related to the functional status of p53. The sequestration of p53 protein in the cytoplasm by HBx abrogates the transcription activity of p53, thereby sensitizing the cells to cytolyis induced by E1B M, 55,000-deleted adenovirus. Furthermore, the E1B-deleted adenovirus exerts antitumor effect, especially when combined with cisplatin, in immunocompetent mice bearing HBx-expressing HCC. Therefore, this study may have clinical implications in exploring E1B M, 55,000-deleted adenovirus for the treatment of HBV-associated HCC or HCC lacking functional p53.

ACKNOWLEDGMENTS

We thank Dr. Mark A. Feitelson (Department of Pathology, Thomas Jefferson University, Philadelphia, PA), Dr. Huan-Yao Lei (Department of Microbiology and Immunology, National Cheng Kung University Medical College), and Dr. Ming-Derg Lai (Department of Biochemistry, National Cheng Kung University Medical College) for providing polyclonal anti-HBx antibodies, ML-1 cells, and p53-luc plasmid, respectively. We also thank Shuen-Huei Li and Ming-Teng Huang for technical assistance.

REFERENCES

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