Hepatitis B virus X protein overcomes all-trans retinoic acid-induced cellular senescence by downregulating levels of p16 and p21 via DNA methylation

Sun-Hye Park, Jin Kyu Jung, Joo Song Lim, Indira Tiwari and Kyung Lib Jang

Department of Microbiology, College of Natural Sciences, Pusan National University, Busan 609-735, Republic of Korea

Despite current molecular evidence suggesting that hepatitis B virus (HBV) X protein (HBx) plays an important role during HBV-mediated hepatocarcinogenesis, the detailed mechanism is still controversial. Here, it was shown that HBx overcomes cellular senescence provoked by all-trans retinoic acid (ATRA) in HepG2 cells, as demonstrated by the impaired induction of irreversible G1 arrest and senescence-associated β-galactosidase activity by ATRA in the presence of HBx. The anti-senescence effect of HBx was also observed in another human hepatoma cell line, Hep3B, but not in Huh-7 cells in which the p16 and p21 proteins are absent. In addition, HBx suppressed ATRA-mediated induction of p16 and p21 in HepG2 cells via promoter hypermethylation, resulting in inactivation of retinoblastoma protein. Furthermore, the ability of HBx to overcome ATRA-induced cellular senescence almost completely disappeared when the levels of p16 and p21 in the HBx-expressing cells became similar to those in the control cells by complementation in the former by exogenous expression, knockdown of their expression in the latter using specific small interfering RNA or treatment with a DNA methylation inhibitor, 5-Aza-2’-deoxycytidine. These results suggest that HBx executes its potential by downregulating levels of p16 and p21 via DNA methylation. As cellular senescence is a tumour-suppression process, the present study provides a new strategy by which HBV promotes hepatocarcinogenesis.

INTRODUCTION

Hepatitis B virus (HBV) is strongly associated with the development of hepatocellular carcinoma (HCC; Tsai & Chung, 2010). HBV X protein (HBx) is encoded by the smallest ORF of the HBV genome, termed X, which is the most frequently integrated viral sequence found in HCCs (Paterlini et al., 1995). As a multifunctional regulatory protein, it can activate several transcription factors, including AP-1, NF-κB, CREB, Oct-1, Myc and TBP (Benn et al., 1996; Maguire et al., 1991; Qadri et al., 1995). In addition, HBx has been implicated in the activation of several cytoplasmic signalling pathways, including PKC, STAT, PI3K, JNK, MAPK and Wnt (Benn et al., 1996; Lee & Yun, 1998; Shih et al., 2000). Moreover, HBx is able to induce HCC in transgenic mice (Kim et al., 1991). Despite current molecular evidence suggesting that HBx plays an important role during HBV-mediated hepatocarcinogenesis, the detailed mechanism is still controversial.

Various genetic and biochemical data suggest that cellular senescence is an important tumour-suppression process, preventing damaged cells from undergoing aberrant proliferation (Campisi, 2005; Ozturk et al., 2009; Schmitt, 2007; Yan & Wajapeyee, 2010). It is characterized by irreversible cell-cycle arrest that can be triggered by many types of intrinsic and extrinsic stress. Other senescence markers include a large flat morphology, induction of a senescence-associated β-galactosidase activity (SA β-gal) (Dimri et al., 1995) and the formation of several heterochromatin domains called senescence-associated heterochromatin foci (SAHF). Numerous studies have elucidated the molecular mechanisms that direct cellular senescence (Ohtani et al., 2001; Ozturk et al., 2009; Schmitt, 2007). Both p16 and p21, whose expression is invariably elevated in senescent cells, act as two critical regulators of senescence. As cyclin-dependent kinase (CDK) inhibitors, p16 and p21 block the activity of G1 and G1/S CDKs, respectively, leading to upregulation of retinoblastoma (Rb) protein activity in the cells. The continuous Rb activation then induces cellular senescence by recruiting heterochromatin proteins such as histone deacetylase to the E2F-responsive promoters to form SAHF, resulting in stable repression of E2F target genes (Narita et al., 2003).
All-trans retinoic acid (ATRA), the most biologically active metabolite of vitamin A, is known to modulate cell proliferation, apoptosis and differentiation (Kastner et al., 1995). In addition, it has been implicated in clinical applications as a cancer therapeutic and chemopreventive agent (Freemantle et al., 2006). Inhibition of tumour cell growth by ATRA mostly involves blockage in the G1 phase of cell-cycle progression. ATRA can exert its effect by directly or indirectly influencing the expression of genes involved in G1-checkpoint regulation. It upregulates levels of CDK inhibitors such as p16, p21 and p27 in various tumour cell lines including human hepatoma cell line HepG2 (Jung et al., 2010; Liu et al., 1996). In addition, it can downregulate levels of positive G1 regulators such as CDK2, CDK4/6 and cyclin D1 (Alisi et al., 2003; Jung et al., 2005; Teixeira & Pratt, 1997). All of these conditions have been shown to arrest cell-cycle progression at the G1 phase through inhibition of E2F transcription factors.

Recently, we have shown that HBx abolishes the potential of ATRA to downregulate levels of G1-checkpoint regulators including p16, p21 and p27 in human HCC cells, resulting in activation of E2F1 in the presence of ATRA (Jung et al., 2010). As a consequence, HBx-expressing cells were less susceptible to ATRA-induced cell growth inhibition compared with control cells. As p16 and p21 serve as key regulators of cellular senescence, in the present study we further investigated whether HBx could overcome ATRA-induced cellular senescence. We found that HBx could downregulate the levels of p16 and p21 under the conditions where ATRA induces cellular senescence. In addition, we demonstrated that HBx induces promoter hypermethylation of p16 and p21 to repress their expression. Finally, we showed that HBx-expressing cells were less susceptible to ATRA-induced cellular senescence compared with control cells. Based on these results, we have proposed a model for the induction of premature senescence initiated by retinoic acid (RA) and its inhibition by HBx.

RESULTS

HBx overcomes ATRA-induced cellular senescence in human hepatoma cells

It is widely known that RA inhibits cell growth and induces cell-cycle G1 arrest (Alisi et al., 2003; Teixeira & Pratt, 1997). To investigate whether HBx suppressed the anti-growth potential of RA in human hepatoma cells, we first compared the growth rate of two HepG2 stable cell lines with or without HBx expression (Kwun & Jang, 2004) in the presence of ATRA. Consistent with our recent report (Jung et al., 2010), HBx-expressing cells were less susceptible to ATRA-induced cell growth inhibition compared with control cells (Fig. 1a). In addition, according to flow cytometry analysis of the two cell lines, the G1 fraction of control cells increased approximately twofold after treatment with ATRA (from 42.6 to 81.5 %), whilst only a 19.4 % increase was observed with the HBx-expressing cells under the same condition (Fig. 1b). Interestingly, the control cells, in contrast to the HBx-expressing cells, barely recovered their growth after release from the ATRA-mediated growth arrest (Fig. 1a), suggesting that ATRA effectively induces irreversible G1 arrest in the control cells but not in the HBx-expressing cells.

Irreversible G1 arrest is one of the major biomarkers of cellular senescence (Yan & Wajapeyee, 2010). Therefore, we postulated that HBx-expressing cells overcome RA-induced cell growth inhibition at least in part by escaping cellular senescence. To test this hypothesis, we examined another important biomarker of cellular senescence, SA β-gal, in the two cell lines with or without ATRA treatment. As shown in Fig. 1(c), treatment with 10 μM ATRA for 72 h induced SA β-gal in 75 % of the control cells but only in 25 % of the HBx-expressing cells. In addition, ATRA markedly increased RNA levels of senescence-related genes such as apolipoprotein J, fibronectin and SM22 (Frippiat et al., 2001) in the control cells but not in the HBx-expressing cells (Fig. 1d, lanes 3 and 4).

To exclude the possibility that the HBx-mediated suppression of ATRA-induced cellular senescence was a cell-line-specific event, we transiently expressed HBx in HepG2 and two other human hepatoma cell lines, Hep3B and Huh-7, followed by SA β-gal analysis. As a result, we found that transient expression of HBx also suppressed ATRA-induced cellular senescence in HepG2 (Fig. 2a) and Hep3B (Fig. 2b) in a dose-dependent manner. However, the effect of HBx was not evident in Huh-7 cells in which ATRA does not induce cellular senescence (Fig. 2c). Taken together, we concluded that HBx inhibits the potential of ATRA to induce cellular senescence to stimulate the growth of human hepatoma cells.

HBx suppresses ATRA-induced cellular senescence by downregulating levels of p16 and p21

We next investigated the molecular pathway by which HBx overcomes ATRA-induced cellular senescence. ATRA upregulated the levels of two key determinants of cellular senescence, p16 and p21, in HepG2 (Fig. 2a) and Hep3B cells (Fig. 2b). In addition, HBx dose-dependently abolished these effects in both cell lines. In contrast, p16 and p21 proteins were undetectable in Huh-7 cells in which both HBx and ATRA had little affect on cellular senescence (Fig. 2c).

To determine the factor responsible for the induction of cellular senescence by ATRA and its suppression by HBx, we first performed RNA interference (RNAi) experiments using small interfering RNA (siRNA) specific for p16 or p21. Knockdown of p16 or p21 in the control cells resulted in upregulation of hyperphosphorylated Rb without affecting its total protein level (Fig. 3a, lanes 3–6). Under the same conditions, the control cells exhibited a much
lower SA β-gal activity (Fig. 3c, columns 3–6), suggesting that both p16 and p21 are involved in the induction of cellular senescence by ATRA.

We also attempted to complement either p16 or p21 in the HBx-expressing cells to identify the factor responsible for the suppression of ATRA-induced senescence by HBx. In the presence of ATRA, HBx upregulated the levels of hyperphosphorylated Rb without affecting its total protein level (Fig. 3a, b, lanes 1 and 2). When either p16 or p21 was complemented by transient transfection of p16 or p21 expression vector into the HBx-expressing cells, levels of hyperphosphorylated Rb decreased to those in the control cells (Fig. 3b, lanes 3–6). As a consequence, HBx-expressing cells became more susceptible to ATRA-induced cellular senescence (Fig. 3c, columns 7–10). These results suggested that HBx overcomes ATRA-induced cellular senescence by downregulating levels of p16 and p21.

**HBx downregulates levels of p16 and p21 via DNA methylation**

Next, we investigated the mechanism by which HBx downregulates levels of p16 and p21. During cellular senescence induced by other stresses such as H2O2, expression of p16 is upregulated by the Ets-1 and Ets-2 transcription factors, which are downstream mediators of the Ras/Raf/MEK pathway (Ohtani et al., 2001). However, levels of Ets-1 and Ets-2 were minimally affected by ATRA (Fig. 4a, lanes 1 and 3). In addition, HBx did not affect their levels in either the presence or the absence of ATRA (Fig. 4a, lanes 2 and 4). These results suggested that neither Ets-1 nor Ets-2 is involved in the ATRA-induced upregulation of p16 and its downregulation by HBx.

In contrast, p21 is a major target of the p53 pathway (El-Deiry et al., 1993). Consistent with our previous reports (Ahn et al., 2002; Kwun & Jang, 2004), levels of p53 were...
upregulated by HBx in both the presence and the absence of ATRA (Fig. 4a, lanes 2 and 4). As a consequence, levels of p21 were upregulated in the HBx-expressing cells in the absence of ATRA (Fig. 4a, lane 2). However, in the presence of ATRA, levels of p21 in the HBx-expressing cells were lower compared with the control cells (Fig. 4a, lanes 3 and 4) because ATRA dramatically upregulated the levels of p21 in the control cells (Fig. 4a, lanes 1 and 3) but not in the HBx-expressing cells (Fig. 4a, lanes 2 and 4). ATRA did not affect the levels of p53 in the presence or absence of HBx (Fig. 4a, lanes 1–4). These results suggest that p53 is not involved in the ATRA-mediated upregulation of p21 and its modulation by HBx.

Interestingly, ATRA elevated the levels of p16 and p21 almost equally in the two cell lines without affecting the levels of Ets-1, Ets-2 and p53 in the presence of a universal DNA methylation inhibitor, 5-Aza-2’-deoxycytidine (5-Aza-2’dC; Fig. 4a, lanes 5 and 6). These results suggested that upregulation of p16 and p21 by ATRA as well as its suppression by HBx involve DNA methylation. To examine this possibility, we first investigated whether ATRA affected DNA methylation of p16 and p21. When methylation-specific PCR (MSP) for amplification of methylated regions on the promoter regions of p16 and p21 was performed, significantly lower frequencies of 5-methylcytosine residues were detected in the ATRA-treated HepG2 cells compared with untreated HepG2 cells (Fig. 4b, lanes 1 and 3). Consistently, ATRA downregulated levels of the DNA methyltransferase DNMT1 and DNMT3a in the HepG2 cells (Fig. 4c, lanes 1 and 3). Next, we investigated whether HBx induced DNA methylation of p16 and p21 and thus could antagonize the potential of ATRA to modulate DNA methylation. According to the results of MSP analysis, HBx induced DNA methylation of p16 in the presence and absence of ATRA (Fig. 4b, lanes 2 and 4). In addition, levels of DNMT1 and DNMT3a were upregulated by HBx in the presence and absence of ATRA (Fig. 4c, lanes 2 and 4), which is consistent with previous reports (Jung et al., 2010; Park et al., 2007). HBx also suppressed the potential of ATRA to inhibit DNA methylation of p21, although it was unable to induce its DNA methylation (Fig. 4b, lanes 2 and 4). The potential of HBx to modulate DNA methylation of p16 and p21 was completely abolished by treatment with 5-Aza-2’dC (Fig. 4b, lanes 5 and 6). In addition, the HBx-expressing cells became more susceptible to ATRA-induced senescence in the presence of 5-Aza-2’dC (Fig. 4c). Therefore, we concluded that HBx overcomes ATRA-induced cellular senescence by antagonizing the potential of ATRA to upregulate levels of p16 and p21 via inhibition of DNA methylation.

**DISCUSSION**

Retinoids are important regulators of normal cellular proliferation and differentiation and suppressors of tumour growth by cell-cycle arrest and apoptosis (Kastner et al., 1995). The present study suggests a new action mechanism of RA: irreversible inhibition of cell growth by inducing cellular senescence. Upregulation of the two key determinants of cellular senescence, p16 and p21, is likely to be essential for this effect. Knockdown of p16 or p21 using a specific siRNA abolished the potential of ATRA to induce...
cellular senescence (Fig. 3c). In addition, ATRA could not induce cellular senescence in Huh-7 cells (Fig. 2c) in which the p16 and p21 proteins are absent because of homozygous deletion of both genes (Hashimoto et al., 2003).

Cancer cells often develop means to abolish the anti-cancer potential of retinoids. For example, abnormalities in RA receptors (RARs) are closely associated with retinoic resistance in various tumours (Hoffman et al., 1996; Hu et al., 1991). The present study also showed that genetic defects in the RA effector molecules can result in retinoid resistance, as demonstrated in Huh-7 cells (Fig. 2c). In addition, post-translational modification of RARs by phosphorylation impairs its function in HCC, leading to uncontrolled cell growth (Matsushima-Nishiwaki et al., 2001). According to our recent report, HBx, the major HBV oncoprotein, downregulates levels of RAR-β2 to suppress ATRA-induced cell-growth inhibition (Jung et al., 2010). The present study further demonstrated that HBx overcomes cellular senescence provoked by ATRA.

Cellular senescence is now considered to be an important tumour-suppression process, preventing damaged cells from undergoing aberrant proliferation (Campisi, 2005; Ozturk et al., 2009; Yan & Wajapeyee, 2010). Developing a means of evading senescence thus seems to be a

Fig. 3. HBx suppresses ATRA-induced cellular senescence by downregulating levels of p16 and p21. (a) HepG2-vector cells were transiently transfected with increasing amounts of p16 RNAi (lanes 3 and 4) or p21 RNAi plasmid (lanes 5 and 6) and treated with ATRA as described in Fig. 1. Protein levels of p16, p21, Rb-P (phosphorylated Rb), total Rb, HBx and γ-tubulin were determined by Western blot analysis. (b) HepG2-HBx cells were transiently transfected with increasing amounts of p16-expressing (lanes 3 and 4) or p21-expressing (lanes 5 and 6) plasmid and treated with ATRA as described in Fig. 1. (c) Cells prepared as in (a) and (b) were subject to SA β-gal analysis as indicated in Fig. 1(c).
fundamental task that all cancer cells should resolve early on. The oncoproteins of several DNA tumour viruses seem to interfere with Rb and p53 to bypass cellular senescence. For example, simian virus 40 large T antigen binds to Rb and p53 to overcome replicative senescence (Shay et al., 1991). Similarly, adenovirus E1A directly targets Rb and prevents senescence induced by oncogenic ras and DNA-damaging agents (Serrano et al., 1997). In addition, downregulation of papillomavirus E6 and E7 triggers cellular senescence through activation of p53 and Rb (DeFilippis et al., 2003). According to the present study, HBx inactivated Rb by downregulating the levels of p16 and p21, resulting in suppression of cellular senescence provoked by RA (Fig. 3). Therefore, a common strategy seems to be adopted by DNA tumour viruses to bypass senescence during tumorigenesis, although the detailed mechanisms appear to be different.

DNA methylation provides an alternative mechanism for inactivation of tumour-suppressor genes during tumorigenesis (Narimatsu et al., 2004; Zhu et al., 2007). The genes frequently methylated in HCC are those encoding APC, GSTP1, RASSF1A, p16, COX-2 and E-cadherin (Park et al., 2007; Zhu et al., 2007, 2010). In addition, HBx induces promoter hypermethylation of these tumour-suppressor genes (Jung et al., 2010; Tong et al., 2009; Zhu et al., 2010). However, its mechanism and biological significance are largely unknown. Recently, Zheng et al. (2009) demonstrated that HBx recruits DNMT3a via direct interaction to the regulatory promoters of target genes, resulting in repression of their transcription via de novo DNA methylation. In addition, HBx upregulates levels of DNMT1 and DNMT3a (Jung et al., 2007; Park et al., 2007). The present study also showed that HBx upregulated levels of DNMT1 and DNMT3a in human hepatoma cells, resulting in promoter hypermethylation of p16 and p21 and subsequent downregulation of their expression (Fig. 4). As levels of p16 and p21 in the HBx-expressing cells were recovered by either exogenous expression or treatment with a DNMT inhibitor, the potential of HBx to overcome ATRA-induced senescence was severely impaired. Therefore, epigenetic modulation of p16 and p21 seems to be an essential mechanism of HBx to overcome cellular senescence provoked by RA.

It has been demonstrated that ATRA modulates the expression of several G1 regulators including p16 and p21, resulting in inhibition of cell-cycle progression (Jung et al., 2010). Therefore, the present study also showed that HBx upregulated levels of DNMT1 and DNMT3a in human hepatoma cells, resulting in promoter hypermethylation of p16 and p21 and subsequent downregulation of their expression (Fig. 4). As levels of p16 and p21 in the HBx-expressing cells were recovered by either exogenous expression or treatment with a DNMT inhibitor, the potential of HBx to overcome ATRA-induced senescence was severely impaired. Therefore, epigenetic modulation of p16 and p21 seems to be an essential mechanism of HBx to overcome cellular senescence provoked by RA.

It has been demonstrated that ATRA modulates the expression of several G1 regulators including p16 and p21, resulting in inhibition of cell-cycle progression (Jung et al., 2010). Therefore, the present study also showed that HBx upregulated levels of DNMT1 and DNMT3a in human hepatoma cells, resulting in promoter hypermethylation of p16 and p21 and subsequent downregulation of their expression (Fig. 4). As levels of p16 and p21 in the HBx-expressing cells were recovered by either exogenous expression or treatment with a DNMT inhibitor, the potential of HBx to overcome ATRA-induced senescence was severely impaired. Therefore, epigenetic modulation of p16 and p21 seems to be an essential mechanism of HBx to overcome cellular senescence provoked by RA.
et al., 2010; Liu et al., 1996); however, the mechanism remains unknown. Normally, RAR as either a homodimer or heterodimer activates expression of target genes in the presence of ATRA by acting on the RA response element located in the promoter regions (Kastner et al., 1995). In addition, ATRA can exert its effects indirectly by affecting the expression of genes that do not contain a distinct RA response element sequence. In the latter case, a common regulator(s) may mediate the potentials of RAR/retinoid X receptor complexes to regulate expression of target genes. Interestingly, RA downregulates both expression and activity of DNMT during acute promyelocytic leukaemia blast cell differentiation in vitro and in vivo (Fazi et al., 2005). The present study also showed that ATRA downregulates the levels of DNMT1 and DNMT3a, resulting in promoter hypomethylation of p16 and p21 (Fig. 4). More extensive studies are required to claim the role of RA as an epigenetic regulator.

Although HBx downregulates the levels of p16 and p21 via DNA methylation, the detailed mechanisms appear to be different. HBx induced promoter hypermethylation of p16 in the presence and absence of ATRA (Fig. 4b). As ATRA could induce promoter hypermethylation of p16 only in the control cells and not in the HBx-expressing cells, the effects of HBx on the promoter methylation and expression of p16 were more dramatic in the presence of ATRA. In contrast, DNA methylation of the p21 promoter was minimally affected by HBx in the absence of ATRA (Fig. 4b). Under these conditions, HBx upregulated the levels of p21 via activation of p53 (Fig. 4a), which is consistent with our previous reports (Ahn et al., 2002; Kwun & Jang, 2004). HBx may stabilize p53 by protecting it from MDM2-mediated degradation (Kwun & Jang, 2004). In the presence of ATRA, however, the levels of p21 were higher in the control cells compared with the HBx-expressing cells (Fig. 4a). This is probably because ATRA induced promoter hypermethylation of p21 in the control cells but not in the HBx-expressing cells (Fig. 4b). Based on these results, we have proposed a model for the induction of premature senescence by ATRA and its inhibition by HBx (Fig. 5). According to this model, ATRA upregulates levels of p16 and p21 via induction of promoter hypermethylation, resulting in the activation of Rb via inhibition of G1- and G1/S CDKs and induces G1 arrest leading to cellular senescence. HBx can antagonize the potential of ATRA to modulate DNA methylation by upregulating levels of DNMTs. This model may provide a new action mechanism of retinoid for tumour suppression. It might be also useful to understand how HCC acquires RA resistance during tumorigenesis.

**METHODS**

**Plasmids.** Plasmids pCMV-3 × HA1-HBx (HBx; Kwun & Jang, 2004) and pCMV-3 × HA1-p16 (p16; Jung et al., 2007) encode HBx and p16, respectively, downstream of three copies of the influenza virus haemagglutinin (HA) epitope. The expression vector for p21 has been described previously (El-Deiry et al., 1993).

**Cell lines and transfection.** HepG2 (KCLB 58065), Hep3B (KCLB 88064) and HuH-7 (KCLB 60104) were obtained from the Korean Cell Line Bank. HepG2 stable cell lines were established by transfection with either pCMV-3 × HA1 or pCMV-3 × HA1-HBX3 followed by selection with 500 μg G418 (Gibco) ml⁻¹ (Kwun & Jang, 2004). For transient expression, 2 × 10⁵ cells per 60 mm dish were transfected with 1 μg of appropriate plasmid(s) using WelFect-EX PLUS (WelGENE) following the manufacturer’s instructions. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FCS. Cells were either mock treated or treated with 10 μM ATRA (Sigma) and/or 5 μM 5-Aza-2’dC (Sigma) for the indicated period.

**Cell proliferation assay.** For the determination of cell growth, cells were seeded at 1 × 10⁶ per well in 96-well plates and incubated under the indicated conditions. A 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed as described previously (Liu et al., 1996). The MTT-derived formazan developed by cells was quantified by measuring absorbance at 550 nm.

**Cell-cycle analysis.** Cell-cycle profile was analysed using flow cytometry. Briefly, 2 × 10⁶ cells were trypsinized, fixed in 80% ethanol and resuspended in 50 μg propidium iodide (Sigma) ml⁻¹ containing 125 U RNase A (Sigma) ml⁻¹. DNA contents were analysed by flow cytometry using Cell-FIT software (Becton Dickinson Instruments).
SA β-gal assay. The proportion of HepG2 cells positive for SA β-gal activity was determined as described by Dimri et al. (1995). Briefly, cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde for 10 min. The presence of SA β-gal activity was determined by incubation with SA β-gal staining solution (Cell Signalling) at 37 °C for 18 h. The cells were rinsed twice with PBS and washed with methanol. The results were expressed as means ± SD in three independent experiments.

Semi-quantitative RT-PCR. Total RNA was extracted using a total RNA isolation kit (Qiagen). DNase I-digested RNA (3 μg) was reverse transcribed with the corresponding reverse primer. One-quarter of the reverse-transcribed RNA was amplified with Taq polymerase (95 °C for 5 min; 30 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 30 s; 72 °C for 5 min) using the following primers: apolipoprotein J, 5'-GGGGTTGGAAGAACGATAAG-3' and 5'-TCGG-GTTCACATTGATC3A-3'; fibronectin, 5'-GATTGGCTTCTGTCTC-3' and 5'-TGGGTTGACTTTCCTACT-3'; and SM2, 5'-TGGGC-TGTATTCTGAGCAA-3' and 5'-CTGGCAAGCTGCCAAAGG-3' (Frippiat et al., 2001). The PCR conditions for HBx and GAPDH have been described previously (Jung et al., 2007).

Western blot analysis. Cells were lysed in buffer [50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40] supplemented with protease inhibitors. Cell extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Hybond PVDF; Amersham). Membranes were then incubated with antibodies against DNMT1 (1:500 dilution), DNMT3a (1:200 dilution), Ets-1 (1:200 dilution), H2A (1:1000 dilution), Roche), Rb (1:500 dilution), Calbiochem), γ-tubulin (1:5000 dilution; Sigma) and phosphorylated Rb (1:1000 dilution; Sigma) for 2 h at room temperature and subsequently with the appropriate HRP-conjugated secondary antibodies: anti-mouse IgG (H+L)-HRP and anti-rabbit IgG (H+L)-HRP (both diluted 1:2000; Bio-Rad) for 1 h at room temperature. A chemiluminescent ECL kit (Amersham) was used to visualize protein bands on X-ray films.

REFERENCES


This study was supported by a grant from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A084399).


