Carcinogenesis vol.25 no.10 pp.2023–2032, 2004
doi:10.1093/carcin/bgh207

Pre-S mutant surface antigens in chronic hepatitis B virus infection induce oxidative stress and DNA damage

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Ground glass hepatocytes (GGHs) are the historic hallmarks for the hepatocytes in the late and non-replicative stages of hepatitis B virus (HBV) infection. We have identified type I and type II GGHs that contain two mutant types of large HBV surface antigens (HBsAg) with deletions over the pre-S_1 and pre-S_2 regions, respectively. These pre-S mutant HBVsAg accumulate in endoplasmic reticulum (ER), resulting in strong ER stress. Type II GGHs often appear in hepatic nodules in the late phases of HBV infection and proliferate in clusters, suggesting that these mutant pre-S_1/S_2 HBsAg may be involved in HBV-related hepatocarcinogenesis, associated with ER stress. In this study, we investigated the potential genomic instability imposed by pre-S mutant HBsAg. Based on the analysis of comet assays, we found that the pre-S_1 and pre-S_2 mutant HBsAg caused oxidative stress and DNA damage. The DNA repair gene *ogg1* was greatly induced by over-expression of pre-S mutant HBsAg. Induction of the DNA repair gene *ogg1* was also detected in the pre-S_2 HBsAg transgenic mice, as well as the type II GGHs from patients with hepatocellular carcinoma, strongly suggesting that the pre-S mutant HBsAg contributes to the oxidative DNA damage to hepatocytes. In addition, the mutation rates in the X-linked *hprt* gene were enhanced in mouse hepatoma ML1-4a cells, which constitutively expressed the pre-S_1/S_2 HBsAg. These results indicate that pre-S_1/S_2 mutant HBsAg, which make up GGHs, induce oxidative DNA damage and mutations in hepatocytes in the late stages of HBV infection.

Introduction

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer (1). Chronic hepatitis B virus (HBV) infection is a major global cause of HCC (2). Chronic HBV carriers have a >100-fold increase in relative risk of developing HCC (3–6). Expression of HBV surface proteins is strongly associated with hepatic injury. The HBV surface antigen (HBsAg) is able to up-regulate the transforming growth factor-alpha (TGF-α) gene, the most important growth factor for hepatocytes. In the chronic phase of HBV infection, the expression of HBsAg is associated with hepatic nodular formation, a prerequisite for hepatocellular carcinogenesis (7).

Expression of the various types of HBsAg is dependent upon the replicative status of the HBV genome. In the acute phase of HBV infection, the major (i.e. small) HBS is the predominant form and constitutes the envelope of the virion. In the chronic phase, the HBV genome integrates into the host genome, and the large form becomes dominant. The large form of HBsAg includes an additional pre-S region that is the upstream promoter region for small HBsAg (7,8). A number of truncated surface gene mutants with a partially deleted pre-S region have already been identified. Recent research (9,10) shows that pre-S mutant HBS antigens contribute to two histological patterns designated 'ground glass hepatocyte' (GGH) types I and II (9,10). Type I GGHs display an inclusion-like pattern of HBsAg, which is deleted in the pre-S_1 promoter region. Type II GGHs display HBsAg at the margins. This type of mutant HBS gene is deleted of nucleotides 4–57 over the pre-S_2 region and contains a point mutation at the start codon of the pre-S_2 region, which leads to a dramatic decrease in the synthesis of small and middle surface antigens (Figure 1A). Both types of GGHs show accumulation of HBsAg in endoplasmic reticulum (ER) (11). Thus, type II GGH has a high correlation with the progression of cirrhosis and HCC. This mutant form of the HBS gene, designated pre-S_2 mutant HBS, emerges only in the late or non-replicative phase of chronic HBV infection and eventually becomes a dominant HBV gene product in hepatocytes. Hepatocytes expressing this type of mutant consistently cluster into groups due to clonal and integrated expansion (9,10).

Accumulation of proteins in ER induces ER stress signaling pathway, including ER overload response, unfolded protein response (UPR) and sterol starvation. These cellular responses are associated with cell cycle exit and apoptosis (12,13). Our previous study found that the GGHs, which contain specific mutant pre-S_1 or pre-S_2 mutant surface antigens, exhibit significant ER stress (11). Here we characterize the molecular and genetic changes caused by these mutant HBS forms. We found that pre-S_1 and pre-S_2 mutant HBsAg cause oxidative stress-induced DNA damage and mutation, suggesting that the pre-S mutant HBsAg contributes to genomic instability in HBV-infected hepatocytes.

Materials and methods

Cell lines and reagents

The human Huh-7 and mouse ML1-4a hepatoma cell lines were used for *in vitro* cell culture studies. The Huh-7 cells were stably co-transfected with the pERV3 plasmid, which contains the expression cassette for ecdysone promoter function, and the pEGSH plasmid, which contains the large wild-type, pre-S_1

Abbreviations: BER, base excision repair; ER, endoplasmic reticulum; FPG, formamidopyrimidine-DNA glycosylase; GGH, ground glass hepatocyte; HCC, hepatocellular carcinoma; HBsAg, HBV surface antigen; HBV, hepatitis B virus; ROS, reactive oxygen species; TMB-8, 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate.

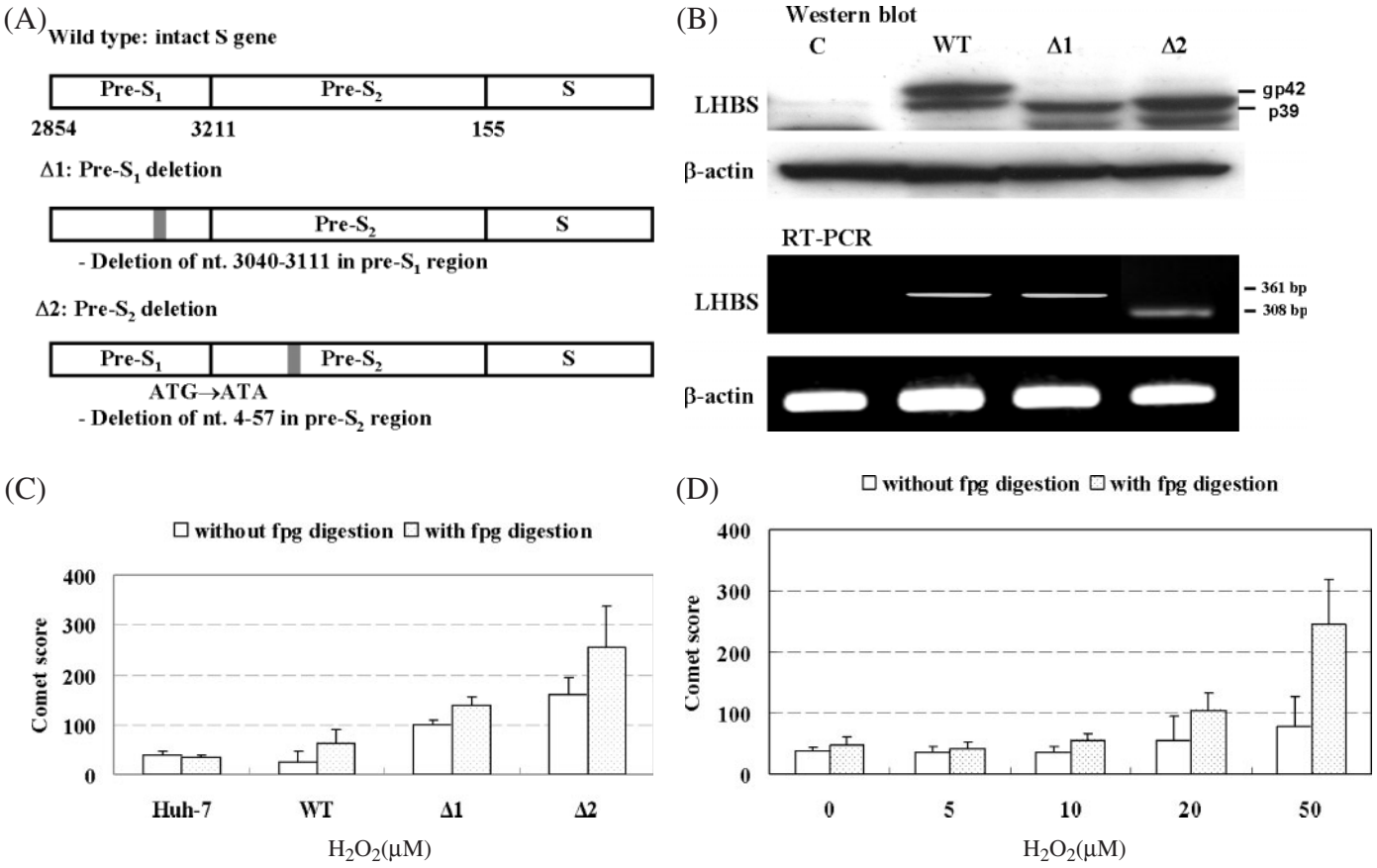

Fig. 1. Oxidative DNA damages in the Huh-7 cells with inducible HBS genes by comet assay. (**A**) Representatives of the pre-S_1 and pre-S_2 DNA regions. The shaded boxes are the regions deleted in the pre-S_1 and pre-S_2 mutant HBS antigens. (**B**) Expression of the wild-type, pre-S_1 and pre-S_2 mutant HBS antigens. WT, the wild-type large HBS antigen. Δ1 and Δ2, the pre-S_1 and pre-S_2 mutant HBS antigens. C, control, the mock-transfected Huh-7 cells. By western blots, the wild-type large HBS antigen presents the p39 and glycosylated p42 protein products. The Δ1 and Δ2 deletion mutants present truncated forms of large HBS antigens. By RT–PCR using the PCR primers derived from the pre-S regions, the wild-type and Δ1 mutant HBS constructs present the 361-bp cDNA product. The Δ2 mutant HBS construct presents the truncated 308-bp product. LHBS, large HBS antigen. (**C**) Results of comet assays in the Huh-7 cells stably transfected with various types of HBS genes. The comet scores presented prior to FPG treatment indicate the DNA strand breaks, induced by various types of HBsAg (the open boxes). After the treatment of the FPG DNA repair enzyme, the sites of 8-hydroxyguanine DNA lesions are specifically cleaved to generate strand breaks (the dotted boxes). The comet tails were separated by alkaline electrophoresis, which detects both single- and double-stranded breaks. The comet scores indicate the mean comet scores (±1 SD)/slide of the total five slides examined. (**D**) The positive control, the oxidative DNA damages after treatments with various doses of H_2O_2.

or pre-S_2 mutant HBS genes under control of the inducible ecdysone promoter (11). The mouse ML1-4a hepatoma cells, which constitutively expressed the large wild-type, pre-S_1 or pre-S_2 mutant HBS genes were constructed by stable transfection of one of these three types of HBS genes. These cells were maintained in regular Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1× non-essential amino acid and 1× of antibiotic/antimycotic mixture (Gibco BRL, Grand Island, NY). The cells were grown at 37°C with 5% CO_2.

Most of the reagents used for the reverse transcription and PCR amplifications were purchased from Promega (Madison, WI). Low-melting-point agarose, used for comet assays, was purchased from Gibco BRL. SYBR Green II, the staining reagent for comet DNAs, was purchased from Molecular Probes (Eugene, OR). The DNA repair enzyme formamidopyrimidine-DNA glycosylase (FPG), used for detection of 8-hydroxyguanine DNA lesions, was purchased from Trevigen (Gaithersburg, MD). The HBsAg ELISA assay kit, used for quantification of the HBS proteins in Huh-7 as well as ML1-4a cells, was purchased from General Biological (Taiwan). Antibodies used for immunological stainings were purchased from DAKO (Carpinteria, CA). Common chemicals used were purchased from Sigma Chemical (St Louis, MO).

Comet assays

Levels of oxidative DNA damage were measured by single-cell alkaline electrophoretic comet assays. Huh-7 cells in which inducible wild-type or pre-S_1/S_2 mutant HBS genes were transfected were used for the study. After induction of each type of HBS gene by ponasterone A for 48 h, the cells were harvested by trypsinization, centrifuged and re-suspended in PBS, then cell numbers were counted using a hemacytometer. To prepare a glass slide for comet assays, PBS (pH 7.4) with 1% agarose was first coated on the slides, and then covered with 1.5% low-melting-point agarose gel in which 4×10^4 cells were embedded. Next, another layer of 1% low-melting-point agarose gel was placed on top. After the coatings, the slides were covered with cover slips and placed on ice for 5 min. The cells on slides were then lysed with ice-cold lysis buffer (5 M NaCl, 100 mM EDTA, 100 mM Tris–HCl, 1% Triton X-100, 10% dimethylsulfoxide) at 4°C for 1 h. After lysis, the slides were immersed in PBS (pH 7.4) for 10 min and then subjected to enzymatic digestion.

To detect 8-hydroxyguanine DNA lesions, the FPG DNA repair enzyme was used to specifically cleave the DNA strand at 8-hydroxyguanine sites (14). For these slides, 2 U of FPG were incubated with the slides at 37°C for 1 h. After FPG treatment, the slides were washed a few times in PBS (pH 7.4). For single-cell alkaline electrophoresis, the slides were first immersed in alkaline electrophoresis buffer (0.3 M NaOH, 1 mM EDTA) for 20 min, followed by electrophoresis at 23 V and 30 mA for 25 min. After alkaline electrophoresis, the slides were transferred to a neutralization solution of 0.4 M Tris–HCl (pH 7.5) and then stained with SYBR green II for 3 min in the dark. Next, 100 cells on each slide were examined using fluorescence microscopy and scored for the fluorescent tail length from the cell nuclei. Each cell was assigned a score on an arbitrary five-point scale (0 = no DNA damage; 4 = extensive DNA damage), based on comet tail length migration. The total comet score for each slide equalled the sum of the grades for each evaluated cell (maximum possible score: 400) (15,16).

RT–PCR of DNA repair genes

Human Huh-7 cells expressing inducible HBS and mouse ML1-4a cells constitutively expressing HBS genes were analyzed for expression of DNA repair genes. Huh 7-cells were treated with ponasterone A for 12, 24 or 48 h (or mock-treated) and then subjected to RNA extraction. RT–PCR was performed in a mixture of 1 μg of cellular RNA, 0.5 μg of random hexamer, 2 mM of each dNTP, 0.6 μl of RNase inhibitor (Takara, Tokyo, Japan), 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol and 200 U of Moloney murine leukemia virus reverse transcriptase (MMLV-RT). The reaction was performed at 37°C for 90 min and then stopped at 95°C for 5 min.

For each PCR reaction, 5 μl of cDNA product of the reverse transcription reaction was used as the template. The PCR primers used were as follows: *ogg1*, forward: 5′-cactgcactgtgtaccgagg-3′, and reverse: 5′-gctgctgcagccaggctagc-3′; *xrcc1*, forward: 5′-atgccggagatccgcctccg-3′, and reverse: 5′-catcattccccaatgtccaca-3′; and the housekeeping gene β-actin, forward: 5′-atcatgtttgagaccttcaa-3′, and reverse: 5′-catctcttgctcgaagtcca-3′. For the GGH cells isolated by LCM from the HCC patients, nested PCR was performed. The primers for nested PCR were forward: 5′-gtgtgcgactgctgcgacaa-3′, and reverse: 5′-gctgctgcagccaggctagc-3′. The PCR mix contained 25 mM *N*-[Tris (hydroxymethyl) methyl]-3-aminopropanesulfonic acid (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol, 200 mM of each dNTP, 0.25 μg/μl activated calf thymus DNA and 2 U of SuperTherm Fold DNA polymerase (JMR Holdings, Kent, UK). The PCR reaction was performed in the following order: *ogg1* at 94°C for 10 min, then 35 cycles (*xrcc1*, 26 cycles) with each cycle of 94°C for 60 s, 54°C for 45 s and 72°C for 120 s. Finally, the PCR tubes were incubated at 72°C for 10 min. The PCR products were examined by agarose gel electrophoresis.

Host-cell reactivation assay

Host-cell reactivation activities on H_2O_2-damaged plasmids were used to detect DNA repair activity in cells. To construct the plasmid substrate, $pCMV^{luc}$ DNA was treated in the dark with 5% H_2O_2 at room temperature for 30 min. The treated plasmid DNA was purified by ethanol precipitation. To test the DNA repair activity in Huh-7 cells expressing various types of HBS genes, H_2O_2-damaged $pCMV^{luc}$ as well as wild-type, pre-S_1 or pre-S_2 HBS genes in the p(3A)-S plasmid vector were co-transfected into the Huh-7 cells. After transfection for 48 h, cell lysates were collected and the luciferase activities were detected using a luciferase assay kit (ABI; Applied Biosystems).

hprt gene mutation assays

ML1-4a cells constitutively expressing wild-type, pre-S_1 or pre-S_2 HBS genes were analyzed for gene mutation rates in the hypoxanthine phosphoribosyltransferase (*hprt*) gene. Cells were seeded into 96-well plates in concentrations of 2×10^3 cells/well containing 5 μg/ml 6-thioguanine (Sigma) for selection of *hprt* mutants. After selection for 14 days, the surviving colonies were counted for the mutation rates of the *hprt* gene. Those cell colonies resistant to 6-thioguanine treatments were further grown and sub-cultured in DMEM growth medium with 3 μg/ml of 6-thioguanine. After the cells became confluent in the culture wells, the cellular RNAs were isolated. The *hprt* cDNA was amplified by RT–PCR and then sequenced to identify the mutations in the gene. The PCR primers used were: forward, atgccgacccgcagtcccagcgtcg, and reverse, ttaggctttgtatttggcttttcca.

Pre-S_2 HBS transgenic mice

The plasmid p(3A)SAg-r2, which contains the large HBS surface gene with an internal deletion on the pre-S_2 region (nt 4–57) (10), was used to construct transgenic mice by the animal facility in our medical center. The plasmid was microinjected into embryos of FVB/N mice. All microinjected embryos were then implanted into pseudo-pregnant female mice. Mice carrying the transgenes were selected by PCR detection of the pre-S mutant HBS gene. Genomic DNA was purified from whole blood using a whole blood kit (InstaGene; Bio-Rad Laboratories, Hercules, CA). Mice with the HBV transgene were tested for HBsAg expression in sera by commercial ELISA kit (General Biological, Taiwan). Transgenic mice were fed standard laboratory chow and water *ad libitum* in the animal facility. The animals were raised and cared for according to the guidelines set up by the National Science Council of the Republic of China.

Detection of large HBS mRNAs and proteins

The specific mRNAs of the large HBsAg were detected by RT–PCR using the primers derived from the pre-S region. The forward primer was derived from the pre-S_1 region, whose sequence was 5′-gctacaaactttgccagcaa-3′; the reverse primer was derived from the pre-S_2 region, whose sequence was 5′-ctgcggtattgtgaggattc-3′. The full-length and pre-S_1 mutant HBS genes were expected to present the 381-bp cDNA product. And the pre-S_2 mutant HBS gene was expected to present the truncated 308-bp cDNA product.

The protein products of the large HBsAg were detected by western blots, using the monoclonal antibody MA18/7, which is specifically against the pre-S_1 region (a generous gift from W.H.Gerlich, Institute of Medical Virology, Justus Liebig University Giessen, Giessen, Germany). The protocols for western blots basically followed those described in the study by Wang *et al*. (11).

HBS immunohistochemical staining

Five-micrometer sections of frozen liver tissues from pre-S_2 HBS transgenic mice were fixed in ice-cold acetone, blocked with 3% H_2O_2 in PBS, and immunostained with monoclonal goat anti-HBS antibody. After they had been washed in PBS, slides were incubated with biotinylated anti-goat secondary antibody and then followed by peroxidase-conjugated streptavidin. The HBsAg was then stained with 3-amino-ethyl-carbazole, counterstained with Mayer's hematoxylin, and examined under a microscope.

Immunofluorescent staining of 8-oxoguanine glycosylase in HCC patients

Five-micrometer sections of frozen liver tissues from HCC patients were immunostained with mouse anti-HBS antibody and rabbit antibody against anti-8-oxoguanine glycosylase (Novus Biologicals, Littleton, CO). The slides were then incubated with anti-mouse secondary antibody conjugated with tetramethylrhodamine (TRITC) as well as anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (FITC). Finally, the cell nuclei were counterstained with Hoechst 33342. The sections were visualized by confocal microscopy at a wavelength of 547 nm for TRITC, 480 nm for FITC and 345 nm for Hoechst 33342.

Results

Induction of oxidative DNA damage by pre-S_1/pre-S_2 HBS mutant antigens

We have shown previously that the pre-S_1 and pre-S_2 mutant HBsAg induce reactive oxygen species (ROS) production in Huh-7 cells (11). To test for the HBsAg-induced oxidative DNA damage, in this study we performed comet assays on Huh-7 cells stably transfected with inducible constructs of wild-type, pre-S_1 or pre-S_2 large HBS genes. Upon induction by ponasterone A, these three types of HBS antigens were expressed in approximately equal amounts, as shown by western blot using the antibody specifically against the pre-S_1 region. The full-length large HBS was in the sizes of 39 and 42 kDa (glycosylated form). In the cells expressing the pre-S_1/pre-S_2 mutant HBsAg, the truncated products were seen (Figure 1B). To specifically detect the amounts of the 8-hydroxyguanine, the major DNA lesion induced by oxidative stress, the DNA repair enzyme FPG was employed. FPG is a DNA glycosylase/lyase that cleaves the DNA strand at the sites of 8-hydroxyguanine (17). The comet scores, presented in Figure 1C, were the mean comet scores/slide of the five slides analyzed for each sample. Expressions of wild-type, pre-S_1 or pre-S_2 HBS mutant antigens all resulted in an increase of comet scores in Huh-7 cells. Treatment with FPG raised comet scores, indicating that 8-hydroxyguanine DNA lesions were present in these cells. The comet scores were increased even before the addition of FPG, suggesting that the wild-type and mutant HBsAg analyzed also created strand breaks, which, alternatively, might represent the intermediate product in DNA excision repair pathways. After treatment with FPG to create strand breaks at the 8-hydroxyguanine sites, the comet scores were further elevated. The comet score for over-expression of pre-S_2 HBsAg was significantly higher than the comet scores for over-expression of wild-type or pre-S_1 HBsAg, suggesting that pre-S_2 mutant HBsAg triggers a higher level of oxidative DNA damage than the other two.

Induction of DNA repair by pre-S_2 HBS mutant antigen

The base excision repair (BER) pathway is the major pathway for oxidative DNA damage (17). We therefore hypothesized that the DNA damage induced by pre-S HBS mutant antigens,

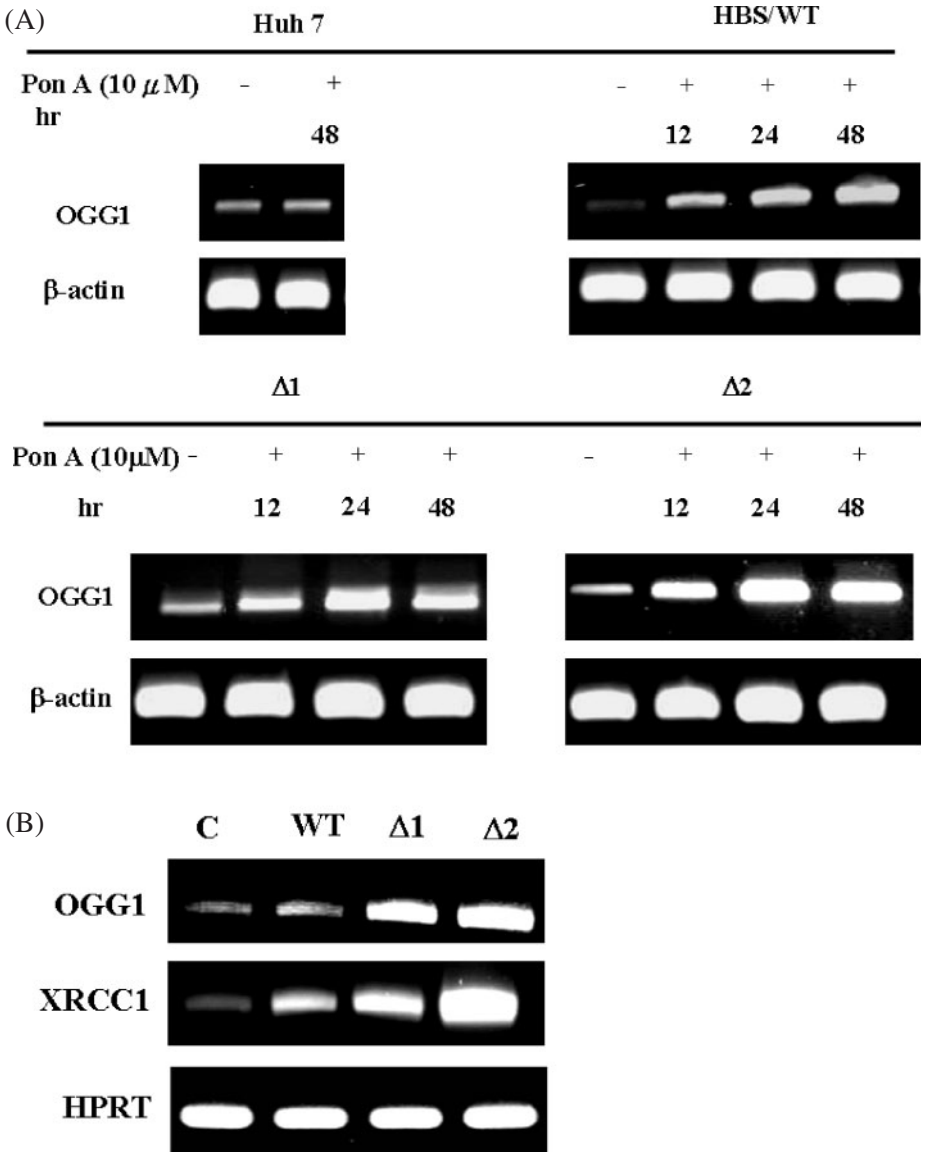

Fig. 2. Stimulation of the DNA repair in cells expressing the pre-S mutant HBS genes. (**A**) The Huh-7 cells with inducible HBS genes. Cells carrying the wild-type or pre-S_1/S_2 mutant large HBS genes were treated with ponasterone A (10 μM) for 12, 24 or 48 h to induce expression of the HBS genes. (–) Negative control, addition of alcohol, the solvent control for ponasterone A. The *ogg1* gene was greatly induced in a time-dependent manner in the Huh-7 cells carrying the wild-type or pre-S_1/Pre-S_2 mutant HBsAg. (**B**) The mouse ML1-4a cells with various constitutively expressed HBS genes. WT, the wild-type large HBS gene. The *ogg1* and *xrcc1* DNA repair genes were significantly induced in the cells carrying the pre-S_1/pre-S_2 mutant HBsAg. Δ1, the pre-S_1 mutant HBS gene. Δ2, the pre-S_2 mutant HBS gene. HPRT, hypoxanthine phosphorylribosyltransferase, the house-keeping gene. (**C**) HCR analysis for the DNA repair activities stimulated by the pre-S mutant HBS genes. The pre-damaged luciferase reporter plasmid $pCMV^{luc}$ and various HBS genes were transfected in the Huh-7 cells, in which the recovery of luciferase activities were analyzed. Cs and Ce, vector controls for the HBsAg and HBeAg genes. The levels of HBsAg and HBeAg in cell free extracts, detected by ELISA, are shown underneath the results of luciferase activities (±1 SD), averaged from three independent sets of experiments. (**D**) Luciferase activities normalized with the HBsAg levels in the cell free extracts. The pre-S_2 mutant HBsAg appears to stimulate highest DNA repair activity as compared with the wild-type of pre-S_1 mutant HBsAg.

especially pre-S_2 HBS, could activate BER. We detected the expression levels of 8-oxoguanine glycosylase 1 (*ogg1*), the major recognition factor for oxidative DNA lesions, by RT–PCR (18). After inductions of the large wild-type, pre-S_1, and pre-S_2 HBS genes by ponasterone A, an ecdysone analog, Huh-7 cells exhibited higher levels of *ogg1* mRNAs (Figure 2A). Expression levels of *ogg1* increased along with the increasing time lengths of induction by ponasterone A, whereas the alcohol, the solvent control, did not exhibit similar effects. This finding indicates that accumulation of HBsAg causes oxidative damage to the genome and consequently up-regulates the BER pathway. In mouse hepatoma ML1-4a cell lines that constitutively express wild-type large HBS, pre-S_1, or pre-S_2 HBS mutant genes, the BER genes *ogg1* and X-ray cross-complementation 1 (*xrcc1*) were significantly induced (Figure 2B). The cell line carrying the pre-S_2 type of HBS gene exhibited a higher induction level than the cell lines carrying the wild-type or pre-S_1 HBS genes. This indicates that the pre-S_1/S_2 mutant HBsAg stimulates BER in human Huh-7 cells and mouse ML1-4a hepatoma cells.

The stimulation of BER by the pre-S_1 and pre-S_2 mutant HBsAg was further confirmed by host cell reactivation (HCR) assays on the luciferase reporter plasmid $pCMV^{luc}$ pre-treated with H_2O_2 (20). Huh-7 cells, transiently co-transfected with

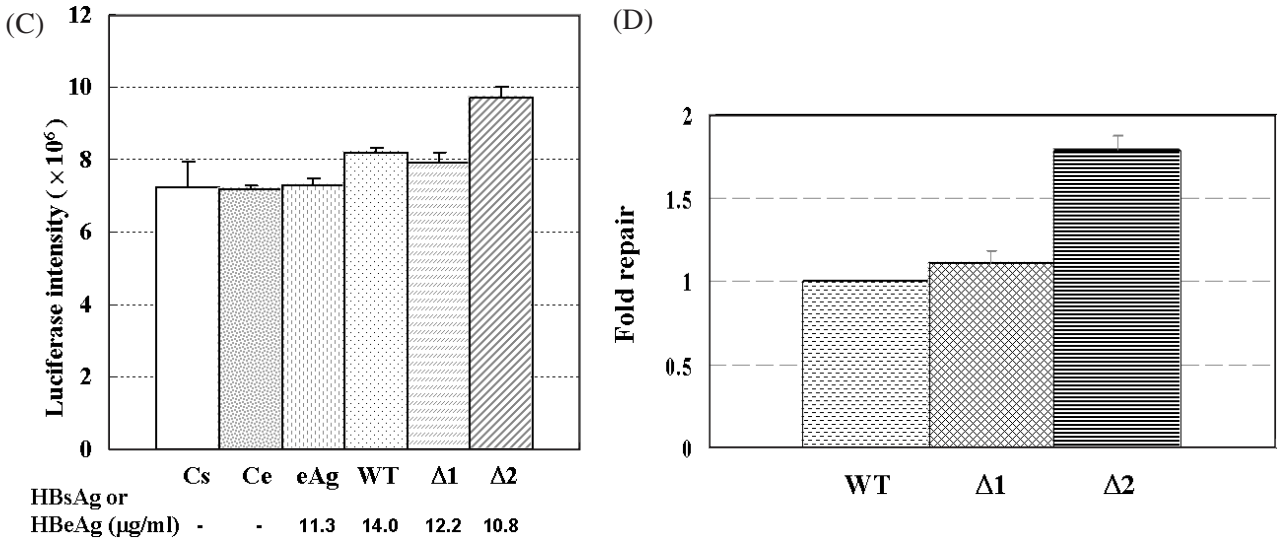

Fig. 2. Continued.

H_2O_2-damaged $pCMV^{luc}$ plasmid as well as the various types of HBS genes, were applied to the luciferase measurements. Each type of HBsAg was able to stimulate DNA repair activity for removal of oxidative DNA lesions on the reporter plasmids (Figure 2C). Pre-S_2 HBsAg appears to exhibit a higher stimulatory effect than either wild-type or pre-S_1 HBsAg. This stimulatory effect was specific to the surface antigens as the HBeAg was unable to induce luciferase activity. To verify that the pre-S_2 HBsAg had contributed the strongest DNA repair activities, the luciferase activities obtained from cells expressing each type of HBsAg was normalized with the respective intracellular HBS antigen level, measured by ELISA assays. Pre-S_2 HBsAg was indeed capable of inducing the highest activity of DNA repair (Figure 2D). These data are consistent with our other findings that pre-S_2 mutant HBsAg cause the strongest oxidative stress for DNA lesions (Figure 1C).

Oxidative DNA damages in the pre-S_2 HBS transgenic mice and GGHs in HCC patients

We established the FVB/N mouse model containing the pre-S_2 mutant HBS transgene. A total of 25 mice at the ages of 10–18.5 months were killed for the study. Data depicted in Figure 3A–C are based on analysis on the representative 14.5-month-old mice, carrying the pre-S_2 mutant HBS transgene or the control vector p(3A)-S. The sera in the pre-S_2 HBS transgenic mice exhibited high levels of HBV surface antigens, as detected by ELISA analysis (Figure 3A). The hepatic tissues display ~40% of HBS-positive hepatocytes in male transgenic mice and 10% or so in females (Figure 3B). In hepatocytes of both the male and female pre-S_2 HBS transgenic mice, the BER factor *ogg1* was up-regulated, as shown in Figure 3C. This finding indicates that the pre-S_2 mutant HBS antigen induces oxidative DNA damages in the mouse hepatocytes as in the *in vitro* cell cultures.

Pre-S_2 mutant HBsAg has been reported to display tumor-promoting phenotypes, e.g. enhanced proliferation and clonal expansion abilities (11). To test the oxidative stress induced by the pre-S_2 mutant HBS antigen in the HCC patients, type II GGHs expressing pre-S_2 HBsAg were isolated from hepatic tissues of six HCC patients by laser capture microdissection. *ogg1* gene induction was detected in these cells using nested RT–PCR (Figure 3D). Type II cells usually presented as hepatic nodules, which made them accessible for LCM sample collection; while type I GGHs scattered as individual cells, most of which were not successfully extracted by LCM. Using immunofluorescence staining in frozen hepatic tissues, the 8-oxoguanine glycosylase protein was not detectable in normal hepatocytes, and only a low level of its expression was detectable in type I GGHs (Figure 3E). In type II GGHs, however, a high level of 8-oxoguanine glycosylase protein was detected. The regions over-expressing 8-oxoguanine glycosylase were those expressing HBsAg, as seen in the merged images of the individual staining for these two proteins. The 8-oxoguanine glycosylase protein was localized in cell nuclei, as it co-localized with genomic DNA, counterstained by Hoechst 33342 fluorescent dye. Taken together with the association of pre-S_2 HBsAg and 8-oxoguanine glycosylase induction, we suggest that pre-S_2 mutant HBS expression causes strong oxidative stress on the hepatic genome.

Induction of oxidative stress by ER stress

To investigate whether the oxidative DNA damages contributed by the pre-S mutant HBS antigens were processed through ER stress, the Huh-7 cells were treated with ER inducers thapsigargin or tunicamycin and tested for oxidative DNA damage. The data depicted in Figure 4A indicate that the treatment of either the thapsigargin (5 μg/ml) or tunicamycin (5 μM) indeed caused significant comet formation. To confirm that the oxidative damage on DNA was indeed induced by ER stress, the ER stress inhibitors 8-(*N*,*N*-diethylamino)octyl 3,4,5-trimethoxybenzoate (TMB-8) (250 μM) and vomitoxin (150 ng/ml) were employed. The data shown in Figure 4B indicate that after treatment of either the TMB-8 or vomitoxin, the over-expression of the *ogg1* gene was no longer seen. And such an effect on *ogg1* was caused by ROS generated through ER stress, as the addition of the anti-oxidant melatonin (10 nM) could completely abrogate the induction of the *ogg1* gene. Therefore, our data demonstrate clearly that the pre-S HBS mutant antigens induce oxidative DNA damage through ER stress.

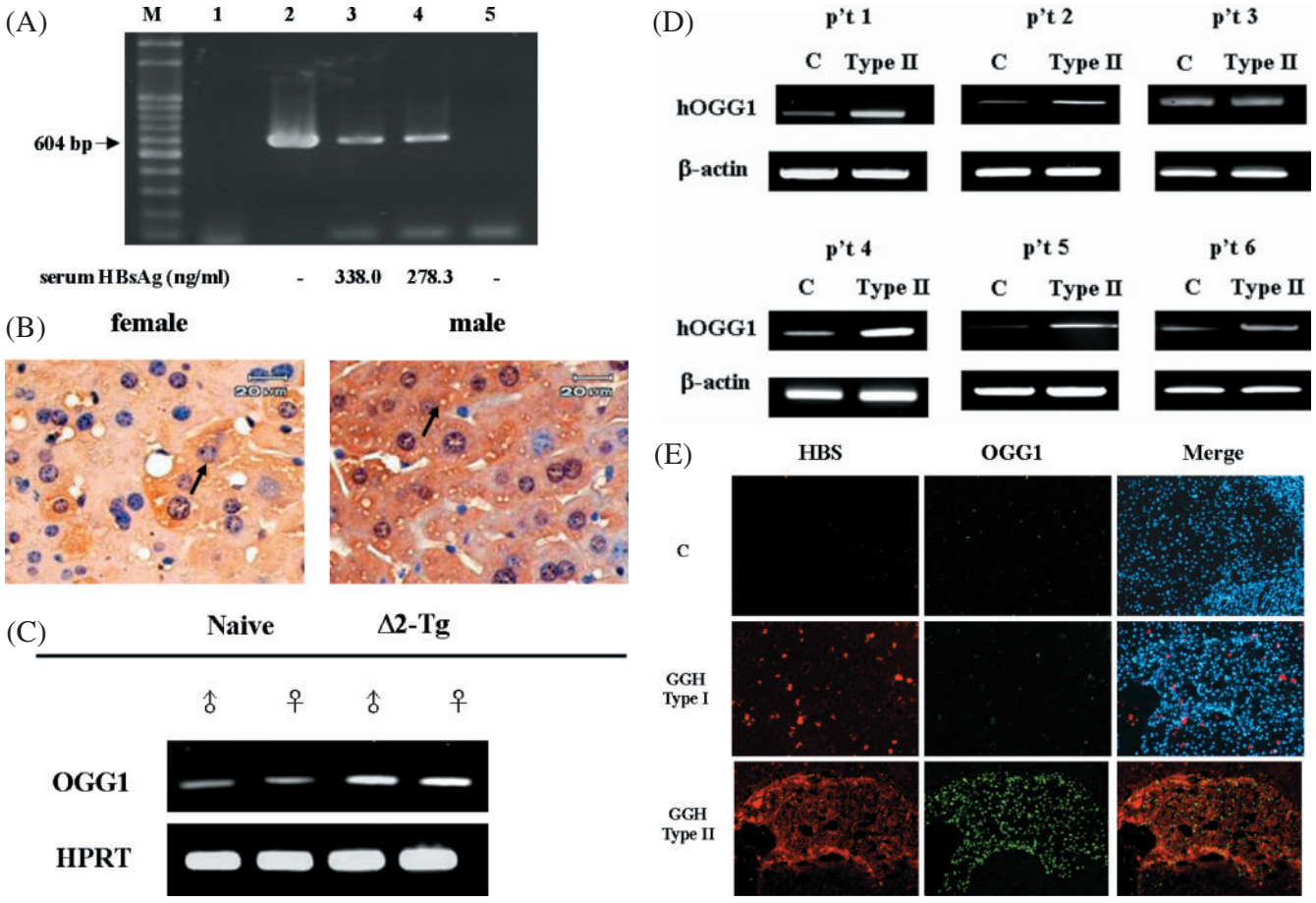

Fig. 3. Oxidative DNA damage in the pre-S_2 mutant HBS transgenic mice and GGHs. (**A**) PCR and ELISA confirmation of the representative 14.5-month-old mice carrying the pre-S_2 mutant HBS transgene. A total of 25 mice in the ages 10–18.5 months were tested. The pre-S_2 mutant HBS gene (604 bp) was amplified by PCR in the genomic DNA of the transgenic mice. M, 100 bp DNA ladder; lane 1, no template control; lane 2, p(3A)SAg r2, 1 ng; lane 3, DNA from male transgenic mouse; lane 4, DNA from female transgenic mouse; lane 5, DNA from naive FVB/N mouse. The HBsAg expression levels in the mouse sera, detected by ELISA, are shown underneath the PCR results. (**B**) The HBS immunohistochemical staining in hepatic tissues of the male and female mice. The brown-color cells pointed by the arrows are the cells expressing HBsAg. (**C**) The RT–PCR results of mouse *ogg1* gene in transgenic mouse hepatic tissues. Δ2-Tg, the pre-S_2 HBS transgenic mice; naive, the mock-transfected mice. (**D**) The *ogg1* gene induction in the type II GGHs of the HCC patients, analyzed by nested RT–PCR. The GGHs were isolated by laser capture microscopy, based on their morphologies identified by immunohistochemical staining of HBS protein. A total of six HCC patients were tested. Five out of six patients exhibit over-expression of the *ogg1* gene. (**E**) *In situ* immunofluorescent staining of the HBS antigen (red) and 8-oxoguanine glycosylase protein (green), and the merged images between the HBS and 8-oxoguanine glycosylase proteins. Cell nuclei of the control (normal hepatocytes) and the type I GGH cells were detected by counter-staining with the dye Hoechst 33324 (blue). The type II GGH cells exhibit significantly higher level of 8-oxoguanine glycosylase protein than the control or type I GGH cells do.

Hprt gene mutations induced by the pre-S_1/S_2 mutant HBsAg

Oxidative stress on DNA often induces gene mutations. Accumulation of gene mutations is surely a significant factor for carcinogenesis. In the mouse ML1-4a cells constitutively expressing various types of large HBS genes, the gene mutation rates in the X-linked *hprt* gene was estimated as a marker for global mutation rates. The 6-thioguanine (6-TG), a nucleotide analog, was used to select for *hprt* mutant cells (21). The cell colonies surviving in the selective medium containing 6-TG were counted for the mutation frequencies of the *hprt* gene. Both pre-S_1 and pre-S_2 mutant HBsAg were able to enhance *hprt* mutation rates (Table I). Cells carrying the pre-S_1 and pre-S_2 mutant HBS genes exhibited 4.5- and 6.2-fold gene-mutation rates, respectively, as opposed to the 1-fold gene mutation rate in cells over-expressing the wild-type HBS gene. Thus, pre-S_2 mutant HBsAg appears to be a stronger mutagen than pre-S_1 HBsAg. The mutation spectra in these 6-TG-resistant cells were analyzed. The majority of mutation types were point mutations, one-base additions, or deletions (data not shown). Thus, the majority of them did not present the G to T transversion, the typical mutation type caused by oxidative stress (22). This finding suggests that the pre-S_1/S_2 mutant HBS antigens induce not only oxidative stress, but also overall genomic instability.

Discussion

The mechanism of hepatocellular carcinogenesis associated with the pre-S HBV mutant surface proteins was investigated in this study. We have demonstrated that these pre-S mutant HBS antigens induce oxidative DNA damage and mutagenesis. The proposed model presented in Figure 5 shows that the pre-S mutant HBsAg, mis-folded in conformation, accumulates in ER and induces ER stress. The ER stress then causes oxidative stress and oxidative DNA lesions, such as 8-hydroxyguanine. The DNA repair mechanism is activated in response to the DNA damages. Meanwhile, the DNA damages induce mutagenesis and potentially result in genomic

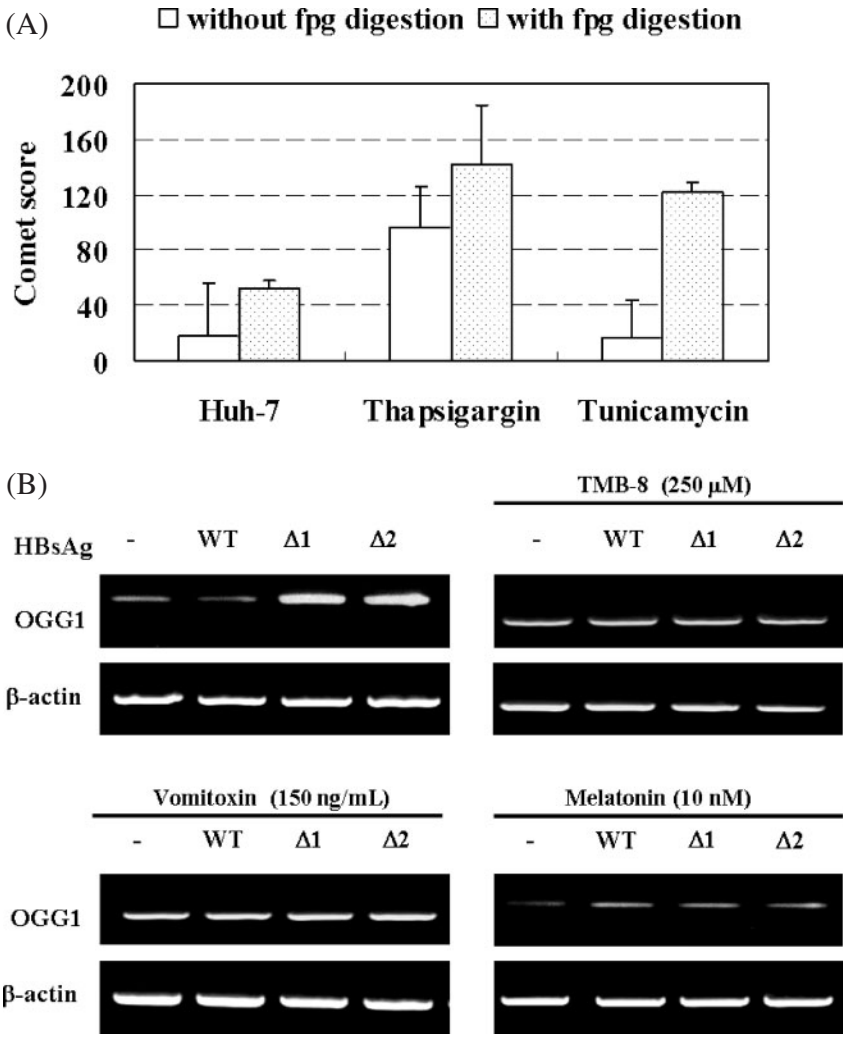

Fig. 4. Oxidative DNA damages induced by ER stress. (**A**) Comet assays with treatment of Huh-7 cells with ER stress inducers tunicamycin (TM) or thapsigargin (TG). The mean comet scores (±1 SD)/slide of the five slides detected were indicated. The thapsigargin (5 μg/ml) as well as tunicamycin (150 ng/ml) significantly induces formation of comet tails. (**B**) Treatment of the ER stress inhibitors TMB-8 or vomitoxin in the Huh-7 cells carrying various types of HBS genes. After treatment of TMB-8 (250 μM) or vomitoxin (150 ng/ml), the *ogg1* gene induction in any cell tested was completely abolished. In an independent experiment, the antioxidant melatonin (10 nM) was added to the Huh-7 cells carrying various types of HBS genes. The induction of *ogg1* gene was also nearly completely abolished, indicating that the induction of the *ogg1* gene was solely caused by the ROS generated by accumulation of the pre-S mutant HBsAg in ER.

Table I. *hprt* mutation frequencies in the ML1-4a cells over-expressing the pre-S mutant HBS antigens

Cell	*hprt*$^-$ cells	*hprt* mutation frequency	Fold increase in mutation frequency
ML1-4a	$0/6 \times 10^6$ cells	0	
ML1-4a$^{HBS/WT}$	$2/7 \times 10^6$ cells	2.9×10^{-7}/cell	1
ML1-4a$^{HBS/D1}$	$8/6 \times 10^6$ cells	1.3×10^{-6}/cell	4.5
ML1-4a$^{HBS/D2}$	$11/6 \times 10^6$ cells	1.8×10^{-6}/cell	6.2

instability. In addition to the oxidative stress-induced DNA damage, the pre-S_2 deletion in type II GGH also defines a cytotoxic T lymphocyte immune escape. This type of mutation probably transforms the HBS antigen to become an immune escape mutant, which emerges in the non-replicative phase of chronic HBV infection. The produced pre-S mutant HBsAg accumulate in cytoplasm due to significant decrease in their secretion out of cells (11). Ultimately, accumulation of the oxidative adducts in cells may inactivate certain tumor suppressors or oncogenes, leading to cellular transformation and hepatocellular carcinogenesis.

In this study, cells carrying the pre-S_1/pre-S_2 mutant HBsAg exhibit oxidative stress-induced mutagenesis, as shown by the significant increase in the *hprt* gene mutation rates. Such induced mutations are presumably important pre-requisites for hepatic tumorigenesis. A recent study by Yeh *et al*. has reported that chromosomal allelic imbalance was identified in most of the cirrhotic nodules examined, indicating that the genetic aberrations occur frequently in early hepatocarcinogenesis (23). This is consistent with our finding in that induced mutagenesis of the host genome plays an important role for evolution of HBV-related HCC.

The type I and II GGHs represent cells with pre-S mutant HBS proteins accumulated in ER (9–11). Therefore, it is suggested that the HBS mutant proteins, partially deleted at the N-terminal pre-S_1/S_2 regions, are folded into improper conformations. We reported recently that the pre-S_1 and pre-S_2 mutant HBS proteins activate the UPR and induce production of ROS (11). Based on the data in this study, the pre-S

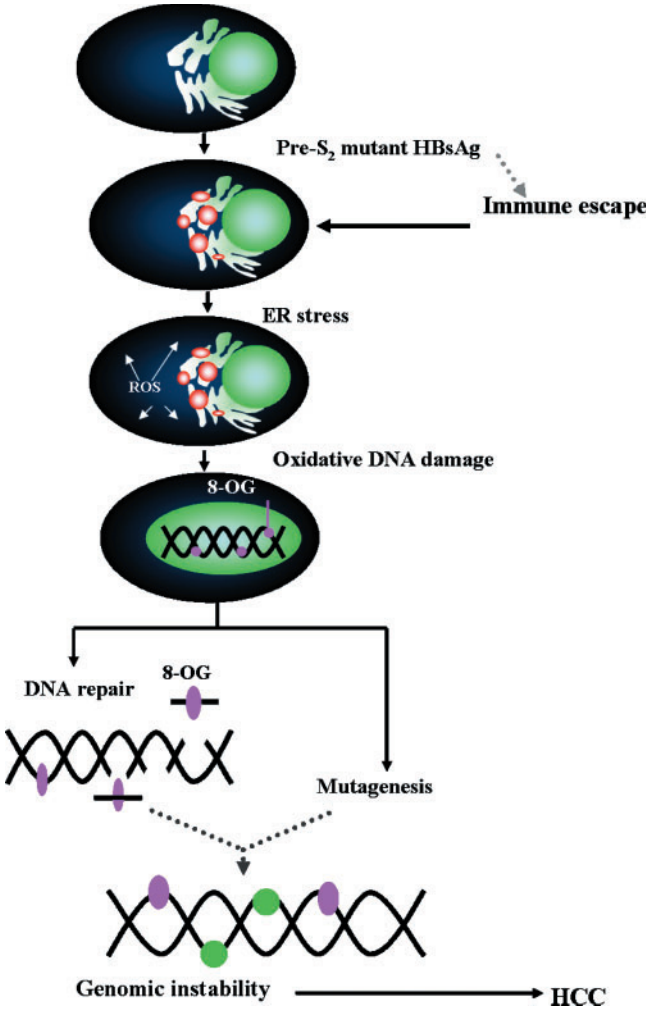

Fig. 5. The proposed model for hepatocellular carcinogenesis associated with the HBV pre-S mutant proteins. The pre-S mutant HBsAg accumulates in ER and causes ER stress. The pre-S_2 mutant type of HBsAg possibly could escape from immune surveillance in hepatocytes and persists in residing in ER. Through ER stress, large amounts of ROS are generated and cause oxidative DNA damage, such as the 8-oxoguanine (8-OG) DNA lesions. Presence of the oxidative DNA lesions stimulates DNA repair activity; in the meantime, the induced mutagenesis occurs in genome. The genomic instability is therefore likely to occur and ultimately results in HCC.

HBsAg-induced oxidative DNA damage is contributed by ER stress, as the ER stress inhibitors TMB-8 and vomitoxin could nearly completely abrogate the HBsAg-induced *ogg1* gene over-expression. The ER stress contributed by the pre-S mutant HBsAg is fairly strong. The levels of oxidative DNA damage caused by the pre-S_1/pre-S_2 HBsAg are higher to those caused by addition of a concentration as high as 50 μM of ER-stress inducers thapsigargin or tunicamycin in the studies of comet assays and *ogg1* gene induction (data not shown). Besides the DNA damages, the ER stress response induces a series of signal transduction pathways, leading to either caspase 12-mediated apoptosis, or to a growth proliferation mediated through the NF-κB pathway, which is highly associated with tumorigenesis (24–26). ER stress also induces cytoplasmic localization of p53 and blocks p53-dependent apoptosis (27). A recent study reported that the fusion protein between the HBS protein and a non-degradable fragment of cyclin A_2 resides in the ER membrane and eventually leads cells to transformation (28), suggesting that the ER stress does play a role in carcinogenesis. In our preliminary study, in fact, we found that the Huh-7 cells carrying the pre-S_2 mutant HBsAg exhibited higher levels of cyclin A_2, as compared with those carrying the wild-type HBsAg (data not shown). Therefore, it is possible potentially that the cyclin A_2 is associated with the pre-S mutant HBsAg and resides in ER, promoting hepatic carcinogenesis.

In the present study, we found that oxidative DNA lesions occur in GGHs. We also demonstrated that induction of the BER gene *ogg1* was consistently detected in all the model systems applied, suggesting that the *ogg1* level be a biomarker for oxidative DNA damage in hepatocytes with chronic HBV infection. Recent study by Rusyn *et al*. reported that expression of base excision DNA repair genes, including *ogg1*, is a sensitive *in vivo* biomarker for oxidative stress to DNA that can be used for the identification of the molecular source of radicals responsible for DNA damage *in vivo* (29). This is consistent with our findings. In this study, the pre-S_2 mutant HBsAg causes stronger induction of a number of BER repair proteins than pre-S_1 and wild-type large HBsAg do. This finding suggests that the pre-S_2 mutant HBsAg causes a higher level of DNA damage than the wild-type or pre-S_1 mutant HBsAg do. Such a high level of DNA lesions induced by pre-S_2 HBS mutant proteins shall be an important contributing factor to hepatic carcinogenesis. Although the DNA repair activities are stimulated in the cells expressing the pre-S mutant HBsAg, they do not seem to function quickly enough to recover all of the DNA lesions to avoid mutation occurrence. Therefore, efficient DNA repair for oxidative DNA damages should play an important role in cancer prevention. Other studies have reported that a defect in the *ogg1* DNA repair gene is involved in various types of human carcinogenesis (30). A number of other cancer-related viral proteins have also been documented to exhibit ER retention, suggesting that the ER stress has high association with carcinogenesis (31). These studies are consistent with our findings in GGHs.

To date, a few mechanisms of HBV-induced HCC have been proposed. Early studies proposed that insertional mutagenesis of the HBV genome into human chromosomes might cause inactivation of tumor suppressor/proto-oncogenes (32–35). However, later studies have shown that integration of HBV genome is genome-wide and unlikely attacks a specific tumor suppressor or proto-oncogene (36–38). Recently, the HBVX protein (pX) and surface proteins have also been proposed to play oncogenic roles. pX initiates transactivation as well as induction of signal transduction pathways such as Ras/Raf-1 (39–41). The large surface protein has been shown to induce HCC in the transgenic mouse model (42,43). In our recent studies on HCC in Taiwan, we identified the pre-S_1/S_2 mutant HBsAg in GGHs, the pathological hallmarks for late phases of chronic HBV infection. These pre-S mutant HBsAgs exhibit viral oncoprotein features, including growth advantage and immune escape. Whether these pre-S mutant HBsAg functionally target on certain tumor suppressor/oncogene remains to be identified. Our preliminary studies have found (unpublished data) that the p53 gene is mutated in approximately half of the patient hepatocytes expressing the pre-S_2 mutant HBS gene, indicating that the p53 gene is probably the direct or indirect target for pre-S_2 mutant HBS protein. However, the over-expression of pre-S_2 mutant HBS protein does not appear to affect the transactivation activity of p53 (data not shown), indicating that the effect of pre-S mutant surface proteins on p53 is probably through mutagenesis of p53 gene.

In conclusion, the pre-S mutant HBsAg induce oxidative DNA damage and mutagenesis, potentially resulting in genomic instability. The induced genomic instability surely enhances HCC development. This is the first study to demonstrate the association of genomic mutagenesis induced by ER response and virus-related carcinogenesis. Our study suggests that identification of the pre-S HBS genes or proteins in the chronic HBV carriers would serve as a useful diagnostic approach for detecting the risks they stand for HCC.

Acknowledgement

This work was supported by the National Health Research Institute, Taiwan.

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Received May 19, 2004; accepted May 28, 2004