Radiation-Induced Hepatitis B Virus Reactivation in Liver Mediated by the Bystander Effect from Irradiated Endothelial Cells

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Abstract

Purpose: Hepatitis B virus (HBV) reactivation is one unique pathogenesis in Asian carriers with liver toxicity after radiotherapy for hepatobiliary malignancies. This study attempts to delineate the biological mechanism of radiation-induced HBV reactivation.

Experimental Design: Primary cultures of hepatocytes (PCC) were prepared from the noncancerous liver tissue removed perioperatively from 12 HBV carriers with hepatocellular carcinoma (HCC). The conditioned medium of irradiated PCCs, HCC, and endothelial cells from patients was transferred to PCCs or HepG2.2.15 cells (a human hepatoblastoma cell line transfected with HBV DNA) before subsequent irradiation. Forty-eight hours after irradiation, HBV DNA was measured by real-time quantitative PCR. Specific cytokines were determined by cytokine array and ELISA analysis. Preradiotherapy and postradiotherapy sera from 10 HBV carriers and 16 non-HBV carriers were analyzed for viral loads and cytokine activities.

Results: Radiation induced HBV DNA replication in (*a*) irradiated PCCs cultured with the conditioned medium from irradiated PCCs (2.74-fold; $P = 0.004$) and endothelial cells (9.50-fold; $P = 3.1 \times 10^{-10}$), but not from HCCs (1.07-fold), and in (*b*) irradiated HepG2.2.15 cells (17.7-fold) cocultured with human umbilical vascular endothelial cells. Cytokine assay revealed increased expression of interleukin-6 (IL-6) in conditioned medium from irradiated human umbilical vascular endothelial cells. All 16 patients with liver irradiated had the increased serum IL-6 compared with 3 of 10 patients with irradiation excluding liver ($P < 0.001$). All nine HBV carriers with liver irradiated had postradiotherapy increases in both HBV DNA and IL-6.

Conclusions: Radiation-induced liver toxicity with HBV reactivation is from a bystander effect on irradiated endothelial cells releasing cytokines, including IL-6.

Because radiotherapy was added to the treatment regimen for gastrointestinal malignancies, radiation-induced liver disease (RILD) has been one of the most serious treatment-related complications (1, 2). Probability models have been used to limit the risk of RILD to a clinically acceptable level (3, 4), and dosimetric analysis has shown that the volumetric fraction of noncancerous liver exposed to radiation was associated with the risk of RILD (5). The threshold dose of RILD in hepatitis B virus (HBV) carriers was much lower than that in noncarriers, indicating a difference in pathogenesis (6). Because the rate of chronic HBV infection in Asian patients is high and HBV reactivation causes additional RILD, the occurrence of RILD is an important concern in Asians with gastrointestinal malignancies (7, 8).

Mechanisms about radiation-induced HBV reactivation remain unknown. Radiation can either directly activate HBV in the infected hepatocytes or via an indirect effects from neighboring nonhepatocytes. The bystander effect of radiation is prominent within a few millimeters of the target (9) and may be of systemic origin (10) to produce cytokines, which have been linked to HBV reactivation (11). Moreover, irradiation has been shown to increase the susceptibility of hepatocytes to tumor necrosis factor-α–mediated apoptosis, which may be related to RILD (12), and to affect endothelial cell survival, tumor growth, and its associated angiogenesis (13).

In this study, the bystander effect of radiation was investigated by transferring conditioned medium from irradiated primary culture noncancerous liver cells (PCC), patient-specific endothelial cells isolated from PCCs, and human umbilical vascular endothelial cells (HUVEC) to nonirradiated PCCs or a human HBV DNA-transfected hepatoblastoma cell line (HepG2.2.15) before subsequent irradiation. Sera from HBV/non-HBV carrier patients were also analyzed for preradiotherapy/postradiotherapy viral loads and cytokine activities.

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Materials and Methods

Cell culture and isolation. PCCs were obtained using a modification of a previous procedure (14). Liver fragments from patients who underwent surgery for hepatocellular carcinoma (HCC) and gave informed consent were washed, incubated, and treated with 0.05% collagenase until a cell suspension was obtained. The isolated hepatocytes were resuspended (5×10^5/mL) in plating medium, dispensed, and incubated. Fresh preparations contained an average of 85% viable hepatocytes (14).

Patient-specific endothelial cells were isolated from fresh human liver tissue using immunomagnetic (anti-CD31–coated Dynabead) purification. The isolated hepatocytes were resuspended in 1 mL of cold M199 medium containing 0.3 mg/mL magnetic tosyl-activated M450 Dynabeads, which had been coated with mouse anti-human CD31, and incubated at room temperature for 30 min with gentle agitation. The endothelium cells bound to the magnetic beads were removed from the unbound nonendothelial cells by magnetic isolation using an MPC-1 magnet (Dynal, Oslo, Norway). The cell bound beads were resuspended and cultured in endothelial cell growth medium. Finally, the purity of endothelial cells was confirmed by immunostaining for CD31 and quantified by flow cytometry.

HepG2.2.15 cells (15), derived from HepG2 cells and stably transfected with a head-to-tail HBV DNA dimer, were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air in DMEM with 10% heat-inactivated bovine serum plus penicillin-streptomycin.

HUVECs were cultured in M199 medium supplemented with 20% fetal bovine serum, endothelial cell growth supplement, 20 IU/mL heparin, and 2 mmol/L L-glutamine. To prevent genetic variation in the sample due to differences in individual specimens, HUVECs from five or more different donors were pooled. HUVECs (passages 1-5) were used for experiments.

Human fibroblast cell line MRC-5 was grown in Eagle's minimal essential medium with 2 mmol/L L-glutamine and 10% fetal bovine serum. A human monocytic leukemia cell line, THP-1, was grown in RPMI 1640 containing 25 mmol/L HEPES, 2 mmol/L L-glutamine, and 10% fetal bovine serum. Normal human embryonic liver cell line CL-48 and human hepatoma cell line HepG2 were cultured in high glucose DMEM with 10% fetal bovine serum. All media contained 1% penicillin/streptomycin.

Irradiation of culture cells. The cells in the culture flask were irradiated with 4 Gy using a cobalt-60 unit. The distance from the source to the bottom of the flask was 80 cm, and the dose rate was 1 Gy/min.

Preparation of conditioned medium. Cultures of various cells were rinsed twice with PBS and stored in 5 mL of fresh DMEM containing 10% fetal bovine serum before 4 Gy of radiation. Conditioned medium was collected after a 48-h incubation, and 25 mmol/L HEPES buffer (pH 7.4), 1 mg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L EDTA, 0.02% NaN_3, and 0.1% bovine serum albumin (Sigma, St. Louis, MO) were added.

Treatment of nonirradiated hepatocytes with conditioned medium of irradiated cells. PCCs, HCC cells, patient-specific endothelial cells, or HUVECs were treated with 4 Gy of radiation. After 48 h, the conditioned medium was collected and transferred to monolayers of nonirradiated hepatocytes. After a 24-h incubation with the transferred conditioned medium, the nonirradiated hepatocytes were irradiated (4 Gy) and incubated for 48 h, and the irradiated cells and conditioned medium were collected for HBV DNA measurement and cytokine array detection. The experimental design of this medium transfer strategy is shown in Fig. 1.

Irradiation of cocultured HepG2.2.15 cells and HUVECs. Boyden chamber coculture system was used with 1×10^6 HepG2.2.15 cells and 1×10^6 HUVECs. The cocultured cells were irradiated (4 Gy) and incubated for 48 h. The irradiated cells were then collected for HBV DNA measurement. The experimental design of this coculture strategy is shown in Fig. 1.

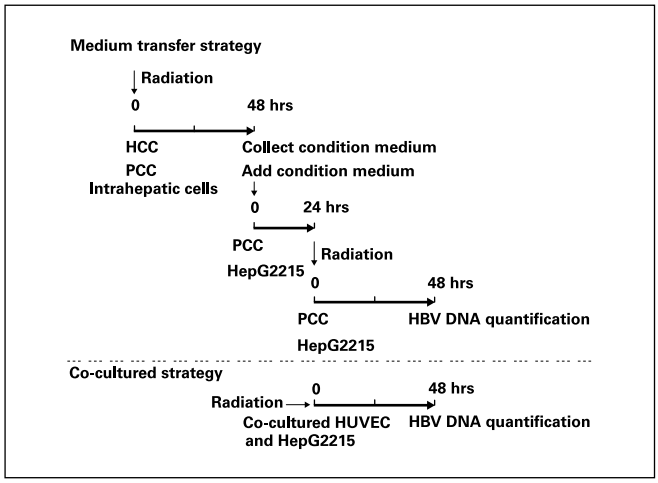


Fig. 1. Experimental design. Conditioned media from irradiated cells, including HCC cells, PCC cells of liver, and intrahepatic cells, were used as inducers of subsequently irradiated HBV-containing PCCs and HepG2.2.15 cells (medium transfer strategy). Cocultured HUVEC and HepG2.2.15 cells were irradiated for the HBV DNA quantification (coculture strategy).

Southern blot analysis. Intracellular core-associated HBV DNA was extracted using a modified version of a previously described method (16). Briefly, 1×10^6 cells were transferred to Eppendorf tubes, vortexed, and allowed to stand on ice for 15 min. Nuclei were pelleted by centrifugation for 1 min at 14,000 rpm. The supernatant was treated with 100 μg/mL DNase I for 30 min at 37°C and treated with EDTA to stop the reaction. Proteins were digested and nucleic acids were purified using phenol-chloroform (1:1) extraction and ethanol precipitation. DNA isolated from the cytoplasmic core particles was separated on a 1.3% agarose gel, blotted onto a Hybond-N+ nylon membranes, and hybridized with a digitonin-labeled full-length HBV fragment (provided by Dr. S-L. Doong, Graduate Institute of Microbiology, National Taiwan University College of Medicine, Taipei, Taiwan). The specific bands were detected by horseradish peroxidase–labeled anti-digitonin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized with an enhanced chemiluminescent detection system. HBV DNA signals were visualized by exposure to Kodak BioMax film (Kodak Biomax, Rochester, NY).

Northern blot analysis. Total cellular RNAs were isolated by Trizol reagent according to the manufacturer's instructions. The isolated RNAs were digested with RNase-free DNase I. RNAs (20 μg) were electrophoresed on a 1.2% agarose gel containing 0.7 mol/L formaldehyde and transferred to Hybond-N+ nylon membranes. After prehybridization for 2 h at 65°C, the digitonin-labeled HBV DNA was added and hybridized overnight at the same temperature. The specific bands were detected using horseradish peroxidase–labeled anti-digitonin antibody and visualized with an enhanced chemiluminescent detection system. Transcript signals were detected on Kodak BioMax film (Kodak BioMax, Rochester, NY).

Quantitative PCR for HBV DNA copy number. Real-time PCR was done using a previously described method (17). The LightCycler PCR amplification (denaturation at 95°C for 10 min, 45 cycles of denaturation at 95°C for 5 s, annealing at 53°C for 10 s, and extension at 72°C for 20 s) was carried out at a programmed temperature transition rate of 20°C/s for denaturation/annealing and 5°C/s for extension. After PCR amplification, a melting curve was generated by measuring fluorescence at 640 nm, whereas the reaction was at 95°C for 60 s, cooled to 45°C (transition rate 0.1°C/s), at 45°C for 120 s, and heated (transition rate 0.05°C/s) to 80°C. The melting curve and quantitative analysis were conducted using LightCycler analysis software version 3.5. The plasmid pHBV-48 (derived from subtype *adw1*) for generating the copy number standard curves was used to quantify HBV DNA (18). The plasmid DNA was purified using a

plasmid purification kit (Qiagen GmbH, Hilden, Germany); the corresponding concentration was determined spectrophotometrically and expressed as the number of copies per mL.

Assay of cytokines in conditioned medium from irradiated PCCs and endothelial cells. The conditioned medium of irradiated or nonirradiated cells (1 mL) was incubated 1 to 2 h with human cytokine antibody array membranes (RayBiotech, Inc., Norcross, GA) pretreated with 1× blocking buffer for 30 min. The membranes were washed a few times with specific buffer, treated with biotin-conjugated antibodies for 1 to 2 h, washed, and treated with diluted horseradish peroxidase–conjugated streptavidin for 2 h and with specific detection buffer at room temperature for 1 min. X-ray film and a chemiluminescence imaging system were used to detect the cytokine array signals.

Serum analysis of viral loads and cytokine from HBV/non-HBV carrier patients. Sera were collected from nine HBV carrier patients (seven with HCC and two with low-third esophageal cancer) before and 2 weeks after radiotherapy to part of the liver (median dose, 45 Gy; range, 40-50.4 Gy). Viral loads and interleukin (IL)-6 activities were measured. Sera of one HBV carrier patient with irradiation (40 Gy) to upper-third esophageal cancer excluding liver were compared. None of these HBV carriers had antiviral agents during the interval of serum collection. Seven non-HBV carrier patients (two hepatitis C carriers with HCC, four noncarriers with low-third esophageal cancer, and one noncarrier with cholangiocarcinoma) with irradiation to part of the liver (median dose, 40 Gy; range, 40-60 Gy) were included with preradiotherapy/postradiotherapy serum IL-6 activities. Nine noncarrier patients (three with upper-third esophageal cancer and six with cervical cancer) undergoing radiotherapy to nonliver sites (median dose, 45 Gy; range, 34.2-61.2 Gy) were analyzed for preradiotherapy/postradiotherapy IL-6 activities. None of these patients had clinical evidence of malignant disease progression in or outside the liver during the interval of serum collection. Viral loads were measured by quantitative PCR method. The activities of cytokine were measured by ELISA analysis.

Statistical analysis. Data are presented as the mean ± SD for the indicated number of separate experiments. Statistical analyses of continuous variables were done using the Student's *t* test. Comparisons of categorical variables were done by χ^2 or Fisher's exact test. *P* value of <0.05 was considered significant.

Results

Conditioned medium from irradiated PCC is required for radiation-induced HBV reactivation. Primary cell cultures (patient-specific HCC cells and PCCs) were prepared from fresh tissue of 12 HBV carrier patients. None of these patients had evidence of HBV reactivation at the time of tissue collection. After irradiation (4 Gy), cells remained viable (Fig. 2A) for 24 h and morphologically intact (Fig. 2B) for 48 h. Subsequent study failed to determine any HBV reactivation in these irradiated PCCs (0.89 ± 0.56–fold the HBV DNA copy number of irradiated versus nonirradiated cells) or HCC cells (0.92 ± 0.19–fold). It suggested that radiation to the HBV-infected PCCs could not directly activate HBV DNA replication. Therefore, we explored the possible effects from irradiated bystander cells.

After 48 h, the conditioned media of irradiated HCC cells and PCCs were transferred to cultures of the various nonirradiated PCCs (to induce HBV DNA replication). Twenty-four hours after this transfer, these PCCs were irradiated (4 Gy) or not (sham). Quantification of HBV DNA (copies per mL) 48 h after irradiation showed that conditioned medium from irradiated HCC cells did not induce HBV DNA replication ($1.84 \pm 0.89 \times 10^6$/mL for irradiated PCCs versus 1.71 ±

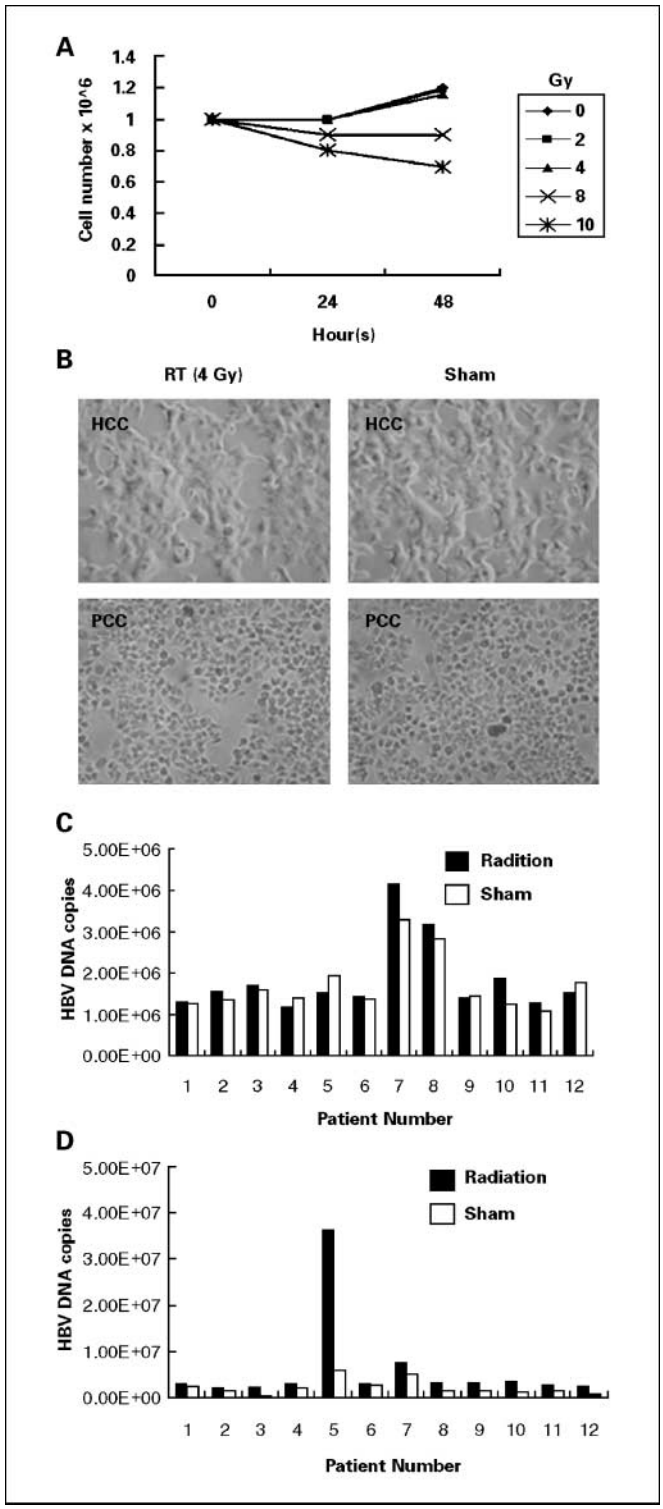
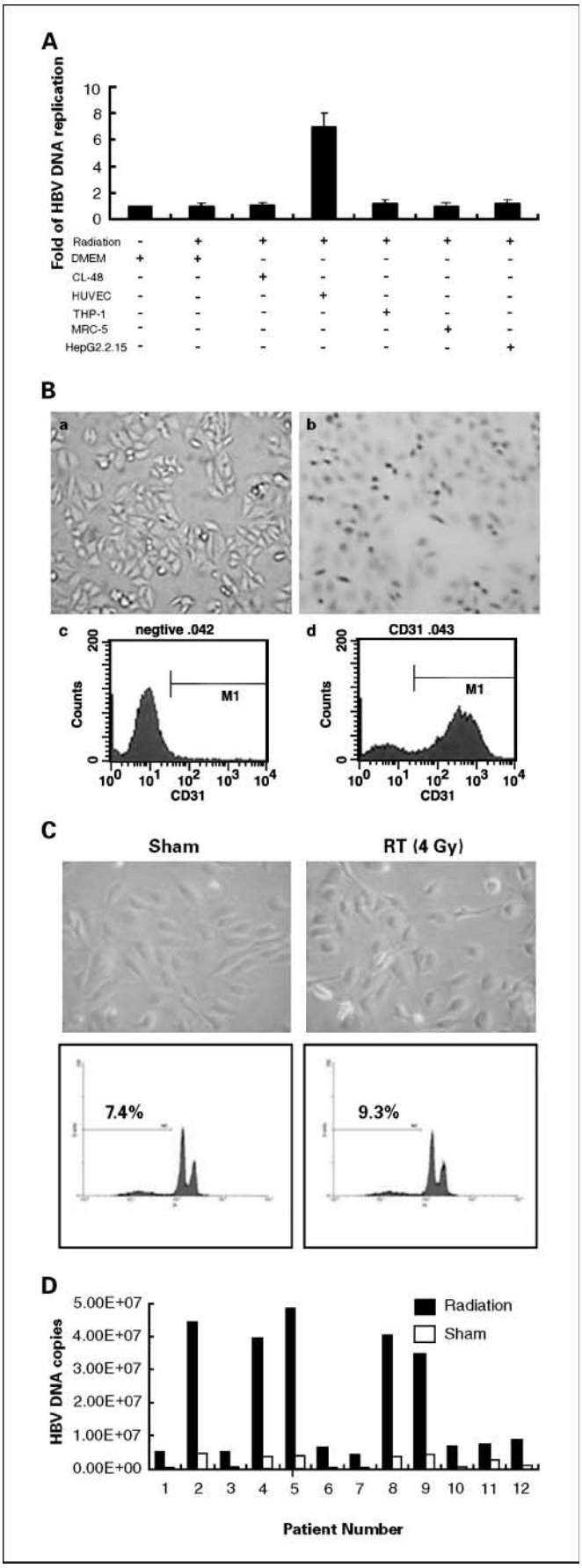


Fig. 2. Biological effect of noncytotoxic dose of radiation on HBV replication in HBV-infected cells. *A,* PCCs (1×10^6/dish) were irradiated with different doses as indicated. After 24 and 48 h, the viable cells were counted by the trypan blue exclusion method. *B,* PCCs or HCC cells were irradiated with 4 Gy (*RT*) or not irradiated (*Sham*). After 48 h, cellular morphology was determined by microscopy and documented by digital photography. Magnification, ×100. Data are 1 representative case from the following 12 cases. *C,* conditioned medium from primary culture of 1×10^6 HCC cells or (*D*) PCCs from HBV carriers was collected 48 h after irradiation (4 Gy) and added to PCCs from HBV carriers for 24 h. The PCCs were either irradiated (4 Gy) or not irradiated. After 48 h, the total genomic DNA was purified for HBV DNA quantification. Data are the number of HBV DNA copies per mL.



0.68 × 10^6/mL for nonirradiated PCCs; Fig. 2C). In contrast, conditioned medium from irradiated PCCs significantly induced HBV DNA replication (6.01 ± 0.96 × 10^6/mL for irradiated PCCs versus 2.13 ± 0.17 × 10^6/mL for nonirradiated PCCs; Fig. 2D).

Irradiated endothelial cells mediate radiation-induced HBV reactivation. Different intrahepatic cell types (including HUVECs, human fibroblast cell line MRC-5, human monocytic leukemia cell line THP-1, human embryonic liver cell line CL-48, and HBV-containing human hepatoma cell line HepG2.2.15) were irradiated and their conditioned media were collected after 48 h. Nonirradiated HepG2.2.15 cells were first incubated 24 h with the conditioned medium from different irradiated cells and then irradiated (4 Gy). After 48 h, assay of HBV DNA (copies per mL) in these irradiated HepG2.2.15 cells found that only the conditioned medium from irradiated HUVECs significantly induced HBV DNA replication (6.95 ± 1.04–fold the copy number for conditioned medium from nonirradiated cells; Fig. 3A).

The morphology of isolated patient-specific endothelial cells was revealed (Fig. 3B, *a*) and verified by immunostaining (Fig. 3B, *b*), and the percentage of isolated endothelial cells was quantified by flow cytometry (Fig. 3B, *c* and *d*). The morphology and analysis of apoptotic cell distribution (Fig. 3C) showed no significant change (7.4% in sham group versus 9.3% in radiation group). The results showed that 4 Gy of radiation dosage did not cause the cytotoxicity of the isolated endothelial cells. The conditioned medium from the irradiated (4 Gy) isolated endothelial cells was used as an inducer of HBV DNA replication in the corresponding, subsequently irradiated (4 Gy) PCCs. HBV reactivation was significantly induced with conditioned medium from irradiated endothelial cells (2.09 ± 1.85 × 10^7/mL for irradiated PCCs versus 2.21 ± 1.74 × 10^6/mL for nonirradiated PCCs; Fig. 3D).

Taken together, HBV reactivation was significantly induced in PCC by conditioned medium from irradiated PCCs (2.74 ± 1.75–fold) and endothelial cells (9.50 ± 2.71–fold; measured relative to conditioned medium from nonirradiated cells in fold increase in HBV DNA copy number) but not from HCC cells (1.07 ± 0.20–fold). The differences between PCCs and HCC cells (P = 0.004) and between endothelial cells and

Fig. 3. Cell type specificity on the effect of radiation-induced HBV replication. *A,* conditioned medium or culture medium control (*DMEM*) was collected from the indicated types of cells 48 h after irradiation (4 Gy) and added to the HBV-infected cell line HepG2.2.15 for 24 h. The HepG2.2.15 cells were irradiated (4 Gy) or not irradiated (sham). After 48 h, the total genomic DNA was purified for HBV DNA quantification. Columns, mean of the fold increase in HBV replication calculated by comparing the irradiated and sham groups in three independent experiments; bars, SD. *B, a*, morphology of endothelial cells isolated from primary culture of noncancerous liver tissue; *b*, the homogeneity of the cells was verified by immunostaining with endothelial cell–specific surface marker CD31 and documented by digital photography. Magnification, ×100. Percentage of endothelial cells was quantified by flow cytometry without (*c*) or with (*d*) CD31 staining. *C,* isolated endothelial cells were irradiated (4 Gy; *RT*) or not (*Sham*). Top, after 48 h, cellular morphology was determined by microscopy and a digital photograph of one representative case is shown. Magnification, ×100. Bottom, apoptotic cells were quantified by propidium iodide–based flow cytometry, and the ratio of apoptotic cells induced by radiation was calculated by comparing the irradiated and not irradiated groups. Data presented in (*B*) and (*C*) are 1 representative case from 12 cases. *D,* conditioned medium from isolated endothelial cells (1 × 10^6) was collected 48 h after irradiation (4 Gy) and added to PCCs from HBV carriers for 24 h. The PCCs were irradiated (4 Gy) or not (sham). After 48 h, the total genomic DNA was purified for the HBV DNA quantification. Data are the number of HBV DNA copies per mL.

HCC cells ($P = 3.1 \times 10^{-10}$) were both statistically significant. Besides, irradiation to cocultured HepG2.2.15 cells and HUVECs induced 17.7 ± 1.4–fold increase in HBV DNA copies. The induction of HBV DNA and RNA in irradiated HepG2.2.15 cells by conditioned medium from irradiated HUVECs was shown by Southern blot analysis (Fig. 4A) and Northern blot analysis (Fig. 4B), respectively.

IL-6 released from irradiated endothelial cells is associated with radiation-induced HBV reactivation. The cytokine arrays to assay conditioned medium from HUVECs obtained 48 h after irradiation showed significant increase in the expression of IL-6 (Fig. 5A). Pretreatment with IL-6 neutralizing antibody of this conditioned medium partially inhibited this induction (7.74 ± 1.69–fold versus 2.96 ± 0.83–fold; $P = 0.01$; Fig. 5B), and absence of pretreatment and pretreatment with nonspecific IgG had no effect. ELISA analysis revealed significantly more IL-6 in the conditioned medium from irradiated than nonirradiated HUVECs (161.3 ± 12.1 pg/mL versus 42.3 ± 4.2 pg/mL; $P = 0.00085$; Fig. 5C). Additionally, the IL-6 concentrations in the supernatant of irradiated and nonirradiated cocultured HepG2.2.15 cells and HUVECs were 206.6 ± 9.4 pg/mL and 123.9 ± 12.5 pg/mL ($P = 0.0007$), respectively. Thus, a radiation-induced bystander effect on endothelial cells (the release of cytokines, including IL-6) seems to cause HBV reactivation (Fig. 6).

Increase in serum viral loads and IL-6 activities in HBV carrier patients undergoing radiotherapy to part of the liver. All nine HBV carrier patients irradiated to part of the liver had the increase in serum HBV DNA copies, with a median post-radiotherapy/preradiotherapy ratio of 4.8 (range, 1.2-5,044.3). Similar increase in serum IL-6 concentration was shown in these patients with a median postradiotherapy/preradiotherapy ratio of 1.14 (range, 1.09-1.41). Non-HBV carrier patients with irradiation to the liver also had the increased serum IL-6 concentration, with a median postradiotherapy/preradiotherapy ratio of 1.19 (range, 1.04-1.72). In contrast, six of nine non-HBV carriers irradiated for nonliver sites had the decreased serum IL-6 concentration after radiotherapy. The only HBV carrier patient undergoing radiotherapy to upper-third esophageal cancer excluding liver presented with simultaneous reduction in serum IL-6 concentration and HBV DNA copy numbers after treatment. Totally, all 16 patients with part of the liver irradiated, compared with 3 of 10 patients irradiated for nonliver sites, showed the increased IL-6 concentrations after radiotherapy ($P < 0.001$).

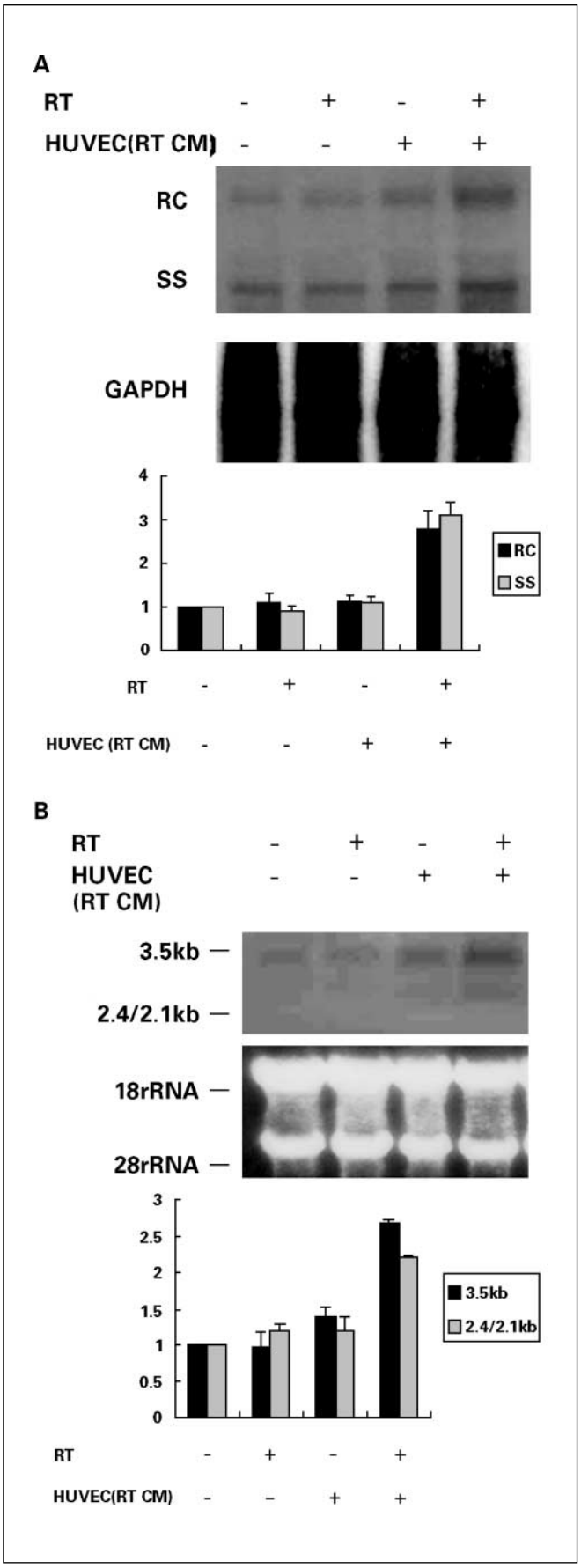


Fig. 4. Conditioned medium from irradiated HUVECs induces HBV DNA replication in irradiated HBV-infected HepG2.2.15 cells. *A*, conditioned medium (*CM*) from HUVECs was collected 48 h after irradiation (4 Gy) and added to HBV-infected HepG2.2.15 cells for 24 h. The HepG2.2.15 cells were then irradiated with 4 Gy or not (sham). After 48 h, the total genomic DNA was purified for Southern blot analysis of HBV DNA. Bands corresponding to the relaxed circular (*RC*) and single-stranded (*SS*) HBV DNA replicative forms. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as the loading control. Bottom, the hybridization signals for the viral core – associated DNA were quantitatively evaluated by digital imaging analysis software. Columns, mean relative to the control group value (*lane 1*) in three independent experiments; bars, SD. *B*, HepG2.2.15 cells were treated under the same conditions as described above. Total RNA was extracted from HepG2.2.15 cells for Northern blot analysis of HBV mRNA. Numbers on the left, positions of major HBV transcripts. The 28S and 18S rRNAs were visualized under UV light and used as equal loading controls. Bottom, the hybridization signals for the viral transcript were quantitatively evaluated by image analysis software. Columns, mean relative to the control group value (*lane 1*) in three independent experiments; bars, SD.

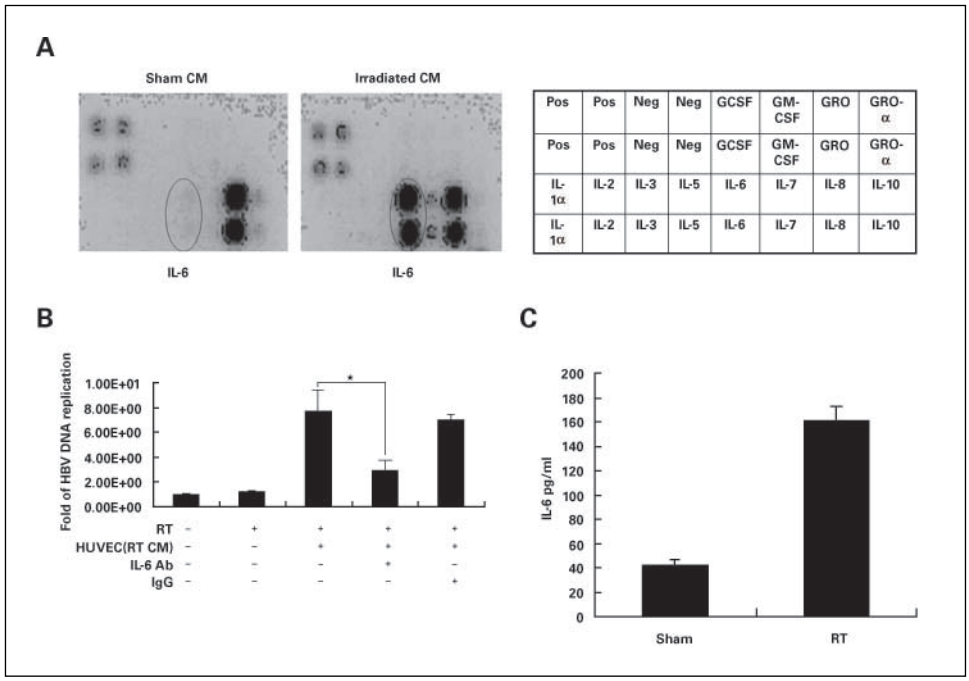


Fig. 5. Cytokines in condition medium from irradiated endothelial cells stimulate HBV DNA replication in irradiated HBV-infected cells. *A,* conditioned medium from HUVECs were collected 48 h after irradiation (4 Gy) and added to the HBV-infected cell line HepG2.2.15 for 24 h. Then, the HepG2.2.15 cells were irradiated with 4 Gy or not (sham). Cytokine assay of the conditioned media showed that expression of IL-6 was increased in the radiation group compared with the sham group. Data presented were one of the results from three independent experiments. Right, relative position of each cytokine. *B,* the conditioned medium from irradiated HUVECs (as described above) was pretreated with anti-IL-6 neutralizing antibody or nonspecific IgG for 30 min. The conditioned medium was added to the HBV-infected cell line HepG2.2.15 for 24 h. Then, the HepG2.2.15 cells were irradiated with 4 Gy or not (sham). After 48 h, the total genomic DNA was purified for the HBV DNA quantification. Columns, mean of fold increase in HBV replication calculated from a comparison of the radiation and sham groups; bars, SD. *C,* HUVECs were irradiated with 4 Gy or not (sham). After 48 h, conditioned medium from HUVECs [48 h after irradiation (4 Gy) or not (sham)] was quantified for IL-6 concentration using ELISA analysis. Columns, mean of three independent experiments; bars, SD.

Discussion

Although dose escalation in hepatic malignancy was proven beneficial in hepatic tumor control and even survival (19), it also increases the risk of RILD. The presentations of RILD in Asian HBV carriers are strikingly different from those reported in the Western countries (20). Furthermore, our previous work showed that the HBV reactivation was the underlying etiology of RILD in certain cases (3).

Chemotherapy-related HBV reactivation has been well documented in the literature; however, the precise mechanism is not fully understood (21). Immunosuppression-enhanced viral replication during chemotherapy, with destruction of infected hepatocytes on restoration of the immune system, is considered the pathogenesis. Nevertheless, this hypothesis may not apply to the pathogenesis of radiation-induced HBV reactivation. Conformal radiotherapy usually focuses radiation on only a small part of the liver. Therefore, local radiotherapy may have less effect on immune function than systemic chemotherapy. Chronic HBV infection with acute exacerbation activates the humoral immune response and promotes antibody production (11). ILs, IFN-γ, and tumor necrosis factor-α contribute to viral clearance. Our current data showed that the conditioned medium from irradiated PCCs or endothelial cells facilitated HBV DNA replication. The cytokine assay of the conditioned medium showed that radiation induced the release of these cytokines. Therefore, cytokines may be key factors in the process of radiation-induced reactivation of HBV.

Radiation spreading from the main radiation path encounters hepatocytes, Kupffer cells, lymphocytes, stellate cells, fibroblasts, and endothelial cells. We have shown that irradiation directly to PCCs or HepG2.2.15 cells themselves did not significantly induce HBV DNA replication. However, in the presence of conditioned medium from irradiated PCCs or endothelial cells, it did, implying that HBV reactivation was

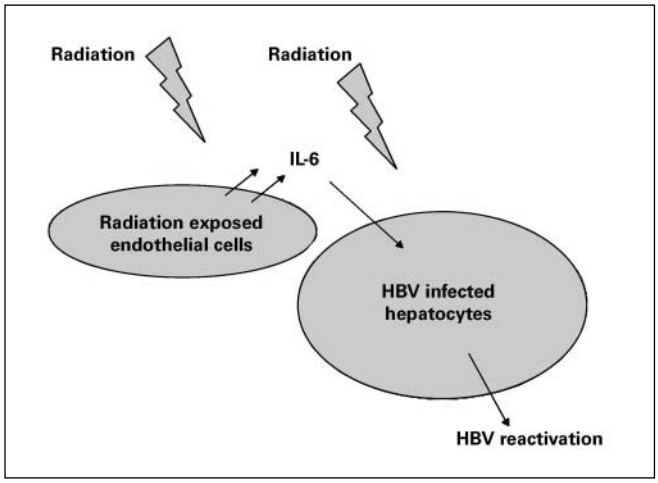


Fig. 6. This schema shows the proposed bystander mechanism of radiation-induced HBV reactivation. The release of IL-6 from radiation-exposed endothelial cells is suggested to be the cause HBV DNA replication.

mediated by an indirect effect (such as factor release) of the irradiated PCCs or endothelial cells. Similarly in radiation pneumonitis, transforming growth factor-β has been strongly linked to the induction of radiation-induced lung injury (22).

Bystander injury dependent on tumor necrosis factor-α and IFN-γ was reported by Bowen et al. (23), with selective accumulation of transgenic $CD8^+$ T cells in liver and hepatitis induction. They pointed out that this etiology is associated with some forms of biologically significant hepatitis, such as autoimmune hepatitis and hepatitis associated with extrahepatic diseases. Radiation-reactivated HBV is also likely to occur via a bystander mechanism. The dose distribution due to the basic physics of commonly used photon radiation inevitably triggers some effects on noncancerous cells outside the tumor. Christiansen et al. (12) showed increased apoptosis of hepatocytes exposed to radiation, with the addition of supernatant from irradiated liver macrophages. They proposed that tumor necrosis factor-α in the supernatant mediates this susceptibility and that the interaction between macrophages and hepatocytes is the important step in developing RILD.

This study found levels of several cytokines increased in the conditioned medium of irradiated endothelial cells. Among them, IL-6 was the cytokine that most affected HBV reactivation. Radiation exposure results in an inflammatory reaction, with endothelial cells playing the key role and involving IL-6, IL-8, and expression of intercellular adhesion molecule 1 (24). IL-6 was increased in plasma from intestine- and liver-irradiated mice (25). The development of radiation-induced inflammatory and thrombotic processes in total body–irradiated mice involved endothelial cells and increased plasma and lung IL-6 (26). Serum IL-6 was significantly increased in advanced liver cancer patients 24 and 48 h after treatment with yttrium-90 microspheres (27). Moreover, IL-6 has long been known to facilitate HBV infection (28, 29). Therefore, it is likely that radiation-induced release of cytokines, including IL-6, from endothelial cells reactivates HBV and causes clinical liver toxicity. We did not find the significant increase of previously reported tumor necrosis factor-α in the conditioned medium of irradiated HUVECs. However, partially but not totally inhibited HBV DNA replication with the IL-6 antibody implied that other cytokines involve this bystander reaction in the *in vivo* conditions.

Our serum analyses confirmed the nonspecific increase of IL-6 in patients undergoing radiotherapy to part of the liver. The simultaneous increase in HBV DNA correlated with this cytokine activity change in HBV carriers. In contrast, serum IL-6 did not increase in most patients irradiated for nonliver sites. It is of note that serum concentration of systemic IL-6 may be much more diluted than that in the paracrine circumstance of irradiated part of the liver. Besides, serum IL-6 may not only reflect the reaction from irradiated liver but also present more complicated malignant disease status. Animal model with liver irradiated and stained for IL-6 is in preparation and may more accurately answer this question.

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