

Fig. 3. Follow-up study of liver histology on asymptomatic hepatitis C virus carriers whose alanine aminotransferase levels became persistently abnormal during the follow-up period. Fourteen patients who developed continuously abnormal serum aminotransferase levels submitted to repeat liver biopsies after 4.0–10.3 years. Progression of the stage of liver disease was noted in six patients, of whom four progressed from F0 to F1 and two from F1 to F2 after 4.0–10.3 years. The left side edge of the large bar indicates the initial recording of abnormal serum aminotransferase during follow-up period.

Serum HCV RNA levels were similar to patients with symptomatic chronic hepatitis, however, the frequency of HCV genotype 2 was significantly higher in HCV carriers with PNAL than those with chronic hepatitis C (data not shown here, of 123 patients with chronic hepatitis C in our clinic 75% had genotype 1 and 22% were genotype 2). Females were predominant among the HCV carriers with PNAL compared with chronic hepatitis [4] which is similar to other reports [5,33–35]. Female HCV carriers with continuously normal ALT had significantly lower ALT levels at entry as shown in Table 6. Of the 105 female patients, 44 had ALT levels ≤ 19 U/L and showed mild liver injury compared with carriers with whose ALT levels were 20–30 U/L. However, the progression rate of fibrosis was not significantly different.

The serum ferritin and serum thioredoxin (TRX) levels in HCV carriers with PNAL showed normal ranges and were significantly lower than in chronic hepatitis C patients, as we have reported previously [26]. The frequency and grade of fatty liver and iron loading were quite low compared with

chronic hepatitis C patients, also as reported previously [26]. Liver histology was minimal to mild and moderate chronic hepatitis was noted in only around 8% of subjects. Long-term follow-up study demonstrated that 29% of HCV carriers with PNAL developed chronic hepatitis with persistently high serum ALT within 5 years, 57% showed transient elevation of serum ALT, and 14% had continuously normal ALT. There are many reports concerning the natural course of liver fibrosis in chronic hepatitis C patients including patients with normal serum ALT level [5,33–41]. More than half of chronic hepatitis C patients show progression of stage of liver fibrosis from F1 to F2–4 within 10 years and it was previously reported that progression of liver fibrosis in HCV carriers with PNAL was more rapid compared with the present result [5]. The main reason for this discrepancy between the previous reports and the present result might be due to the difference in the definition of the normal range of serum ALT. Poynard et al. [37] reported that the median rate of progression of fibrosis per year was 0.1333 fibrosis unit, which was 1.5–3 times faster than the present results in HCV carriers with PNAL.

These results indicate that HCV carriers with PNAL are in a condition with less oxidative stress [26] and they have a lower risk of cirrhosis and hepatocarcinogenesis compared to chronic hepatitis patients [13,22].

It is well known that the rate of the development of hepatocellular carcinoma (HCC) is correlated with the progression of liver fibrosis; the stage of liver disease [9,11,13]. Sustained low serum ALT also lowers the rate of the development of HCC [9,13,42]. No HCC was detected during the follow-up period in any of the HCV carriers in this study, reflecting the results of previous clinical studies.

Peginterferon and ribavirin administration for 48 weeks resulted in sustained virological response in around 40% of patients with genotype 1 [15], however, this therapy is expensive and induces various side effects.

The present results indicate that most HCV carriers with persistently normal serum ALT have a good prognosis with a low risk of developing hepatocellular carcinoma. Antiviral treatment for these patients should take into consideration the follow-up results of blood chemistry and liver histology.

Table 6
Characteristics of 61 female patients in groups A–C followed over 5 years

	Group A (N=8)	Group B (N=34)	Group C (N=19)
Age (y.o.)	49.6 ± 12.9	44.9 ± 12.5	48.2 ± 8.9
BMI (kg/m ²)	20.8 ± 2.9	20.6 ± 2.1	21.8 ± 2.5
Ferritin (ng/ml)	73.4 ± 33.7	59.3 ± 56.8	76.8 ± 47.1
ALT (U/L)*	15.8 ± 3.2	22.4 ± 4.6	23.9 ± 4.9
HCV RNA (KIU/ml)**	186.5 ± 141.8	474.6 ± 486.0	454.0 ± 575.2

Values were expressed as mean ± SD. There were no significant differences in their age, BMI, ferritin, and HCV RNA levels in three groups. *Serum ALT level was significantly lower in group A compared with group B (group A vs group B; $P=0.0045$) and with group C (group A vs group C; $P=0.0003$), however, no significant difference was noted between group B and C ($P=0.0758$). **There were no significant differences in serum amount of HCV RNA between group A and B ($P=0.3529$) and group A and C ($P=0.8676$).

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A green tea polyphenol, epigallocatechin-3-gallate, induces apoptosis of human hepatocellular carcinoma, possibly through inhibition of Bcl-2 family proteins

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Background/Aims: A major polyphenol of green tea, epigallocatechin-3-gallate (EGCG), has previously been shown to induce cell-cycle arrest and apoptosis in various cancers. However, little is known about its effects on hepatocellular carcinomas (HCCs).

Methods: Four HCC cell lines, HLE, HepG2, HuH-7 and PLC/PRF/5, were treated with EGCG or vehicle. Cell viability was assessed by trypan blue staining and WST-8 assay. Cell-cycle, apoptosis and apoptosis-related proteins in HLE cells were evaluated by flow cytometry and Western blotting. The effect of EGCG was also studied in vivo using a xenograft model. The effect of co-treatment with EGCG and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was also assessed.

Results: EGCG inhibited the growth of all HCC cell lines at concentrations of 50–100 µg/ml. In HLE cells, EGCG induced apoptosis but not cell-cycle arrest and appears to have down-regulated Bcl-2 α and Bcl-xl by inactivation of NF- κ B. Oral administration of EGCG showed similar effects in HLE xenograft tumors. Co-treatment with EGCG and TRAIL synergistically induced apoptosis in HLE cells.

Conclusions: EGCG induced apoptosis in HLE cells, both in vitro and in vivo. Moreover, it enhanced TRAIL-induced apoptosis. Therefore, EGCG treatment may be useful for improving the prognosis of HCCs.

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Keywords: EGCG; Apoptosis; HCC; NF- κ B; Bcl-2 family; TRAIL

1. Introduction

Epidemiological studies have shown that green tea has anticarcinogenic and anticancer effects [1–5]. A major polyphenol of green tea, epigallocatechin-3-gallate (EGCG), is thought to be the main active ingredient. It is considered to suppress tumor growth indirectly by anti-angiogenic action and activation of immune function [6,7], and directly through cell-cycle arrest and induction of

apoptosis mediated by various proteins including p53 and nuclear factor-kappa B (NF- κ B) [8,9].

During the progression of hepatocellular carcinoma (HCC), cancer tissues gradually obtain various malignant features in a multistep fashion [10]. Most early-stage HCCs are small tumors with ill-defined boundaries, and consist of well-differentiated cancerous tissues. During the progression, the less differentiated lesions with more malignant properties occasionally arise and replace the preexisting parts. This process increases the malignant potential of a tumor. For this multistep dedifferentiation, HCCs in an advanced stage show poor prognosis, especially when tumors are too large to be treated by surgery or local interventions such as percutaneous ethanol injection therapy (PEIT) and radiofrequency ablation (RFA).

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HCCs with poor prognosis are characterized by rapid cell proliferation and strong expression of antiapoptotic genes [11], which suggests that they are mainly due to incomplete cell-cycle arrest and apoptosis-resistance under conventional therapies. To overcome these problems a new or additional therapeutic strategy is needed. In addition, little is known about the anticancer effects and the basic mechanisms of EGCG against HCCs [12,13]. In the present study, we examined the effects of EGCG on growth of human HCC cell lines using an in vitro culture system and an in vivo animal xenograft model, and elucidated the mechanism of growth suppression by analysis of cell-cycle regulation and profiles of proapoptotic and antiapoptotic proteins.

2. Materials and methods

2.1. Cell lines and cell culture

Human HCC cell lines, HLE (an undifferentiated cell line) and HepG2, HuH-7 and PLC/PRF/5 (differentiated cell lines) were purchased from the Health Science Research Resources Bank, Osaka, Japan. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin.

2.2. Antibodies and inhibitors, ligand and EGCG

Antibodies for Bid, Bax, Bcl-2 α , Bcl-xl, c-FLIP, AIF, and β -actin were purchased from Lab Vision Corporation (Fremont, CA). Antibodies for survivin, XIAP, cIAP-1/2 and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) were from R and D Systems Inc. (Minneapolis, MN). An antibody for RIP was from BioVision Research Products (Mountain View, CA). Antibodies for nuclear factor- κ B (NF- κ B) p65 and phospho-NF- κ B p65 (Ser536) were from Cell Signaling Technology, Inc. (Beverly, MA). An NF- κ B inhibitor, Bay 11-7085 was from Calbiochem (San Francisco, CA). A purified preparation of EGCG (>98% pure) was a kind gift from Dr Yukihiko Hara (Mitsui Norin Ltd, Shizuoka, Japan).

2.3. Cell growth assay

Cells were incubated in 6-well plates (1×10^5 cells/well) for 24 h, then treated with 50 μ g/ml EGCG or vehicle. The time of EGCG addition was defined as 0 and cell numbers were counted by trypan blue staining every 24 h (24–96 h). Medium containing the appropriate concentration of EGCG or vehicle was changed every 2 days.

2.4. Cell cytotoxicity assay

Cells were incubated in 96-well plates (5×10^3 cells/well) for 24 h, then treated with EGCG (10, 25, 50 and 100 μ g/ml), TRAIL (100 ng/ml) or vehicle. After 24 or 48 h of treatment, the number of viable cells was measured using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] assay (Nacalai Tesque, Kyoto, Japan). Ten microliters WST-8 solution was added into each well and the cells were incubated for another 1 h. The absorbance was measured at a test wavelength of 450 nm and a reference wavelength of 600 nm using a microplate reader (Benchmark, Bio-Rad Laboratories, CA). Cell cytotoxicity was evaluated as the ratio of the absorbance of the sample to that of the control.

2.5. Cell cycle analysis

Cells were incubated for 24 h and then treated with EGCG (10, 25, 50 and 100 μ g/ml) or vehicle. Cells (1×10^6) were fixed with 70% ethanol at -20°C for 24 h, centrifuged, suspended in 1 ml PBS containing RNase A (10 μ g/ml), incubated at 37°C for 30 min, stained with propidium iodide (40 μ g/ml), incubated on ice for 30 min and examined with a flow cytometer using Modfit LT software (Becton Dickinson, San Jose, CA).

2.6. Analysis of apoptosis

Cells (5×10^5) that had been treated for 24 h with EGCG (10, 25, 50 and 100 μ g/ml), TRAIL (100 ng/ml) or vehicle were labeled with annexin V-FITC and PI using an apoptosis detection kit (BioVision Research Products, Mountain View, CA) according to the manufacturer's protocol. The distribution of apoptotic cells was identified by a flow cytometer using CellQuest software (Becton-Dickinson, San Jose, CA). Cells that were annexin V(–) and PI(–) were considered viable cells. Cells that were annexin V(+) and PI(–) were considered early-stage apoptotic cells. Cells that were annexin V(+) and PI(+) were considered late-stage apoptotic cells or necrotic cells.

2.7. Caspase colorimetric assay

Cytoplasmic protein was extracted from 2×10^6 cells of each cell line that had been treated with EGCG (10, 25, 50 and 100 μ g/ml) or vehicle for 24 h. Cytoplasmic protein (150 μ g) and 5 μ l fluorogenic substrate were added to 50 μ l reaction buffer. The mixtures were placed in wells of a 96-well plate and assayed for caspase with a caspase-3, 8, 9 colorimetric assay kit (BioVision Research Products, Mountain View, CA) according to the manufacturer's protocol.

2.8. Western blot

Cells that had been treated with EGCG (10, 25, 50 and 100 μ g/ml), Bay 11-7085 (2.0 and 4.0 μ g/ml), TRAIL (100 ng/ml) or vehicle for 24 h were lysed using standard procedures. The supernatant was used as cytoplasmic protein. Twenty to fifty micrograms cytoplasmic protein was electrophoresed in a 4–12% bis-tris gel and transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk and incubated with primary antibody for 1 h at room temperature. After incubation with horseradish peroxidase-conjugated secondary antibody for 1 h, antibody-stained bands were detected with SuperSignal West Pico Chemiluminescent Substrate (PIERCE, Rockford, IL). The density of each band was measured using densitography software (ATTO, Tokyo).

2.9. Immunoprecipitation

Cells were treated with 100 μ g/ml EGCG for 0, 3, 6, 12 and 24 h. Five hundred microgram of cytoplasmic protein, prepared as described above, was incubated with 60 μ l Protein G Sepharose beads (Amersham Biosciences Corp, Piscataway, NJ) for 1 h and centrifuged. The supernatant was incubated with 2 μ g anti-Bax antibody overnight and with 60 μ l Protein G Sepharose beads for another 1 h. The beads were washed 4 times and boiled with 40 μ l sample buffer. The immunoprecipitated Bcl-2 α and Bcl-xl which bound to Bax were analyzed by Western blot analysis.

2.10. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from cells that had been treated with EGCG (10, 25, 50 and 100 μ g/ml) or vehicle for 24 h and subjected to RT-PCR [14]. Transcripts of the gene for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used as an internal control. The primer sequences and amplicon sizes were as follows: bcl-2 α forward: 5'-TTGTGGCCTTCTTTGAGTTCG-3', bcl-2 α reverse: 5'-TAC TGCTTTAGTGAACCTTTT-3' (332 bp product), bcl-xl forward: 5'-GGAGCTGGTGGTTGACTTCT-3', bcl-xl reverse: 5'-CCGGAA

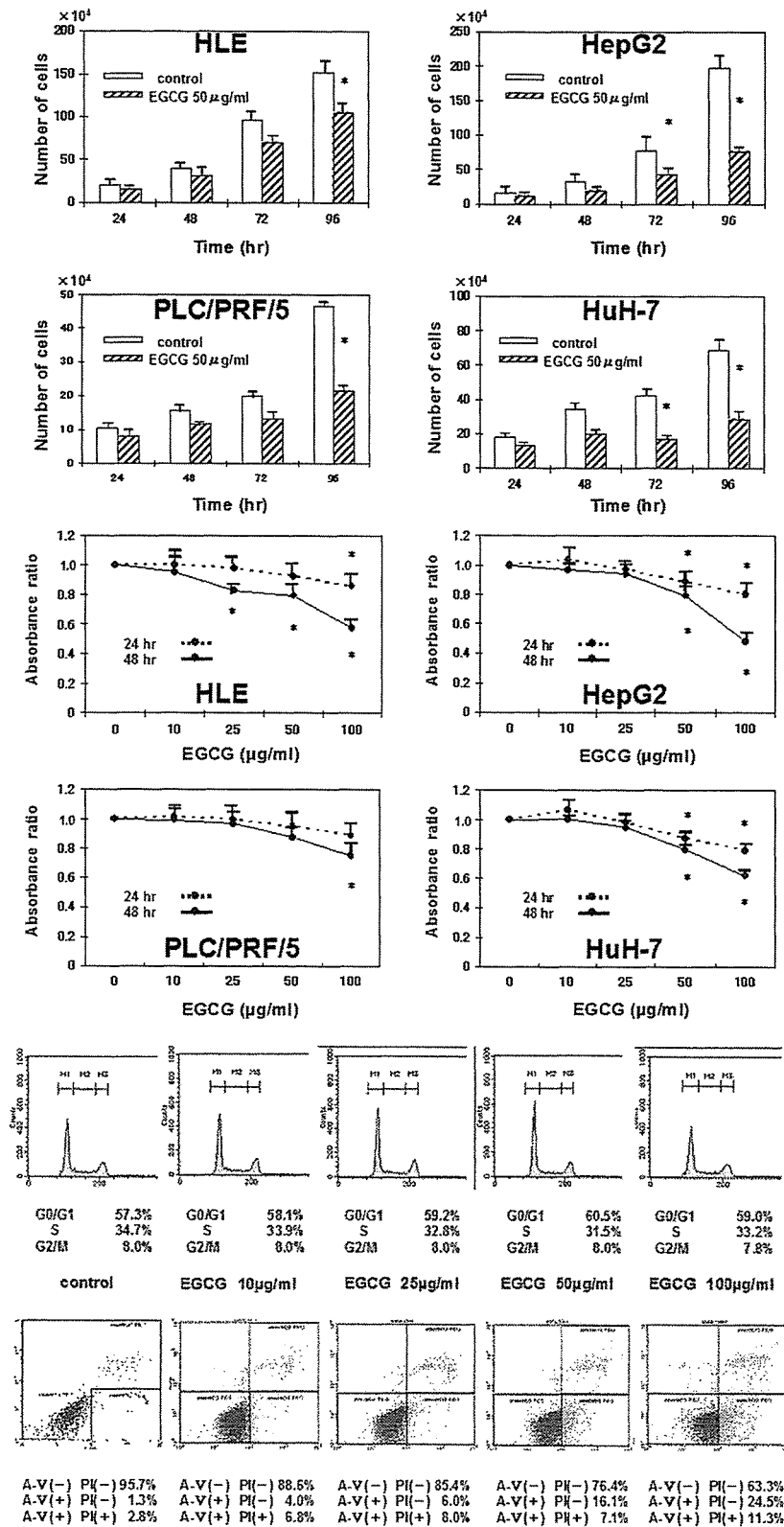


Fig. 1. EGCG inhibits growth of human hepatocellular carcinoma (HCC) cells in vitro and induces apoptosis. (a) Proliferation was assessed in human HCC cell lines, HLE, HepG2, PLC, and HuH-7. The cells were treated with 50 μg/ml EGCG or vehicle for 24, 48, 72 and 96 h, and counted by trypan blue staining. The data represent the mean and SD from five independent experiments. **P* < 0.05. (b) Cell cytotoxicity was assessed using the WST-8

GAGTTCATTCACACTAC-3' (379 bp product), G3PDH forward: 5'-GTCAACGGATTGGTCGTATT-3', G3PDH reverse: 5'-AGTCTTCTGGGTGGCAGTGAT-3' (540 bp product). The PCR protocol for bcl-2 α or bcl-xl consisted of denaturation at 94 °C for 3 min, 32 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, final extension at 72 °C for 5 min. The protocol for G3PDH was the same except for annealing at 54 °C for 45 s and amplification for 25 cycles. Amplification was exponential throughout all cycles. The PCR products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide.

2.11. Growth inhibition of HCC by EGCG in a xenograft model

HLE cells (1×10^6) were injected into the dorsal subcutaneous tissue of twenty 8-week-old nude mice (male BALB/c Slc-nu/nu, Clea Japan, Inc., Tokyo, Japan) to form tumors. Tumor volumes were calculated daily with the formula $(4/3)\pi[(\text{major axis}/2) \times (\text{minor axis}/2)]^2$. When the tumor volumes reached 50–250 mm³, the mice were randomly divided into 4 groups (groups A, B, C, D, $n=5$). The four groups were given sterile water containing 0, 0.8, 2.5 and 7.5 mg/ml EGCG ad libitum, respectively. Water consumption of each group was measured daily. The first day of EGCG water administration was defined as day 0. The mice were weighed every day until day 25 when they were sacrificed. None of the mice in any of the groups showed any obvious changes in body weight, in water consumption or in behavior throughout the experiment. Tumor tissues were collected and fixed in 4% buffered paraformaldehyde. Apoptotic cells were detected with a TUNEL assay and expressions of Bcl-2 α and Bcl-xl in HLE cells were assessed with immunohistochemical staining [14]. Animals were treated humanely following the United Kingdom Coordinating Committee on Cancer Research Guidelines.

2.12. Statistical analysis

Differences between groups treated with each concentration of EGCG were assessed using Student's *t*-test. The synergistic effect of EGCG on TRAIL treatment was tested by ANOVA.

3. Results

3.1. EGCG inhibits growth of human HCC cell lines in vitro and induces apoptosis in HLE cells

EGCG treatment inhibited the growth of all of four HCC cell lines in a time-dependent manner (Fig. 1a). The cytotoxicity of EGCG was also observed in all of the cell lines in a dose- and time-dependent manner (Fig. 1b). We selected HLE cells for further investigations because undifferentiated HCC cells such as HLE cells generally show poor prognosis and require a new strategy in clinical practice [15]. EGCG treatment had no effect on cell-cycle progression but induced early-stage apoptosis in a dose-dependent manner (Fig. 1c). The number of cells that were either apoptotic in the delayed stage or necrotic also increased similarly by EGCG treatment. These results

suggest that the anti-tumor effect of EGCG in HLE cells is mainly induced by apoptosis.

3.2. EGCG activates caspases and decreases expressions of Bcl-2 α and Bcl-xl in HLE cells

EGCG treatment activated caspases-8, 9 and 3 in a dose-dependent manner (Fig. 2a). The caspase-9 pathway is mainly regulated by proteins of the bcl-2 family and inhibitor of apoptosis proteins (IAP) family. The expressions of Bcl-2 α and Bcl-xl were gradually inhibited as the EGCG concentration increased. The expressions of Bid and c-IAP2 decreased at higher concentrations of EGCG (50, 100 μ g/ml), the former probably due to the cleavage by activated caspase-8. EGCG had no influence on Bax, survivin, XIAP or c-IAP1 (Fig. 2b), c-FLIP and RIP (factors that control the activation of caspase-8), or Fas, the death receptor, or AIF, caspase-independent apoptosis-inducing factor (Fig. 2c). EGCG decreased the bindings of Bcl-2 α and Bcl-xl to Bax, which may activate caspase-9 (Fig. 2d).

3.3. EGCG down-regulates the mRNAs of bcl-2 α and bcl-xl by inhibition of NF- κ B

The mRNA expressions of bcl-2 α and bcl-xl decreased following EGCG treatment in a dose-dependent manner (Fig. 3a). Thus, EGCG decreases the expressions of Bcl-2 α and Bcl-xl at the level of transcription. EGCG did not affect the protein level of the NF- κ B p65 subunit in the cytoplasm but it decreased the active form, phospho-NF- κ B p65 (Ser536), (Fig. 3b). When HLE cells were treated with an NF- κ B inhibitor, Bay 11-7085 [16], the expressions of Bcl-2 α and Bcl-xl decreased in a dose-dependent manner (Fig. 3c). These results appear to show that EGCG down-regulates both Bcl-2 α and Bcl-xl expressions via NF- κ B inhibition. To examine the time course of these effects, HLE cells were exposed to EGCG for up to 24 h. EGCG treatment inhibited the activation of NF- κ B (P65) within 3 h and the effect continued for 24 h (Fig. 3d). The expressions of Bcl-2 α and Bcl-xl decreased remarkably after 6 and 3 h of exposure, respectively and continued to decrease, while those of Bax and Fas showed no obvious changes.

3.4. EGCG inhibits growth of HLE in vivo, induces apoptosis and down-regulates Bcl-2 α and Bcl-xl

The growth of HLE xenograft tumors in nude mice was suppressed by administration of EGCG compared with the control (Fig. 4a). The inhibitory effect was observed

assay. The cells were treated with EGCG at the indicated concentrations (0–100 μ g/ml) and the absorbance ratio was measured in 24 and 48 h. The data represent the mean and SD from ten independent experiments. * $P < 0.05$. (c) Effects of EGCG on cell cycle progression and apoptosis in HLE cells were examined by a flow cytometer. The cells were treated as described under Section 2. The proportion of each phase of the cell cycle was analyzed using the Modfit LT software, and apoptosis was assessed using the CellQuest software. The data are from a representative experiment repeated three times with similar results. [This figure appears in colour on the web.]

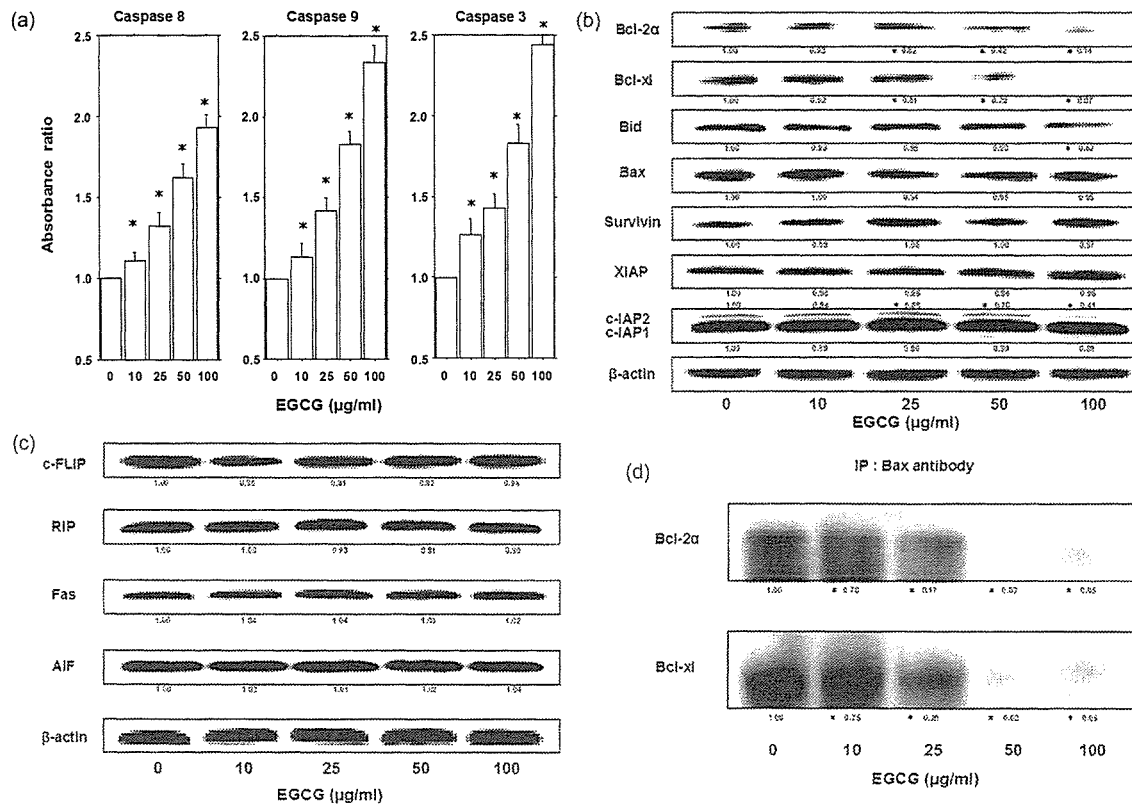


Fig. 2. EGCG activates caspases and decreases expressions of Bcl-2α and Bcl-xl in HLE cells. (a) Activation of caspase-8, -9 and -3 by EGCG in HLE cells. The cells were treated with EGCG at the indicated concentrations (0–100 μg/ml) for 24 h and caspase activities in the cell lysates were measured with a colorimetric assay kit. The data represent the mean and SD from five independent experiments. * $P < 0.05$. (b, c) Western blots showing expressions of Bcl-2 family proteins and IAP family proteins (b) and c-FLIP, RIP, Fas and AIF (c) in cells treated with EGCG at the indicated concentrations (0–100 μg/ml) for 24 h. (d) The quantities of Bcl-2α and Bcl-xl binding to Bax were analyzed by immunoprecipitation and Western blot assay. The data shown in each of (b,c) and (d) are representative of three experiments with similar results. Image intensities of a band for a protein p were defined as $(I_p^x/I_c^x)/(I_p^0/I_c^0)$ where I_p^x and I_c^x are the band intensities of the protein and the control (β-actin) at EGCG concentration x and I_p^0 and I_c^0 are the band intensities of the protein and control at EGCG concentration 0. * $P < 0.05$.

significantly from day 3 and increased as the concentration of EGCG increased. None of the mice showed any significant difference in body weight, water consumption, or behavior throughout the experiment. The tumor sections showed a large number of apoptotic cells and weak staining of Bcl-2α and Bcl-xl in EGCG-treated mice. In contrast, few apoptotic cells and strong staining of Bcl-2α and Bcl-xl were observed in the control mice (Fig. 4b). These results indicate that oral administration of EGCG induced apoptosis of tumor cells in vivo, probably by down-regulation of Bcl-2α and Bcl-xl.

3.5. EGCG sensitizes HLE cells to TRAIL-mediated apoptosis

HLE cells treated with TRAIL (100 ng/ml) for 24 h showed a weak cytotoxic effect compared with control. However, co-treatment with EGCG and TRAIL significantly enhanced the cytotoxicity in an EGCG dose-dependent manner (Fig. 5a). This effect was significantly different from that of treatment with EGCG alone, and

was synergistic ($P < 0.0001$ by ANOVA). An apoptosis assay demonstrated similar results (Fig. 5b). EGCG and TRAIL also had a synergistic effect on down-regulating the expressions of Bcl-2α and Bcl-xl in HLE cells (Fig. 5c).

4. Discussion

Advanced HCCs often show poor prognosis even following transcatheter arterial embolization (TAE) or surgical resection [17,18]. Our finding that EGCG inhibited the growth of not only well-differentiated cell lines but also an undifferentiated cell line, HLE (Fig. 1a and b) suggests that EGCG has the potential to improve the prognosis of HCCs even in the advanced stage.

Although EGCG can induce apoptosis in various cancers, little is known about its effect on HCC. EGCG was shown to induce cell-cycle arrest and apoptosis through the activation of p53 and Fas-Fas ligand pathways in HepG2 cells [19]. In our experiments, EGCG failed to induce cell-cycle arrest

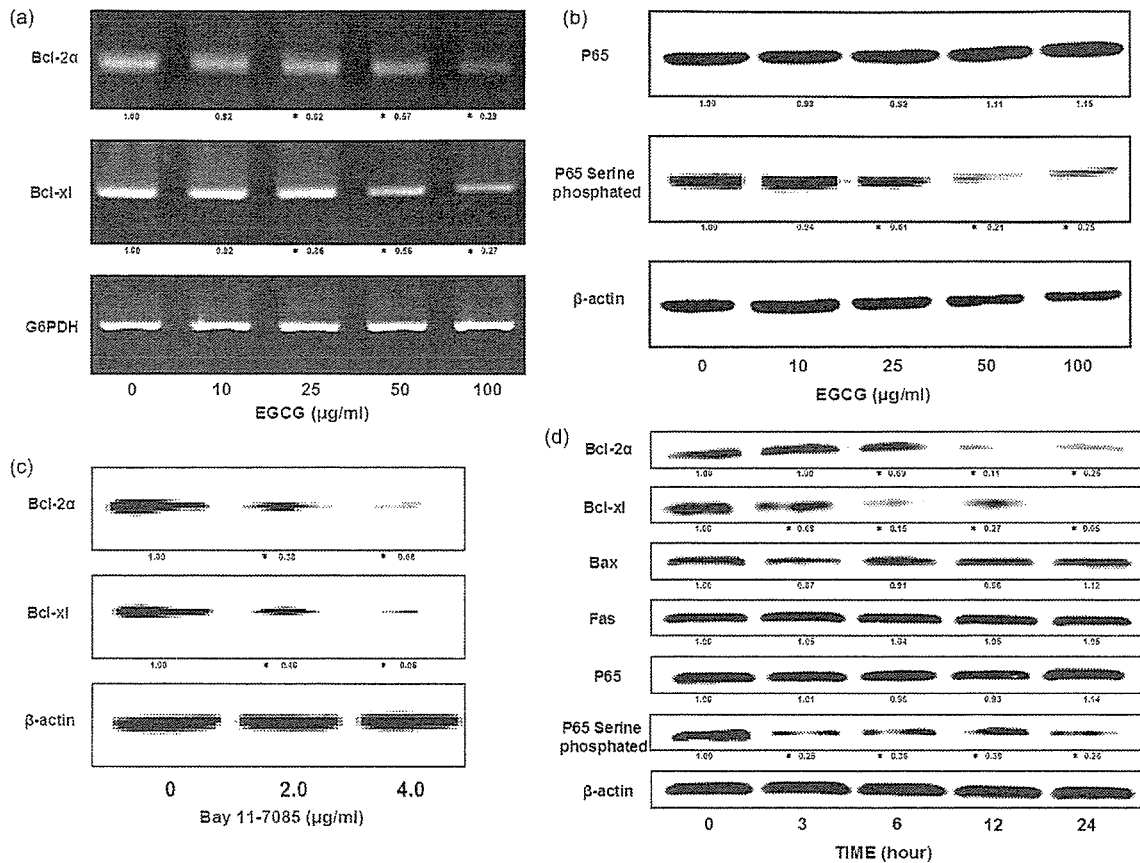


Fig. 3. EGCG inhibits mRNA transcriptions of Bcl-2 α and Bcl-xl in HLE cells. (a) Both mRNA expressions of Bcl-2 α and Bcl-xl in the cells treated with EGCG at the indicated concentrations (0–100 μ g/ml) for 24 h were analyzed by RT-PCR. (b) Activation of NF- κ B in the cells was assessed by Western blot assay using antibodies for NF- κ B p65 and phospho-NF- κ B p65 (Ser536). The cells were treated with EGCG at the indicated concentrations (0–100 μ g/ml) for 24 h. (c) Expressions of Bcl-2 α and Bcl-xl in the cells treated with the NF- κ B inhibitor, Bay 11-7085 at the indicated concentrations (0–4.0 μ g/ml) for 24 h. Those expressions were assessed by Western blot assay. (d) Western blot assay showing expressions of Bcl-2 α , Bcl-xl, Bax, Fas, NF- κ B p65 and phospho-NF- κ B p65 (Ser536) in cells treated with 100 μ g/ml EGCG for 0, 3, 6, 12 and 24 h. The data shown in each of (a)–(d) are representative of three experiments with similar results. See Fig. 2 for explanation of band intensities. * $P < 0.05$.

but induced apoptosis in HLE cells (Fig. 1c). This is probably because p53 protein in HLE cells is dysfunctional due to a mutation of the p53 gene, whereas it is still functional in HepG2 cells. Additionally, EGCG treatment had no effect on expression of Fas in HLE cells (Figs. 2c and 3d). These results indicate that EGCG has more than one mechanism for inducing apoptosis in HCC. A Western blot assay of pro- and anti-apoptotic proteins showed that EGCG inhibits the expressions of Bcl-2 α and Bcl-xl in HLE cells (Fig. 2b), which suggests that activation of caspase-9 by inhibition of Bcl-2 α and Bcl-xl is a major mechanism for induction of apoptosis. The inhibition of c-IAP-2 also might contribute to activation of caspase-9. EGCG also activated caspase-8 in HLE cells (Fig. 2a) though it had no effect on the expressions of cFLIP, RIP and Fas (Fig. 2c). The overexpression of Bcl-2 in HCC cells was shown to reduce Fas-mediated apoptosis [20]. EGCG may activate caspase-8 through reactivation of the Fas-Fas ligand pathway by down-regulation of Bcl-2 α expression. Another possibility

is that EGCG directly activates caspase-8 through its binding to Fas [21].

NF- κ B partially regulates the transcriptions of Bcl-2 α and Bcl-xl [22]. In addition, our results suggest that EGCG down-regulates the expressions of Bcl-2 α and Bcl-xl by inhibition of NF- κ B (Fig. 3a–c). NF- κ B is a transcriptional factor that is associated with various cellular processes, such as cytokine production, cellular adhesion, cell-cycle activation, apoptotic resistance, oncogenesis, and is activated in various solid tumors [23]. Thus, the inhibitory effect of EGCG on NF- κ B may suppress the growth of various cancer cells in multifunctional ways. NF- κ B activation is induced by reactive oxygen species [24] and inhibited by antioxidants [25]. Thus, the inhibitory effect of EGCG on NF- κ B activation may be due to its strong ability to scavenge reactive oxygen species. Bcl-xl overexpression is frequently found in HCC tissues and is significantly correlated with a good prognosis [26,27]. On the other hand, Bcl-2 α is not frequently expressed in HCCs [28], but TAE

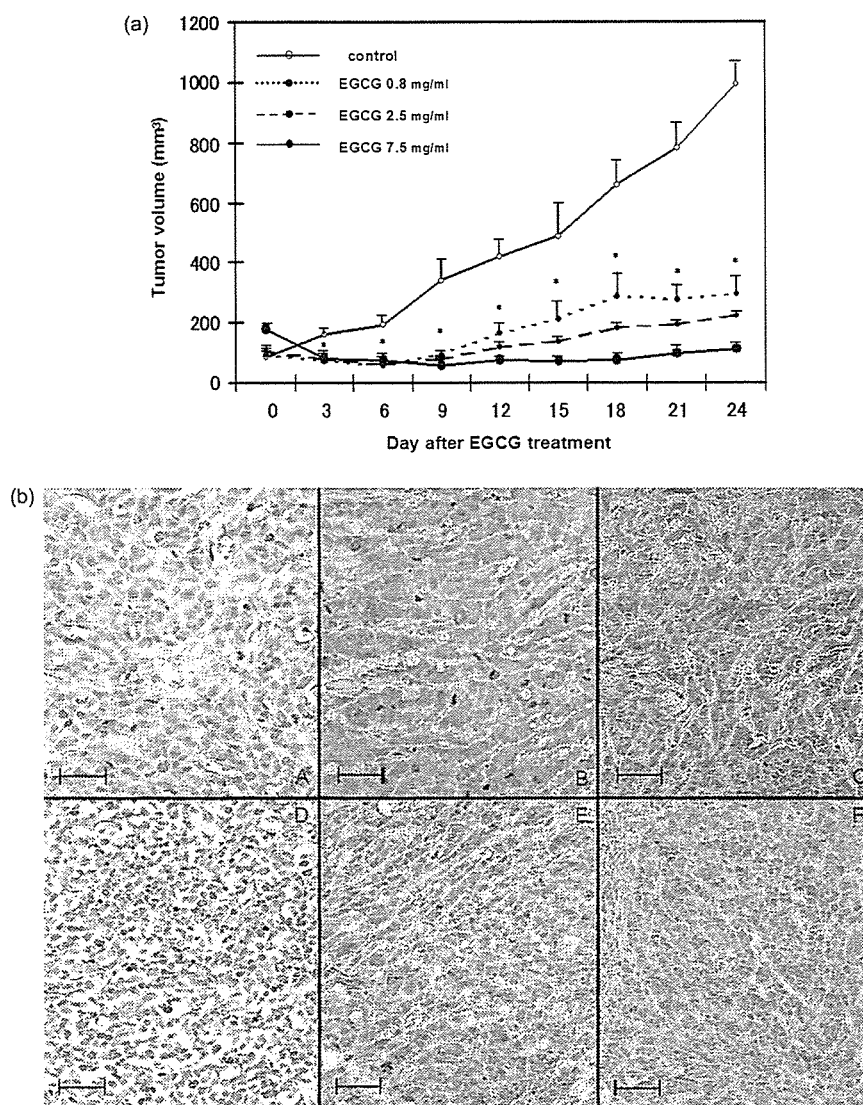


Fig. 4. (a) Growth curve of HLE xenografts in nude mice. Nude mice were injected with approximately 1×10^6 HLE cells into the dorsal subcutaneous tissue. When the tumor volumes reached 50–250 mm³, the mice were divided into four groups and fed with sterile water or 0.8, 2.5, 7.5 mg/ml EGCG water respectively. The data represent the mean and SD from five nude mice. * $P < 0.05$. (b) Apoptosis and expressions of Bcl-2 α and Bcl-x1 in HLE xenografts. Apoptosis was assessed by TUNEL assay (A, D) and expressions of Bcl-2 α (B, E) and Bcl-x1 (C, F) were assessed by immunohistochemistry. The upper row shows tumor sections in the control group and the lower row shows tumor sections in the group receiving 7.5 mg/ml EGCG. Each picture was taken at a magnification of 400 \times . Scale bars, 50 μ m.

increases its expression in HCC tissues [29]. The induction of Bcl-2 α by TAE may allow the cancer cells to escape from hypoxic injury. Furthermore, these antiapoptotic proteins are associated with resistance to chemotherapy [30] and to endogenous apoptotic signals such as TRAIL [31,32] both in vitro and in vivo. In our experiment, EGCG treatment down-regulated both Bcl-2 α and Bcl-x1 in HLE cells (Figs. 2b and 4b) both in vitro and in vivo and sensitized HLE cells to TRAIL-induced apoptosis in vitro (Fig. 5a and b). The sensitizing effect of EGCG appears to result from the down-regulation of Bcl-2 α and Bcl-x1 through inhibition of NF- κ B (Fig. 5c).

Our findings that EGCG suppresses the growth of HLE cells (Fig. 4a) and down-regulates the expressions of Bcl-2 α and Bcl-x1 in vivo (Fig. 4b) raise the possibility that oral administration of EGCG can help overcome the chemotherapy resistance of HCCs. However, the concentrations of EGCG that were needed to significantly down-regulate *anti*-apoptotic proteins and induce apoptosis in vitro (50–100 μ g/ml) were much higher than the concentration that is physiologically achieved by ordinary green tea consumption. Consumption of six or seven cups of green tea per day (corresponding to a dose of ~ 30 mg EGCG/kg per day) will result in a plasma EGCG concentration of approximately 1 μ g/ml [33]. However,

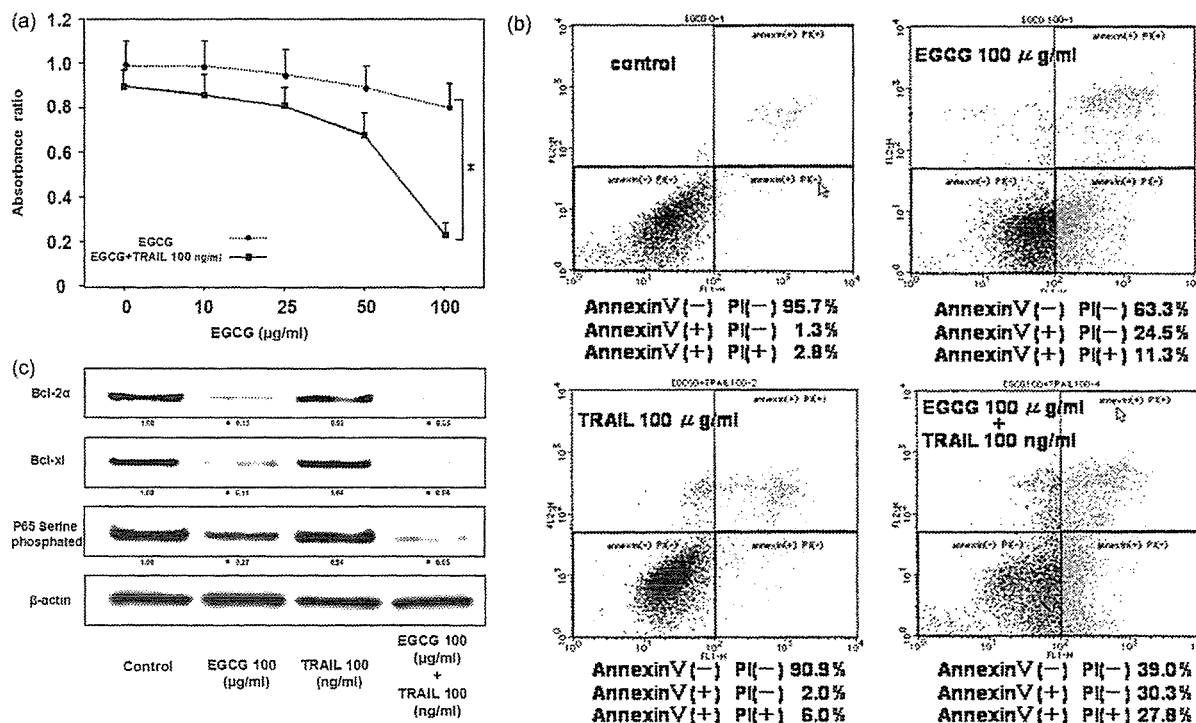


Fig. 5. EGCG sensitizes HLE cells to TRAIL-mediated apoptosis. (a) Cell cytotoxicity was assessed using the WST-8 assay in the cells treated with vehicle or 100 μg/ml EGCG or 100 ng/ml TRAIL or combinations of these agents for 24 h. The combination treatment significantly enhanced the cell cytotoxicity compared with EGCG treatment alone. The data represent the mean and SD from five independent experiments. $P < 0.0001$ by ANOVA. (b) Apoptosis in the cells treated as described above was examined by flow cytometry. The experiment was repeated three times with similar results. (c) Western blot assay showing expressions of Bcl-2α, Bcl-xl and phospho-NF-κB p65 (Ser536) in cells treated with vehicle or 100 μg/ml EGCG or 100 ng/ml TRAIL or combinations of these agents for 24 h. The data shown in (c) is representative of three experiments with similar results. See Fig. 2 for explanation of band intensities. $P < 0.05$. [This figure appears in colour on the web.]

higher plasma EGCG concentrations can be achieved by taking EGCG supplements [34]. In our study, EGCG had no obvious adverse effects on the growth or behavior of mice, even at a dose of 1125 mg/kg per day. EGCG also has an antiangiogenic effect and activates an immune response against tumors, which may increase its antitumor effect in vivo.

In conclusion, our results show that EGCG treatment inhibited the growth of HLE cells in vitro and in vivo. The inhibition was caused by the induction of apoptosis as a result of the activations of caspase-8, -9 and -3. These caspases appear to be activated by the down-regulation of Bcl-2α and Bcl-xl through inhibition of NF-κB. Our results indicate that EGCG treatment may be useful in clinical cases to improve the prognosis of advanced HCCs by direct induction of apoptosis and by enhancing the effects of other factors such as TRAIL.

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In vivo selection of transduced hematopoietic stem cells and little evidence of their conversion into hepatocytes in vivo[☆]

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Background/Aims: FMEV-type retroviral vector provides high transgene expression in hepatocytes and hematopoietic stem cells (HSCs). Here, we examined whether these vectors could provide a sufficient drug-resistance gene expression in HSCs and whether transduced HSCs could differentiate into hepatocytes in vivo.

Methods: The CD45⁺/Lin⁻ cells were transduced in vitro by FMEV-type vectors containing human O⁶-methylguanine–DNA methyltransferase (MGMT)/reporter genes and transferred into recipient mice. After the treatment with temozolomide and O⁶-benzylguanine (TMZ/BG) in vivo, we analyzed the transgene expression in peripheral blood cells by flow-cytometry. Immunohistochemistry was performed on the liver slices in partial hepatectomized recipient mice.

Results: After TMZ/BG treatment, transduced host cells were enriched in recipient mice. In the liver, we observed the efficient transgene expression in many small cells along sinusoids. However, only few large cells in hepatic lobules expressed albumin. They also expressed both a transgene and a recipient marker gene, suggesting the fusion of donor HSCs with recipient hepatocytes.

Conclusions: This vector expressed a drug-resistance gene in HSCs highly enough to protect them from the drugs. But, the conversion of HSCs into hepatocytes in vivo might be a rare event in this model.

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Keywords: FMEV-type retroviral vector; Hematopoietic stem cell; In vivo selection; Transdifferentiation; Hepatocytes

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Abbreviations: HSC, hematopoietic stem cell; MGMT, O⁶-methylguanine–DNA methyltransferase; TMZ, temozolomide; BG, O⁶-benzylguanine; MDR, multidrug resistance; GFP, green fluorescent protein; PBNC, peripheral blood nucleated cell; Wt, wild-type.

1. Introduction

Retroviral vectors are the most commonly used gene transfer vehicles for various cell types [1] and the liver is one of the attractive targets for gene therapy, since it synthesizes pivotal proteins for metabolism or hemostasis [2]. We have already reported that a FMEV-type retroviral vector containing the polycythemic strain of spleen focus-forming virus (SFFVp) long terminal repeats (LTR) and the mouse embryonic stem cell virus (MESV) leader sequences efficiently express transgenes in HSCs [3–5] as well as hepatocytes [6,7].

The human O⁶-methylguanine–DNA methyltransferase (MGMT) gene is one of the multidrug resistance (MDR) genes. The MGMT protein removes alkyl adducts from the O⁶ position of guanine before the

drug-induced reaction that leads to irreversible cell death. Most of tumor cells can resist alkylating agents, such as nitrosoureas, through increased wild-type MGMT expression. But this resistance is inhibited by the treatment with *O*⁶-benzylguanine (BG), which acts by inactivating wild-type MGMT. P140K [8] gene is one of the BG-resistant MGMT variants. Retroviral vectors carrying these MDR genes are used for the protection of hematopoietic stem cells (HSCs) from anti-cancer drugs [9].

In the treatment of liver primary and metastatic tumors, intensive chemotherapy cannot be performed because a pancytopenia or severe liver failure is frequently seen in the patient of the decompensated liver cirrhosis stage. Transfer of a MDR gene into HSCs would enable to protect cancer patient from drug-induced myelosuppression. Furthermore several studies have demonstrated that bone marrow-derived adult stem cells are capable of transdifferentiation into multiple organs, including the liver [10,11]. This indicates the possibility that the transfer of a MDR gene into HSCs would enable the protection from not only myelosuppression but also the liver damage.

In the present report, we have examined whether the vectors express a MGMT gene in HSCs highly enough to protect them from TMZ/BG combined therapy in vivo. We also examined whether the transduced HSCs could transdifferentiate into hepatocytes and whether the transdifferentiate hepatocytes express the transgene in vivo.

2. Materials and methods

2.1. Reagents and antibodies

BG was purchased from Sigma and TMZ was provided from Shering-Plough Research Institute (Kenilworth, NJ). Recombinant murine stem cell factor, thrombopoietin and Flt-3 ligand were purchased from Chemicon International, R&D Systems and Genzyme/Techno, respectively.

Anti-mouse monoclonal antibodies (MoAbs) of peridinin chlorophyll *a* protein cyanine 5.5-OSu-conjugated CD45 (PerCPCy5.5-CD45, 30-F11), phycoerythrin-conjugated Thy-1 (PE-Thy-1, G7), biotinylated B220 (B-B220, RA3-6B2) and Gr-1 (B-Gr-1, RB6-8C5) were purchased from BD Biosciences, streptavidin-PC5 (st-PC5) from Coulter Japan. A MoAb of anti-mouse c-kit (ACK2) was purified from hybridoma culture supernatant and conjugated with fluorescein isothiocyanate (FITC-c-kit). These were used for the study using a fluorescein-activated cell sorter or flow-cytometric analysis.

MoAbs of anti-human MGMT (MT3.1), anti-actin (C4), biotinylated anti-F4/80 (BM8), anti-green fluorescein protein (GFP, GFP-20) or PE-conjugated anti-mouse IgG kappa were purchased from Kamiya Biomedical, Chemicon International, Acris Antibodies, Sigma or Cell Lab, respectively, and a goat anti-luciferase, anti-CD68 (sc-5474) or FITC-conjugated anti-albumin antibodies from Promega Japan, Santa Cruz or Berhyl lab, respectively. A horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, a F(ab')₂ fragment of a FITC-conjugated goat anti-mouse IgG(H+L), streptavidin-FITC (st-FITC) and FITC-, PE- or Alexa647-conjugated anti-goat IgG(H+L) antibodies were from Dako Japan, Immunotech, Coulter Japan, Caltag, IMGENEX or InVitrogen, respectively. These were used for the studies of immunoblotting and immunohistochemistry.

2.2. Plasmids

Based on a vector plasmid, pSF1N/CMV [12], we have constructed retroviral vector plasmids, pSF-GFP/MGMT and pSF-luc/MGMT, by ligating a *NotI*-*HindIII* fragment containing a reporter gene, GFP, internal ribosome entry site (IRES) of NF- κ B repressing factor [13] and a variant of human MGMT (P140K) or a luciferase gene into a vector plasmid, pSF1N/CMV, respectively.

2.3. Mice

We purchased 8–10-week-old C57BL/6 mice from Shimizu Experimental Animal Farm (Shizuoka, Japan) and GFP transgenic mice (GFP-Tg mice), TgN(β -act-EGFP)Osb [14], were kindly provided by Dr. M. Okabe of Osaka University.

We hepatectomized mice (70% partial hepatectomy) 3 weeks before immunohistochemical analysis of the liver.

2.4. Cells

A human hematopoietic cell line, K562, and an amphotropic retrovirus packaging cell line, Phoenix-ampho [15], were maintained in DMEM supplemented with 10% fetal calf serum and an ecotropic retrovirus packaging cell line, GP+E86 [16], with 10% calf serum. A murine stromal cell line, MS-5 [17], was maintained in alpha-medium with 20% horse serum.

We established retroviral vector producing clones by transfecting plasmids of pSF-GFP/MGMT or pSF-luc/MGMT into GP+E86 cells followed by limited dilution.

2.5. Transduction and transplantation of cells

After transfecting pSF-GFP/MGMT into Phoenix-ampho cells, we used the supernatant containing viral vectors to transduce K562 cells.

After depleting hematopoietic differentiation marker-positive bone marrow cells using SpinSep (Stem Cell Technologies), we stained these cells (Lin⁻) with FITC-c-kit and PerCPCy5.5-CD45, followed by separation of CD45-positive cells (Fig. 2B, region "A") using a fluorescence-activated cell sorter, Epics Elite (Coulter Japan). We transduced these sorted cells (Lin⁻/CD45⁺) using essentially the same protocol as previously described [5]. In brief, we overlaid cells on top of mixed culture of irradiated virus producing clones of GP+E86 cells and MS-5 and cultured them in the presence of stem cell factor (50 ng/ml), thrombopoietin (25 ng/ml) and Flt-3 ligand (200 ng/ml) for 10 days. We transplanted 2×10^4 these transduced cells into a 850 cGy-irradiated recipient mouse via tail vein.

2.6. Enrichment of transduced cells with TMZ and BG

Transduced K562 were exposed to 40 μ M BG for 1 h, followed by culture in the presence of 100 μ M or 400 μ M TMZ for 2.5 h. After washing, we additionally exposed cells to BG for 12 h and evaluated the proportion of cells expressing GFP on a flow-cytometer (Epics XL, Coulter Japan) after 7 days of culture.

Nine weeks after transplantation, mice were treated twice with TMZ and BG intraperitoneally (0.6 mg of TMZ and 1 mg of BG per mouse per day) for 5 days, in each of treatment courses separated by a 4-week interval as schematically shown in Fig. 3. We stained peripheral blood nucleated cells (PBNCs) with PE-Thy-1 or B-B220, B-Gr-1, followed by st-PC5, and evaluated the proportion of cells expressing GFP.

2.7. Immunoblotting

Immunoblotting was performed as previously described [18] using anti-MGMT, anti-luciferase or anti-actin antibodies and a HRP-conjugated secondary antibody.

2.8. Immunohistochemical analysis

Immunohistochemical analysis was performed as previously described [18] using anti-GFP, anti-F4/80, anti-CD68, anti-luciferase, anti-MGMT or anti-albumin antibodies and FITC- or PE-conjugated secondary antibodies or a secondary reagent, st.-FITC.

3. Results

3.1. Enrichment of transduced hematopoietic K562 cells in vitro

We constructed a series of retroviral vectors containing the SFFVp LTR and the MESV leader sequences. These vectors carry a variant gene of human methylguanine methyltransferase, P140K [8], at the downstream position of IRES. First, one of these vectors, SF-GFP/MGMT (Fig. 1A), was examined in vitro whether the transduced cells can be protected from the combination of BG, a drug which depletes wild-type cells of MGMT activity, and TMZ, a DNA-methylating drug. Human hematopoietic K562 cells were transduced by SF-GFP/MGMT and exposed to the combination of these drugs (BG/TMZ). Proportions of cells expressing a GFP gene were small without transduction or without selection (0.2% or 4.2%, respectively), while the cells expressing GFP were efficiently selected by the exposure to BG/TMZ in a dose-dependent manner (Fig. 1A). To confirm the efficient expression of transgenes by the vector, we did Western blotting. Expression of MGMT was undetectable in cells untransduced or unexposed to BG/TMZ, while clear bands were detected in cells after the selection by BG/TMZ (Fig. 1B). These results indicated that transgenes were efficiently expressed by using the vector.

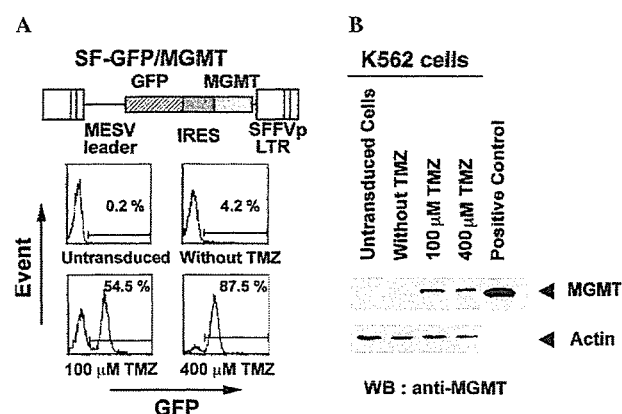


Fig. 1. In vitro selection of transduced cells. (A) Selection of transduced K562 cells in vitro. We evaluated the proportion of transduced K562 cells expressing GFP using a flow-cytometer after 7 days of culture. (B) A human MGMT protein in K562 cells was detected by Western blotting after in vitro selection.

3.2. Enrichment of transduced HSCs in vivo

To examine whether these vectors protect HSCs from the exposure of BG/TMZ in vivo, $\text{Lin}^-/\text{CD45}^+$ cells from bone marrow cells of GFP-Tg mice or wild-type (Wt) were sorted (Fig. 2B, region A). We transduced these sorted cells with the vectors, SF-luc/MGMT and SF-GFP/MGMT, carrying MGMT and a luciferase gene or a GFP gene, respectively (Fig. 2A), and transferred into sub-lethally irradiated (850 cGy) recipient mice (Fig. 2A). After transplantation, we treated mice twice with TMZ/BG as schematically shown in Fig. 2C.

Fig. 3A shows the number of nucleated cells in peripheral blood (PBNCs) during the treatment. Although the number of PBNCs was drastically decreased after the first treatment with BG/TMZ, the number was not changed significantly after the second treatment (Fig. 3A), suggesting that gene-modified HSCs were enriched by the first treatment and HSCs were protected from the second treatment.

To examine the proportion of retrovirally transduced cells during the treatment, we transduced $\text{Lin}^-/\text{CD45}^+$ cells of Wt mice with SF-GFP/MGMT vector and transferred into irradiated Wt mice (Fig. 2A, Wt > Wt). Flow-cytometric analysis showed that proportion of GFP-positive (GFP+) cells in PBNCs was 8.8% before the treatment and increased to 20.4% and 74.1% after the first and second treatment, respectively (Fig. 3B). Next, we examined the proportion of donor-derived cells in PBNCs. Fig. 3C shows the proportion of GFP+ cells in B220-positive (B cells), Thy-1-positive (T cells) and Gr-1-positive (granulocytes) populations during the treatment. When we transferred transduced $\text{Lin}^-/\text{CD45}^+$ cells from a GFP-Tg mouse into Wt mice (GFP > Wt), proportion of GFP+ cells in B, T cells and granulocytes increased after the BG/TMZ treatment. While the proportion in these cells decreased when we transferred the cells from a Wt mouse into GFP-Tg mice (Wt > GFP), indicating successful enrichment of transduced donor HSCs in vivo. Results of Western blotting of PBNCs in Wt > GFP mice demonstrated that PBNCs expressed detectable amount of luciferase (Fig. 3D) or MGMT (Fig. 3E) after the treatment, confirming the enrichment of transduced cells in vivo. All of these data indicate that FMEV-type vectors express a drug-resistance gene in HSCs highly enough to protect them from the treatment of drugs in vivo.

3.3. Little evidence of transdifferentiation of $\text{Lin}^-/\text{CD45}^+$ hematopoietic cells into hepatocytes in vivo

We examined the expression of transgenes in the liver of recipient mice seven days after partial hepatectomy and found GFP+ cells in the wall of blood vessels and along sinusoid in the liver lobules of Wt > Wt mice (Fig. 4A, lower column) and GFP > Wt mice

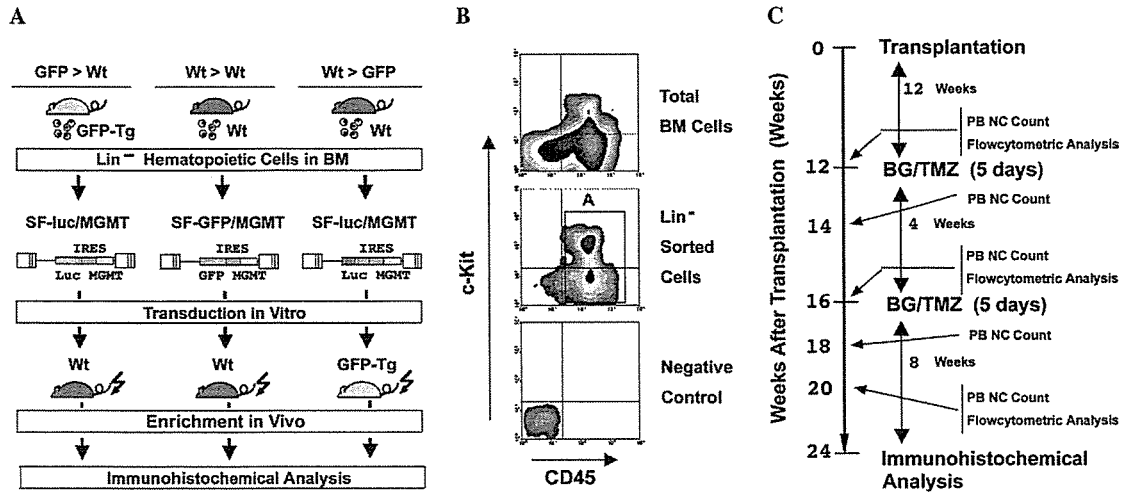


Fig. 2. (A) Schematic illustration of the experimental design. We used Lin⁻/CD45⁺ cells of GFP-Tg mice or wild-type mice as a source of donor HSCs for irradiated Wt recipients (GFP > Wt or Wt > Wt, respectively), or Lin⁻/CD45⁺ cells of Wt for GFP-Tg (Wt > GFP). We transduced these cells by retroviral vectors, SF-luc/MGMT and SF-GFP/MGMT, and transferred into recipient mice via tail vein. (B) Enrichment of Lin⁻/CD45⁺ hematopoietic cells. After enrichment of Lin⁻ cells, we stained and sorted cells with anti-c-kit and anti-CD45 monoclonal antibodies and separated CD45⁺ cells (region “A”). (C) Schematic illustration of the experimental design of in vivo enrichment of transduced hematopoietic cells.

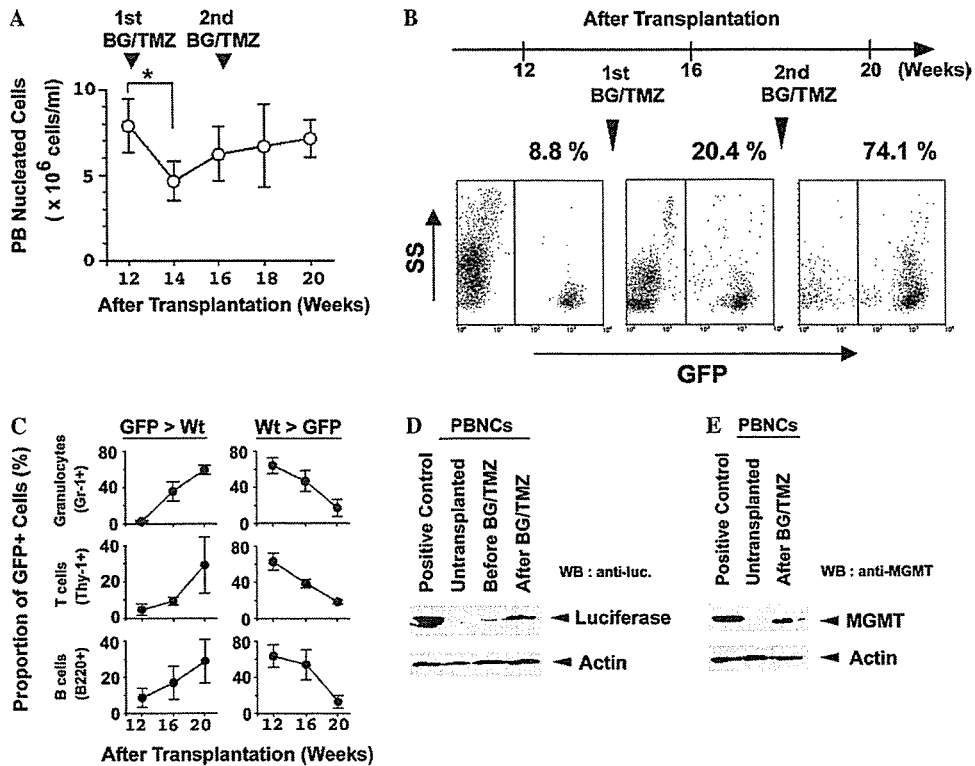


Fig. 3. In vivo enrichment of transduced hematopoietic cells. (A) PBNC counts during the treatment. Each circle and bar represents the mean value and standard deviation of 10 mice. *Statistically different ($P < 0.01$, unpaired Student *t*-test). (B) Proportion of transduced cells in PBNCs during the treatment. We examined the proportion of cells expressing GFP by a flow-cytometer. Data show one of representative mice. (C) Proportion of cells derived from donor mice in PBNCs during the treatment. We transduced Lin⁻/CD45⁺ cells of GFP-Tg or Wt mice by SF-luc/MGMT vector, transplanted into irradiated Wt or GFP-Tg mice (GFP > Wt or Wt > GFP, respectively) (A) and examined the proportion of cells expressing GFP in B220, Thy-1 or Gr-1-positive population by a flow-cytometer. Each circle and bar represents the mean value and standard deviation of three mice. (D) A luciferase protein and (E) a human MGMT protein in PBNCs of Wt > GFP mice were detected by Western blotting.

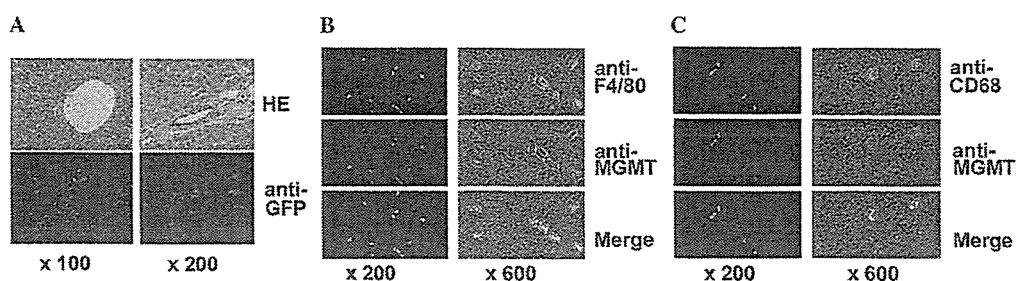


Fig. 4. Expression of a transgene in small cells along hepatic sinusoids in the liver of recipient mice (Wt > Wt). (A) Many small GFP+ cells were seen along hepatic sinusoids and several GFP+ cells in the wall of blood vessels (lower panels). The same slice of the liver was stained with hematoxylin–eosin (upper panels). (B) Expression of both F4/80 antigen and a transgene (MGMT) in small cells. (C) Expression of both CD68 and a transgene (MGMT) in small cells. [This figure appears in colour on the web].

(Fig. 5A). The latter cells were smaller than hepatocytes, were negative for endothelial marker, CD31 (data not shown), but positive for both a transgene, MGMT, and Kupffer cell markers, F4/80 [19] or CD68 [20] (Figs. 4B and C), indicating the expression of a transgene in Kupffer cells. To characterize the GFP+ cells in the liver more in detail, we stained the liver specimens of Wt > Wt mice with anti-MGMT and anti-albumin antibodies. While the small cells along sinusoid were positive for MGMT but not for albumin, a small number of large cells showed the immunoreactivity with both anti-GFP and anti-albumin antibodies (2–3 cells per one section, Fig. 5B). Thus, these large cells expressed both a donor-derived transgene, GFP, and a hepatocyte specific gene, albumin, although the proportion was very low (one cell per 10^5 – 10^6 hepatocytes). These results show that a FMEV-type vector provides an efficient transgene in the liver as well as HSCs in vivo.

To examine whether transduced HSCs transdifferentiate into albumin-positive cells or fuse with hepatocytes

in vivo, we examined liver specimens of Wt > GFP mice. In these specimens, we also found that the large cells in the hepatic lobule (2–3 cells per one section) expressed a transgene, MGMT (Fig. 5C, left column), or luciferase (Fig. 5C, right column) and that these cells were also GFP-positive (Fig. 5C, middle and lower column). We could not find the cells which were GFP-negative and expressed MGMT or luciferase. These results indicate that a donor-derived MGMT-positive or luciferase-positive cells fused with recipient hepatocytes which were GFP-positive and synthesize albumin. Although the vectors provide efficient expression of a transgene in the liver, we could not find the evidence of transdifferentiation but the fusion of hematopoietic cells with hepatocytes.

4. Discussion

In this report, we showed that a FMEV-type vector provides a transgene expression in HSCs highly enough

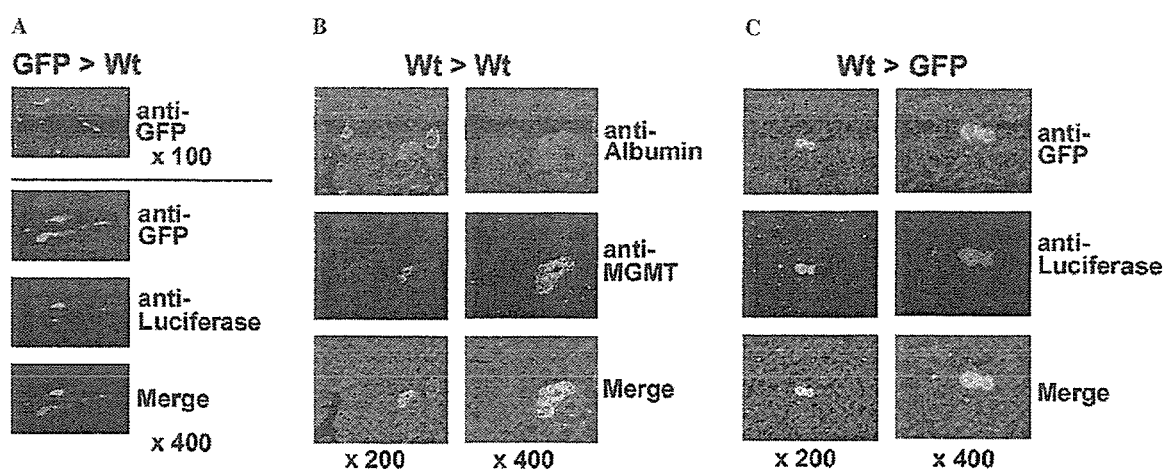


Fig. 5. Expression of transgenes in the liver. (A) Expression of a transgene, luciferase, in the liver of recipient mice (GFP > Wt). (B) Expression of a transgene, MGMT, in albumin-positive cells of recipient mice (Wt > Wt). (C) Expression of both a transgene (luciferase) and a recipient cell marker (GFP) in the liver of recipient mice (Wt > GFP). [This figure appears in colour on the web].

to protect them from a DNA-methylating drug. Thus, this type of vector would be useful for the protection of HSCs from intensive chemotherapy toward cancers, such as hepatocellular carcinoma, and also for the enrichment of gene-modified HSCs in vivo. We also showed that even after the successful replacement of hematopoietic cells with transduced cells, these cells did not efficiently transdifferentiate into hepatocytes, suggesting that they rescued the injured hepatocytes temporarily through the fusion. Although we could detect fused cells, the proportion of these cells in the liver was extremely low (one cell per 10^5 – 10^6 hepatocytes). Recent study also demonstrated that the proportion of fused cells is rare in murine immune-mediated hepatitis model [21]. The low frequency of the fusion would suggest little therapeutic value, although fused cells expressed not only hepatocyte specific genes such as albumin but also a transgene of MDR. Thus transfer of a MDR gene into HSCs would enable the protection from myelosuppression but not the liver damage. Fused cells also suggest the possibility of the fusion events of hepatocellular carcinoma cells with hematopoietic cells expressing a drug-resistant gene. Such a fused cell would become a malignant cell which would be highly drug-resistant. Thus it should be very careful to transfer a MDR gene into HSCs in hepatocellular carcinoma patients.

Over past years, studies have been shown that stem cells of hematopoietic lineage can also transdifferentiate into nonhematopoietic tissue including the liver [10,11]. In contrast, some studies demonstrated that this so-called “plasticity of HSCs” appears to be rare event [22] and HSCs only fuse with hepatocytes in murine chemical hepatitis models [23,24]. Particularly, myelomonocytic cells are the source of hepatocytes fusion partner in murine model [25,26]. Our data are in good accordance with these reports, confirming them by a different approach.

Recent studies have revealed that multipotent adult progenitor cells [27] or unrestricted somatic stem cells [28] differentiate into hepatocytes in vivo. Human mesenchymal stem cells have been reported to differentiate into hepatocytes without fusion [29]. These cells but not HSCs would be good candidates as a target to achieve efficient protection of the liver from intensive chemotherapy or gene-modified long-term cell therapy for the liver.

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The minimum number of clones necessary to sequence in order to obtain the maximum information about hepatitis C virus quasispecies: a comparison of subjects with and without liver cancer

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SUMMARY. Most studies of hepatitis C virus (HCV) quasispecies have reported the results of sequencing only three to five clones per sample. The possibility that sequencing so few clones might not provide a representative picture of the quasispecies present in a sample has never been evaluated. The present study was conducted to evaluate whether sequencing greater numbers of clones results in better information about the HCV quasispecies number and distribution, and to compare the HCV quasispecies in liver cancer cases and controls. RNA was extracted from serial serum samples from six subjects with HCV-associated liver cancer and 11 age- and sex-matched HCV-infected controls without liver cancer. The hypervariable region 1 (HVR1) of the HCV genome was amplified, cloned, and sequenced. For further studies of 12 serum samples from two liver

cancer cases and two matched controls, successive groups of 10 additional clones were sequenced up to a total of 50 clones per serum sample. When only 10 clones were sequenced from each specimen, no consistent differences were seen between the number of HCV quasispecies in the six liver cancer cases and the 11 controls. However, sequencing 40 clones from each of 12 samples from two liver cancer cases and two controls revealed a greater number of quasispecies in liver cancer cases than in controls. Testing an additional 10 clones (50 clones per sample) did not significantly increase the number of quasispecies detected.

Keywords: hepatitis C virus, hepatocellular carcinoma, quasispecies.

INTRODUCTION

One of the important characteristics of hepatitis C virus (HCV) is that its genome exhibits significant genetic heterogeneity as a result of the accumulation of mutations during viral replication. This high mutation rate, which is characteristic of RNA viruses, can be attributed to an error-prone RNA-dependent RNA polymerase that lacks proof-reading activity. HCV, like other RNA viruses, circulates in

an infected individual as a population of closely related, yet heterogeneous, quasispecies. The quasispecies population is generally composed of one dominant viral sequence (the master sequence) and a number of other sequences differing from the master sequence to various extents. The HCV hypervariable region 1 (HVR1) undergoes extensive variation during the course of chronic infection, and some of these changes may correspond to the emergence of immune-escape mutants, a proposed mechanism of HCV persistence.

In the present study, the genetic diversity of the HCV genome was evaluated in serum samples obtained prospectively in individuals with HCV infections who later developed liver cancer, and in matched control subjects. The impact of the number of clones sequenced on the number of quasispecies detected was further studied by sequential sampling of a large number of clones from selected serum samples.

Abbreviations: 5'-NCR, 5'-noncoding region; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HVR1, hypervariable region 1; PCR, polymerase chain reaction.

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