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Interaction of endothelial progenitor cells expressing cytosine deaminase in tumor tissues and 5-fluorocytosine administration suppresses growth of 5-fluorouracil-sensitive liver cancer in mice

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The drug delivery system to tumors is a critical factor in upregulating the effect of anticancer drugs and reducing adverse events. Recent studies indicated selective migration of bone marrow-derived endothelial progenitor cells (EPC) into tumor tissues. Cytosine deaminase (CD) transforms nontoxic 5-fluorocytosine (5-FC) into the highly toxic 5-fluorouracil (5-FU). We investigated the antitumor effect of a new CD/5-FC system with CD cDNA transfected EPC for hepatocellular carcinoma (HCC) in mice. We used human hepatoma cell lines (HuH-7, HLF, HAK1-B, KYN-2, KIM-1) and a rat EPC cell line (TR-BME-2). *Escherichia coli* CD cDNA was transfected into TR-BME-2 (CD-TR-BME). The inhibitory effect of 5-FU on the proliferation of hepatoma cell lines and the inhibitory effect of 5-FU secreted by CD-TR-BME and 5-FC on the proliferation of co-cultured hepatoma cells were evaluated by a tetrazolium-based assay. In mouse subcutaneous xenograft models of KYN-2 and HuH-7, CD-TR-BME was transplanted intravenously followed by 5-FC injection intraperitoneally. HuH-7 cells were the most sensitive to 5-FU and KYN-2 cells were the most resistant. CD-TR-BME secreted 5-FU and inhibited HuH-7 proliferation in a 5-FC dose-dependent manner. CD-TR-BME were recruited into the tumor tissues and some were incorporated into tumor vessels. Tumor growth of HuH-7 was significantly suppressed during 5-FC administration. No bodyweight loss, ALT abnormality or bone marrow suppression was observed. These findings suggest that our new CD/5-FC system with CD cDNA transfected EPC could be an effective and safe treatment for suppression of 5-FU-sensitive HCC growth. (*Cancer Sci* 2012; 103: 542–548)

In 1997, putative endothelial progenitor cells (EPC) were isolated from peripheral blood and shown to be incorporated into the vasculature in adults.[1] The EPC in adults originate from the bone marrow and selectively home to sites with ongoing vascular formation.[2,3]

Vascular development is essential for the growth of solid tumors.[4,5] Accumulating evidence suggests that circulating bone marrow-derived cells, including EPC, migrate into tumor-associated stroma to support vascular formation and tumor development.[6,7] Stromal-derived factor-1 (SDF-1) mainly recruits bone marrow-derived cells to tumor tissues from the peripheral circulation.[8]

Cytosine deaminase (CD) in bacteria and fungi are known to deaminate 5-fluorocytosine (5-FC) to the highly toxic 5-fluorouracil (5-FU).[9,10] Normal mammalian cells do not have CD and are relatively resistant to 5-FC. Gene transfer of *Escherichia coli* CD to mammalian cells renders these cells selectively sensitive to the toxic effects of 5-FC. In many reports of cancer gene therapy with this suicide gene/prodrug system, the most interesting property is the ''bystander effect'', the death of unmodified tumor cells by 5-FU secretion from CD cDNA transfected cells.[11] Most of these reports have demonstrated the efficacy of the suicide gene/prodrug system.

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the tropics and Far East, including Japan.[12] Hepatocellular carcinoma develops multifocally in the cirrhotic liver. Hepatocellular carcinoma is a highly angiogenic tumor, ultimately supplied with neoarteries in parallel with tumor development.[13,14] For advanced non-resectable HCC, chemotherapy with 5-FU is sometimes selected.[15,16] However, the therapeutic efficacy of 5-FU is not fully satisfactory due to liver dysfunction, leukocytopenia and thrombocytopenia caused by the associated liver cirrhosis. To improve the outcome of chemotherapy with 5-FU, it seems important to establish a new drug delivery system to supply a sufficient amount of 5-FU to HCC tissue only without severe adverse effects.

In the present study, we injected rat-derived endothelial progenitor cells, which were transfected with *E. coli* CD cDNA, and administered 5-FC to tumor-bearing mice to evaluate the antitumor effect of the new CD/5-FC system for HCC.

Materials and Methods

Reagents, cells and animals. HUVEC and human hepatoma cell lines (HuH-7, HLF) were obtained from CAMBREX Bio Science Walkersville Inc. (Walkersville, MD, USA). Human hepatoma cell lines (KYN-2, KIM-1, HAK1-B) were provided from the Department of Pathology, Kurume University School of Medicine (Kurume, Japan). TR-BME-2 cells, a cell line derived from rat bone marrow EPC were provided by the Department of Pharmaceutics, Keio University (Tokyo, Japan).[17] Male 5-week-old nude mice (BALB/c nu/nu, Kyudou KK, Fukuoka, Japan) were acclimatized and placed in separate cages. All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Institute of Health.[18] The experimental protocol was approved by the Laboratory Animal Care and Use Committee of Kurume University.

Plasmid construction and *in vitro* transfection. A retroviral vector, containing the entire coding sequence of the *E. coli* CD

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gene (pLXSP-CD), was provided by D. F. Condorelli (Department of Chemical Science, Section of Biochemistry and Molecular Biology, University of Catania, Catania, Italy). Transfection of plasmid was performed according to the report by Barresi *et al.*[19]

***In vitro* inhibition of cell proliferation by addition of 5-FU.** Approximately 1000 HUVEC and TR-BME-2 cells transfected with CD cDNA (CD-TR-BME) were added with EGM-2 (Clonetics, San Diego, CA, USA) supplemented with 5% FBS to 96-well plates coated with human fibronectin (Gibco Invitrogen Co., Grand Island, NY, USA) and type 1 collagen (Gibco Invitrogen Co.). Then, 1×10^3 HuH-7, HLF, KIM-1, KYN-2 and HAK1-B in DMEM (Gibco Invitrogen Co.) supplemented with 10% FBS were added. HUVEC, CD-TR-BME cells and human hepatoma cells were incubated at 37°C. After 24 h, 5-FU at 0, 5, 10, 50, 100, 500 or 1000 ng/mL was added to the medium and incubated for 72 h. The cytotoxicity was then evaluated by a tetrazolium-based assay (Cell Count Reagent SF; Nakalai Tesque Inc., Kyoto, Japan).

5-Fluorouracil production by CD-TR-BME cells after the addition of 5-FC. Next, 5×10^4 CD-TR-BME cells were cultured with 1 mL of EBM-2 supplemented with 5% FBS for 24 h at 37°C. Then, 5-FC at 0, 1, 10, 100 or 1000 μg/mL was added to the medium and incubated for 72 h. The concentration of 5-FU in the media was measured using HPLC.

***In vitro* "bystander effect" experiment.** Next, 5×10^3 hepatoma cells (HuH-7, HuH-7, HLF, KIM-1, KYN-2, HAK1-B) were cultured with DMEM supplemented with 10% FBS for 24 h. After confirming that CD-TR-BME cells could not migrate through a 2-μm pore size filter of Chemotaxicell cell-culture chambers (Kurabo Inc., Osaka, Japan), CD-TR-BME cells (5×10^4) were cultured in the chambers with EBM-2 medium containing 5% FBS for 24 h at 37°C. The chambers with the CD-TR-BME cells were then co-cultured with hepatoma cells with EBM-2 medium containing 5% FBS and 5-FC for 72 h. The cytotoxicity against the hepatoma cells was evaluated by a tetrazolium-based assay.

Protocols of treatment with a combination of CD-TR-BME cells and 5-FC. Male nude mice were injected subcutaneously with 5×10^6 HuH-7 cells or KYN-2 cells. After the tumor volume reached 50 mm^3, the mice were divided at random into three groups: the PBS-treated group; the CD-TR-BME cell-injected group; and the CD-TR-BME cells treated with 5-FC group, respectively. After tumor formation, mice of the CD-TR-BME and CD-TR-BME + 5-FC groups received injections of 100 μL of PBS containing 1×10^6 CD-TR-BME cells via the tail vein for 5 days. Mice of the CD-TR-BME + 5-FC group received an intraperitoneal injection of 500 mg/kg of 5-FC for 10 days. All mice were then bred without any treatment for 7 days. Tumor size was measured by calipers in two dimensions every 3 days. The mice were killed at day 21. Tumor volume was calculated by the following equation: length $\times$ width^2 $\times$ 0.52.

Total RNA extraction and RT-PCR. For total RNA isolation, CD-TR-BME cells or 100 mg of tumor tissues were extracted with Isogen (Nippon Gene, Tokyo, Japan). cDNA was synthesized using 2 μg total RNA. The 20-μL RT reaction consisted of 5× first strand buffer, 0.5 mM dNTP, 50 nM random primers and 20 U SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The RNA and primers were mixed and denatured by heating at 70°C for 10 min; the reverse transcription reaction mixture was then incubated for 30 min at 50°C, followed by 15 min at 70°C. The resulting cDNA was amplified by PCR with primer pairs specific for CD, SDF-1, CXCR4 and GAPDH (Table 1). The PCR products were resolved in 1.5% agarose gels and visualized by ethidium bromide staining and ultraviolet trans-illumination.

Migration of CD-TR-BME cells to tumor tissue and confocal laser scanning microscopy. The CD-TR-BME cells were labeled with PKH26-red (Sigma Chemical Co., St Louis, MO, USA).

Table 1. Primers used in RT-PCR

Gene	Annealing T (°C)	Primer sequences	PCR products (bp)
CD	54	5′-GGA GGCTAACAATGTCGAAT 3′-ATGTTTGCAACTTGCTGACC	1302
Murine SDF-1	60	5′-GGACGCCAAGGTCGTCGCCGTG 3′-TTGCATCTCCCACCCATGTCAG	335
Rat CXCR4	54	5′-ATGGGTTGGTAATCCTGGTC 3′-AGAGTAGGACCGGAAGTAGT	224
GAPDH	60	5′-ACCACAGTCCATGCCATCAC 3′-ATGTCGTTGTCCCACCACCT	452

CD, cytosine deaminase; SDF-1, stromal cell-derived factor-1.

Sections of tumor tissues were fixed with acetone and incubated overnight with rat anti-mouse CD 31 antibody (Research Diagnostics Inc., Flanders, NJ, USA) or rabbit anti-human SDF-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4°C. The sections were then incubated with FITC-conjugated anti-rabbit IgG (DAKO Japan Inc., Kyoto, Japan) or FITC-conjugated anti-rat IgG (CHEMICON INTERNATIONAL, Temecula, CA, USA) for 30 min with TO-PRO-3 iodide (Invitrogen) for nuclei labeling at room temperature. Each incubation was followed by three washes with PBS. Four color imaging was performed (Z-series, 63× oil magnification, Zeiss LSM 510-Meta Confocal Microscope; Carl Zeiss Inc., Jena, Germany). Two independent hepatologists counted the number of CD31-positive vessels of tumor tissues obtained from mice treated with PBS at day 15 (n = 6), only CD-TR-BME cells at day 15 (n = 6) and CD-TR-BME cells plus 5-FC at day 8 (n = 6) and day 15 (n = 6). In each group, 30 random fields were selected blindly.

Tissue and serum 5-FU concentrations and serum α-fetoprotein (AFP) levels. At days 8 and 15 of the experiment, tumor tissues of HuH-7 cells and sera were collected from tumor-bearing mice injected with CD-TR-BME cells and treated with 5-FC (n = 6). Then, 1 g of wet tumor tissue was homogenized with 1 mL of PBS. The 5-FU concentrations in the tumor tissues and sera were measured on days 8 and 15 using HPLC. In addition, to measure the serum AFP levels, tumor-bearing mice treated with CD-TR-BME cells (n = 6) and mice injected with CD-TR-BME cells and treated with 5-FC (n = 6) were killed at day 15.

Alanine aminotransferase (ALT) levels, leukocytes, hemoglobin (Hb), platelet and bodyweight. Serum ALT activity was measured using a standard UV method. Bodyweight, peripheral leukocyte count, Hb level and platelet count were also measured. The above parameters were measured at day 15.

Statistical analysis. Data were expressed as mean ± SD. Differences between groups were examined for statistical significance using the Mann–Whitney U-test and the Kruskal–Wallis rank test. A P-value <0.05 denoted the presence of a statistically significant difference.

Results

5-Fluorouracil inhibits cell proliferation *in vitro*. 5-Fluorouracil inhibited the proliferation of HUVEC (IC_{50}, 100.4 ng/mL) and CD-TR-BME (IC_{50}, 99.8 ng/mL) in a dose-dependent manner. However, the proliferation of HUVEC and CD-TR-BME cells was not inhibited in the presence of up to 50 ng/mL of 5-FU concentration (Fig. 1A,B). In five hepatoma cell lines, HuH-7 (IC_{50}, 10.1 ng/mL) was the most sensitive to 5-FU, followed by HAK1-B (IC_{50}, 100 ng/mL), HLF (IC_{50}, 100.4 ng/mL), KIM-1 (IC_{50}, 449.7 ng/mL) and KYN-2 (IC_{50}, 449.8 ng/mL).

CD-TR-BME cells produce 5-FU after addition of 5-FC. CD-TR-BME cells secreted 5-FU into the media and the production level was 5-FC dose dependent (Fig. 1C). After 72 h, the final

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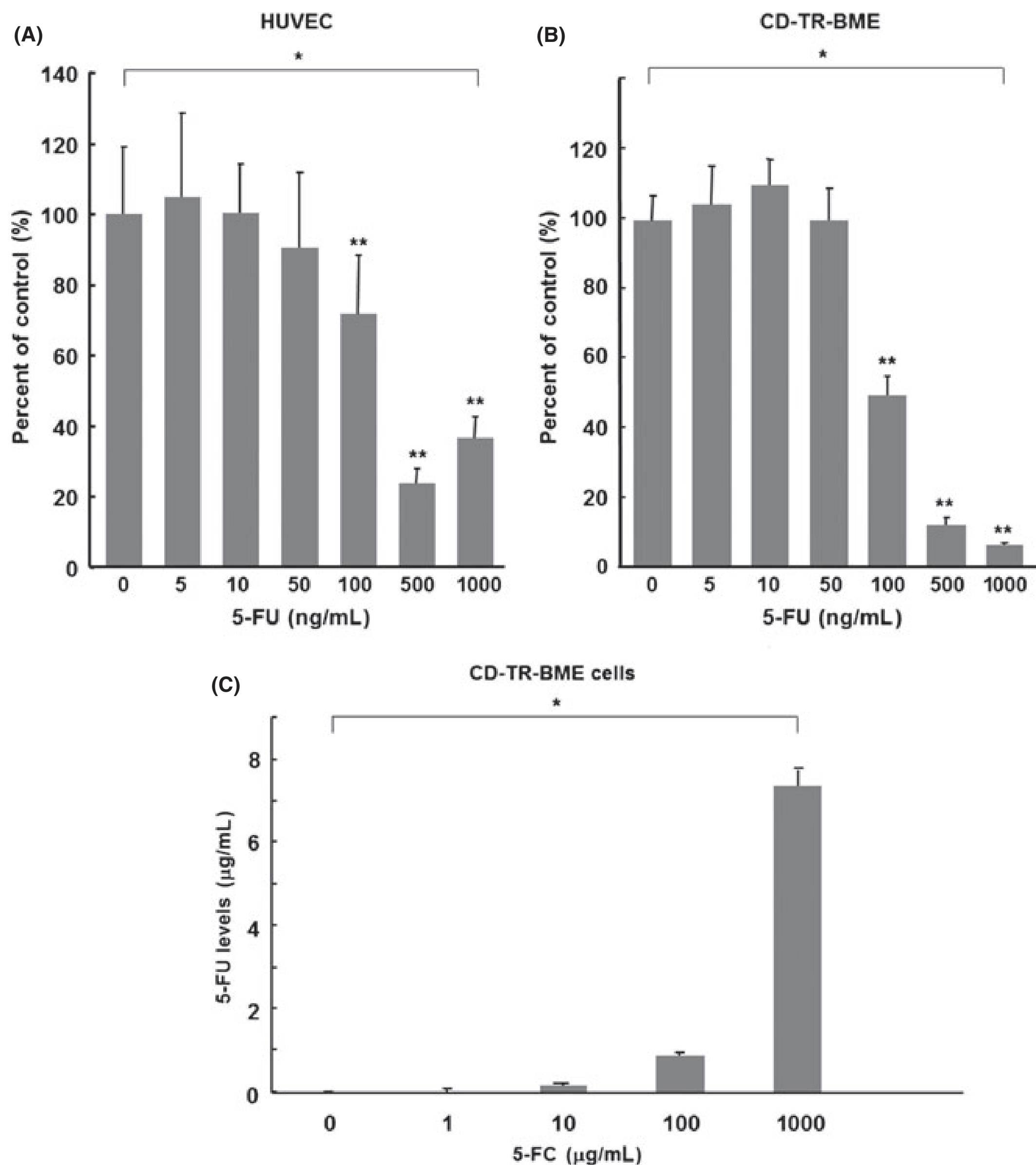

Fig. 1. Inhibition of cell proliferation by 5-fluorouracil (5-FU). (A) HUVEC, (B) CD-TR-BME. These cells were cultured with 10 mL of media containing 0–1000 ng/mL of 5-FU for 72 h. Cell proliferation was evaluated using a tetrazolium-based assay. Data are expressed relative to the control (n = 12). *P < 0.0001, using the Kruskal–Wallis test. **P < 0.0001, compared with the control group, using the Mann–Whitney U-test. (C) Secretion of 5-FU by CD-TR-BME cells with 5-fluorocytosine (5-FC). CD-TR-BME cells were cultured with 1 mL of the media containing 0–1000 mg/mL of 5-FC for 72 h. The concentration of 5-FU was measured. Data are expressed as mean ± SD of 12 samples. *P < 0.0001, using the Kruskal–Wallis test.

concentration of 5-FU secreted by the CD-TR-BME cells with 5-FC into the media was 7.4 ± 0.4 μg/mL.

"Bystander effect" of CD-TR-BME cells *in vitro*. After culture of the hepatoma cells with the chambers containing CD-TR-BME cells, the media significantly inhibited the proliferation of hepatoma cells in a 5-FC dose-dependent manner. HuH-7 was the most sensitive to 5-FC (IC_{50}, 0.89 μg/mL), followed by HAK1-B (IC_{50}, 10.3 μg/mL), KIM-1 (IC_{50}, 10.7 μg/mL), HLF (IC_{50}, 11.0 μg/mL) and KYN-2 (IC_{50}, 100.5 μg/mL) (Fig. 2). Proliferation of CD-TR-BME cells was suppressed in a 5-FC dose-dependent manner (data not shown). However, the proliferation of TR-BME cells was not suppressed with the addition of up to 1000 μg/mL 5-FC (data not shown).

Combination treatment with CD-TR-BME cells and 5-FC for a HuH-7 or KYN-2 cell xenograft model. HuH-7, the most sensitive to 5-FU, and KYN-2 cells, the most resistant, were used for *in vivo* experiments. In the HuH-7 cell xenograft model, at days 0, 3 and 6 of the initial treatment, there was no significant difference in tumor volume among the three groups. Since then, tumor volumes of the PBS-treated and CD-TR-BME groups continued to increase until day 21. From days 9 to 15, tumor growth in the CD-TR-BME + 5-FC mice was significantly suppressed compared with those of the other two groups. After completion of the 5-FC treatment, tumor volumes of CD-TR-BME + 5-FC mice started to increase rapidly and there was no significant difference in tumor volume of the three groups at day 21 (Fig. 3A,B). Serum AFP levels of the CD-TR-BME and CD-TR-BME + 5-FC mice at day 15 were 413 843 ± 203 129 and 113 436 ± 47 910 ng/mL, respectively. However, in the KYN-2 xenograft model, there was no significant difference in tumor volume among the three groups (Fig. 3C).

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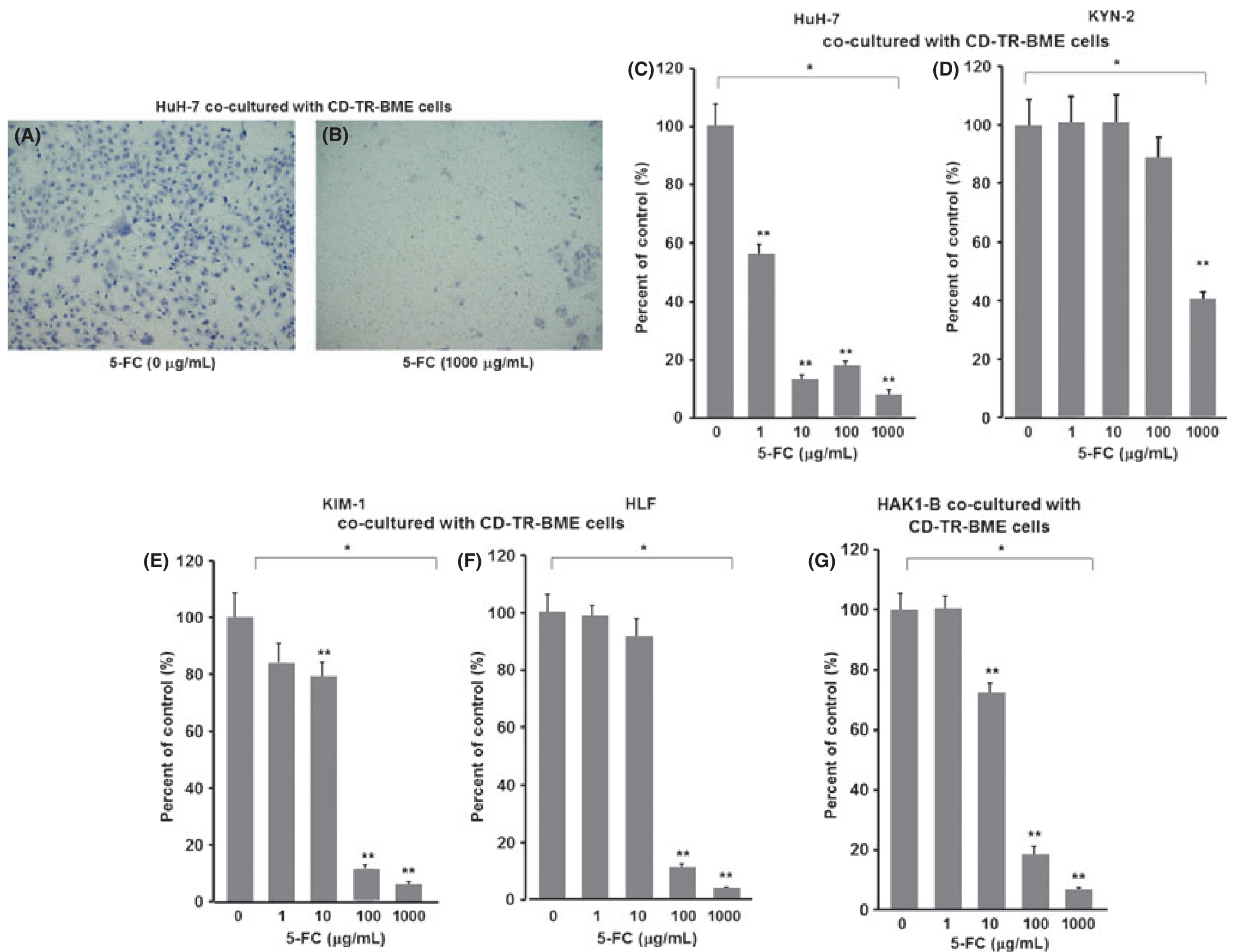

Fig. 2. Bystander effect of CD-TR-BME cells with 5-fluorocytosine (5-FC). Hepatoma cells were cultured in media containing 0–1000 mg/mL of 5-FC for 72 h with CD-TR-BME cells in chemotaxicell cell-culture chambers. (A) HuH-7 cells cultured with CD-TR-BME cells without 5-FC. (B) HuH-7 cells cultured with CD-TR-BME cells with 1000 mg/mL of 5-FC. (C–G) Cell proliferation was evaluated using a tetrazolium-based assay. Data are expressed relative to the control (n = 12) (C, HuH-7; D, KYN-2; E, KIM-1; F, HLF; G, HAK1-B). **P* < 0.0001, using the Kruskal–Wallis test. ***P* < 0.0001, compared with the control group, using the Mann–Whitney *U*-test.

Migration of CD-TR-BME cells to tumor tissues and vascular density in tumor tissues. RT-PCR analysis showed SDF-1 expression in tumor tissues of HuH-7 cells and CXCR4 expression in the CD-TR-BME cells (Fig. 4A,B). Immunohistochemical analysis showed recruitment of the injected CD-TR-BME cells close to the SDF-1-expressing hepatoma cells (Fig. 4C). Examination at a higher magnification showed the incorporation of some CD-TR-BME cells into new blood vessels within the tumor tissues (Fig. 4D). However, most of these cells were localized in the interstitial tissues around the vessels in the tumor tissues (Fig. 4E). At 200-fold magnification, the numbers of CD31-positive vessels in tumor tissues of mice treated with PBS on day 15, CD-TR-BME cells on day 15 and CD-TR-BME cells plus 5-FC on days 8 and 15 were 7.2 ± 2.1, 7.2 ± 2.1, 6.9 ± 0.9 and 7.1 ± 2.1, respectively. There was no significant difference in vessel density among the groups.

5-Fluorouracil concentration in tumor tissues and sera. The 5-FU concentrations in the tumor tissues of HuH-7 cells at days 8 and 15 were 25.2 ± 14.2 and 22.4 ± 12.4 ng/mL, respectively. In all but one sample, serum 5-FU levels were not detected at days 8 and 15. Serum 5-FU concentrations of samples with detectable levels at days 8 and 15 were 8.4 and 7.3 ng/mL, respectively. These cases showed the highest tissue 5-FU concentrations at days 8 and 15, respectively.

Effect of CD-TR-BME and 5-FC treatment on leukocyte count, Hb, platelet count, serum ALT and bodyweight. Leukocyte count, Hb level and platelet count of CD-TR-BME-injected mice and CD-TR-BME + 5-FC-treated mice were 1600 ± 352/μL, 13.2 ± 1.9 g/dL, 46.3 ± 21.7 × 10^4/μL and 1800 ± 593/μL, 13.9 ± 1.2 g/dL, 48.8 ± 22.9 × 10^4/μL, respectively. Serum ALT levels of CD-TR-BME-injected mice and CD-TR-BME + 5-FC-treated mice were 38.5 ± 8.9 and 41.2 ± 7.8 U/L, respectively. The bodyweights of CD-TR-BME-injected mice and CD-TR-BME + 5-FC-treated mice were 25.2 ± 2.4 and 26.2 ± 2.9 g, respectively. There were no significant differences in leukocyte count, Hb level, platelet count, serum ALT level and bodyweight between CD-TR-BME-injected mice and CD-TR-BME + 5-FC-treated mice.

Discussion

In the *in vitro* study, HuH-7 cells were the most sensitive to 5-FU among five tested hepatoma cell lines. Furthermore, HuH-7 was more sensitive to 5-FU than CD-TR-BME cells and

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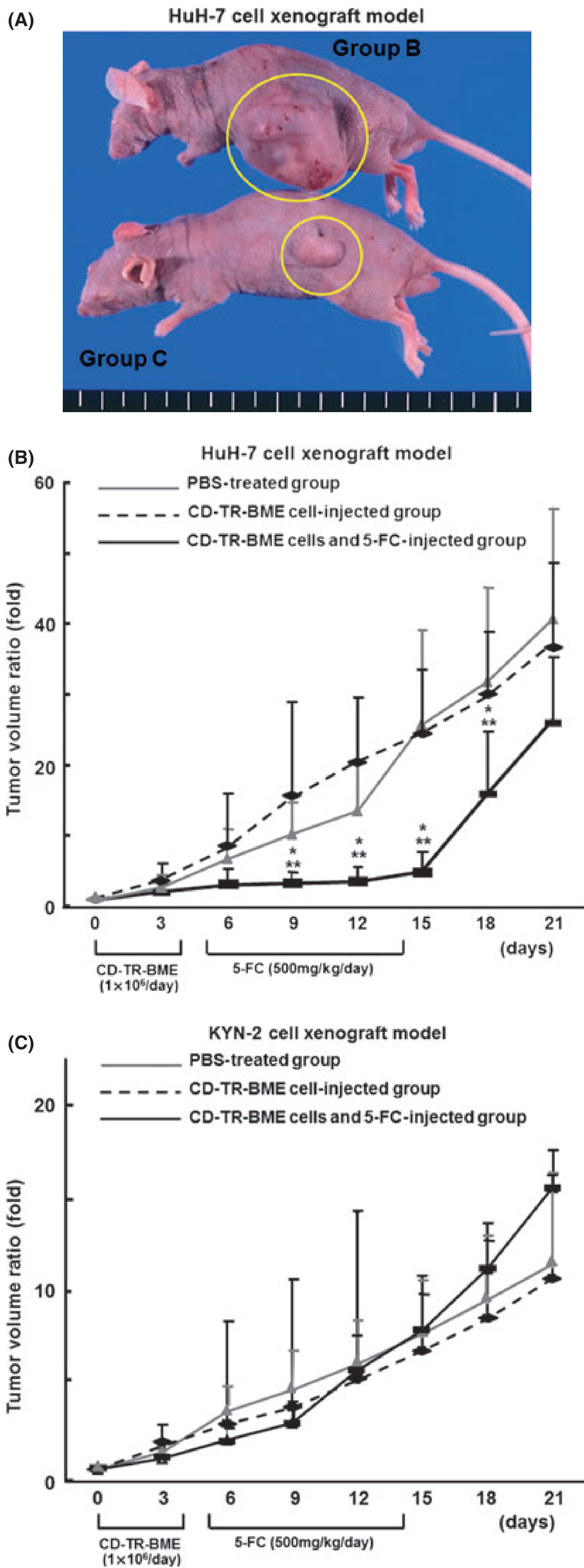

Fig. 3. Treatment of subcutaneous tumor with a combination of CD-TR-BME cells and 5-fluorocytosine (5-FC). (A) Tumors of HuH-7 cells at day 21. Group B, CD-TR-BME cells; Group C, CD-TR-BME cells + 5-FC. (B) Time-course observation of tumor volumes (HuH-7). Data are expressed relative to the control (day 0) ($n = 6$). *$P < 0.05$, compared with the CD-TR-BME cell-injected group, using the Mann–Whitney *U*-test. **$P < 0.05$, compared with PBS-treated cells, using the Mann–Whitney *U*-test. (C) Time-course observation of tumor volumes in the dorsal portion of mice (KYN-2). Data are expressed relative to the control (day 0) ($n = 6$).

HUVEC. The CD-TR-BME cells produced 5-FU into the media in a 5-FC dose-dependent manner. 5-Fluorocytosine and 5-FU are small and highly water-soluble molecules; they penetrate well into most body sites and do not require cell–cell contact for intercellular transfer.[20] CD-TR-BME cells seem to incorporate 5-FC into the cytoplasm, then produce and secrete 5-FU into the media without cell damage at relatively low concentrations of 5-FC. At higher concentrations of 5-FC, high concentrations of 5-FU in CD-TR-BME cells induce their apoptosis.

In the process of EPC homing from the bone marrow into the tumor microenvironment, vascular endothelial growth factor (VEGF) and SDF-1 undoubtedly play critical roles through specific interactions with CXCR4 and VEGFR-1 and VEGFR-2, respectively.[8,21] Recent data show that among these cytokines, VEGF mainly induces mobilization of EPC from the bone marrow into the circulation while SDF-1 recruits EPC to the tumor tissues.[22–31] In the present study, tumor tissues expressed SDF-1 and CD-TR-BME cells expressed CXCR4. Injected CD-TR-BME cells migrated to tumor tissues. Of course, there seems to be a possibility that some of the injected CD-TR-BME cells have migrated to other tissues such as non-cancerous liver tissue and bone marrow. Tamura *et al.*[23] reported that intravenously injected TR-BME cells were homed to tumor tissue with significantly higher specificity. Injected CD-TR-BME cells seemed to migrate mainly to tumor tissues through SDF-1 and CXCR4 interaction.

Interestingly, some of the injected CD-TR-BME cells were incorporated into vascular formation and others were distributed in the interstitial space of tumor tissue. Furthermore, intravenous injection of CD-TR-BME cells did not increase the vascular density in tumor tissues. These distribution patterns were also observed in tumor tissues with the injection of wild type EPC in our preliminary study. Another group reported that only some of the systemically injected EPC were incorporated into tumor vessels.[8] A wide range of cell types including endothelial cells were shown to transdifferentiate into tumor-associated-fibroblasts (TAF).[24] Residual CD-TR-BME cells might migrate to the interstitial space due to the enhanced permeability of tumor vessels and transdifferentiate into TAF.[25] In general, tumor tissues produce VEGF and SDF-1 to recruit an appropriate number of bone marrow-derived EPC for vasculogenesis.[2,4] We might have injected too many CD-TR-BME cells than what was required for vasculogenesis.

The concentrations of 5-FU in tumor tissues on days 8 and 15 of the treatment were 25.4 ± 13.0 and 22.4 ± 12.4 ng/g wet tissue, respectively. Serum 5-FU was not detected in any of the samples except for one on days 8 and 15, respectively. As the CD-TR-BME cells and EPC selectively migrate to tumor tissues, we assume that 5-FU was selectively produced by the CD-TR-BME cells in tumor tissues. Therefore, no adverse effects were observed. Treatment with 5-FC after CD-TR-BME cell injection did not reduce the vascular density in tumor tissues. In the *in vitro* study, proliferation of HUVEC and CD-TR-BME cells was not suppressed in the presence of up to 50 ng/mL of 5-FU, while proliferation of HuH-7 cells was suppressed by 36 and 52% at 10 and 50 ng/mL of 5-FU, respectively. The CD/5-FC system mainly suppressed tumor growth of HuH-7 cells in

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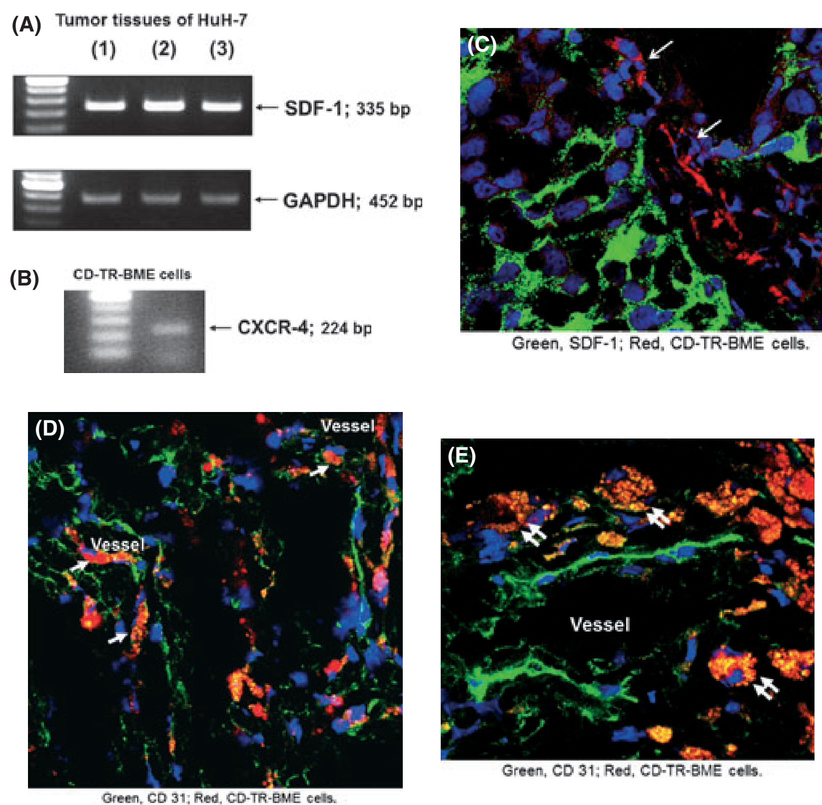

Fig. 4. Migration of CD-TR-BME cells to tumor tissues. (A) Expression of stromal-derived factor-1 (SDF-1) transcripts in tumor tissues (RT-PCR). Lanes 1–3, representative tumor tissues of HuH-7 cells. (B) Expression of CXCR4 transcripts in CD-TR-BME cells (RT-PCR). (C–E) Immunostaining of SDF-1 (green) and CD31 (green) with nuclear staining (blue) in tumor tissues of mice injected with CD-TR-BME cells labeled with red fluorescent marker PKH26-red. CD-TR-BME cells (red) (arrows) were recruited to tumor tissue close to SDF-1-expressing hepatoma cells (green) (C). CD-TR-BME cells (red) were incorporated into vessel walls (arrows) (D) or distributed within stromal tissue near a blood vessel (double arrows) (E).

the xenograft model by inhibiting tumor cell proliferation rather than an anti-angiogenic effect. However, The CD/5-FC system seems to be unable to maintain enough concentration of 5-FU to suppress the tumor growth of KYN-2 cells, which are 5-FU resistant. In the *in vitro* study, HuH-7 cells were more sensitive to 5-FU than KYN-2 cells. Since cell proliferation of KYN-2 cells was not suppressed in the presence of up to 100 ng/mL of 5-FU, the 5-FU concentration in tumor tissues seemed too low to suppress the tumor growth of KYN-2 cells *in vivo*. Miller *et al.*[26] reported that inherent 5-FU sensitivity is an important factor in determining efficacy of the CD/5-FC system. These findings indicate that diffusible 5-FU from CD-TR-BME cells in tumor tissues is at an optimal concentration to suppress the proliferation of HuH-7 cells. Furthermore, as the concentration of 5-FU in tumor tissues on day 15 of the treatment was maintained similar to that on day 8, the number of injected CD-TR-BME cells in tumor tissues did not seem to be decreased by the 5-FU produced by these cells.

Our CD/5-FC system with carrier cells enabled gene delivery to tumor tissues and repeated treatment by the escape from anti-vector immunity. However, for clinical application, the following issues must be resolved. First, the injected cells must be improved for more selective migration to tumor tissues. To improve selective migration to tumor tissues, gene transfection of CXCR4 or VEGF receptor (VEGFR) to CD-TR-BME cells might be necessary. Second, production of 5-FU in tumor tissues must be upregulated. To upregulate 5-FU production in tumor tissues, it might be necessary to increase the number of injected CD-TR-BME cells or to transfect more CD cDNA to TR-BME cells. However, high concentrations of 5-FU in tumor tissues induced apoptosis of CD-TR-BME cells as well as tumor cells. Repeated injections of CD-TR-BME cells will be required. The other possible way is to use a replication-competent oncolytic virus as the vector.[27,28] The replication-competent virus vector will be able to transfect CD cDNA to tumor cells and other kinds of cells in tumor tissues and upregulate 5-FU production. Third, tumor cell proliferation by injected CD-TR-BME cells must be inhibited. There were no significant differences in tumor volume and vascular density between the non-treated tumor group and the CD-TR-BME cell-injected tumor group. Furthermore, Sasajima *et al.*[29] reported that injection of vascular proangiogenic cells caused vascular remodeling and delay of tumor growth as well as a reduction of factors involved in drug resistance. As EPC produce VEGF, EGF, TGF-α and HGF,[30,31] these growth factors might stimulate tumor cell proliferation and angiogenesis.

In conclusion, we have demonstrated that injected CD cDNA-transfected endothelial progenitor cell lineage migrated to the tumor tissues of hepatoma cells and suppressed tumor growth by producing 5-FU in tumor tissues with intraperitoneal 5-FC injection. Our CD/5-FC system did not cause any severe adverse effects, suggesting that selective 5-FU production in tumor tissues with the character of EPC to home to tumor tissues might be a suitable strategy in the treatment of human HCC.

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Disclosure Statement

The authors have no commercial affiliations and no financial relationships to disclose.

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