Metronomic S-1 potently inhibited tumor growth compared with MTD S-1 ($P < .01$). The mean tumor volumes were 4810.5 ± 1440.9 cm^3 in the control group, 3212.6 ± 1364.7 cm^3 in the MTD S-1 group, 1927.1 ± 652.9 cm^3 in the metronomic S-1 group, and 2331.4 ± 662.1 cm^3 in the vandetanib group, respectively. The mean tumor volumes in the MTD S-1 plus vandetanib group and metronomic S-1 plus vandetanib group were 2026.7 ± 1106.7 and 1383.7 ± 697.5 cm^3, respectively. The greatest inhibition of tumor growth was induced by the metronomic S-1 in combination with vandetanib (Figure 3*A*). In addition, we evaluated toxicity in each of Huh-7 subcutaneous tumor treatment groups (Figure 3, *B–D*). In leukocyte count, there were no significant differences in the groups (Figure 3*B*). In Hb concentration, the control group was 12.84 ± 1.82 g/dl, the MTD S-1 group was 9.77 ± 3.63 g/dl, the metronomic S-1 group was 11.73 ± 3.27 g/dl, and the vandetanib group was 12.34 ± 2.77 g/dl. For the combination treatments, the MTD S-1 plus vandetanib group was 8.24 ± 1.64 g/dl, and for the metronomic S-1 plus vandetanib group, it was 11.74 ± 1.55 g/dl (Figure 3*C*). With respect to rate of body weight loss, in the MTD S-1 monotherapy and MTD S-1 with vandetanib groups, the values observed were 10.48% ± 6.85% and 8.59% ± 5.02% reduction compared with the control group, respectively. Vandetanib, metronomic S-1, and the combination therapy resulted in 5.64% ± 4.23%, 3.04% ± 2.23%, and −0.51% ± 5.56% reduction compared with the control group, respectively (Figure 3*D*). Both the MTD S-1 and MTD S-1 plus vandetanib treatment groups experienced severe body weight loss and reduced Hb concentrations compared with the control group (Figure 3, *C* and *D*). In marked contrast, the metronomic S-1 monotherapy and metronomic S-1 with vandetanib groups did not manifest any overt toxicity (Figure 3, *B–D*).

Evaluation of Antitumor Efficacy Using Metronomic S-1 Chemotherapy in an Orthotopic Liver Transplant Model

For tumor volume assessments, all treatments except MTD S-1 monotherapy were effective compared with the control group (Figure 4*A*). Tumor volumes at sacrifice were 4186.0 ± 1128.0 cm^3 in the control group, 3259.0 ± 788.7 cm^3 in the MTD S-1 group, 1501.3 ± 1002.2 cm^3 in the metronomic S-1 group, and 1582.0 ± 354.9 cm^3 in the vandetanib group. There was a significant difference between metronomic S-1 and MTD S-1 in tumor growth inhibition ($P < .05$; Figure 4*A*). For the combination treatment groups, tumor volumes were 931.1 ± 331.7 cm^3 in the MTD S-1 plus vandetanib group and 875.0 ± 369.4 cm^3 in the metronomic S-1 plus vandetanib group. There was no significant difference between the metronomic S-1 plus vandetanib group and the MTD S-1 plus vandetanib group. However, the greatest inhibition of tumor growth was detected in the metronomic S-1 plus vandetanib treatment group ($P < .001$; Figure 4*A*).

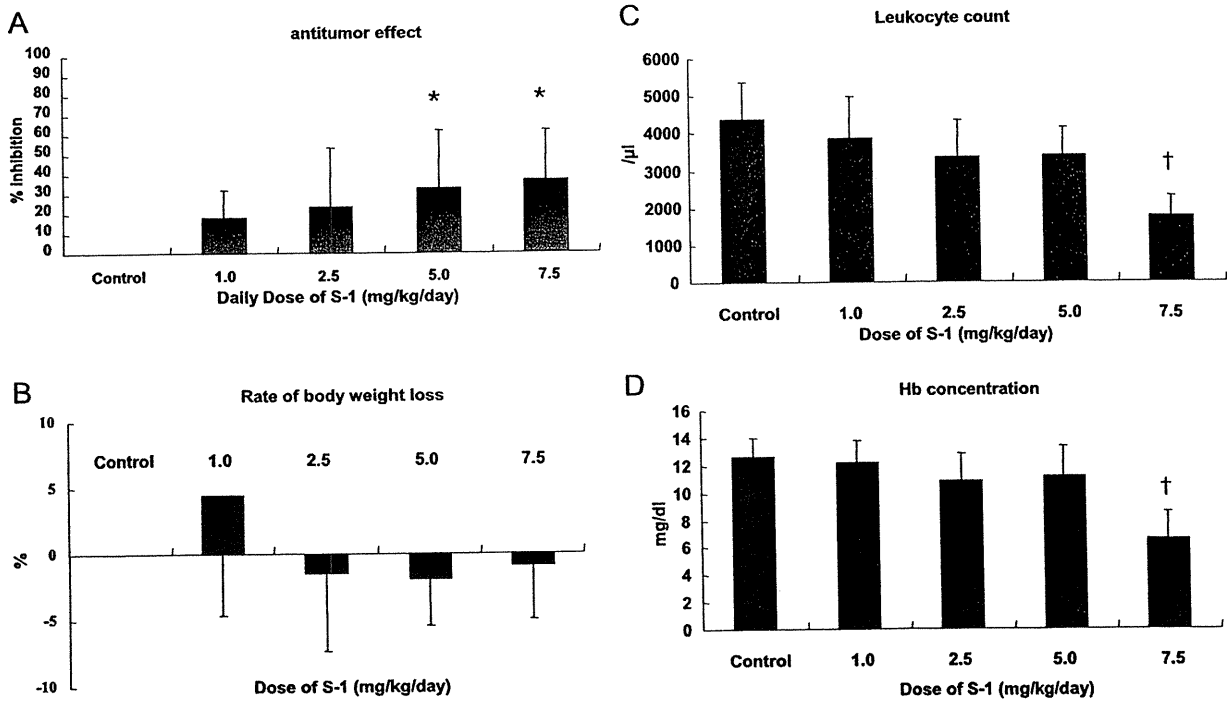


Figure 2. Determination of the optimal dose of S-1 in metronomic chemotherapy. Huh-7 subcutaneous tumor models were treated daily with either HPMC or different metronomic doses of S-1 (1.0, 2.5, 5.0, or 7.5 mg/kg per day) for 14 consecutive days. (A) Inhibition rates of tumor volumes (%) are expressed as mean ± SD ($n = 10$ per group). Dosages of 5.0 and 7.5 mg/kg per day S-1 groups statistically inhibited tumor growth compared with the control group (*$P < .05$). (B–D) Toxicity parameters are represented as mean ± SD. (B) Body weight (BW) changes on killing were calculated according to the following formula: BW change (%) = [(BW on sacrifice) − (BW on day 0)] × 100. (C) Hb concentration. (D) Leukocyte count. Each different dose of S-1 did not show body weight loss. However, the only 7.5-mg/kg-per-day S-1 group represented severe myelosuppression, such as decreased Hb concentration or leukocyte count. †$P < .001$ by compared with the control group.

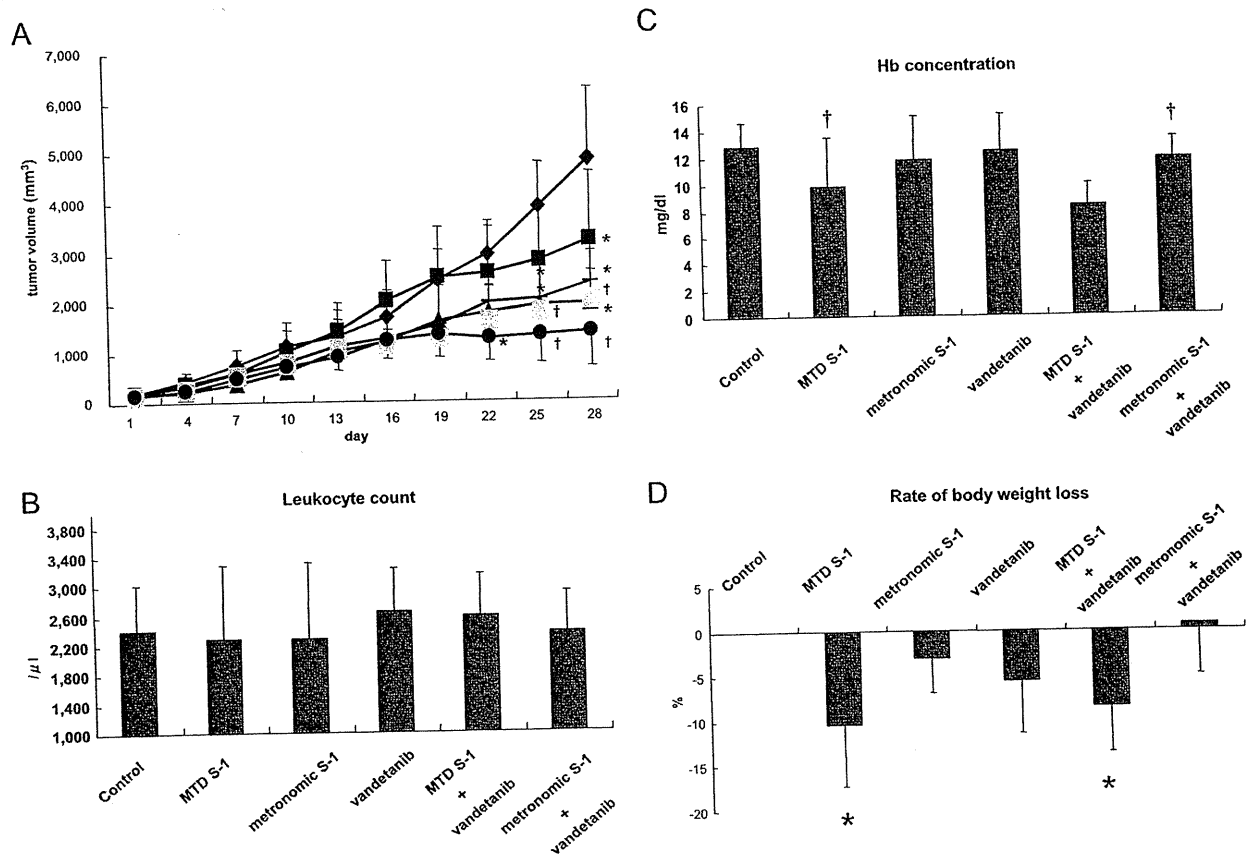


Figure 3. Therapeutic effects of metronomic S-1 chemotherapy in the Huh-7 subcutaneous tumor transplant model. (A) Tumor-bearing nude mice (n = 10 per group) were treated in the following six groups: 1) HPMC as the control group (blue); 2) MTD S-1 15 mg/kg per day for 1 week, followed by a 1-week break period (purple); 3) metronomic S-1 5 mg/kg per day for 2 weeks without break period (green); 4) vandetanib 25 mg/kg per day for 2 weeks (red); 5) MTD S-1 with vandetanib (yellow); or 6) metronomic S-1 with vandetanib (black). All treatments were performed for 4 weeks in total. Tumor volume changes are expressed as mean ± SD. All treatments showed efficacy compared with the control group (*P < .05), and the metronomic S-1 therapy was more effective than the MTD S-1 treatment. The metronomic S-1 with vandetanib significantly inhibited tumor growth compared with the control group (†P < .001). (B–D) Toxicity parameters are expressed as mean ± SD. (B) Hb concentration. (C) Leukocyte count. (D) Rate of body weight loss. MTD S-1 and the MTD S-1 with vandetanib showed severe body weight loss (*P < .01) and decreased Hb concentration (†P < .05) compared with the control group. Metronomic S-1 and the metronomic S-1 with vandetanib did not show any overt toxicities.

Evaluation of Survival Using Metronomic S-1 Chemotherapy in an Orthotopic Liver Transplant Model

The mean survival time in the control group was 28.9 ± 6.4 days. MTD S-1 did not prolong survival (mean survival time, 29.6 ± 3.9 days). In contrast, metronomic S-1 significantly prolonged survival (mean survival time, 34.3 ± 4.8 days). The mean survival time in the vandetanib group was 33.6 ± 5.0 days. MTD S-1 plus vandetanib treatment did not prolong survival times compared with vandetanib monotherapy (mean survival time, 37.6 ± 5.5 days). However, the metronomic S-1 plus vandetanib group provided the greatest prolonged survival times among all the treatment groups (mean survival time, 49.6 ± 11.5 days; Figure 4*B*).

Effect of Metronomic S-1 Chemotherapy Alone and in Combination with Vandetanib on Parameters of Tumor Angiogenesis

The results in Figure 5 show the MVD count in each treatment group. There was no significant difference in the MVD count between the control and the MTD S-1 group (control 41.1 ± 9.2, MTD S-1 35.8 ± 5.5; Figure 5*B*). However, tumor MVD was decreased in the metronomic S-1 group (17.2 ± 4.1) compared with the control group (P < .001) and the MTD S-1 group (P < .001; Figure 5*B*). Tumor MVD in mice treated with vandetanib was 13.7 ± 5.1. In the MTD S-1 plus vandetanib group, the MVD count was 18.8 ± 7.4. Metronomic S-1 plus vandetanib group showed the greatest reduction of tumor MVD (P < .01 compared with MTD S-1 plus vandetanib group, 8.2 ± 1.6; Figure 5*B*).

Detection of Proliferation and Apoptotic Cells in Tumor Tissues

To further investigate the mechanism of the observed antitumor effect, we examined the effect of metronomic S-1 and in combination with vandetanib on tumor cell proliferation and apoptosis (Figure 5). With respect to tumor cell proliferation, there were no differences between the control and all treated groups. The mean

number of apoptotic tumor cells (apoptotic index) measured in the control group was 6.2 ± 2.6. The MTD S-1 group did not show any significant difference (6.1 ± 4.9). However, the metronomic S-1 and vandetanib groups showed a significant increase in the apoptosis index (26.0 ± 5.4 and 18.4 ± 8.8, respectively, $P < .0001$). A significant increase in the tumor cell apoptosis index was also observed in the metronomic S-1 plus vandetanib group with 42 ± 3.5 ($P < .0001$).

Expression of VEGF and TSP-1 in Tumor Tissues

The results in Figure 6 show the expression of TSP-1 and VEGF in treated tumor tissues. The expression level of TSP-1 was significantly upregulated by approximately two- to three-fold in both the metronomic S-1 and the metronomic S-1 plus vandetanib treatment groups ($P < .05$ compared with the control group; Figure 6, *A* and *B*). With respect to expression levels of VEGF, there were no differences between the control and the MTD S-1 and metronomic S-1 groups (Figure 6, *C* and *D*). In contrast, the vandetanib and the metronomic S-1 plus vandetanib groups showed significantly upregulated the VEGF expression compared with the control group ($P < .05$; Figure 6, *C* and *D*). There was a significant difference between the vandetanib monotherapy group and the metronomic S-1 plus vandetanib treatment group ($P = .045$).

Discussion

Our results add to an expanding body of literature reporting the therapeutic benefit of metronomic chemotherapy, especially when it is combined concurrently with a targeted antiangiogenic drug [5,12,13]. Moreover, to our knowledge, this is the first preclinical report of using S-1 in a metronomic dosing and administration schedule for HCC preclinical model. Also noteworthy is that we undertook a comparative

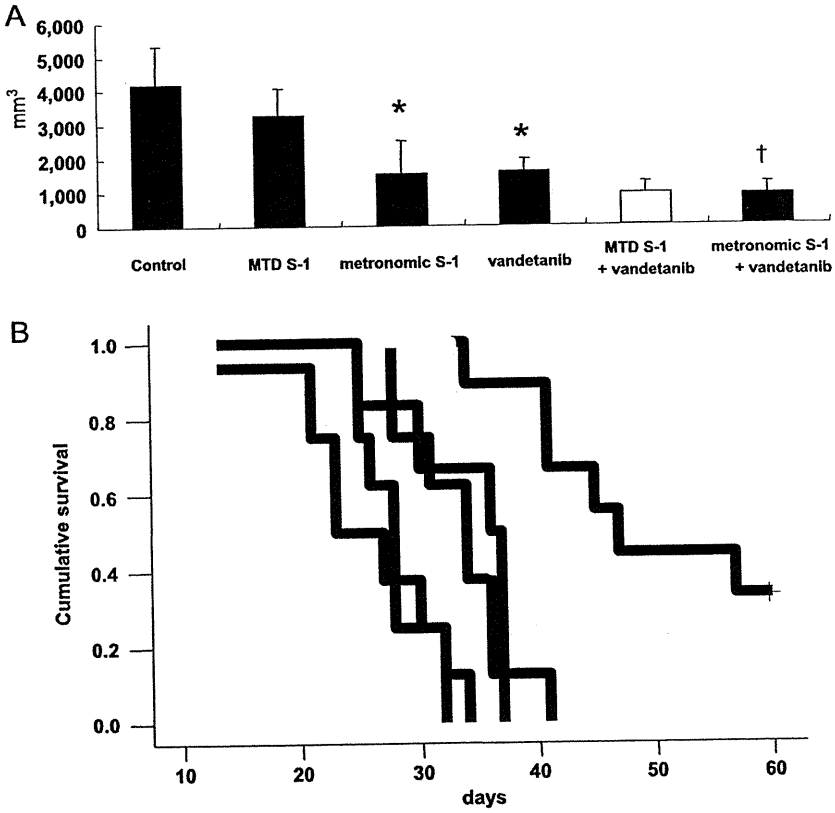
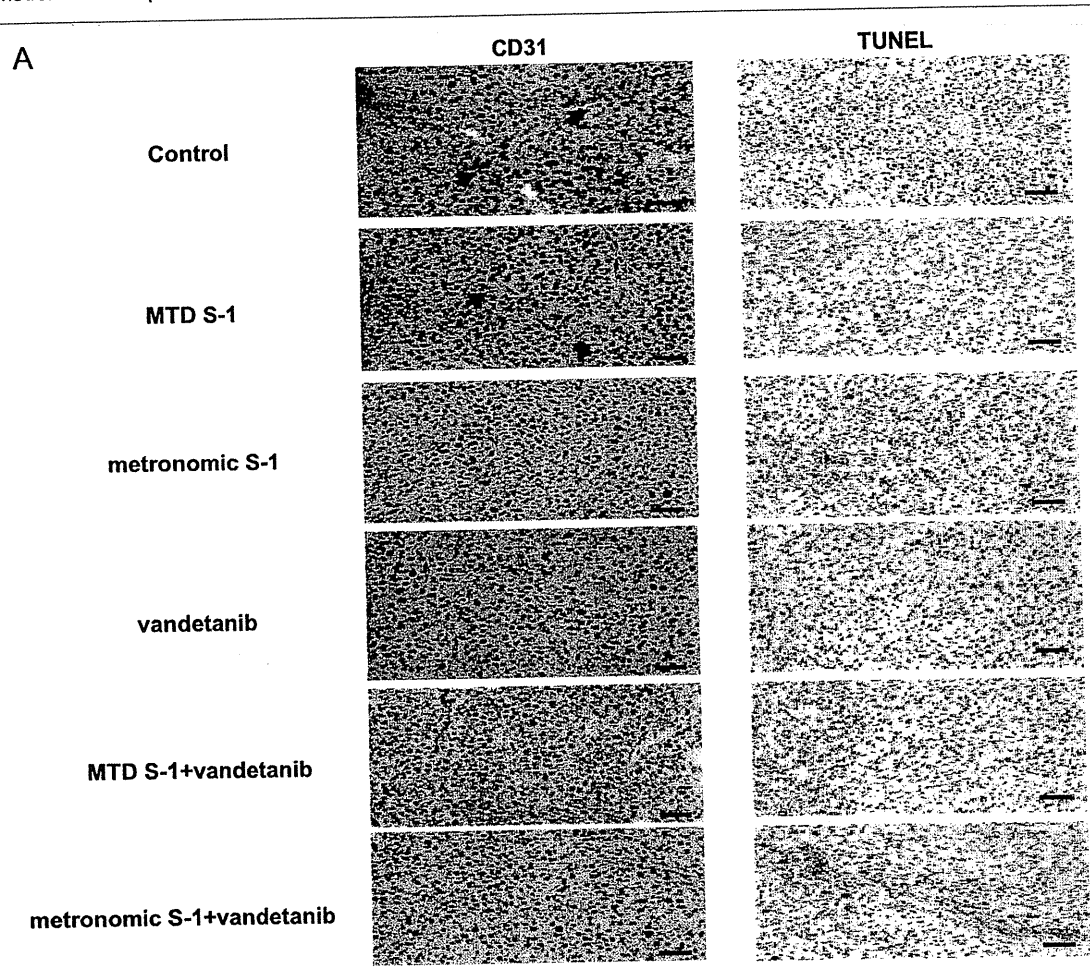


Figure 4. Assessment of therapeutic effects in KYN-2 liver transplant model. Tumor-bearing nude mice were treated in the following six groups: 1) HPMC as the control group (blue); 2) MTD S-1: 15 mg/kg per day for 1 week, followed by 1 week break period (purple); 3) metronomic S-1: 5 mg/kg per day for 2 weeks without break period (green); 4) vandetanib 25 mg/kg per day for 2 weeks (red); 5) MTD S-1 with vandetanib (yellow); or 6) metronomic S-1 with vandetanib (black). (A) Inhibition of tumor growth for KYN-2 liver transplant model. All treatments were performed 4 weeks in total. There was no significant difference between the control and the MTD S-1 groups. The metronomic S-1 group contributed to obvious inhibitory effect of tumor growth (*$P < .05$ compared with the control and the MTD S-1 groups). The metronomic S-1 with vandetanib treatment group showed the greatest inhibitory effect of tumor growth among all the groups (†$P < .001$). (B) Survival of mice treated with MTD S-1 or metronomic S-1 and in combination with vandetanib ($n = 10$ per group). Treatment was continued until mice were moribund, and days of life were recorded. Survival data were compared for significance with the log-rank test. MTD S-1 did not prolong survival compared with the control group. In contrast, metronomic S-1 prolonged survival compared with the control and MTD S-1 groups. The metronomic S-1 with vandetanib group provided the most effective therapy with longest survival times among all the groups ($P < .001$).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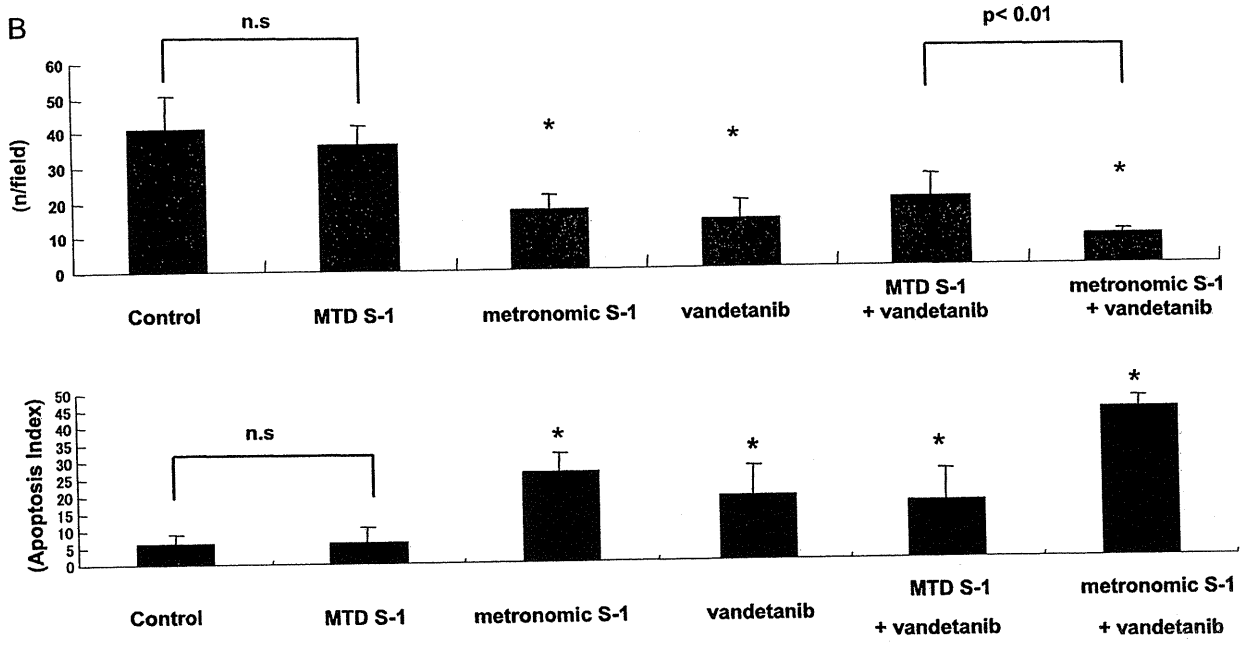
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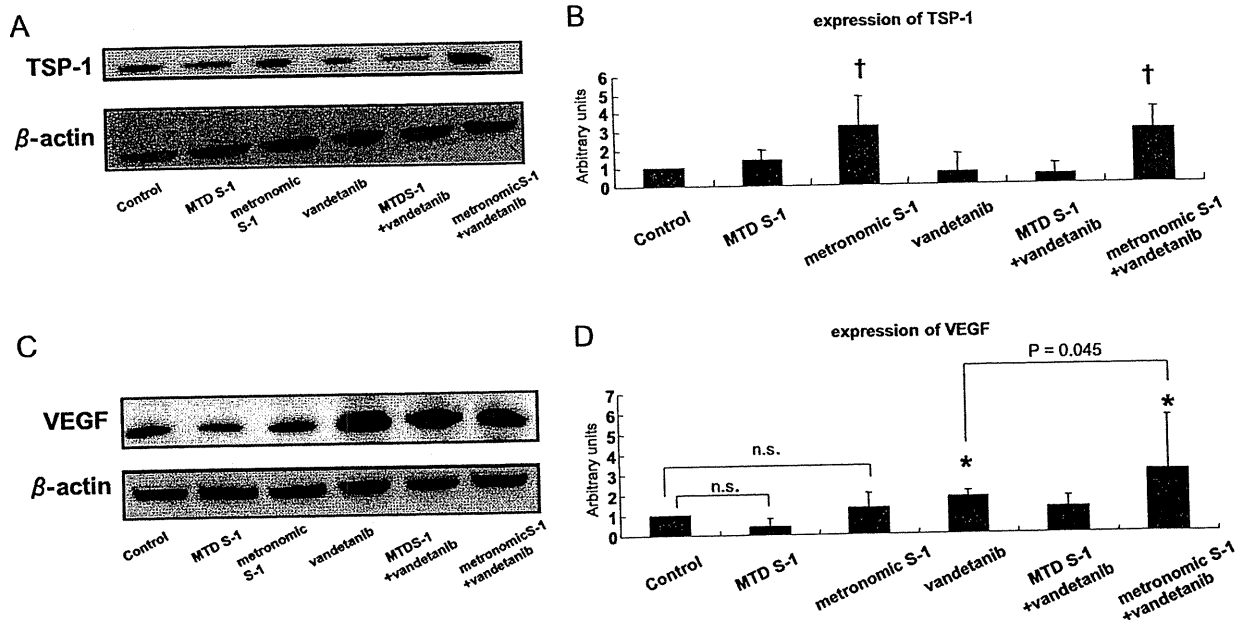

Figure 6. Western blot analysis of TSP-1 and VEGF. The band intensities of both TSP-1 and VEGF in treatment groups were measured and calibrated with each protein in control group and β-actin. (A and B) The metronomic S-1 and metronomic S-1 with vandetanib groups showed strongly upregulated the expression of TSP-1 ($^{\dagger}P < .001$ compared with the control group). (C and D) The expression of tumor VEGF was increased by the vandetanib and the metronomic S-1 with vandetanib groups. There were no differences between the control and the MTD S-1, metronomic S-1 group ($^{*}P < .05$ compared with the control group). There was a significant difference between the vandetanib and metronomic S-1 with vandetanib group ($P = .045$).

analysis of the effects of S-1 given in a more conventional MTD schedule with metronomic S-1, and our results consistently showed the metronomic dosing/schedule was superior to the MTD protocol, both in terms of increased antitumor efficacy and reduced toxicity. Importantly, in this regard, the metronomic protocol we used involved a cumulative dose over time that was 30% less than the corresponding MTD protocol. Below we discuss a number of different aspects of our results and some of the translational/clinical implications.

Antiangiogenic Effects Mediated by Metronomic S-1 Chemotherapy

Previous studies during the last decade have indicated that metronomic chemotherapy regimens using cytotoxic agents inhibit tumor growth by various mechanisms, namely, antiangiogenic effects, direct tumor cell targeting effects, or anticancer immune responses [4,19, 20]. Our results with metronomic S-1 would seem to confirm the antiangiogenic effect findings. First, we found that exposure of 5-FU in a metronomic-type protocol *in vitro* brought about a greater antiproliferative effect at distinctly low concentrations not only of 5-FU on two different tumor cell lines but also, especially, HUVECs, compared with an MTD-like exposure. This is similar to the results of other studies such as that of Bocci et al. [16] using paclitaxel or the active metabolite of cyclophosphamide. Second, we found reduced MVD and increased number of apoptotic tumor cells in mice treated with the metronomic S-1 schedule but not the MTD protocol. Third, we observed an increased expression of TSP-1, which has been reported previously using other cytotoxic drugs administered in a metronomic fashion *in vivo*, including cyclophosphamide [21]. Fourth, we noted that a tumor cell line (KYN-2) that is intrinsically resistant *in vitro* to high concentrations of 5-FU—the major metabolite of S-1—nevertheless responds to metronomic S-1 *in vivo* but not to MTD S-1, suggesting that a target other than the tumor cell population *per se* is likely involved in the *in vivo*

Figure 5. MVD and apoptosis in tumors tissues. The sections of tumors from the KYN-2 liver transplant model were stained by anti-CD31 antibody and Terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick-end labeling (TUNEL). Original magnification, ×200. The density of CD31-positive vessels (arrow) and TUNEL in a tumor field are represented as mean ± SD ($n = 30$ per group). (A) Representative sections for each treatment are shown. Bar, 10 μm. (B) There was no significant difference in MVD between the control and the MTD S-1 groups. Tumor vessel numbers were reduced by metronomic S-1. The metronomic S-1 with vandetanib group showed the most inhibitory effect of tumor vessel count among all the groups ($^{*}P < .001$ compared with the control group and the MTD S-1 group). The MTD S-1 group did not show any significant difference in the number of tumor cell apoptosis index (6.1 ± 4.9). However, the metronomic S-1 and vandetanib groups significantly increased in the number of apoptosis index, respectively (26.0 ± 5.4 and 18.4 ± 8.8, $P < .0001$). A significant increase of tumor cell apoptosis index was also observed in the metronomic S-1 with vandetanib group with 42 ± 3.5 ($P < .0001$).

antitumor activity that was observed using metronomic S-1. Fifth, O'Reilly et al. [22] have reported that the antiangiogenic effect mediated by endogenous antiangiogenic factors induces increased apoptosis of tumor cells, likely a secondary effect due to decreased MVD, whereas proliferation of tumor cells was not affected. Similarly, tumor apoptotic cell numbers were increased, whereas proliferation of tumor cells was not inhibited by metronomic S-1 chemotherapy in our study. On the basis of all of the aforementioned data and information, the antitumor effect of metronomic S-1 chemotherapy was likely to be mainly through antiangiogenesis mediated by inhibiting the proliferation of endothelial cells and inducing the expression of TSP-1, although some additional mechanisms cannot be entirely excluded. The mechanism of antiangiogenesis of metronomic S-1 chemotherapy is thought to be quite different from that of vandetanib. Inhibiting VEGFR by vandetanib resulted in increased VEGF production in tumor tissues, paradoxically, whereas metronomic S-1 chemotherapy did not increase VEGF production. Ebos et al. [23] reported that this difference of production of VEGFs influenced to achieving malignant potential of cancer cells. Also, at this point, metronomic chemotherapy is thought to be a promising strategy of long-term treatment of cancer.

Translational/Clinical Implications of the Metronomic S-1 + Vandetanib Preclinical Results

There are several potentially important implications of our results with respect to how they might conceivably be exploited for the future treatment and management of HCC patients. It is well known that there are no effective chemotherapy regimens for the treatment of advanced HCC using conventional chemotherapy regimens. One reason for this is the frequent underlying liver dysfunction [2]. As a consequence, using MTD given in conventional schedules is often contraindicated because of possible excessive toxicity. However, chemotherapy drugs given in a metronomic, less toxic fashion may be an alternative strategy to circumvent this problem. In this regard, there is conflicting evidence regarding the clinical benefit of metronomic UFT, another 5-FU prodrug, at least in the postoperative adjuvant use for HCC [24]. However, some aspects regarding the negative clinical findings should be taken into consideration. One is the dosing. The daily dose used in the aforementioned adjuvant study was less than the dose used for a positive phase 3 adjuvant UFT clinical trial for non–small cell lung cancer patients [9]. The second is the benefit that might be gained by using an antiangiogenic drug in combination with metronomic UFT. For example, a recent report by Tang et al. showed that neither metronomic UFT nor antiangiogenic drug therapy alone had overt antitumor activity in a model of locally advanced HCC, whereas these drugs when combined showed significant antitumor activity [25]. Also, in our study, combining with vandetanib resulted in enhanced antitumor effects for S-1 chemotherapy; nevertheless, MTD S-1 monotherapy did not show any effective antitumor effects. VEGFR is related to chemoresistance for tumor endothelial cells through surviving [26]. Inhibiting VEGFR by vandetanib might have contributed to enhanced chemosensitivity for tumor endothelial cells. And EGFR is associated with resistance to 5-FU [15]. Inhibiting EGFR by vandetanib might have enhanced chemosensitivity to 5-FU. In addition, it is notable in our study that not only the combination with vandetanib but also metronomic S-1 monotherapy showed significant antitumor effects. Because S-1 may be superior to UFT in antitumor effect by virtue of its biochemical modulators [7], S-1 might be an even more suitable agent for metronomic chemotherapy.

In summary, we have demonstrated preclinically that metronomic S-1 chemotherapy showed effective therapeutic outcomes without overt toxicity for treatment of HCC, mainly by suppressing tumor angiogenesis, and the activity of which is amplified by concurrent combination with vandetanib. Metronomic S-1 and the concurrent combination treatment with an antiangiogenic agent might be a promising treatment strategy for HCC.

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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*, doi: 10.1111/j.1349-7006.2011.02182.x, 2012)

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 × width^2 × 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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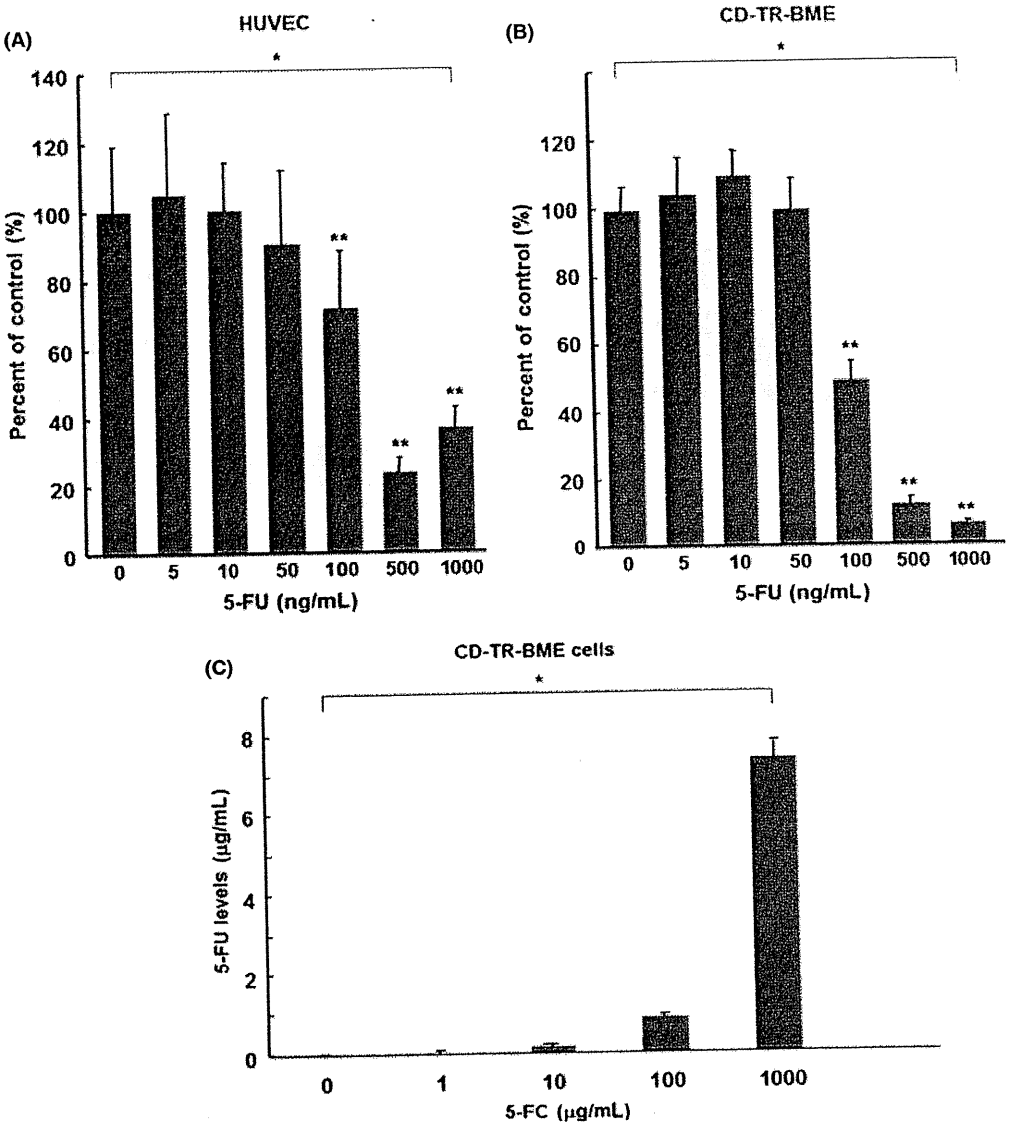

Fig. 1. Inhibition of cell proliferation by 5-fluorouracil (5-FU). (A) HUVEC, (B) CD-TR-BME. These cells were cultured with 10 mL of media containing 0–1000 ng/mL of 5-FU for 72 h. Cell proliferation was evaluated using a tetrazolium-based assay. Data are expressed relative to the control (n = 12). *P < 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).

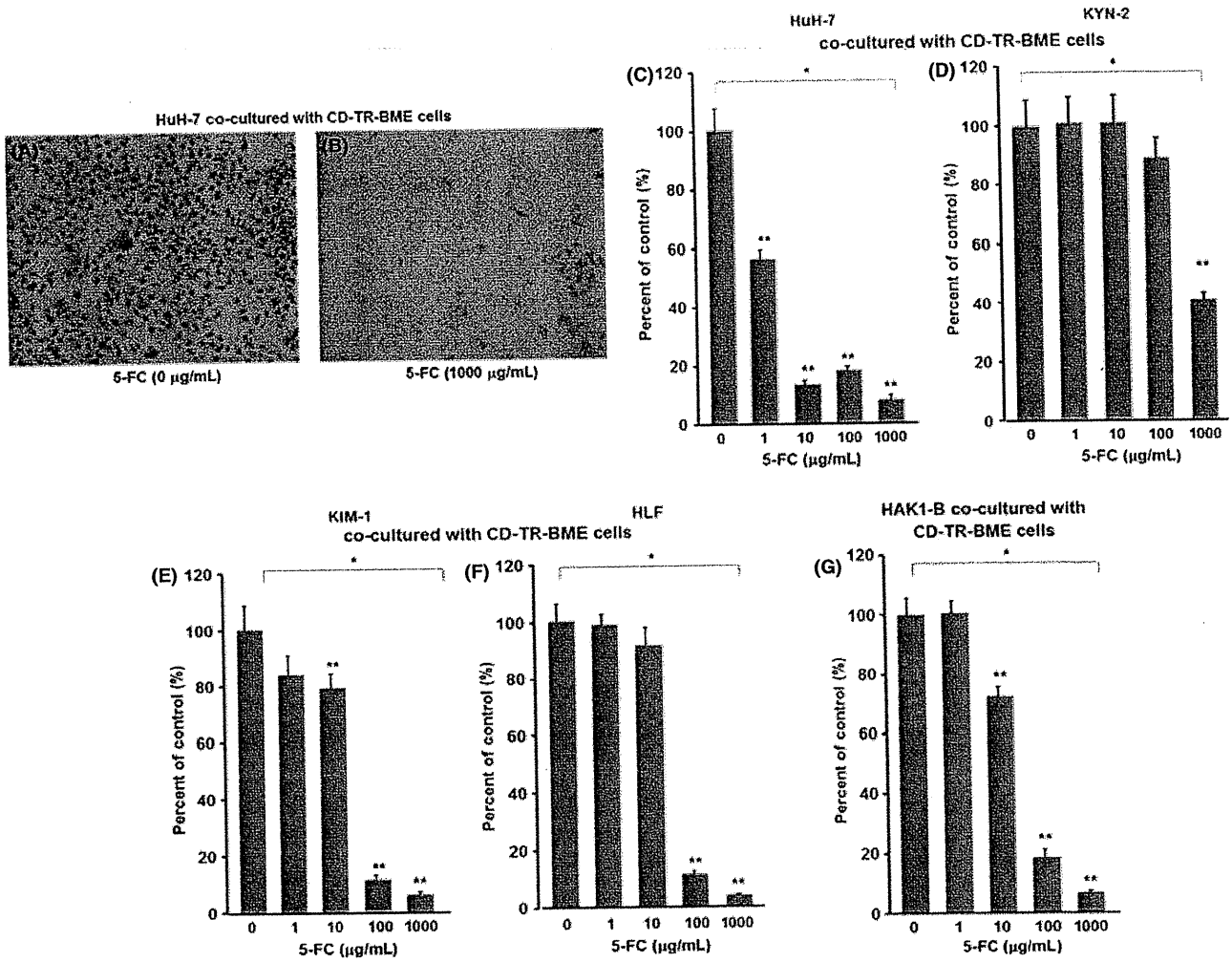

Fig. 2. Bystander effect of CD-TR-BME cells with 5-fluorocytosine (5-FC). Hepatoma cells were cultured in media containing 0–1000 mg/mL of 5-FC for 72 h with CD-TR-BME cells in chemotaxicell cell-culture chambers. (A) HuH-7 cells cultured with CD-TR-BME cells without 5-FC. (B) HuH-7 cells cultured with 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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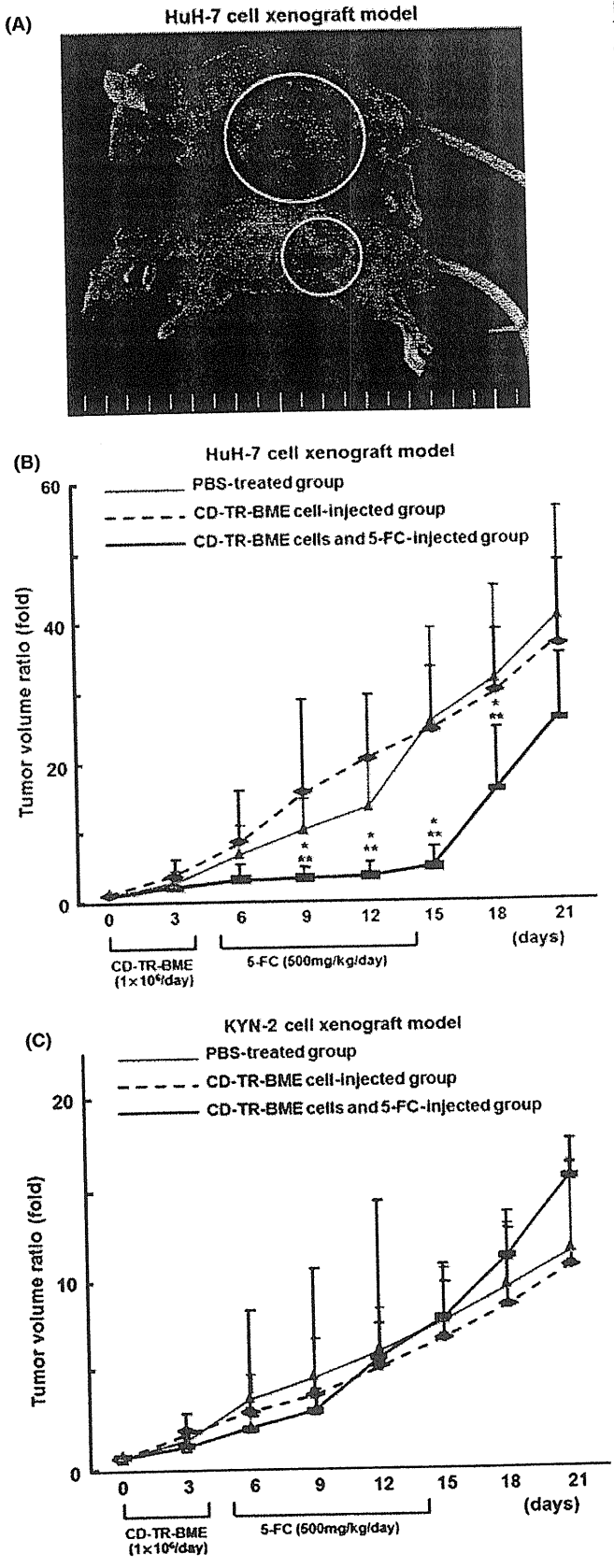

Fig. 3. Treatment of subcutaneous tumor with a combination of CD-TR-BME cells and 5-fluorocytosine (5-FC). (A) Tumors of HuH-7 cells at day 21. Group B, CD-TR-BME cells; Group C, CD-TR-BME cells + 5-FC. (B) Time-course observation of tumor volumes (HuH-7). Data are expressed relative to the control (day 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

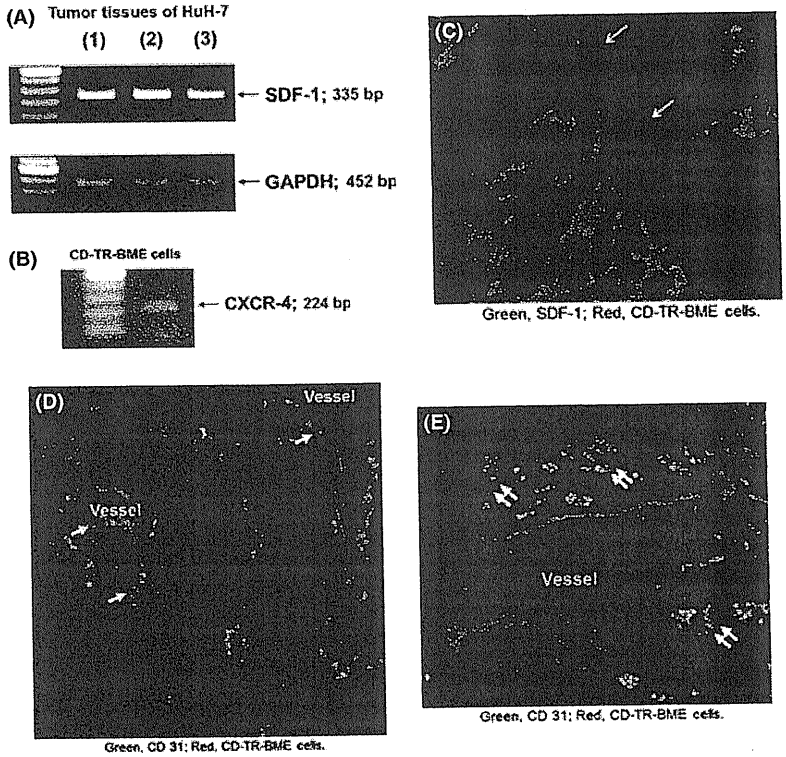

Fig. 4. Migration of CD-TR-BME cells to tumor tissues. (A) Expression of stromal-derived factor-1 (SDF-1) transcripts in tumor tissues (RT-PCR). Lanes 1–3, representative tumor tissues of HuH-7 cells. (B) Expression of CXCR4 transcripts in CD-TR-BME cells (RT-PCR). (C–E) Immunostaining of SDF-1 (green) 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

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Session 5

ソラフェニブ投与中急速に悪化したstageⅣ-B肝細胞癌症例に対する動注化学療法

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はじめに

進行肝細胞癌(HCC)に対する治療において，stageⅣ-Aに対する肝動注化学療法の有用性が報告されている[1)2)]。当院ではstageⅣ-Aに対してnew FP肝動注療法(NFP)[3)]を中心に行い，肝外転移がみられた時点でソラフェニブ[4)]へ変更している。しかし，ソラフェニブで効果がみられなかった場合，そのまま継続するべきか，再度ほかの治療へ変更するべきか判断に苦慮することがある。今回ソラフェニブ投与中，急速に悪化したstageⅣ-BのHCC症例に対し肝動注化学療法が有効であった3例を経験したので報告する。

症例

■症例1：64歳，男性(図1)。

病　歴：肝左葉に腫瘍径170mm，右葉に32mm，左門脈一次分枝に腫瘍塞栓を伴うstageⅣ-AのHCCに対して2005年8月からNFPを開始した。PRが得られ同年12月にはリザーバー抜去し肝左葉切除を施行した。2009年9月，腹膜播種が出現しS-1内服などsystemic chemotherapy(SC)を行うも効果はなく，2010年3月からソラフェニブの800mg/日投与へ変更した。腹膜播種は放射線治療にてコントロールできたが，6月より急速に肝内病変の悪化がみられソラフェニブを中止し，肝動注療法目的に2010年6月入院となった。

血液生化学検査：Alb 3.1g/dL，T-Bil 0.4mg/dL，PT 88.4%，WBC 5,800/μL，Hb 8.9g/dL，PLT 14.1×10⁴/μL，Child-Pugh score 6点，AFP 908ng/mL，AFP-L3 90.8%，PIVKA-Ⅱ 60,700mAU/mL。

臨床経過：肝動脈内リザーバー再留置後NFPを開始，NFPを5クール終了後肝内病変は著明に改善がみられ，PIVKA-Ⅱは6,400mAU/mLと低下した。2010年10月CT上PRが得られ，2011年2月現在生存中である。

■症例2：83歳，男性(図2)。

病　歴：肝右葉に門脈腫瘍塞栓Vp2を伴う腫瘍径122mmのHCCに対して，2008年10月からNFPを中心とした治療を開始し腫瘍縮小がみられた。2009年4月下大静脈腫瘍塞栓を伴ったHCCが再燃したが，下大静脈腫瘍塞栓に対する放射線治療(total 45Gy)と肝動注療法にて肝内病変は改善した。2009年8月に多発肺転移が出現，ソラフェニブを1日800mg/日で投与開始し，その後600mg/日へ減量した。2009年12月にはPRとなり治療継続中であったが，2010年9月肝内病変の急速な増大がみられた。

血液生化学検査：Alb 3.9g/dL，T-Bil 0.6mg/dL，PT 89.4%，WBC 6,200/μL，Hb 12.3g/dL，PLT 16.7×10⁴/μL，Child-Pugh score 5点，AFP 8,945ng/mL，AFP-L3 72.8%，PIVKA-Ⅱ 8,945mAU/mL。

臨床経過：入院後ソラフェニブ投与を中止し，2010年9月簡易リザーバー挿入後NFPを2クール施行した。2010年11月腹部CTで肝内病変は改善し，ソラフェニブを600mg/日で再開，2011年2月現在生存中である。

■症例3：77歳，女性(図3)。

病　歴：肝S8腫瘍径32mmのHCCに対して行った2007年12月のTACE(CDDP 30mg)が初回治療であった。2008年11月と2009年8月にはHCCが多発性に再発し，TACE(EPI 30mg)を施行した。2010年7月多発肺転移，左腸骨転移がみられ，ソラフェニブを400mg/日で投与開始し1週後に600mg/日へ増量するも，副作用の

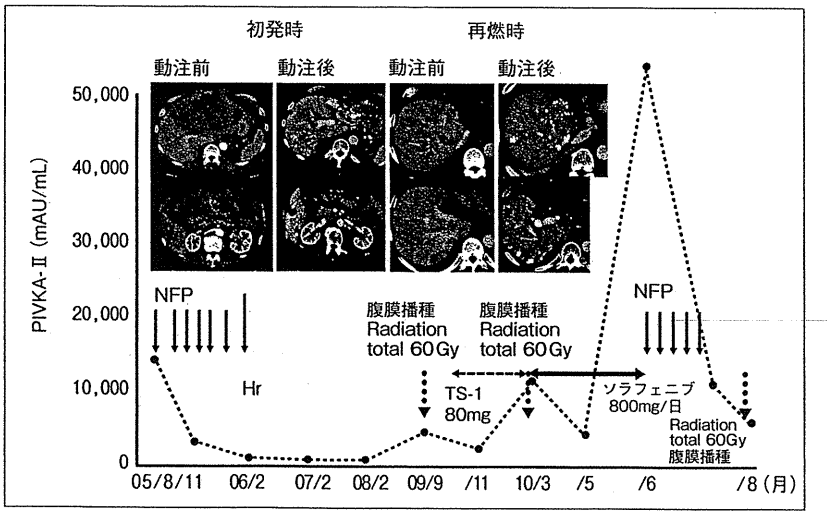

図1．【症例1】経過

ため400mg/日で維持した。骨転移へは放射線治療(total 45Gy)を併用した。同8月急速に骨転移病変が増大し疼痛増強，オピオイド鎮痛薬にて鎮痛を試みるも効果乏しく歩行困難となった。

血液生化学検査：Alb 4.2g/dL，T-Bil 1.0mg/dL，PT 92.0 %，WBC 4,800/μL，Hb 13.5g/dL，PLT 16.7×10^4/μL，Child-Pugh score 5点，AFP 37,720ng/mL，AFP-L3 9.7%，PIVKA-Ⅱ 1,860mAU/mL。

臨床経過：内腸骨動脈へ簡易リザーバーを留置しNFPを2クール施行，疼痛の著明な改善がみられ歩行可能となり治療開始1ヵ月で退院となった。2010年11月骨盤部CTでは骨盤部腫瘍の著明な縮小がみられPRと判断された。その後2010年12月，肝内病変の再燃と肺転移の悪化がみられ，肝内へ簡易リザーバーを用いてNFPを2クール施行，PRが得られた。多発肺転移に対してはソラフェニブ投与を開始し，2011年2月現在SDの状態で生存中である。

まとめ

当院では進行HCC 22例(stageⅢ／Ⅳ-B：1／21例)へソラフェニブ投与し，病状進行や副作用のため8例は治療法を変更した。8例中5例はS-1内服[5]へ変更，3例は局所コントロールのためNFPを行い効果がみられた。ソラフェニブ治療中に病変の悪化がみられた場合，継続投与に関する有効性を示す報告は現時点ではなく，SHARP study[4]でも中止とされている。本邦の「肝がんの新規治療法に関する研究班」においては264例のソラフェニブ投与症例のうち185例が中止となっている。投与中止の理由は，病状進行が63%，副作用は22%であった。投与中止例では，後治療が行われていなかった症例が60%と半数以上であった。今回の症例検討から，状況に応じてNFPを組み合わせることは有効と考えられ，予後をさらに延長する可能性がある。この場合，動注療法NFPへ切り替えるタイミングを損なわないようにしなければならない。エビデンスがあるわけではないが，当院ではソラフェニブ投与開始1ヵ月経過の時点で画像評価し，明らかに病状が進行した場合は，症例の状況に応じて治療変更を検討している。HCCがどのような状況にあっても最善の治療を常に模索し続けることが重要である。

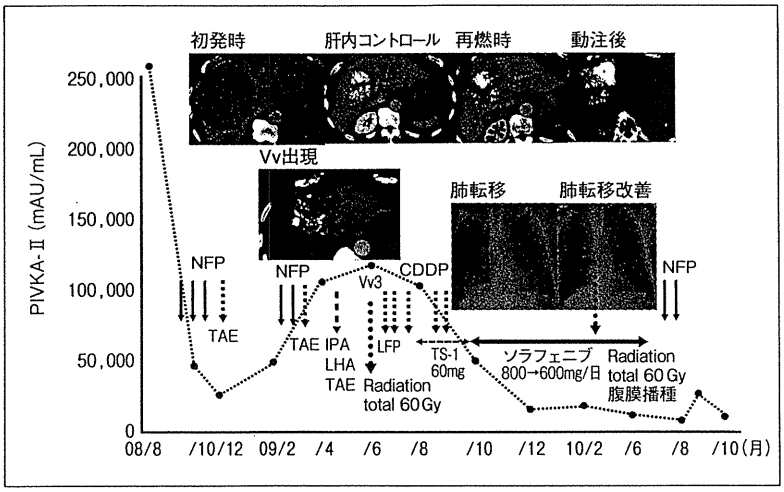

図2．【症例2】経過

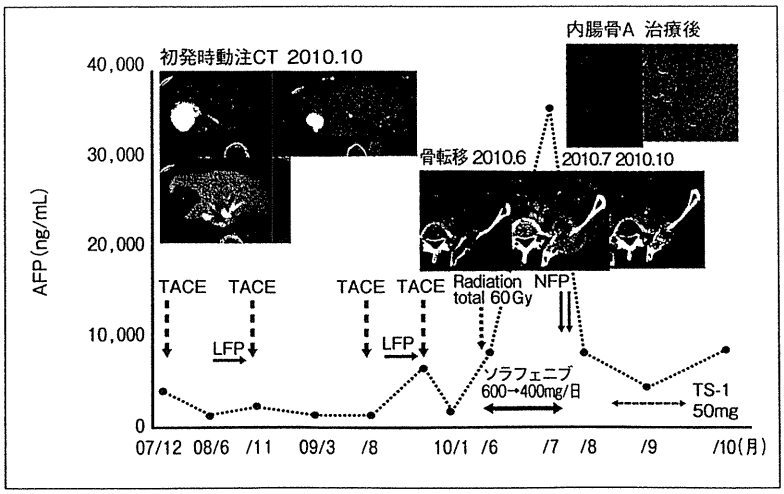

図3．【症例3】経過

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Original Article

Hepatitis C virus infection causes hypolipidemia regardless of hepatic damage or nutritional state: An epidemiological survey of a large Japanese cohort

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Aim: Infection with hepatitis C virus (HCV) is the leading cause of liver cirrhosis that develops into hepatocellular carcinoma. Previous studies have shown *in vitro* that lipids within hepatocytes are crucially important for a series of HCV infection–proliferation–release processes. On the other hand, in the patients with HCV, the serum total cholesterol (Total-C) and low-density lipoprotein cholesterol (LDL-C) levels have been reported to be lower. We conducted an epidemiological survey of a large cohort and investigated whether the lower serum lipid levels were caused by a direct or the secondary effects of HCV infection (i.e. hepatic damage or nutritional disorder).

Methods: Among 146 857 participants (male, 34%; female, 66%) undergoing public health examinations between 2002 and 2007 in Ibaraki Prefecture, Japan, the HCV positive rates determined by HCV antibody/antigen and/or RNA tests were 1.37% and 0.67% in males and females, respectively.

Results: In addition to Total-C and LDL-C, serum high-density lipoprotein cholesterol and triglyceride concentrations were also significantly lower in the HCV positive subjects compared with the negative subjects, regardless of sex, age or nutritional state evaluated by body mass index. Multivariate analysis showed that HCV infection was the strongest among the factors to be significantly associated with the lower level of these lipids. Particularly, the hypolipidemia was also confirmed in the HCV positive subjects with normal aminotransferase levels (alanine aminotransferase ≤30 and aspartate aminotransferase ≤30).

Conclusion: This epidemiological survey in a large Japanese cohort suggests that the HCV infection itself might directly cause hypolipidemia, irrespective of host factors including age, hepatic damage and nutritional state.

Key words: health examination, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, total cholesterol, triglyceride

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is the leading cause of liver cirrhosis and the consequent development of hepatocellular carcinoma over time. The World Health Organization (WHO) estimates that there are approximately 180 million HCV carriers worldwide, namely, 3% of the world population, with 3–4 million new cases appearing every year, 70% of whom develop chronic hepatitis.[1,2]

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Previous studies have shown that the life cycle of HCV is strongly associated with host lipids. The HCV forms lipo-viro-particles that are transported into hepatocytes via the low-density lipoprotein (LDL) receptor.[3–6] The replication of HCV occurs where the viral replicase is assumed to localize, on the phospholipid membrane of the endoplasmic reticulum (ER) or ER-associated membrane matrix.[7] The dynamic movement of lipid droplets to the ER has been confirmed to be involved in the production of HCV particles through core protein recruitment of non-structural proteins and in some steps of virus assembly.[8] Furthermore, HCV secretion from hepatocytes is closely associated with triglyceride (TG)-rich very low-density lipoproteins.[9–11]

Several epidemiological cohort studies reported that the serum total cholesterol (Total-C) and LDL cholesterol (LDL-C) levels in HCV carriers were significantly lower than those in uninfected control subjects.[12,13] Although the reason has not been elucidated, the lower levels of serum Total-C and LDL-C were specific in HCV carriers, but not in hepatitis B virus carriers.[14–18] Recently, we have estimated that the associated parameters in the public health examination for the HCV infection based upon multivariate analysis of data from over 25 000 individuals.[19] In the result, the greatest two negatively-associated parameters for HCV carriers were serum levels of Total-C and TG, while the most positively-associated parameters were serum aminotransferase levels. Here, a question has arisen whether the hypolipidemia in the HCV carriers was caused by the impaired liver function or not, because the liver is the central organ in lipid metabolism and the decreased level of serum cholesterols has been observed in the patients with liver cirrhosis due to lower ability of cholesterol synthesis and/or malnutrition.[20,21] However, previous studies have not shown whether the hypolipidemia would occur in asymptomatic HCV carriers with normal aminotransferase levels.[22–24] Furthermore, the effects of other factors, including age, sex, nutritional state and past history of HCV infection, on serum lipid levels have not been studied in HCV carriers.

In the present study, we investigated the relations between the serum lipid profiles and the above host factors in a large cohort in public health examination with over 140 000 participants including significant numbers of asymptomatic HCV carriers without any therapies. The results showed that the hypolipidemia was a characteristic feature in HCV carriers irrespective of aminotransferase levels or nutritional states.

METHOD

Cohort study and population

THE HCV TESTING was conducted during the annual public health examination for community residents, based in part on a project for urgent comprehensive countermeasures against hepatitis and hepatocellular carcinoma at the ages of 40, 45, 50, 55, 60, 65 or 70 years, from 2002–2006, and was supported by the Japanese Ministry of Health, Labor and Welfare. Additionally, the Ibaraki Prefecture extended the project of HCV testing for an additional year to 2007, and the present study used data from a 6-year period. The present cohort study used the data from a total of 146 857 individuals (50 399 males, 34%; 96 458 females, 66%) who participated in the annual public health examinations from 2002–2007 in Ibaraki Prefecture. The HCV test was conducted with HCV antibody/antigen and/or RNA testing in accordance with the guideline for the medical HCV examination, as summarized in our previous report.[19] In the flow chart for the determination of HCV infection, using a cut-off index (COI) of the HCV antibody titer obtained with the HCV antibody test (Lumipulse; Fujirebio, Tokyo, Japan), subjects were initially divided into the HCV negative with COI of less than 1, the HCV positive candidates with COI of $1 \leq \text{COI} < 50$ and the HCV positive with COI of 50 or more. The HCV positive candidates were finally determined to be HCV negative and positive based upon the HCV antigen test for the HCV core protein and the nucleic acid amplification test (NAT) for HCV RNA.

The health examination involved measurements of serum lipid levels, including Total-C, high-density lipoprotein cholesterol (HDL-C) and TG, as well as age, height, weight and serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). According to the general health examination, serum was collected on fasting. Serum LDL-C levels were calculated using the Friedewald formula, as follows: LDL-C (mg/dL) = Total-C (mg/dL) − HDL-C (mg/dL) − 0.2 × TG (mg/dL).[25] Over 802 mg/dL (8.8 mmol/L) of TG level was excluded from the calculation of LDL-C.[26] The lipid levels were diagnosed as indicating normal, hypolipidemia or hyperlipidemia based on the respective reference value for Japanese clinical laboratory examination.[27,28] Body mass index (BMI) was calculated by dividing the weight (Wt) in kilograms by the square of the height in meters.[29] All of the health examinations, including HCV tests and serum biochemical analyses, were conducted in the Ibaraki Health Service Association and Ibaraki Prefectural Institute of Public Health (Mito, Japan), and the data of health examination were analyzed anonymously, after informed consent was obtained from community representatives to conduct an epidemiological study based on the guidelines of the Council for International Organizations of Medical Science.[30]

Classification by factors

In the present study, both HCV negative and positive subjects were further divided into subgroups based upon different factors: (i) sex; (ii) age; (iii) serum HCV

antibody titer; (iv) serum markers of liver damage; and (v) nutritional state. The age classification was established by the age range, and was divided into 5-year increments. In the classification by serum HCV antibody titer, the HCV negative subjects were divided into two subgroups, HCV antibody titer COI of less than 1 and COI of 1 or more, and the subjects with COI of 1 or more were finally decided as being HCV negative by the HCV antigen test and NAT.[19] For classification by liver damage, the HCV negative and positive subjects were further divided into the two groups, based upon the healthy limits of serum aminotransferases (ALT and AST): "normal" was less than 30 IU of both, and "abnormal" was over 30 IU of either or both aminotransferases. In Japan, the healthy limits of both serum aminotransferase levels for diagnosis of liver damage in public health examinations were re-established to be under 30 IU, based on the recent guideline for antivirus therapy for HCV.[31] The nutritional status was evaluated by BMI, and the classification was conducted along with the WHO-defined BMI class: under Wt was BMI of less than 18.5, normal Wt of $18.5 \leq \text{BMI} < 25$, over Wt of $25 \leq \text{BMI} < 30$ and obese class according to obese classes 1–3 (BMI >30).

Statistical analysis

Data are expressed as the mean ± standard error of the value or percentage. Significant differences between the two groups were determined by unpaired Student's *t*-test or Mann–Whitney *U*-test depending upon the number of subjects and variations in the groups compared. Comparison of the percent distribution between the two groups was estimated by Pearson's χ^2-test analysis. Multivariate logistic regression analysis was performed to determine factors including HCV positive, age, BMI, ALT and AST associated with serum level of each lipid diagnosed as the hypolipidemia (Total-C ≤119 mg/dL, HDL-C ≤39 mg/dL in males and ≤44 mg/dL in female, LDL-C ≤64 mg/dL, TG ≤49 mg/dL). The strength of association was described with an odds ratio with 95% confidence intervals and *P*-value. The statistical analysis was performed using SPSS II software version 11.0.

RESULT

HCV positive rate and profile of serum lipids between HCV positive and negative

AMONG THE 146 857 individuals who participated in the health examination from 2002–2007, the HCV positive rates were 0.90%, 1.37% and 0.67% in all (sum of the sexes), males and females, respectively. There were no significant differences in BMI between the HCV negative (male, 23.9 ± 0.01; female, 23.1 ± 0.01) and positive (male, 23.3 ± 0.1; female, 23.1 ± 0.1) subjects. Table 1 shows the average serum lipid levels (Total-C, HDL-C, LDL-C and TG) by sex between the HCV positive and negative subjects. Among all subjects, all serum lipids in the HCV positive subjects were significantly lower than in the HCV negative subjects, regardless of sex.

The lipid levels in both HCV negative and positive subjects were divided into hypolipidemia, normal lipid and hyperlipidemia, based upon whether they were below, within and above the normal ranges of the respective reference values for Japanese (Fig. 1). Among both sexes, the proportion that were above the normal range for all examined lipids was significantly lower in the HCV positive compared to those in the HCV negative subjects (χ^2-test analysis $P < 0.0001$ in all: Total-C, 29% in the HCV negative vs 6% in the HCV positive for males, 41% vs 21% in females; HDL-C, 3% vs 1% in males, 6% vs 4% in females; LDL-C, 24% vs 7% in males, 34% vs 20% in females; TG, 35% vs 18% in males, 21% vs 14% in females).

The HCV negative subjects were also divided into those with HCV antibody titer of 1 or more and less than 1, and the former and latter were considered as having a prior infection and never infected.[17] The percentages of HCV negative subjects with prior infection were 0.91%, 1.28% and 0.72% for all, males and females, respectively, and the number of subjects was similar to the HCV positive subjects for each sex. Significant differences in the serum lipids were observed when the HCV positive subjects were compared regarding the presence or absence of a prior infection (Table 1). Among the HCV negative subjects, the examined lipids tended to be lower in those with prior infection compared with those who had never been infected, particularly in males, but there were no statistically significant differences.

Table 2 shows the multivariate logistic regression analysis of risk factors for lower level of serum lipids. In the parameters including HCV positive, age, ALT, AST and BMI, the significances were recognized in almost all analyses for the respective lower level of serum lipids in both sexes, while there were no significances in age for Total-C in male, ALT and BMI for Total-C in female, and both aminotransferases for LDL-C in female. In the HCV positive parameter of both sexes, the odds ratios in all examined lipids were remarkably higher than other analyzed