

M-CSF Receptor Antagonists Inhibit the Initiation and Progression of Hepatocellular Carcinoma in Mice

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Abstract. *Background/Aim:* The aim of this study was to investigate the effects of the macrophage colony-stimulating factor (M-CSF) receptor antagonist on hepatic carcinogenesis in mice. *Materials and Methods:* Mice were injected with diethylnitrosamine (DEN) and treated with M-CSF receptor antagonist GW2580 (GW) or a saline vehicle just after (early treated group) or 2 weeks after (late treated group) DEN injection. Animals were sacrificed after 28 weeks and incidence of tumor was assessed. Isolated Kupffer cells were co-cultured with M-CSF in the presence or absence of GW, and the concentration of VEGF was measured. *Results:* The incidence of tumors was significantly blunted both in the early- and the late-treated groups. In addition, angiogenesis within the tumor was also suppressed in both groups. The concentration of VEGF increased in Kupffer cells treated with M-CSF compared to those cultured without M-CSF. This increase was blunted by GW. *Conclusion:* M-CSF and its receptor could be novel molecular targets for hepatocellular carcinoma.

Macrophage colony-stimulating factor (M-CSF), which is a cytokine inducing differentiation and activation of monocytes and macrophages, has a strong migratory activity on macrophages, vascular endothelial cells and fibroblasts (1-4). Recently, it was reported that expression of M-CSF in non-tumor liver tissues was associated with disease recurrence, and poor survival after hepatectomy in human hepatocellular carcinoma (HCC), indicating the importance of the peritumoral tissue environment in the intrahepatic recurrence of HCC (5, 6).

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There are two subtypes of macrophages, M1-type and M2-type. M1-type macrophage is an inflammation-inducing macrophage stimulated with a Th1 type cytokine. In contrast, M2-type macrophages are stimulated by Th2 type cytokines and are thought to be strongly related to tumor growth (6). Many of the tumor-associated macrophages are thought to be M2-type macrophages expressing CD163, CD204 and/or CD206. M2-type macrophage secrete growth factors which are essential for tumor growth and invasion (6, 7). Indeed, M-CSF induces vascular endothelial growth factor (VEGF) production and angiogenic activity in human monocytes (6). Furthermore, inhibition of M-CSF decreased significantly infiltration of macrophages, resulting in a significant delay of tumor progression. In contrast, overexpression of M-CSF or supplementation with recombinant M-CSF resulted in enhancement in the recruitment of macrophages and correlated with accelerated tumor growth, progression, and angiogenesis (6, 8-10). Previously, it was reported from this laboratory that occurrence and progression of chemically-induced hepatocellular carcinoma (HCC) were significantly lower in M-CSF-deficient mice than in wild-type mice (11, 12). Furthermore, it was also reported that recurrence of HCC after curative resection was much faster in patients with high expression of M-CSF in non-tumor liver tissue than patients with low expression of M-CSF (6, 13). In that study, intrahepatic expression of M-CSF was associated with M2-type macrophages and angiogenesis (6, 13). Thus, M-CSF is involved in induction and progression of HCC by inducing angiogenesis via M2-type macrophages and could be a novel molecular target for treatment of HCC (4, 6). Therefore, the specific purpose of this study was to investigate effects of M-CSF receptor antagonist on HCC in mice.

Materials and Methods

Animals. Male C3H/HeN (2 weeks old, obtained from Slc, Shizuoka, Japan) mice were housed in a clean, temperature-controlled environment with a 12 h light-dark cycle and were given free access to regular laboratory water and chow diet for several days. All animals received humane care, and the study protocols

were approved by the Committee of Laboratory Animals at the University of Yamanashi according to institutional guidelines.

M-CSF receptor antagonist GW2580. M-CSF receptor antagonist GW2580 was dissolved in 0.5% hydroxyethyl cellulose/0.1% Tween 80.

Mice (2 weeks old) were given diethylnitrosamine (DEN) (20 mg/kg) intraperitoneally. Then, animals were divided into two groups. One group was treated immediately with GW2580 [80 µg/body weight (g)] or vehicle (control group) by gavage twice a day, and another group was started treatment at 4 weeks after DEN injection (Figure 1). Mice were sacrificed at 28 weeks after DEN injection and the number of tumors and tumor size were evaluated. The expression of CD31 as a marker of angiogenesis was evaluated by immunohistochemistry, and positive area was determined by image analysis. Distribution of F4/80 (as a marker of macrophages)-positive and CD206 (as a marker of M2-type macrophages)-positive macrophages were analyzed by immunohistochemistry. In addition, distribution of CD68 (as a marker of macrophages)-positive and CD163 (as a marker of M2-type macrophages)-positive macrophages were analyzed by fluorescence double immunohistochemistry. Then, ratio of M2-type macrophage against all macrophages was assessed.

Kupffer cells were isolated from male C3H/Hen mice (10–12 weeks old), resuspended at a concentration of 5×10^6 cells/ml in D-MEM media in the presence or the absence of M-CSF (100 ng/ml) and/or GW2580 (1mg/ml) and seeded at 1×10^4 cells/ml per well. Cells were then incubated for the designated times at 37°C in 5% CO₂, and supernatants were collected and stored at –80°C for further analysis. The first group was only Kupffer cells, and the second group was Kupffer cells cultured in the presence of M-CSF, the third group was Kupffer cells cultured in the presence of M-CSF and GW2580, and the fourth group was Kupffer cell cultured in the presence of GW2580. Concentration of VEGF was determined at cell culture media using ELISA kits according to the manufacture's protocol (Ray Biotech, Peachtree Corners, GA, USA) at day 1, day 3, day 5.

The murine HCC cell line, MH134, which was originally induced by carbon tetrachloride in C3H/He mice, was generously provided by Chugai (Tokyo, Japan). MH134 cells were cultured with different concentrations of M-CSF (0, 1, 10 and 100 µg/well) to assess direct effects of M-CSF against tumor growth *in vitro*, and the number of tumor cells was counted at day 1, day 3, and day 5.

Isolation of Kupffer cells. Kupffer cells were isolated from mice as detailed elsewhere with minor modifications (6, 14). Briefly, mice were anesthetized with inhaled diethyl ether, the abdomen was opened, the portal vein was cannulated with a small length of polypropylene tube, ligated the inferior vena cava above the diaphragm, and the inferior vena cava was dissected. The liver was perfused *in situ* for 5 min with Ca²⁺/Mg²⁺-free liver perfusion medium 1 (LPM-1: 8,000 mg/l NaCl, 400 mg/l KCl, 88.17 mg/l NaH₂PO₄·2H₂O, 120.45 mg/l Na₂HPO₄, 2380 mg/l HEPES, 350 mg/l NaHCO₃, 190 mg/l EDTA, 900 mg/l glucose, 6 mg/l Phenol red; pH 7.4, 37°C), and was then perfused with complete liver perfusion medium 2 (LPM-2: same as LPM-1 except without EDTA and glucose, but with 560 mg/l CaCl₂·2H₂O; pH 7.4, 37°C) containing 0.06% collagenase type IV (Sigma, St. Louis, MO, USA) for additional 15 min. After perfusion, the liver was removed, cut into small pieces, and homogenized. After passing through a 70 µm nylon mesh to eliminate nondigested material, cells were washed twice with warm Gey's balanced salt solution (GBSS-B: 370 mg/l KCl, 210 mg/l MgCl₂·6H₂O, 70 mg/l MgSO₄·7H₂O, 150 mg/l

NaH₂PO₄·2H₂O, 30 mg/l KH₂PO₄, 1,090 mg/l glucose, 227 mg/l NaHCO₃, 225 mg/l CaCl₂·2H₂O, 6 mg/l phenol red, 8,000 mg/l NaCl, 100 U/l streptomycin, 105 U/l penicillin G; pH 7.4) and centrifuged over 16% (wt/vol) Nycodenz® (Axis-Shields, Oslo, Norway) gradient for 20 min at $1,900 \times g$ at 4°C. Kupffer cells were collected from under the interface, washed with GBSS-B and resuspended in D-MEM media (Invitrogen, Carlsbad, CA, USA) or PBS and used.

Immunohistochemistry for CD31, F4/80, and CD206. Briefly, formalin-fixed, paraffin-embedded tissue specimens were cut into 4-µm sections. Each section was mounted on a silane-coated glass slide, deparaffinized, and incubated in antigen retrieval solution for 40 min at 95°C using Dako REAL Target Retrieval Solution (Dako, Carpinteria, CA, USA). Endogenous peroxidases were quenched by incubation at room temperature in 0.3% H₂O₂, followed by rinsing with PBS. Endogenous biotin was quenched using Dako Biotin Blocking System (Dako). Sections were blocked using 5% normal blocking serum for 20 min. Rabbit polyclonal antibody to CD31 (1:50, Abcam, Cambridge, UK) was applied for 2 h at room temperature. Anti-F4/80 primary antibody (1:100, Serotec, Oxford, UK) was applied for 30 min at room temperature. CD206 (Anti-Mannose Receptor antibody) (1:1,000 Abcam) was applied overnight at 4°C. Immunoperoxidase staining was completed using a Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine (DAB) as a chromogen. The number of positive cells against each antibody was assessed under microscopy in five random (400×) fields for calculation of the proliferation index. Histological samples were evaluated by one of the authors and by an outside expert in rodent liver pathology. Quantitative analysis of the CD31 positive area was calculated from five different fields and indicated as a percentage of the total area of the field using Image J software.

Immunofluorescence double staining for CD68 and CD163. Briefly, formalin-fixed, paraffin-embedded tissue specimens were cut into 4-µm sections. Each section was mounted on a silane-coated glass slide, deparaffinized, and incubated in antigen retrieval solution for 20 min at 95°C using Dako REAL Target Retrieval Solution S1699 (Dako). Sections were blocked using 5% normal blocking serum for 20 min. CD163/M130 polyclonal antibody (1:100; Bioss, MA, USA) or anti-CD68 antibody (FA-11) (1:100; Abcam) were applied for overnight at 4°C. Preabsorbed secondary antibodies Goat Anti-Rat IgG H&L (Alexa Fluor 488) (Thermo Fisher Scientific K.K, Yokohama, Japan) and Goat Anti-rabbit IgG H&L (Alexa Fluor 647) (Thermo Fisher Scientific K.K) were used at a 1/100 dilution for 2 h at room temperature. The number of positive cells against each antibody was assessed and evaluated in four random fields (200×) by BZ-X700 (Keyence, Osaka, Japan).

Statistical analysis. Data are expressed as mean±SEM. The Student's *t*-test was used for the determination of significance as appropriate. *p*<0.05 was considered significant.

Results

In animals administered with DEN, HCC was observed at 28 weeks after administration in the GW2580 non-treated group. On the other hand, tumor incidence was significantly blunted in both the early GW2580 administered and the late administered groups compared to the non-treated group

2 weeks after birth

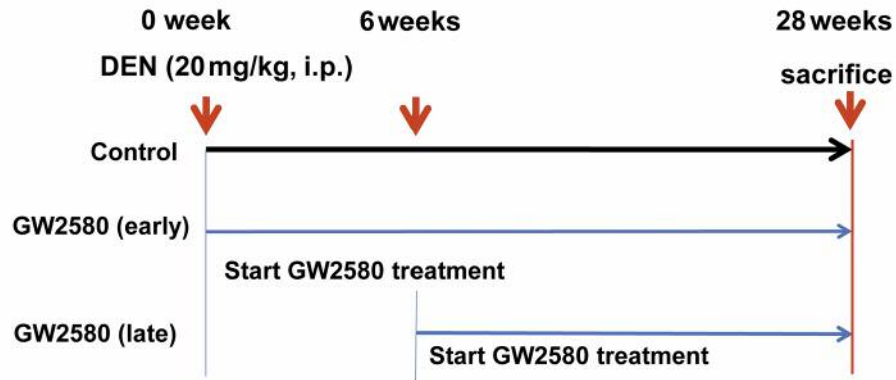


Figure 1. Experimental protocol. Control: Animals were orally administered HPC solution which does not contain GW2580. GW2580 early: animals were immediately administered GW2580 after DEN treatment. GW2580 late: animals started administration of GW2580 4 weeks after DEN treatment.

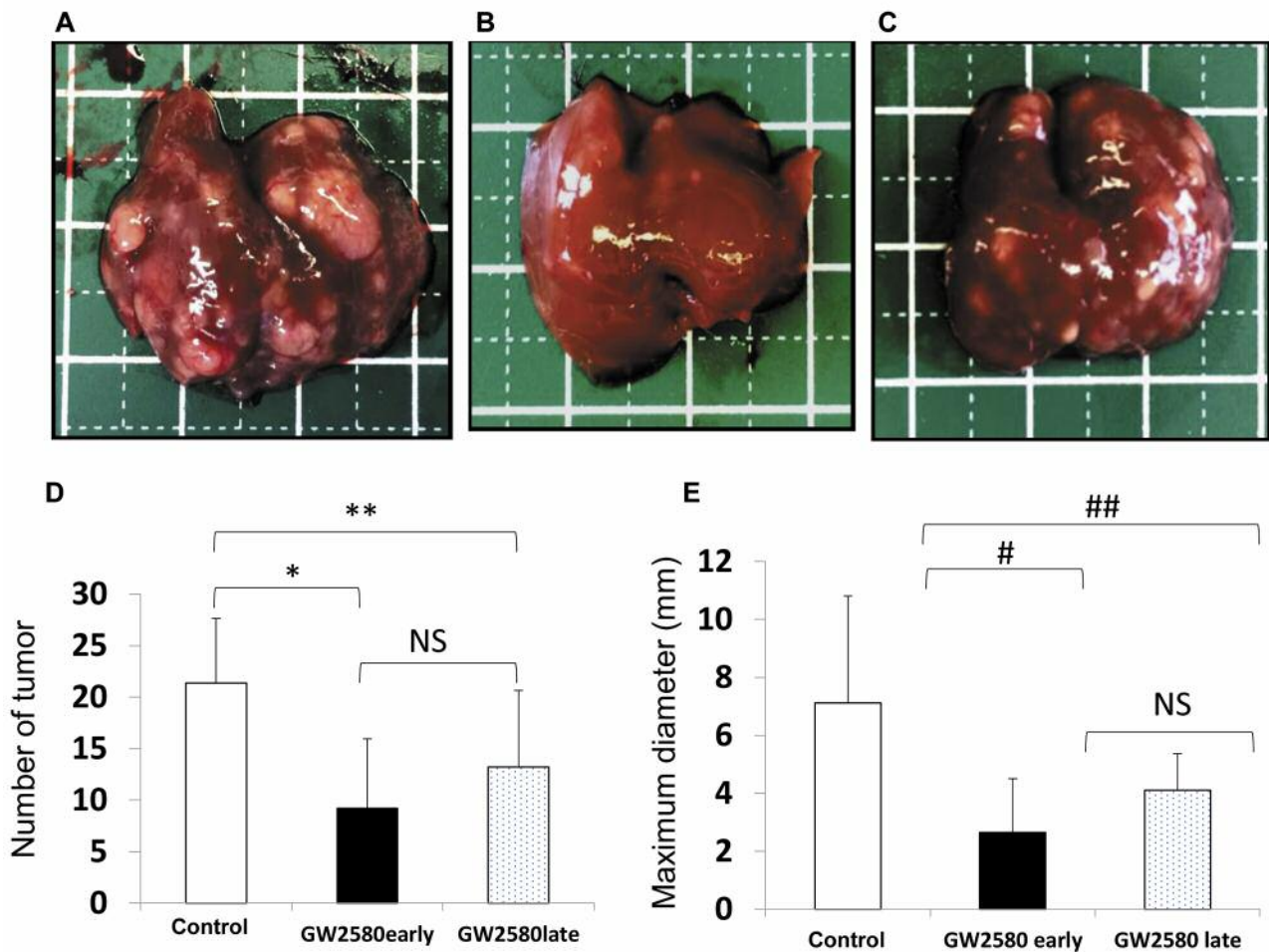


Figure 2. Effects of GW2580 in tumor growth. A; The control group, B; the GW2580 early-administered group and C; the GW2580 late administered group. The number of tumors (D) and maximum tumor size (E) are shown. Data represent means ± SEM. * and # $p < 0.01$ compared to the control mice by the unpaired Student's *t*-test. ** and ## $p < 0.05$ compared to the control mice by the unpaired Student's *t*-test.

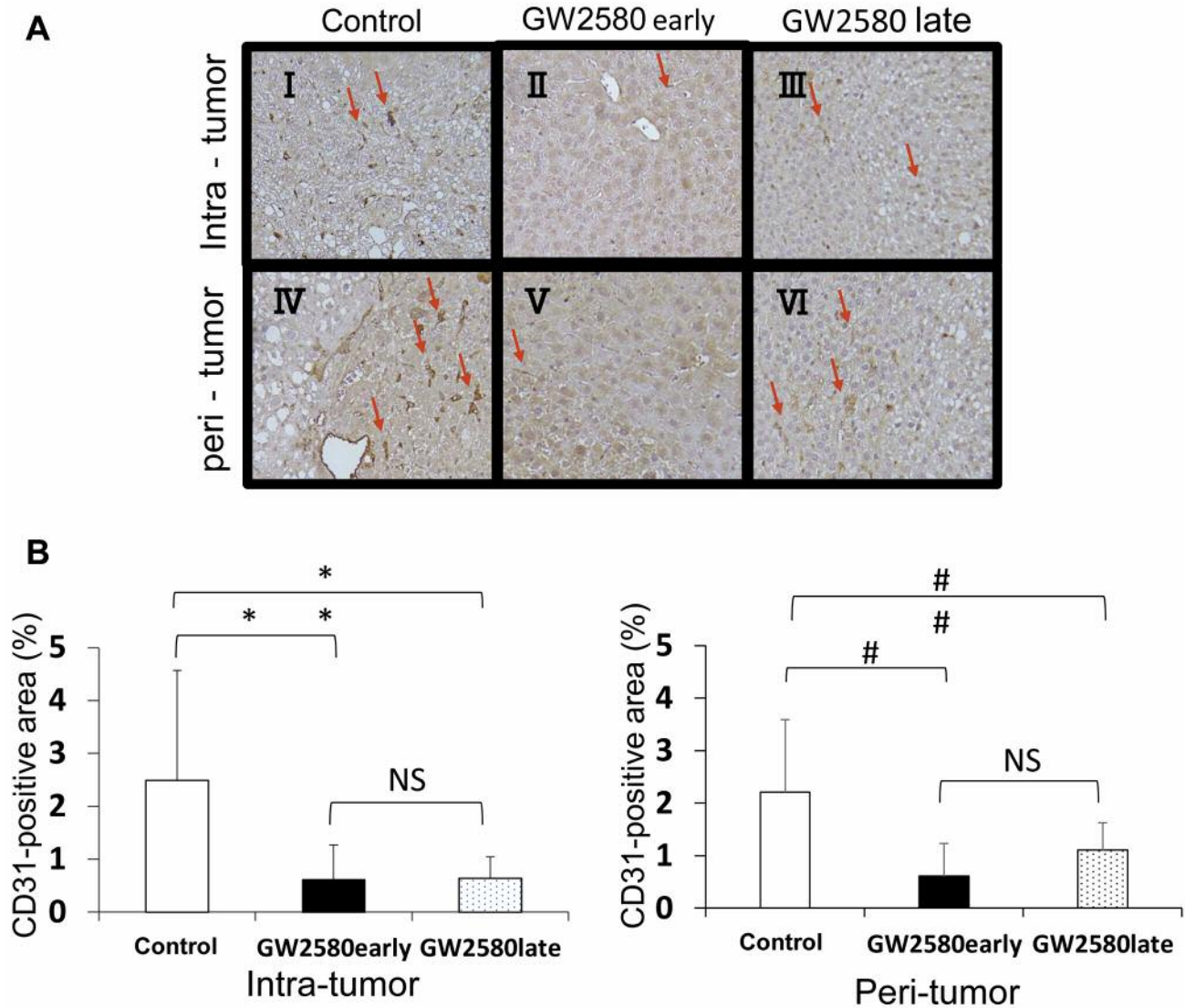


Figure 3. Immunohistochemistry for CD31 in the subcutaneous tissue. A: Representative photomicrographs are shown. B: Open bar, control mice; closed bar, GW2580 early-administered group; patterned bar, GW2580 late-administered group. Original magnification, $\times 400$. *, **, #, and ## $p < 0.01$ compared to the control mice by the unpaired Student's t-test.

(Figure 2A, B and C). Furthermore, the number and the maximum diameter of tumor were significantly blunted in both the early and the late GW2580 administered groups compared to the non-treated group (Figure 2D and E). There were no significant differences between the early group and the late administered group.

Analysis of angiogenesis in intratumor and peritumor tissues after the administration of DEN. To evaluate angiogenesis in the peritumor and intratumor tissues, immunohistochemical staining for CD31 was performed. The number of CD31-positive cells in the peritumor and

intratumor tissues was significantly lower in both the early and late GW2580 administered groups than in the non-treated group. There were no significant differences between early administered group and late administered groups (Figure 3A and B).

Analysis of the phenotype of macrophages in intratumor and peritumor liver tissues after the administration of DEN. The ratio of M2-type macrophages (CD206)/M1-type macrophages (F4/80) in the peritumor tissues was also significantly lower in both the early and late GW2580 administered groups than in the non-treated group (Figure 4A and B).

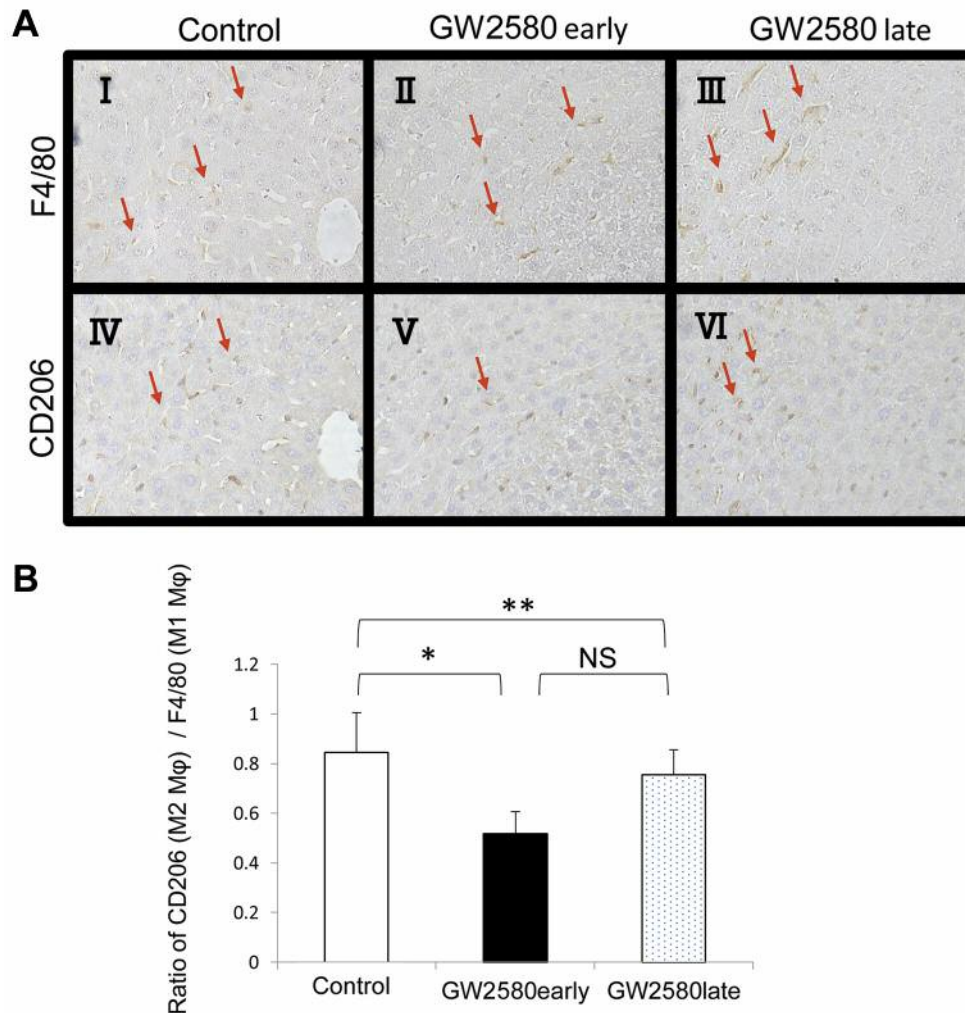


Figure 4. Immunohistochemistry for F4/80 and CD206 in the liver after the administration of DEN. A: Representative photomicrographs are shown. Original magnification, $\times 400$. F4/80-positive cells, M1-type macrophages and CD206-positive cells, M2-type macrophages. B: Open bar, control mice; closed bar, GW2580 early group and patterned bar, GW2580 late group. * $p < 0.01$ compared to control mice by the unpaired Student's *t*-test. ** $p < 0.05$ compared to the control mice by the unpaired Student's *t*-test.

Analysis of the population of hepatic macrophages after the administration of DEN. Double immunofluorescence staining indicated that the ratio of M2-type macrophages (CD163) / all macrophages (CD68) in the peritumor tissue was also significantly lower in both the early and the late GW2580 administered groups than in the non-treated group (Figure 5A and B). There were no significant differences between the early and the late administered groups.

Effects of GW2580 on the production of VEGF by M-CSF-stimulated isolated Kupffer cells *in vitro*. Production of VEGF by isolated Kupffer cells increased in a time dependent manner (Figure 6A). The concentration of VEGF increased significantly in cells cultured in the presence of M-

CSF. This increase was significantly blunted in the presence of GW2580.

Effects of M-CSF on the proliferation of MH 134 cells *in vitro*. There were no significant differences in the number of MH134 among the groups studied, suggesting that proliferation of MH134 was not affected by treatment with M-CSF *in vitro* (Figure 6B).

Discussion

Angiogenesis is strongly associated with oncogenesis and tumor growth (12, 15-17). A previous study from our laboratory showed that macrophages activated by M-CSF

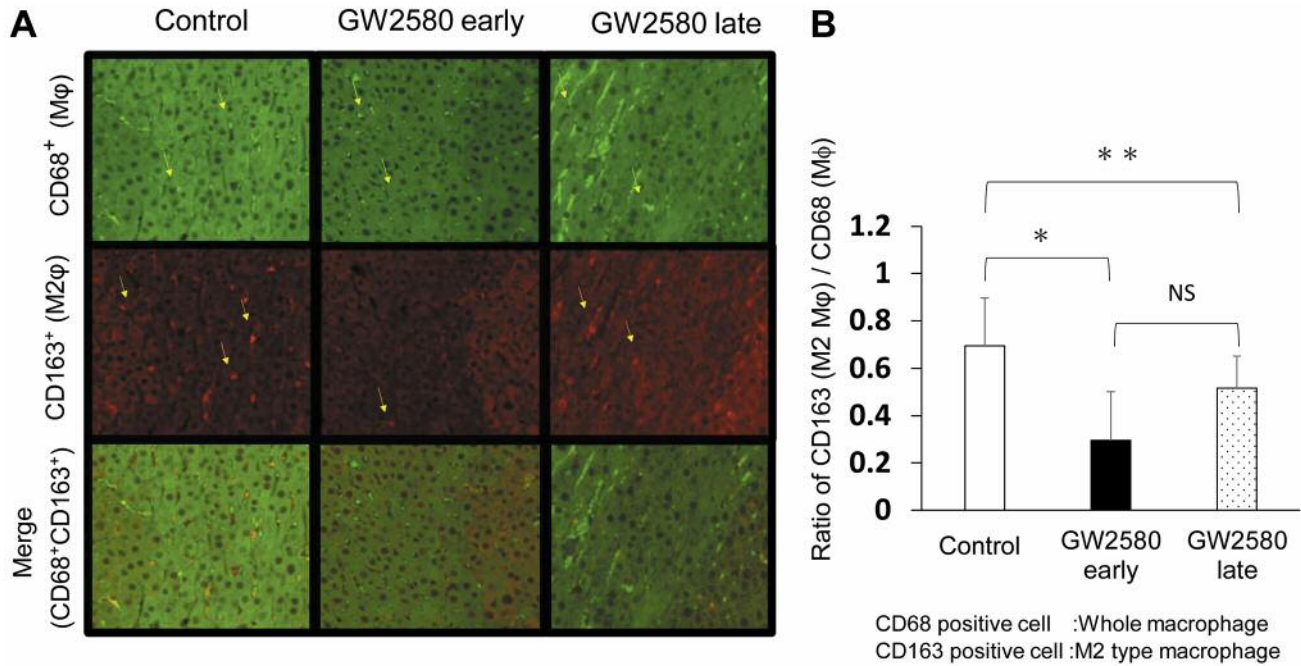


Figure 5. Double immunofluorescence staining for CD68 and CD163. A: Representative photomicrographs are shown. Original magnification, $\times 200$. CD68-positive cells (green); all macrophages and CD163-positive cells (red); M2-type macrophages. B: Open bar; control mice, closed bar; GW2580 early group, and patterned bar; GW2580 late group. * $p < 0.01$ compared with the control mice by the unpaired Student's t-test. ** $p < 0.05$ compared with the control mice by the unpaired Student's t-test.

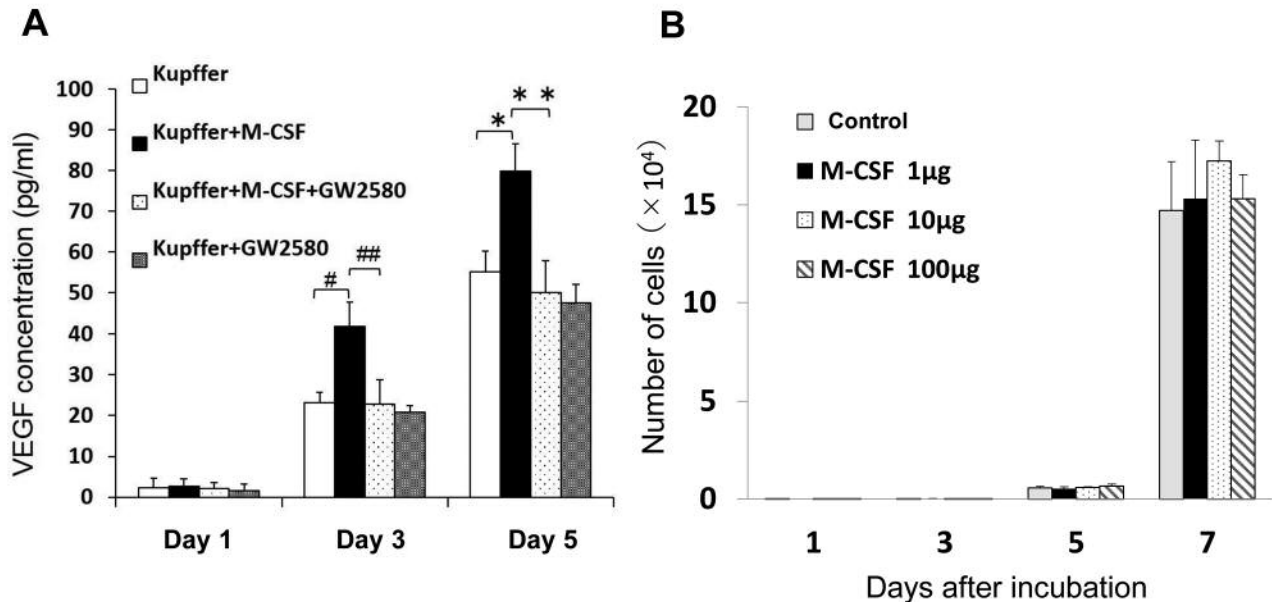


Figure 6. Effects of M-CSF and GW2580 on production of VEGF by isolated hepatic macrophages and proliferation of mouse HCC cell line in vitro. A) Production of VEGF by isolated Kupffer cells and B) proliferation of MH134 cells in vitro. Data are presented as the mean \pm SEM. # $p < 0.01$ compared with the control mice by the unpaired Student's t-test. ## $p < 0.01$ compared to control mice by the unpaired Student's t-test. * $p < 0.01$ compared with the control mice by the unpaired Student's t-test. ** $p < 0.01$ compared to the control mice by the unpaired Student's t-test.

were involved in tumor initiation and progression (18, 19). M2-type macrophages include Kupffer cells expressing the M-CSF receptor. It has been reported that M2-type macrophages activated by M-CSF induce neovascularization by increasing production of VEGF (6), which is an activator of angiogenesis. Importantly, VEGF production by isolated Kupffer cells was inhibited by GW2580 (Figure 6A). These results indicated that M-CSF is involved in tumor progression by inducing production of VEGF by macrophages including Kupffer cells and monocytes.

In this study, occurrence of chemically induced HCC was significantly suppressed by treatment GW2580 *in vivo*, suggesting that the M-CSF receptor antagonists was effective in suppressing induction of hepatocarcinogenesis (Figure 2E). The maximum diameter of the hepatic tumors was also significantly smaller in the GW2580 administered group (Figure 2D), suggesting that GW2580 also inhibited tumor progression. After DEN administration, oxidative stress derived from hepatic macrophages has been shown to be involved in carcinogenesis in the liver (9, 20, 21). Thus, administration of GW2580 may lead to inhibition of activation of macrophages and reduction of oxidative stress.

GW2580 was not found to inhibit HCC cell proliferation, suggesting that it indirectly inhibits tumor growth (Figure 6B). It has previously been reported that Kupffer cells stimulated by M-CSF promote proliferation of vascular endothelial cells by producing VEGF (6, 13). In this study, VEGF production by isolated Kupffer cells was suppressed by GW2580 *in vitro*. After DEN administration, the ratio of M2-type/M1-type macrophages and the area of angiogenesis were reduced in both the early and the late GW2580 administered groups (Figure 3A and B). Thus, M-CSF most likely acts on macrophages to promote production of VEGF, leading to angiogenesis and tumor growth.

The mechanism of induction of HCC involves chronic inflammation caused by viral hepatitis, alcohol and non-alcoholic steatohepatitis, and chemicals, suggesting that prevention of inflammation could be effective in inhibiting occurrence of HCC (22). In these inflammatory conditions, activated Kupffer cells play a critical role. In this study, using a chemically-induced HCC model, oral medication by M-CSF receptor antagonist inhibited activation of Kupffer cells and occurrence or progression of tumor. Since oral medication is appropriate and easy for long-term therapy than intravenous administration, it may be possible to treat HCC using this compound long-term.

In conclusion, M-CSF and/or its receptor may be novel molecular targets for the therapy of HCC.

Conflicts of Interest

The Authors declare that they have no conflict of interest. No Authors disclosed any financial conflicts for this manuscript, and there were no

personal relationships with other people or organizations that may have potentially and inappropriately influenced our work or conclusions.

Authors' Contributions

Yoshihiro Akazawa contributed to the data collection and writing of the paper; Hiroshi Kono contributed to the conception and design of the study; Michio Hara contributed to critical revision of the article; Shinji Furuya contributed to critical revision of the article; Yuuki Nakata contributed to the analysis and interpretation of the study; Hiroyuki Wakana contributed to data collection; Hisataka Fukushima contributed to data collection; Chao Sun contributed to the data collection and writing of the paper; Hideki Fujii contributed to the conception and design of this study, and obtained funding; Daisuke Ichikawa contributed to the conception and design of this study, and obtained funding.

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