Depletion of Tumor-Associated Macrophages Enhances the Effect of Sorafenib in Metastatic Liver Cancer Models by Antimetastatic and Antiangiogenic Effects

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Abstract

Purpose: To investigate the role of macrophages in tumor progression under sorafenib treatment and to explore whether combination of drugs that deplete macrophages improved the antitumor effect of sorafenib.

Experimental Design: Tumor growth, lung metastasis, and tumor angiogenesis were observed in HCCLM3-R and SMMC7721, two human hepatocellular carcinoma xenograft nude mouse models, when treated with sorafenib (30 mg/kg daily, n = 6 per group) or a vehicle as control. Macrophage infiltration was measured in the peripheral blood and in sorafenib-treated tumor by immunohistochemistry and flow cytometry with F4/80 antibody and CD11b antibody. The effect of macrophage depletion on tumor angiogenesis and metastasis after sorafenib treatment, using two drug target macrophages, zoledronic acid (ZA) and clodrolip, was measured in the two models of hepatocellular carcinoma.

Results: Although sorafenib significantly inhibited tumor growth and lung metastasis, it induced a significant increase in peripheral recruitment and intratumoral infiltration of F4/80- and CD11b-positive cells, which was accompanied with elevation of colony-stimulating factor-1, stromal-derived factor 1α, and vascular endothelial growth factor in the tumor and elevation of plasma colony-stimulating factor-1 and mouse vascular endothelial growth factor in peripheral blood, suggesting the role of macrophages in tumor progression under sorafenib treatment. Depletion of macrophages by clodrolip or ZA in combination with sorafenib significantly inhibited tumor progression, tumor angiogenesis, and lung metastasis compared with mice treated with sorafenib alone. ZA was more effective than clodrolip.

Conclusions: Macrophages may have an important role in tumor progression under sorafenib treatment. ZA is promising when combined with sorafenib to enhance its antitumor effect.

Sorafenib treatment increased overall survival and time to progression of patients with advanced hepatocellular carcinoma (HCC) by <3 months compared with patients who received placebo (1, 2). Patients with lung metastasis had a poorer response to sorafenib treatment (3). Emerging clinical evidence as well as preclinical findings revealed that invasiveness and metastatic behavior of tumor cells are increased after vascular endothelial growth factor (VEGF)-targeted therapy (4–6), which may explain why, after a transitory growth delay of primary tumor and prolongation of progression-free survival, clinical responses do not endure and tumors relapse as more invasive metastatic disease, thereby limiting the benefit for overall survival (7).

Tumor-associated macrophages facilitate tumor angiogenesis, and extracellular matrix degradation and remodeling, and also promote tumor cell motility (8). Recent studies reveal the direct communication between macrophages and tumor cells that leads to the invasion and egress of tumor cells into blood vessels (9). Thus, macrophages are at the center of the invasive microenvironment and are important targets for cancer therapy (8). Furthermore, macrophages are derived from myeloid cells, which have been reported as a primary cause of resistance to anti-VEGF therapy, and the elimination of these cells from the tumor microenvironment significantly restricts tumor growth in spontaneous and xenograft murine tumor models (10). Moreover, a phase II study of sunitinib, another
Translational Relevance

Hepatocellular carcinoma (HCC) is currently a major public health problem and the fifth most common cause of cancer-related death among men in the United States. Sorafenib prolonged time to progression and overall survival of HCC patients by only 3 months, and patients with metastasis had a poorer response to sorafenib treatment. Thus, further improvement lies on the combination of sorafenib with other classes of drugs. Zoledronic acid (ZA) is now clinically available as an antimetastatic drug that prevents and treats bone metastases in patients with solid tumors by targeting osteoclasts, which are macrophages homing to the bone. In the current study, we used two HCC cell lines with high and low metastatic potential, and found that sorafenib induced macrophage recruitment and infiltration to HCC xenograft. When combination with sorafenib, ZA reduced the numbers of macrophages induced by sorafenib, decreased tumor angiogenesis, and, above all, further inhibited tumor progression and reduced lung metastasis. This work might be applied to clinical practice by combination of ZA with sorafenib for HCC patients.

inhibitor of multiple tyrosine kinase inhibitors whose spectrum of activity overlaps that of sorafenib, found that a high level of plasma stroma-derived factor-1 (SDF-1α) was associated with rapid progression or high mortality in HCC patients receiving sunitinib treatment (11). Because SDF-1α is a chemokine for progenitor cells (12), it is possible that monocytes/macrophages may take part in tumor progression. It is still unclear, however, whether sorafenib treatment induces increased macrophage infiltration in tumors and whether the antitumor effect of sorafenib could be enhanced by the elimination of macrophages.

Clodronate-containing liposome (clodrolip), in which the bisphosphonate clodronate is encapsulated into liposome, has been shown to significantly reduce the number of infiltrating mononuclear macrophages in tumors (13–15) and peripheral blood (14, 16), and to inhibit survival of macrophages in vitro (14). It was reported that clodrolip depletes macrophages by 80.5% (16) to 93% (13), but it is not clinically available. Zoledronic acid (ZA) is a highly charged hydrophilic molecule that does not readily cross the plasma cell membrane (17); it reaches pharmacologically active concentrations only in cells that exhibit marked fluid-phase endocytosis, such as osteoclasts and macrophages (18). ZA has been used to treat bone metastasis and reduce skeletal tumor burden in many types of cancers by inhibiting the activity of osteoclasts. ZA is well tolerated, either alone or combined with chemotherapy (19), but the combination of ZA with targeted therapies has not been investigated yet. It is reported that ZA has direct antitumor effect (20, 21), but only in tumors such as breast (20) or cervical carcinoma (21) in which macrophage infiltration is prominent, or in bone in which an extremely higher concentration of ZA can be achieved (20). Further, the concentration of ZA at which it presents a direct inhibiting effect on a tumor cell is 12.5- to 50-fold higher than that in a patient or animal study. In tumors without significant macrophage infiltration, ZA has no antitumor effect (17, 22). It is evident that ZA depletes tumor-associated macrophages by reverting their polarization from M2 to M1 phenotype (23) and by suppressing their expression of matrix metalloproteinase-9 (24, 25).

The present study used orthotopic nude mouse model with two human HCC cell lines to investigate the role of macrophages in tumor progression under sorafenib treatment. Using two drugs that specifically target macrophages, clodrolip and ZA, we investigated the combination effects of these drugs with sorafenib on tumor growth, tumor angiogenesis and metastasis.

Materials and Methods

Animals, cell lines, and treatments

Male athymic BALB/c nu/nu mice (5-wk-old) were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Science. All mice were bred in laminar flow cabinets under specific pathogen-free conditions. The experimental protocol was approved by the Shanghai Medical Experimental Animal Care Committee. Two HCC cell lines were used in this study. SMMC7721, which is of low metastatic capacity and was established by the Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai, and HCCLM3, a human HCC cell line with high metastatic potential that originated from MHCC97 (26). Stable red fluorescent protein–expressing HCCLM3 (LM3-R) cells by infection with lentivirus containing full-length cDNA of red fluorescent protein were established by our institute (27). The murine monocyte/macrophage cell line RAW 264.7 cells (mouse leukemic monocyte macrophage cell line) was established by the Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai. The LM3-R, SMMC7721, and RAW 264.7 cells were maintained at 37°C with a 5% CO₂ in air atmosphere in DMEM supplemented with 10% (volume/volume) heat-inactivated fetal bovine serum and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin).

Clodrolip and PBS-containing liposome were gifts of Roche Diagnostics GmbH. ZA was purchased from Novartis. Sorafenib was prepared as previously described (28) and formulated at 4-fold (4×) of the highest dose in a cremophor EL/ethanol (50:50) solution. This 4× stock solution was prepared fresh daily. Final dosing solutions were prepared on the day of use by dilution to 1× with endotoxin-free distilled water (Life Technologies) and mixed by vortexing immediately before dosing. Sorafenib was finally prepared to a 1× solution with cremophor EL/ethanol/water (12.5:12.5:75, the vehicle solution).
Treatment and grouping of nude mice bearing orthotopic HCC tumors

Seven days after orthotopic implantation of tumor cells into the liver, the mice were randomly assigned to receive the following treatments (n = 6 for each group): (a) a daily oral dose of vehicle solution and a daily i.p. injection of PBS-containing liposome (control group), (b) a daily oral dose of sorafenib at 30 mg/kg (sorafenib group), (c) i.p. injection of clodrolip at 100 μg/kg; thrice weekly (clodrolip group), (d) a daily oral dose of sorafenib at 30 mg/kg plus an i.p. injection of clodrolip at 100 μg/kg thrice weekly (sorafenib plus clodrolip group), (e) a daily s.c. injection of ZA at 100 μg/kg (ZA group), or (f) a daily oral dose of sorafenib at 30 mg/kg plus a daily s.c. injection of ZA at 100 μg/kg (sorafenib plus ZA group). All the mice were treated for 5 weeks.

Detection of metastasis by fluorescent microscope and H&E staining

Tumors were excised and measured by largest (a) and smallest (b) diameters to calculate tumor volume by V = ab²/2. The lungs were also excised, and red fluorescent protein–positive metastatic foci were imaged by fluorescent imaging (stereomicroscope: Leica MZ6; C-mount: 0.63/1.25; charge-coupled device: DFC 300FX), and integrated absorbance was quantitated by Image-Pro Plus software (Media Cybernetics, Inc.) as previously described (27). After examination of the images, orthotopic tumors and lungs were fixed with 10% buffered formalin and were paraffin embedded. Serial sections were cut at 5 μm for histologic study. Sections of lung were stained with H&E to pathologically determine the presence of lung metastasis.

Immunohistochemical staining for CD31, F4/80, and CD11b

To assess the distribution of CD31 and F4/80, we stained sections with rat anti-CD31 (1:200; BD Pharmingen), rat anti-F4/80 antibody (1:50; Serotec), and rat anti-CD11b antibody (1:50; Serotec). Rabbit anti-rat IgG–horseradish peroxidase (1:100; Serotec) or rabbit anti-rat FITC antibody (1:10; Santa Cruz) were applied as secondary antibodies. Staining was evaluated by two independent observers. For negative controls, primary antibodies were replaced by PBS. For quantification of mean vessel density in sections stained for CD31, five fields at ×100 magnification in the “hotspot” were captured for each tumor, and microvessel density (MVD) was quantitated as CD31-positive area/total area. Quantitation of F4/80- or CD11b-positive cells in an HCC graft was calculated as F4/80- or CD11b-positive area/total area. The data were expressed as mean ± SEM.

Flow cytometry analysis of number of F4/80-positive cells

Mouse whole blood was obtained with heparin used as the anticoagulation agent, centrifuged to separate plasma, and stored at −80°C until tested. Blood cells of the lower

layer were processed with lysing buffer (BD Pharmingen) for 20 minutes at room temperature and were then centrifuged at 1,500 rpm for 5 minutes. Cells were then incubated with primary rat anti-F4/80 antibody (1:10; Serotec) or rat anti-CD11b (1:10; Serotec) for 1 hour at 37°C in PBS containing 0.5% (w/v) bovine serum albumin. After being washed with PBS, the cells were incubated with goat anti-rat IgG–FITC as a second antibody for 1 hour at 37°C. Negative controls were obtained without adding primary antibody. FITC-labeled cells were enumerated with a FACScalibur cytometer (BD Biosciences), and cell counts were expressed as % gated (percentage of F4/80-positive cells or CD11b-positive cells to mononuclear cells in the peripheral blood).

ELISA of plasma proteins

Levels of plasma VEGF of mouse origin (mouse VEGF) and colony-stimulating factor-1 (CSF-1) of human origin were analyzed by ELISA with the use of Quantikine ELISA kits (R&D Systems). All analyses were carried out in duplicate.

Quantitative real-time PCR analysis

Total RNA was extracted from cell lines and frozen tumor specimens (n = 6 for each group) using Trizol Reagent (Invitrogen). Total RNA (2 μg) was reverse transcribed using a PrimeScript RT reagents kit (TaKaRa). Reverse transcription–PCR was performed before quantitative real-time PCR. Messenger RNA expressions were determined by real-time PCR using SYBR Premix Ex Taq II (TaKaRa). PCR amplification cycles were programmed for 10 seconds at 95°C, followed by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Data were collected after each annealing step. Glyceraldehyde-3-phosphate dehydrogenase was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. Relative expression of genes was calculated and expressed as 2-ΔΔCt, as previously described (25), and fold changes were calculated by normalization mRNA of the sorafenib-treated samples to that of control samples. The primers used for the amplification of human genes were as follows: VEGF, forward 5'-GGGAGCCGGCTGACATTAT-3' and reverse 5'-TTGGTTCATC-GACCAACCA-3'; CSF-1, forward 5'-GGAGTGGACACCTGTGACTCT-3' and reverse 5'-TTGCCAGGGCGCTCACAA-3'; SDF-1α, forward 5'-ATGAAACGCAAAGTCAGTGTCGTC-3' and reverse 5'-CTTTTTTAAAGCTTTCTCCAGGTACT-3'.

Western blotting analysis

Tumor protein was extracted and the protein concentration was determined with the BCA protein assay (Pierce), and equal amounts of protein were subjected to 12% SDS-PAGE. After gel electrophoresis, the proteins were transferred to the polyvinylidene difluoride membranes (Immobilon PVDF; Millipore). The membranes were blocked for 1 hour at room temperature in 5% nonfat
dry milk in TBS containing 0.05% Tween 20, followed by overnight incubation at 4°C with primary antibodies. The membranes were then incubated with horseradish peroxidase-labeled secondary antibody (Chemicon) for 1 hour at room temperature. Peroxidase activity was detected through chemiluminescence (SuperSignal West Femto luminol substrate and peroxide buffer; Pierce). Primary antibodies used include anti-CSF-1 (Santa Cruz Biotechnology), anti-SDF-1α (Santa Cruz Biotechnology), anti-VEGF (Santa Cruz Biotechnology), and anti-glyceraldehyde-3-phosphate dehydrogenase (Kangcheng Technology).

Production of mouse VEGF by macrophage induced by conditioned medium from tumor cells

The LM3-R and SMMC7721 cell lines were seeded in six-well plates, and were allowed to grow to 60% confluence and then washed twice with serum-free DMEM, followed by treatment with serum-free DMEM containing 5 μmol/L sorafenib or same volume of vehicle for 24 hours. Then, DMEM was removed and fresh serum-free DMEM was added into the well. Another 24 hours later, the conditioned medium was collected and added to RAW 264.7 cells (mouse leukemic monocyte macrophage cell line, established by the Shanghai Institute of Cell Biology, Chinese Academy of Sciences, Shanghai), which were seeded in six-well plates. After incubation for 24 hours, the medium was collected and centrifuged to remove cellular debris, and the supernatants were frozen at −80°C until assayed for mouse VEGF by ELISA. The number of RAW cells was also counted to normalize the mouse VEGF protein expression in ELISA. All experiments were done in triplicate.

Statistical analysis

Data were analyzed by the computer program SPSS 15.0 (SPSS, Inc.) by means of an unpaired two-tailed Student’s t test. Continuous data were expressed as mean ± SEM. Results were considered statistically significant at a P value of <0.05.

Results

Clodrolip and ZA inhibit tumor growth and lung metastasis when combined with sorafenib

After 5 weeks of treatment, sorafenib-treated mice had a smaller tumor volume compared with that of mice in the control group in the LM3-R model (1.03 ± 0.05 cm³ versus 3.37 ± 0.23 cm³, P < 0.001) and in the SMMC7721 model (1.02 ± 0.1cm³ versus 1.86 ± 0.3 cm³, P < 0.05). Compared with sorafenib-treated mice, mice treated with a combination of sorafenib and clodrolip reduced tumor volume by 31% (P < 0.01) in LM3-R model and 23% (P < 0.05) in SMMC7721 model, and combination of ZA with sorafenib reduced tumor volume by 67% (P < 0.001) in the LM3-R model and 39% (P < 0.001) in the SMMC7721 model (Fig. 1A and C). In both models, although clodrolip alone did not inhibit tumor growth, it significantly enhanced the antitumor effects of sorafenib (Fig. 1A). Mice treated with ZA alone had a significantly smaller tumor volume compared with that of controls (Fig. 1A and C), which may not rule out mechanisms other than the macrophage depletion effect of ZA, such as an antiangiogenic effect (29, 30) or inhibition of matrix metalloproteinase 9 expression by macrophages other than by reducing the number of macrophages (31).

Lung metastasis was also significantly inhibited by sorafenib in the LM3-R model (integrated absorbance: 58.0 ± 3.65 versus 167.7 ± 15.80 per lung, P < 0.001; Fig. 1B), and the combination of clodrolip or ZA with sorafenib further inhibited lung metastasis compared with sorafenib alone (integrated absorbance: 36.17 ± 6.36 and 3.67 ± 2.28 versus 58.0 ± 3.65, P < 0.05 and P < 0.001, respectively). In the SMMC7721 model, lung metastases were found in two of six mice in the control group; no lung metastasis was found in the sorafenib group or the combination group.

Weight loss was similar in mice treated by sorafenib or ZA, but clodrolip, used either alone or combined with sorafenib, was associated with significant loss of body weight in LM3-R model (Fig. 1D). However, no significant body weight loss was found in mice bearing SMMC7721 tumors.

Clodrolip and ZA reduce F4/80- or CD11b-positive cells in peripheral blood and macrophage infiltration in sorafenib-treated tumors

We examined macrophage infiltration in tumors in response to sorafenib treatment in both models by immunohistologic staining of F4/80 and CD11b antibodies. Histologic studies revealed prominent infiltration of F4/80- or CD11b-positive cells in tumors (F4/80-positive staining area, 1.427% ± 0.47% versus 0.067% ± 0.019%, P < 0.05; Fig. 2C; CD11b-positive staining area, 5.0% ± 0.81% versus 1.06% ± 0.24%, P < 0.01; Fig. 2D), whereas macrophage infiltration was scarce in tumors of the control group. In the SMMC7721 model, significant increased macrophage infiltration in tumor was also found (P < 0.05; respectively; Fig. 2E and F). Macrophage infiltration was significantly suppressed by the combination of sorafenib with clodrolip or ZA, but not in the clodrolip- or ZA-treated tumors (Fig. 2A-D).

We also examined the number of monocytes/macrophages in peripheral blood by flow cytometry with F4/80 antibody and CD11b antibody (32). In the LM3-R model, sorafenib treatment increased the number of F4/80- and CD11b-positive cells in peripheral blood compared with that of controls (16.09% ± 1.29% versus 8.37% ± 0.88% for F4/80-positive cells and 15.68% ± 2.14% versus 11.43% ± 1.62% for CD11b-positive cells, respectively, P < 0.05). Mice treated with sorafenib with clodrolip or ZA had significantly fewer F4/80- or CD11b-positive cells in the peripheral blood than mice treated with sorafenib alone. However, mice treated with clodrolip alone or ZA alone did not significantly decrease the
number of F4/80- or CD11b-positive cells in peripheral blood compared with that of control (Fig. 3A and B). In the SMMC7721 model, sorafenib treatment also increased F4/80- and CD11b-positive cell recruitment in peripheral blood compared with that of controls (3.8 ± 0.48% versus 2.51 ± 0.29% for F4/80-positive cells and 15.8 ± 0.5% versus 10.6 ± 0.57% for CD11b-positive cells, P < 0.05, respectively). Sorafenib-induced recruitment of F4/80-positive cells to peripheral blood were depleted by the combination with clodrolip or ZA (2.56 ± 0.19% and 1.9 ± 0.2% versus 3.8 ± 0.48%, P < 0.05, respectively), whereas sorafenib-induced CD11b-positive cells (15.8 ± 0.5% versus 12.2 ± 1.66%, P < 0.05) were depleted by the combination of sorafenib with ZA.

CSF-1, SDF-1α, and VEGF are elevated after sorafenib treatment

CSF-1, SDF-1α, and VEGF are the important chemokines for macrophages (33, 34). We assessed the expression of three molecules in tumors by Western blotting and/or quantitative real-time PCR, and in the plasma by ELISA in the LM3-R and SMMC7721 model. We found that CSF-1 was elevated by 5.1-fold in the LM3-R tumor and 2.2-fold in the SMMC7721 tumor, and SDF-1α was elevated by 2.1-fold and 1.5-fold in both tumor models, respectively. VEGF mRNA was elevated by 1.65-fold in SMMC7721 tumor. Although the VEGF mRNA was not significantly increased in the LM3-R tumor, the VEGF protein was significantly elevated in this model (Fig. 4A-C).

Fig. 1. ZA and clodrolip enhanced tumor growth inhibition and reduced lung metastasis. A, sorafenib significantly inhibited tumor growth, and clodrolip and ZA enhanced the tumor inhibitory effect of sorafenib. B, sorafenib significantly inhibited lung metastasis, and clodrolip and ZA further reduced lung metastasis in sorafenib-treated mice. IOD, integrated absorbance. C, tumor volume was significantly reduced by the combination of sorafenib with clodrolip or ZA in the orthotopic model of the SMMC7721 cell line. D, clodrolip alone or combined with sorafenib was associated with significant loss of body weight in the LM3-R model (*, P < 0.05; **, P < 0.01; *** , P < 0.001).
The increase of CSF-1, SDF-1α, and VEGF was increased macrophage infiltration in the tumor and increased macrophage recruitment in the peripheral blood. As CSF-1 was reported to be one of the most important chemokines to recruit macrophages and was elevated to a much greater extent than SDF-1α and VEGF, we further assessed CSF-1 level in the plasma by ELISA and found that plasma CSF-1 concentration was elevated by 2.5-fold in sorafenib-treated mice in the LM3-R model ($P < 0.05$) and 1.55-fold ($P < 0.05$) in the SMMC7721 model (Fig. 4D and E), when compared with that of the nontreated mice.

**ZA and clodrolip inhibit tumor angiogenesis in sorafenib-treated mice**

Sorafenib significantly inhibited tumor angiogenesis as indicated by reduced MVD in the LM3-R model ($1.28\% \pm 0.15\%$ versus $3.77\% \pm 0.44\%, P < 0.001$), and sorafenib combined with clodrolip or ZA further inhibited tumor angiogenesis ($0.69\% \pm 0.08\%$ or $0.3\% \pm 0.07\%$ versus $1.28\% \pm 0.15\%, P < 0.01$ or $P < 0.001$, respectively), suggesting that macrophages play a role in tumor angiogenesis (Fig. 5A and B). Mouse plasma VEGF was elevated after sorafenib treatment ($218.2 \pm 18.2$ pg/mL versus $129.4 \pm 5.4$ pg/mL, $P < 0.01$) and was reduced by ZA or clodrolip ($123.0 \pm 2.9$ pg/mL or $93 \pm 21.8$ pg/mL versus $218.2 \pm 18.2$ pg/mL, $P < 0.01$ or $P < 0.001$, respectively; Fig. 5C). Mouse plasma VEGF treated with clodrolip alone did not differ significantly from that of the control group ($112.7 \pm 6.0$ pg/mL versus $129.4 \pm 5.4$ pg/mL, respectively, $P > 0.05$; Fig. 5C).

Because macrophages are one of the major sources of VEGF, we treated RAW 264.7 cells with conditioned media from sorafenib-treated cells or nontreated cells, and examined the mouse VEGF in the supernatants of macrophages. We found that after being treated by the conditioned media from sorafenib-treated LM3-R and SMMC7721 cells, macrophage secreted a higher level of mouse VEGF, compared with that treated with the conditioned media of nontreated tumor cells (Fig. 5D).
Discussion

The present study showed that sorafenib treatment induced a significant increase in the recruitment of F4/80- and CD11b-positive cells in blood and the infiltration of these cells in two orthotopic HCC xenografts. These results were accompanied with an elevated plasma mouse VEGF level, suggesting that macrophages may play a role in the progression of a tumor under sorafenib treatment. When combined with sorafenib, clodrolip and ZA, two drugs that deplete macrophages, significantly reduced intratumoral macrophage infiltration as well as decreased macrophage recruitment in peripheral blood compared with that of mice treated with sorafenib alone. In addition, sorafenib-induced elevation of mouse VEGF was also decreased by clodrolip and ZA, which led to the

![Recruitment of F4/80- and CD11b-positive cells in peripheral blood. A, percentage of F4/80- and CD11b-positive cells in peripheral blood were measured by flow cytometry; the flow cytometry data are representative of five to six samples in each group. B, columns, mean of five to six samples in each group; bars, SEM. *, P < 0.05; **, P < 0.01.](image)
enhanced inhibition of tumor growth, tumor angiogenesis, and lung metastasis.

In the present study, clodrolip alone did not inhibit tumor growth in sorafenib-nontreated mice but did present a synergistic inhibitory effect on tumor growth when combined with sorafenib, indicating the importance of sorafenib-induced macrophages in tumor progression under sorafenib treatment. Macrophages can be attracted into hypoxic, perinecrotic areas along a trail of necrotic debris emanating from such areas (8). Although T cell–mediated cellular immune mechanisms are absent in nude mice, it has been shown that the presence of macrophages can be activated (35). We found that sorafenib caused severe hypoxia and necrosis in the treated tumor, as indicated by pimonidazole and hypoxia-inducible factor 1α staining (see Supplementary Figure). Hypoxia-inducible factor 1α has been found to induce the recruitment of bone marrow–derived vascular modulatory cells, including F4/80- and CD11b-positive cells, to regulate tumor angiogenesis and invasion (36). In the present study, CSF-1, SDF-1α, and VEGF, all chemokines for macrophages (37, 38), were elevated in sorafenib-treated tumor. Plasma CSF-1 also increased after sorafenib treatment. CSF-1 can activate macrophages, thereby contributing to macrophage-tumor interaction, and may finally lead to tumor progression (9). It was also reported that CSF-1 has a direct effect on tumor growth, so we cannot rule out the possibility that CSF-1 may directly participate in tumor progression under sorafenib treatment. However, plasma CSF-1 levels were unchanged or even slightly elevated (not significantly) after the combination of either clodrolip or ZA with sorafenib, indicating that the effect of clodrolip or ZA was by targeting macrophages rather than CSF-1.

Upon activation by cytokines produced by cancer cells, macrophages can release a vast diversity of growth factors, cytokines, and inflammatory mediators; many of these

**Fig. 4.** CSF-1, SDF-1α, and VEGF were elevated in sorafenib-treated tumor. A to C, protein level of CSF-1, SDF-1α, and VEGF by Western blotting of LM3-R tumor (A), and mRNA level of M-CSF, SDF-1, and VEGF of sorafenib-treated tumor by quantitative PCR were normalized to control (B and C). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. CSF-1 was elevated by 5.1-fold in the LM3-R tumor and 2.2-fold in the SMMC7721 tumor. SDF-1α was also elevated by 2.1-fold and 1.5-fold in both tumor models, respectively. VEGF was elevated by 1.65-fold in the SMMC7721 tumor but not elevated in the LM3-R tumor. D and E, plasma CSF-1 level were also significantly elevated in sorafenib-treated mice compared with control in the LM3-R tumor (D) and SMMC7721 tumor (E), and the combination of clodrolip and ZA significantly reduced plasma VEGF in both models and also reduced plasma CSF-1 in the SMMC7721 model. *, P < 0.05; **, P < 0.01; ***P < 0.001.
Factors are key agents in cancer metastasis (39). Macrophage-produced VEGF regulates malignant progression through stimulation of tumor angiogenesis and tumor cell invasion (34). In a mouse model of cervical cancer, inhibition of matrix metalloproteinase 9 in macrophages blocked the release of VEGF and thereby inhibited angiogenesis and tumor growth (21). In the present study, sorafenib treatment increased plasma mouse VEGF concentration, which was accompanied with an increased number of F4/80- and CD11b-positive cells in the peripheral blood and increased macrophage infiltration in the tumor. Because the plasma level of mouse VEGF in the mice treated with clodrolip or ZA alone did not differ from that of control mice, it is highly possible that the decrease of sorafenib-induced plasma mouse VEGF associated with macrophage depletion and MVD decrease, which is consistent with the results from in vitro experiments in LM3-R and SMMC7721 cell lines.

The present study showed that the combination of sorafenib with either clodrolip or ZA had a better antitumor efficacy than sorafenib alone in two orthotopic HCC models, and the main effect of intratumoral-infiltrated macrophage was tumor promoting. However, we do agree that this conclusion needs to be validated in an immunity-competent mouse model. Clodrolip was toxic to mice in the present study and still is not clinically available. ZA has

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**Fig. 5.** Clodrolip and ZA reduced tumor angiogenesis of sorafenib-treated tumor. A, immunohistochemistry analysis of tumors stained with anti-CD31 (BD Pharmingen). B, quantitation of tumor vasculature indicated that clodrolip and ZA inhibited angiogenesis in combination with sorafenib. MVD of CD31 in each section was measured in five images from each treatment (n = 6). Bars, MVD in each treatment. Original magnification, ×100. **, P < 0.01; ***, P < 0.001.

C, combination with clodrolip or ZA was associated with decreased plasma VEGF of mouse origin (mouse VEGF). D, in vitro assessment of mouse VEGF in the supernatant of macrophage culture. Microphages were treated by conditioned medium of sorafenib-treated or nontreated LM3-R and SMMC7721 cells.
been widely used in the prevention of bone metastasis and reduction of skeletal tumor burden in many kinds of cancer, mainly by the inhibition of osteoclasts activity; ZA is well tolerated, either alone or combined with chemotherapy (19). We believe that ZA may also have other mechanism of action in addition to macrophage depletion; however, the antimacrophage effect is one of the major effects of ZA, as shown by our data. Another reason why we used ZA in this study is that ZA is clinically available to further improve the effect of sorafenib in clinical practice.

Conclusions

Although sorafenib significantly inhibited tumor growth and metastasis, it induced the elevation of F4/80- and CD11b-positive cells in peripheral blood and increased the infiltration of intratumoral macrophages, suggesting that macrophages have a role in the progression of tumor under sorafenib treatment. With the depletion of macrophages by clodrolip or ZA, tumor progression, tumor angiogenesis, and metastasis were significantly inhibited. ZA is clinical available and is promising to be combined with sorafenib for HCC patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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