Mechanical Analysis of Tumor Growth Regression by the Cyclooxygenase-2 Inhibitor, DFU, in a Walker256 Rat Tumor Model: Importance of Monocyte Chemoattractant Protein-1 Modulation

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Abstract  Cyclooxygenase (COX)-2 inhibition results in tumor regression; however, little is known about the mechanism. In the present study, using a Walker256 tumor model and a rat bone marrow–derived endothelial cell line TR-BME-2, we analyzed the effects of a new selective COX-2 inhibitor, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2-(5H)-furanone (DFU), on the production of chemokines and growth factors and on the neovascularization. The oral administration of DFU (5 mg/kg/d) significantly suppressed the tumor growth with decreasing microvessel density in vivo, although it showed no direct inhibition of Walker256 cell proliferation in vitro. It was newly found that the recruitment of systemically injected TR-BME-2 cells into the tumor site was significantly inhibited by DFU treatment. In addition, we found that DFU significantly reduced the production of monocyte chemoattractant protein-1 (MCP-1) both in tumor tissues and in the systemic circulation ($P < 0.001$ and $P < 0.001$, respectively). Such reduction was not observed in other chemotactic factors, vascular endothelial growth factor and stromal cell–derived factor-1. The induced chemotaxis of TR-BME-2 by serum of tumor-bearing rats was significantly reduced in DFU-treated rat serum, although DFU showed no direct inhibition for TR-BME-2 cells, either cell growth or chemotaxis. Treatment with neutralizing antibodies to soluble mediators, including MCP-1, significantly suppressed the chemotaxis. Regarding the down-regulation machinery of MCP-1 production in vivo, tumor-associated macrophages seem to play crucial roles, because DFU eliminated MCP-1 production in the activated macrophages remarkably but not in Walker256 tumor cells in vitro. In conclusion, COX-2 inhibitor DFU exerts tumor regression activity in a Walker256 tumor model by suppressing MCP-1 production in tumor tissues and in the circulation.

Postnatal neovascularization does not rely exclusively on sprouting from preexisting blood vessels (angiogenesis); instead, endothelial progenitor cells (EPC) circulate from the bone marrow to incorporate into and thus contribute to postnatal physiologic and pathologic neovascularization, which is consistent with postnatal vasculogenesis (1). Exogenously given EPCs augment the impaired neovascularization in an animal model of experimentally induced limb ischemia (2). In addition, the efficacy of therapeutic neovascularization by vasculogenesis against ischemic disease has been shown clinically. For instance, the mobilization of EPCs into the peripheral blood during acute myocardial infarction in humans (3) and the efficacy and safety of implantation of bone marrow-mononuclear cells (MNC) in ischemic limbs were reported in a pilot study on therapeutic angiogenesis (4, 5).

Neovascularization or angiogenesis plays an important role in the growth, progression, and metastasis of solid tumors (6, 7). It has been suggested that the degree of tumor angiogenesis is related to the clinical outcome, where there is a relationship between the angiogenic properties and tumor aggressiveness (8–10). Various antiangiogenic modalities have been experimentally developed and the inhibitory effects on endothelial cell proliferation or angiogenic factors have been reported (11–14). At present, some antiangiogenic cancer therapies have already shown their clinical efficacies (15, 16). However, further understanding of neovascularization is necessary to obtain greater clinical benefits of antiangiogenic cancer therapies.

Recently, Lyden et al. reported that bone marrow–derived precursors are essential for tumor neovascularization in an experimental model (17). Furthermore, we also reported that our newly established rat bone marrow–derived EPCs (TR-BME-2 cells) specifically accumulated at the tumor site and increased tumor neovascularization and tumor growth (18, 19). In addition, it was also confirmed that the tumor growth was significantly suppressed in rats that underwent myeloablative treatment before cell inoculation (19). The

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above findings suggest that targeting the vasculogenesis would serve as a novel antiangiogenic therapy for human solid malignancies (20).

Several studies described that tumor-derived growth factors promote neovascularization by inducing the production of cyclooxygenase-2 (COX-2)–derived prostaglandin E$_2$ (21). COX-2 inhibitors have been categorized into a new class of antiangiogenic agents and induce tumor-associated endothelial cell apoptosis and inhibition of endothelial cell proliferation by cell cycle arrest (22–24). 5,5-Dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl)phenyl-2-(5H)-furanone (DFU) is also a selective COX-2 inhibitor, a structurally related analogue of rofecoxib containing a 5,5-dimethyl furanone ring. Previously, we reported that DFU had an antitumor effect with an anti-angiogenic effect against human breast cancer xenografts in severe combined immunodeficient mice (25); however, the details are still not clear. In the present study, we examined the antitumor effect of DFU in the rat Walker256 tumor model and determined the antichemotactic effect of DFU and its relevant events with TR-BME-2 cells. Among the many biological features of neovascularization, migration of EPCs toward solid cancers seems to be an extremely crucial process. Vascular endothelial growth factor (VEGF) is certainly considered as an important endothelial cell migration factor, but it is also known that the CXC chemokine, stromal cell–derived factor-1 (SDF-1), contributes to EPC-mediated vasculogenesis in the ischemic tissue model (26) and the CC chemokine, monocyte chemoattractant protein-1 (MCP-1), stimulates chemotaxis (27). Therefore, in the present study, we focused on these soluble mediators when analyzing the tumor regression mechanism driven by DFU.

Materials and Methods

Cells and cell culture. The TR-BME-2 cell line was established from the bone marrow of temperature-sensitive SV40 large T antigen (SV40 ts T-Ag) transgenic rats. TR-BME-2 is a clone of TR-BMEs and has been characterized previously as a progenitor endothelial cell line (18). TR-BME-2 was cultured in EBM-2 medium (Cambrex BioScience Walkersville, Inc., Walkersville, MD) at 33°C, an active temperature for SV40 ts T-Ag. The syngeneic Walker256 breast carcinosarcoma cell line provided by Cell Resource Center for Biomedical Research...
The collected single cells were layered on Ficoll-Hypaque (Pharmacia) and centrifuged at 1,500 rpm at 4°C for 10 minutes at 4°C. The cell pellet was collected by centrifuging the cell suspension at 3,000 rpm for 10 minutes at 4°C. The collected cells were subjected to DNase I (Roche Diagnostics, Mannheim, Germany) at 37°C for 1 hour. The cell pellet was re-suspended in RPMI 1640 (Life Technologies) supplemented with 2 mg/mL collagenase, 300 μg/mL hyaluronidase (Sigma, St. Louis, MO), and 0.2 mg/mL DNase I (Roche Diagnostics, Mannheim, Germany) at 37°C for 1 hour. The cell pellet was collected by centrifuging the cell suspension at 1,500 rpm at 4°C for 5 minutes followed by washing twice with RPMI 1640. The collected single cells were layered on Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 1,600 rpm for 25 minutes at room temperature. The lymphocyte layer was collected and washed thrice with PBS. These isolated cells (1 x 10^6/mL/tube) were subjected to FACScan (Becton Dickinson, San Jose, CA) and the findings were analyzed using CellQuest software.

**DFU treatment study in vivo.** Female Wistar rats were purchased from Charles River Japan (Yokohama, Japan). The protocols of the animal experiments were approved by the Institutional Animal Care and Use Committee. DFU (Merck & Co., Inc., Whitehouse Station, NJ) was dissolved in DMSO (20 mg/mL) as a stock solution for the in vivo study. Rats were s.c. inoculated with 1 x 10^6 Walker256 cells in the posterior aspect of the right flank (day 0). In the in vivo study, 0.5 mL DFU stock solution was dissolved in 600 mL distilled water. The rats in the DFU group were orally given drinking water containing 30 mL distilled water plus DFU (5 mg/kg) everyday from day 0. In the TR-BME-2-treated group, TR-BME-2 cells were labeled with a fluorescent reagent, PKH26 (Zynaxiz, Malvern, PA), according to the manufacturer’s protocol. Labeled TR-BME-2 cells were given systemically to tumor-bearing rats treated with DFU (Ca+DFU) were collected (n = 8 in each group) and the levels of MCP-1 (D), VEGF (E), and SDF-1 (F) were shown. Representative data. Columns, mean; bars, SD. **, P < 0.01;***, P < 0.001.

**Immunostaining and microvessel density.** Frozen tumor tissue was immediately immersed in Tissue-Tek OCT compound (Sakura Fine-technological Co., Ltd., Tokyo, Japan). The tissue was sectioned and 6- to 10-μm slices were fixed in 4% paraformaldehyde. After peroxidase quenching, immunohistochemical staining was done using an electric Polytron homogenizer (Kinematica, Germany). Homogenized samples were centrifuged at 12,000 rpm at 4°C for 20 minutes and the supernatants were stored at −80°C until processing. The total protein of these extracts was quantified using a BCA protein assay kit (Pierce, Rockford, IL).

**Reverse transcription-PCR.** Reverse transcription-PCR (RT-PCR) with specific primers for CCR2 and CXCR4 were done with total cellular RNAs extracted from TR-BME-2 cells. PCR of CCR2 was carried out with the primers 5'-ATTCACCCCTGCTCTTCC-3' and 5'-AGTACCTCCAGGATCAAACT-3' (product size, 391 bp) using
cells and HUVECs were cultured in RPMI 1640 with 10% FBS at 37°C for 24 hours and harvested by trypsinization. These cells were resuspended in serum-free RPMI 1640 and plated on the upper chamber (5 × 10^5/200 μL/well). The lower chamber was filled with of RPMI 1640 (700 μL/well), which was supplemented with 10% rat serum, 50 ng/mL rat MCP-1, 10 ng/mL human SDF-1α (PeproTech, London, United Kingdom), 5 μg/mL anti-rat MCP-1 (IBL, Gunma, Japan), 50 pg/mL rat VEGF, 1 μg/mL anti-rat VEGF (R&D Systems), and 1 μg/mL anti-mouse SDF-1α (Torrey Pines Bioslabs, Inc., Houston, TX). After 2 hours of incubation at 37°C, the cells on the upper side of the chamber were removed with cotton swabs. Cells that migrated to the lower side of the membrane were fixed in 70% ethanol, stained with Giemsa, and counted for five fields (>200) per filter under a microscope.

**MNC preparation and lipopolysaccharide stimulation.** Peripheral blood was collected with heparin and diluted with an equal volume of PBS. Diluted blood was layered on Ficoll-Hypaque and centrifuged at 1,600 rpm for 25 minutes at room temperature. The lymphocyte layer was collected and washed thrice with PBS. Collected MNCs were suspended in RPMI 1640 (supplemented with 10% FBS) and cultured on a six-well culture plate (2 mL/well) at 37°C for 1 hour. These cells were then washed with PBS twice and adherent cells were cultured with or without 10 nmol/L lipopolysaccharide (LPS; Sigma) and 100 μmol/L DFU at 37°C for 24 hours.

**Cell proliferation assay.** Cells were seeded 5 × 10^4 per well in 96-well plates and adhered for 24 hours. Then, medium containing differing concentrations of DFU diluted in DMSO was added to give the final concentrations of 0, 10, and 100 μmol/L. The final concentration of DMSO was 0.1% in all groups. After 24 hours of culture in the presence of DFU, the proliferative activity was determined with [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] (WST-8) assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. A WST-8 solution (10 μL) was added to each well, and after 1 hour of culture, the conversion of WST-8 to formazan was measured in a plate reader at 450 nm.

**Statistical analysis.** All values are expressed as mean ± SD. Statistical significance was evaluated using the unpaired Student’s t test for comparisons between the two means.  

### Results

**Inhibitory effects of DFU on tumor growth and neovascularization.** We evaluated the antitumor effect of the oral administration of DFU against Walker256 tumors in rats (Fig. 1A). The group receiving DFU had a significant decrease in tumor weight compared with the control group (mean ± SD, 0.33 ± 0.15 versus 0.49 ± 0.15 g; n = 8; P = 0.048, respectively). DFU (~100 μmol/L) did not show an antiproliferating effect of Walker256 cells in vitro (Fig. 1B). There was a large reduction in CD31^+ vessels in the tumors of the DFU-treated group compared with the tumors of the control group as evidenced by the results of immunostaining (Fig. 1C). The mean ± SD of the MVD in DFU-treated and nontreated rats were 56.6 ± 7.6 and 143.7 ± 13.5 number of vessels/mm², respectively. The MVD in the group receiving DFU significantly decreased (Fig. 1D).

**Effect of DFU on endothelial recruitment in vivo.** Systemically injected TR-BME-2 cells into tumor-bearing rats specifically accumulated in the inoculated Walker256 tumors (19). Using this model, we investigated whether DFU inhibited the migration of endothelial recruitment in vivo. Fluorescently labeled TR-BME-2 cells were injected into the rats with day 3 tumors, which were then resected on day 7. By using the
different sizes between tumor cells and endothelial cells, small numbers of endothelial cells within the tumor-derived single cells were detected using flow cytometry as reported previously (19). In this study, we analyzed endothelial recruitment in the gate of endothelial cells. Fluorescent cells were hardly detected within the resected tumors, which received no treatment (no treatment, n = 3); however, many fluorescent cells were detected within resected tumors from rats injected with labeled TR-BME-2 cells (TR-BME-2, n = 3). Such accumulation of TR-BME-2 cells was remarkably inhibited by the treatment with DFU (TR-BME-2 + DFU, n = 3; Fig. 2). These results indicated that DFU exerts inhibitory activities on the endothelial cell recruitment from the circulation into peripheral tumor tissues, which may contribute to the tumor growth suppression driven by DFU.

Concentration of MCP-1, VEGF, and SDF-1 in tumor tissue and serum. We looked into the concentrations of major angiogenic and migration factors, VEGF, MCP-1, and SDF-1, in tumor tissue and the serum. Tissue extracts were prepared from the nontreated tumor (control) and the DFU-treated tumor (DFU) and the level of MCP-1, VEGF, and SDF-1 were measured by ELISA. We determined the location of a tumor (DFU) and the level of MCP-1, VEGF, and SDF-1 in tumor tissue and the serum. Tissue extracts were prepared and serum.

Fig. 3C). The final concentration of solvent (DMSO) was 0.1% in all groups. Representative of three independent experiments. Columns, mean compared with the control; bars, SD. **, P < 0.01; ***, P < 0.001. A, chemotaxis assay. TR-BME-2 cells or HUVECs were placed in the upper chamber of a chemotaxis cell. Medium containing 10% serum from healthy rats (Control), tumor-bearing rats (Ca), and DFU-treated tumor-bearing rats (Ca+DFU) was added to the lower chamber. Chambers were incubated for 2 hours at 37°C. Migrated cells were stained and counted for five fields. Representative of three independent experiments. Columns, mean compared with the control; bars, SD. **, P < 0.01; ***, P < 0.001. B, proliferation assay. TR-BME-2 cells and HUVECs were cultured with DFU (0, 10, and 100 μM) for 24 hours at 37°C. The final concentration of solvent (DMSO) was 0.1% in all groups. Representative data. Columns, mean compared with 0 μM/L DFU; bars, SD. **, P < 0.01; ***, P < 0.001; ***, P < 0.001 (n = 6).

Effects of DFU on endothelial cell recruitment into tumor tissue. To investigate the effects of DFU on the recruitment of endothelial cells to tumor site, we examined the chemotactic response and cell proliferation using TR-BME-2 in tumor-bearing and DFU-treated rats. In addition, we also examined those of response on endothelial cells using HUVECs. The chemotactic activity of TR-BME-2 cells and HUVECs increased in medium containing 10% serum from tumor-bearing rats (Ca); however, this decreased in medium containing 10% serum from tumor-bearing rats (Ca + DFU) in vitro (n = 4; P < 0.001 and P < 0.01; Fig. 5A). In the cell proliferation assay, a different response was observed between TR-BME-2 cells and HUVECs. The cell growth inhibition

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was recognized in HUVECs but not in TR-BME-2 cells (n = 6; P < 0.05; Fig. 5B).

Chemotactic response of the TR-BME-2 cells and suppression of cell migration in the serum from tumor-bearing rats by neutralizing MCP-1, VEGF, and SDF-1. We did a migration assay to confirm the chemotactic response of the EPC line, TR-BME-2, against MCP-1, VEGF, and SDF-1. The expression of the MCP-1 chemokine receptor CCR2 and the SDF-1 receptor CXCR4 in TR-BME-2 cells was detected by RT-PCR (Fig. 6A). The detection of VEGF receptors 1 and 2 in TR-BME-2 were reported previously (18). The chemotactic activity of TR-BME-2 cells increased significantly in medium containing 10% serum from tumor-bearing rats with or without neutralizing antibody to MCP-1, VEGF, or SDF-1 was added to the lower chamber. Chambers were incubated for 2 hours at 37°C. Migrated TR-BME-2 cells were stained and counted for five fields. Representative of three independent experiments. Columns, mean compared with the absence of a neutralizing antibody; bars, SD. *** P < 0.001.

Suppression of MCP-1 from tumor-associated macrophages by DFU. Because DFU (100 μmol/L) hardly effected the secretion of MCP-1, VEGF, and SDF-1 from Walker256 cells in vitro (Fig. 4), we speculated that MCP-1 might be released from stroma cells, such as macrophages within tumors, or, more specifically, tumor-associated macrophages (TAM). To confirm this, we prepared plastic adherent MNCs with LPS stimulation as a quasi-TAM. Although the cells strongly produced MCP-1 compared with the controls (n = 3; P < 0.001), DFU significantly suppressed the expression of MCP-1 (n = 3; P < 0.001; Fig. 7A). The VEGF production from macrophages tended to be increased by LPS and by
DFU stimulation; however, no significant difference was detected \((n = 3; \text{Fig. 7B})\). The SDF-1 production from LPS-stimulated macrophages was not significantly suppressed by DFU \((n = 3; \text{NS; Fig. 7C})\).

**Discussion**

Recently, many antiangiogenic therapies have been clinically developed against cancer, because neovascularization is one of the most important therapeutic targets. For instance, the blockade of VEGF and the VEGF receptor pathway has been shown to result in tumor regression and the prolongation of survival \((28, 29)\). In addition, it has also been highlighted that the suppression of vasculogenesis is a promising therapeutic approach in blood vessels perfusing tumors \((17, 20, 30)\). In the therapeutic field of benign disease, the concept of stem cell pharmacogenomics has been advocated primarily for clinical improvement, specifically the regulation of stem cell function through drugs \((31)\). Therefore, it would be reasonable to focus on the process of vasculogenesis, such as the recruitment of endothelial cells from circulation to the peripheral solid tumor site and endothelial chemotaxis, in anticancer treatment. From these points of view, in the present study, we analyzed the activity of a COX-2 inhibitor, DFU, on its antiangiogenic and antivasculogenic aspects.

DFU suppressed tumor growth significantly *in vivo*. However, it was also revealed that DFU had no direct anti–cell proliferation activity on the Walker256 tumor cells *in vitro*, suggesting that other mechanisms are involved. Therefore, we paid attention to the antineovascularization effect of DFU and, in fact, found that DFU significantly reduced the MVD. Although DFU showed no direct activity on the TR-BME-2 cells *in vitro*, in either cell proliferation or cell chemotaxis, it was discovered that the recruitment of systemically injected TR-BME-2 cells into the tumor tissue site was remarkably inhibited in DFU-treated rats compared with the DFU-untreated control rats. In the previous studies, TR-BME-2 cells having biological properties as EPCs were characterized to accumulate to peripheral tumor site specifically and helped to enhance neovascularization and tumor growth when it is injected systemically \((19)\). Therefore, it is possible to consider that the inhibition of endothelial recruitment to the tumor tissue site contributes to the down-regulation of microvessel formation and tumor regression. Although the real number of circulating EPCs was not counted in this study because of technical reasons, many recent studies have shown that EPCs have a significant role in tumor neovascularization and the growth in rat tumor models.

Simultaneously, we measured the concentrations of major endothelial growth or migration factors, such as VEGF, MCP-1, and SDF-1, in tumor tissues and in the circulation of tumor-bearing rats in the presence or absence of DFU. Interestingly, DFU decreased the levels of MCP-1, but not VEGF or SDF-1, in both tumor tissues and the systemic circulation. Because DFU showed no inhibitory activities on the expressions of MCP-1, VEGF, and SDF-1 in Walker256 cells *in vitro*, an indirect mechanism was speculated to explain the down-regulation of MCP-1 expression in tumor tissues. According to the experiments with macrophages, it was shown that MCP-1 production in the activated macrophages was clearly down-regulated, so that the anti-inflammatory activity of DFU might be a key machinery to reduce the MCP-1 expression. In sense, the down-regulation of MCP-1 by celecoxib, a COX-2 inhibitor, has also been reported in a rabbit atherosclerotic balloon injury model \((32)\). In tumor tissues, TAMs might be one of the major targets of DFU. Many studies have elucidated that the suppression of the activities of TAMs results in the regression of tumors. Because MCP-1 is a key regulator in the interaction between tumor cells and TAMs, it is hypothetically possible to consider that the reduction of MCP-1 production interferes with the synergistic protumor interaction between tumor cells and the macrophages and causes the inhibition of tumor growth \((33)\). Another COX-2 inhibitor, NS-398, is known to negatively modulate the production of angiogenic factors, including VEGF, in colon cancer cells *in vitro* \((34)\), indicating that the
mechanism of actions might differ between different COX-2 inhibitors. In the present study, DFU was rather up-regulated in the circulating levels of VEGF. The mechanisms of this paradoxical up-regulation of VEGF by DFU treatment are still unclear.

Subsequently, we analyzed the effect of DFU treatment on endothelial chemotaxis using TR-BME-2 cells. These endothelial cells express chemokine receptors, CCR2 and CXCR-4, as well as VEGF receptors. The importance of VEGF on the mobilization and recruitment of bone marrow–derived EPCs toward an ischemic lesion has already been clarified (35), and it is documented that SDF-1 and MCP-1 exhibit a potent stimulatory effect on EPC kinetics and enhance the neovascularization of ischemic tissues (26, 36–39); however, little is known about the details in the malignant diseases. DFU showed no direct chemotactic and no anti–cell proliferation activity to TR-BME-2 cells, which might indicate a unique profile of DFU, because it is reported that celecoxib can induce endothelial cell apoptosis and inhibit endothelial cell proliferation (23). In the in vitro chemotaxis assay, the serum from the rats treated with DFU showed less chemotactic activity compared with that from control tumor-bearing rats. Neutralizing antibodies to the soluble mediators remarkably reduced the chemokine or growth factor–induced chemotaxis, indicating that all three of these mediators can potentially function as chemotactic factors to the endothelium. The blockade of MCP-1 activity caused the suppression of endothelial chemotaxis, which implies that the down-regulation of the MCP-1 level might result in a similar result. Apart from TR-BME-2 cells, DFU suppressed chemotaxis and cell proliferation of the HUVECs, so that there is the possibility that DFU acts directly on the normal endothelial cells in the tumor tissues. Nevertheless, it would be more reasonable to hypothesize that DFU inhibits the recruitment or chemotaxis of endothelial cells from circulation and, perhaps, the growth of preexisting endothelial cells as well. At the moment, it is difficult to clearly distinguish these two biological events, so-called angiogenesis and vasculogenesis.

In conclusion, a COX-2 inhibitor, DFU, exerts tumor regression activity in the Walker256 tumor model. As a mechanism, the suppression of MCP-1 production in tumor tissues and in the circulation and the indirect suppression of endothelial chemotaxis were indicated. These analyses highlighted that MCP-1 might be a key molecule to deal not only with anti-COX-2 treatment but also for other cancer therapies. In future, it is warranted to consider anti-MCP-1 therapeutic strategies against solid cancers.

References


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