

Evaluation of pro- and anti-tumor effects induced by three colony-stimulating factors, G-CSF, GM-CSF and M-CSF, in bladder cancer cells: Is G-CSF a friend of bladder cancer cells?

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Abstract. Cytotoxic chemotherapy is the standard treatment for patients with advanced bladder cancer. However, this treatment can cause transient and prolonged neutropenia, which can result in fatal infection. Three recombinant human colony-stimulating factors (CSFs), granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), and macrophage CSF (M-CSF), are currently available to reduce the duration and degree of neutropenia. The present study investigated the pro- and anti-tumor effects of these three CSFs and the changes in molecular profiles. Xenograft tumors in athymic

mice were generated by subcutaneously inoculating the human bladder cancer cell lines MGH-U3 and UM-UC-3. A total of 2 weeks after cell inoculation, mice were randomly divided into four groups (control, G-CSF, GM-CSF and M-CSF) and treated thrice a week for 2 weeks. Tumor growth during monitoring and tumor weight at the time of euthanization were significantly higher in mice treated with G-CSF and lower in mice treated with GM-CSF compared with the control mice. Tumors were examined by immunostaining with antibodies against proteins associated tumor proliferation (Ki-67), angiogenesis [CD31 and vascular endothelial growth factor (VEGF)], anti-immunity (CD204) and epithelial-mesenchymal transition (EMT; E-cadherin). Immunohistochemical staining revealed that tumor proliferation, angiogenesis, recruitment of M2 macrophages and EMT were promoted by G-CSF, whereas lymphangiogenesis and recruitment of M2 macrophages were inhibited by GM-CSF. Treatment-associated changes in serum pro- and anti-tumoral cytokines and chemokines were evaluated by enzyme-linked immunosorbent assay (ELISA)-based arrays. In the ELISA for serum, the levels of cytokines associated with angiogenesis (interleukin-6 and VEGF), and EMT (transforming growth factor- β 1 and - β 2) were elevated in mice treated with G-CSF. Treatment with GM-CSF and M-CSF also affected the level of these cytokines characteristically. The current results indicate that administration of exogenous G-CSF to patients with bladder cancer promotes tumor growth through promotion of cell proliferation, angiogenesis, recruitment of M2 macrophages and enhancement of EMT through the modulation of the tumor microenvironment.

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Abbreviations: UCB, urothelial carcinoma of the bladder; MIBC, muscle invasive bladder cancer; FN, febrile neutropenia; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; G-CSFR, granulocyte colony-stimulating factor receptor; MDSC, myeloid-derived suppressor cell; HUVEC, human umbilical vascular endothelial cell; FBS, fetal bovine serum; H&E, hematoxylin and eosin; IHC, immunohistochemistry; ELISA, enzyme-linked immunosorbent assay; EMT, epithelial-mesenchymal transition; VEGF, vascular endothelial growth factor; HPF, high power field; TNF, tumor necrosis factor; IGF-1, insulin-like growth factor-1; IL, interleukin; FGF, fibroblast growth factor; IFN, interferon; TGF, transforming growth factor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; M-CSFR, macrophage colony-stimulating factor receptor; RT-PCR, reverse transcription-polymerase chain reaction; EPC, endothelial progenitor cell; STAT3, signal transducer and activator of transcription 3; JAK, Janus kinase

Key words: bladder cancer, neutropenia, colony-stimulating factors, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, angiogenesis, immunoreaction, immunomodulation, angiogenesis

Introduction

In Japan, urothelial carcinoma of the bladder (UCB) is the eighth most common malignancy in men (1). Worldwide, UCB is the second most frequent malignancy of the urogenital tract and the fourth most common cancer among men (2). In total, ~20-40% of cases of UCB present as or develop to muscle invasive bladder cancer (MIBC) (3). Radical cystectomy is the gold standard treatment for MIBC and the administration of neoadjuvant or adjuvant cisplatin-based chemotherapy

has become common due to improvements of prognosis for patients treated by this method (4-6). Cisplatin-based chemotherapy is also a first-line therapy for patients with advanced UCB, including locally advanced and metastatic disease (7). Chemotherapy-induced myelosuppression is often a problem for patients treated with chemotherapy, resulting in febrile neutropenia (FN) and sometimes leading to infection-associated mortality. The aim of clinical management for advanced UCB is to continue chemotherapy safely while controlling adverse effects like FN, resulting in prolonged survival time.

The administration of granulocyte colony-stimulating factor (G-CSF) is recommended for patients who are treated with strong chemotherapies to reduce the rate of chemotherapy-induced mortality (8,9). In addition, the administration of G-CSF can result in a shorter duration of grade IV neutropenia, antibiotic treatment and hospital stay (10). The effects of administration of granulocyte-macrophage CSF (GM-CSF) and macrophage CSF (M-CSF) on chemotherapy-induced myelosuppression and FN have also studied. The duration of neutrophil recovery and hospital stay was reduced by GM-CSF administration, and the incidence rate and duration of FN was reduced by the administration of M-CSF (11,12).

G-CSF, GM-CSF and M-CSF serve roles in regulating the hematopoiesis of blood cells, modulating the functional response and maintaining immune response (13). G-CSF and M-CSF serve roles in the proliferation and differentiation of macrophage and neutrophils, respectively, at stages of lineage commitment. In addition, GM-CSF regulates the expansion and maturation of primitive hematopoietic progenitors at earlier stages of lineage commitment, induces activation status of macrophages and mediates differentiation to other cells, including dendritic cells, that participate in immune responses (13). G-CSF was initially purified from the human bladder cancer cell line 5637 in 1985 and has been identified to serve a role in promoting the growth of bladder cancer cells, which express the G-CSF receptor (G-CSFR) (14,15). To the best of our knowledge, at present, the association between CSFs and tumor progression remains unclear. Although G-CSF is most frequently used for patients with chemotherapy-induced myelosuppression and FN, G-CSF has also been demonstrated to promote tumor growth, progression and metastasis through the enhancement of tumor angiogenesis, proliferation and migration, and functions in the maintenance of myeloid derived suppressor cells (MDSCs), which promote cancer progression by immune suppression, resulting in poor prognosis (16-18). GM-CSF and M-CSF also affect cancer progression through the modulation of anti-tumor immunity in the tumor microenvironment (19-22). However, the associations between CSFs and tumor progression in patients with UCB remain unclear and changes in the molecular profile in the tumor microenvironment have not been fully understood. The present study used xenograft tumors generated by subcutaneously inoculating mice with human bladder cancer cell lines, MGH-U3 (low-grade) and UM-UC-3 (high-grade) (23). The aim of the present study was to evaluate the pro- and anti-tumor effects of G-CSF, GM-CSF and M-CSF, and the changes in the molecular profiles.

Materials and methods

Cell lines. Two human urothelial carcinoma cell lines, MGH-U3 and UM-UC-3, and two mouse urothelial carcinoma cell lines, MB49 and MBT2, were used in the current study. UM-UC-3 and MBT2 were purchased from the American Type Culture Collection (Manassas, VA, USA) and the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), respectively. MGH-U3 and MB49 cells were kindly provided by Dr Helene LaRue (Laval University Cancer Research Centre, Quebec, Canada) and Dr Tatsuya Nakatani (Osaka City University, Osaka, Japan), respectively. Cancer cell lines were maintained in RPMI-1640 (Nacalai Tesque, Inc., Kyoto, Japan) or Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a standard humidified incubator at 37°C in an atmosphere of 5% CO₂. Human umbilical vascular endothelial cells (HUVECs; Lonza, Tokyo, Japan) were also used in the present study. HUVECs were cultured in EBM-2 basal media supplemented with the EGM-2 MV kit (Lonza) containing 2% FBS in a standard humidified incubator at 37°C in an atmosphere of 5% CO₂. HUVECs were used at passages 4-6.

Animals. Animal care was in compliance with the recommendations of the Guide for Care and Use of Laboratory Animals (National Research Council) and the study was approved by the Animal Facility Committee at Nara Medical University (ID: 11883; Nara, Japan). A total of 42 male athymic BALB/c nu/nu mice (six-week-old; 22 g) were purchased from Oriental Bio Service, Ltd. (Kyoto, Japan). All mice were maintained under pathogen-free conditions and provided with sterile food and water. The mice were kept in a temperature- and humidity-controlled room, with a 12-h/12-h light/dark cycle and food was provided *ad libitum*.

Reagents. To treat tumor-bearing mice, the following recombinant human and mouse CSFs were purchased from Miltenyi Biotec (Auburn, CA, USA); human G-CSF, GM-CSF and M-CSF, and mouse G-CSF, GM-CSF and M-CSF. All reagents were diluted in sterile PBS solution according to the manufacturer's protocol. Sterile PBS was used as a treatment control.

Xenograft model and intraperitoneal administration. Fig. 1A presents a schematic representation of the present study. After allowing mice to acclimate to the facility for 1 week, MGH-U3 and UM-UC-3 urothelial cancer cells (5×10^5 /tumor) in 50 μ l RPMI-1640 medium and 50 μ l growth factor-reduced Matrigel (Corning Inc., Corning, NY, USA) were injected into the flank of each mouse. A total of 2 weeks after cell inoculation, when the tumors reached 5 mm in diameter, the mice were randomly divided into four groups: Control (PBS, n=6), G-CSF (1.25 μ g/kg, n=12), GM-CSF (4.5 μ g/kg, n=12) and M-CSF (145,000 U/kg, n=12). The prescribed dose of each drug was calculated based on the dosage for humans in a clinical setting of neutropenia (8-12). Intraperitoneal administration was initiated from the third week and was performed thrice a week for two weeks. Tumor diameters were measured thrice

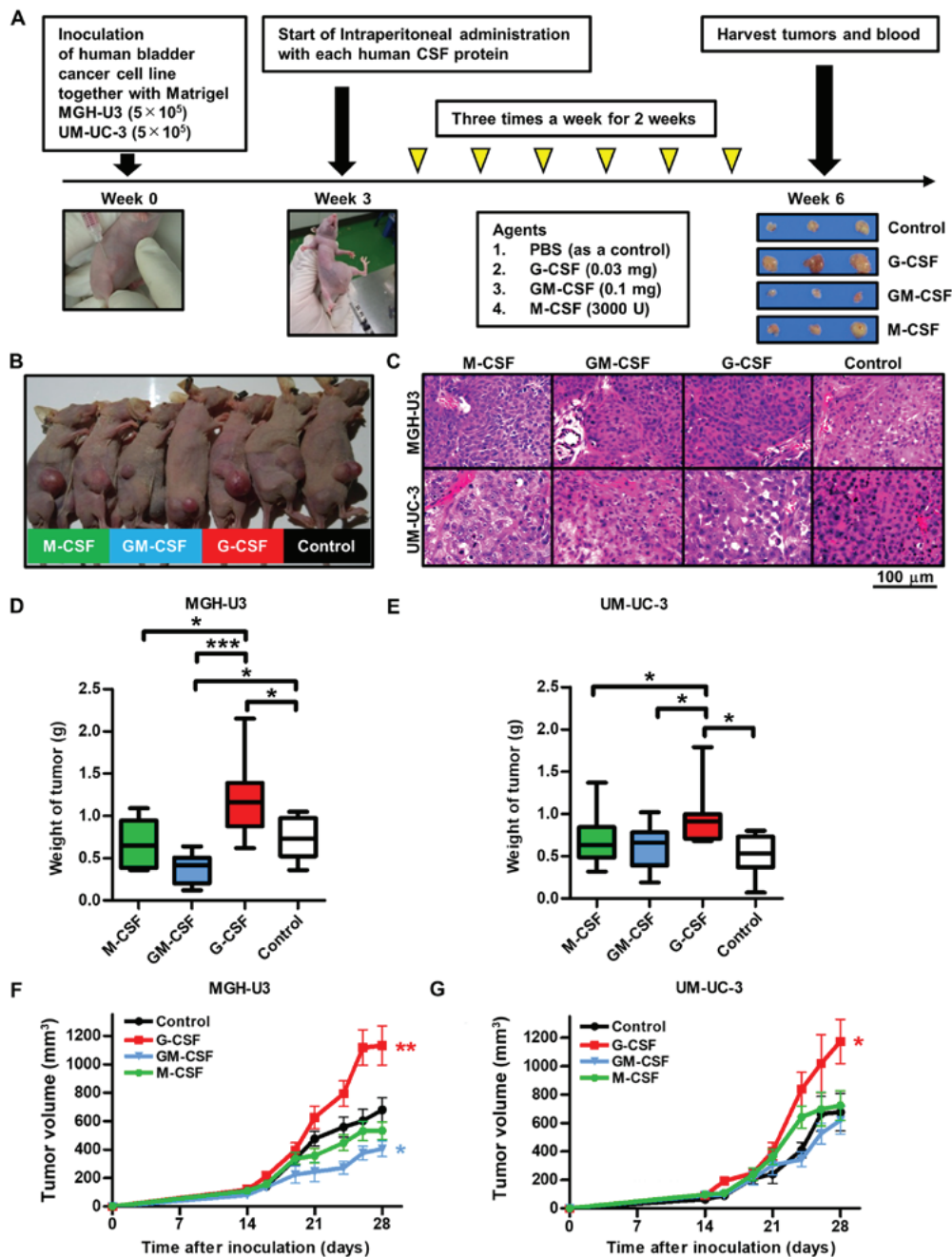


Figure 1. Study treatment scheme and inoculated xenografts. (A) Schematic diagram illustrating the study workflow. Mice were injected with UM-UC-3 cells (5×10^5 /tumor) or MGH-U3 cells (5×10^5 /tumor), together with growth factor-reduced Matrigel. A total of 2 weeks after inoculation, mice were randomly divided into four treatment groups [PBS (control), recombinant human G-CSF, recombinant human GM-CSF and recombinant human M-CSF]. Subsequently, mice were treated thrice a week for two weeks. A week after the last treatment, mice were euthanized, and xenografts and blood were harvested. Anti-Ki-67, CD31, LYVE-1, VEGF, CD204 and E-cadherin antibodies were used to evaluate cell proliferation, angiogenesis, lymphangiogenesis, M2 macrophages and epithelial-mesenchymal transition. Serum was used to perform ELISA. (B) Representative tumor sizes of each group. Xenografts of mice treated with G-CSF were the largest and those of mice treated with GM-CSF were the smallest in the four treatment groups. (C) Representative images of hematoxylin and eosin-stained xenografts from each group. Viable cancer cells were observed in all resected xenografts. (D) Weight of xenografts following inoculation with MGH-U3 cells. Weight of xenografts in mice treated with G-CSF significantly increased compared with the other groups. Weight of xenografts in mice treated with GM-CSF significantly decreased compared with the control. * $P < 0.05$, *** $P < 0.0001$. (E) Weight of xenografts of inoculated UM-UC-3 cells. Weight of xenografts in mice treated with G-CSF significantly increased compared with the other groups. * $P < 0.05$. (F) Tumor growth rate during treatment was significantly higher in mice treated with G-CSF and lower in mice treated with GM-CSF compared with the control in xenografts inoculated with MGH-U3 cells. * $P < 0.05$, * $P < 0.01$ vs. Control. (G) Tumor growth rate during treatment was significantly higher in mice treated with G-CSF compared with the control in xenografts inoculated with UM-UC-3 cells. * $P < 0.01$ vs. Control. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; CSF, colony-stimulating factor; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; VEGF, vascular endothelial growth factor.

a week using electronic calipers and tumor volumes were calculated using the following formula: $(\text{width}^2 \times \text{length}) / 2$. Subsequently, 1 week after the last treatment, all mice were

euthanized by exsanguination under anesthesia with 2-3% isoflurane and tissues (tumor and whole blood by cardiac puncture) were harvested for the subsequent experiments.

Tumors were examined by hematoxylin and eosin (H&E) staining and immunohistochemical (IHC) staining analysis. Treatment-associated changes in pro- and anti-tumoral cytokines and chemokines in serum were evaluated by enzyme-linked immunosorbent assay (ELISA)-based assays. The maximum tumor diameter observed in the present study was 19.9 mm.

IHC staining analysis and quantification. Tumors were examined by IHC staining analysis as previously described (24). Briefly, tumors were fixed in 10% neutral buffered formalin for 48 h at room temperature, embedded in paraffin and then the sections (3- μ m-thick) were subjected to IHC staining for various markers involved in cell proliferation, angiogenesis, lymphangiogenesis, anti-tumor immunity and epithelial-mesenchymal transition (EMT): Ki-67 (proliferation), CD31 (angiogenesis), lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1; lymphangiogenesis), vascular endothelial growth factor (VEGF; angiogenesis), CD204 (as a marker of M2 macrophages; anti-tumor immunity) and E-cadherin (EMT). IHC staining was performed using the Histofine ABC kit (cat. no. 424043; ready-to-use; Nichirei Biosciences, Tokyo, Japan), according to the manufacturer's protocol. Slides were incubated overnight at 4°C with antibodies against Ki-67 (clone MIB-1, ready-to-use; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA), CD31 (cat. no. M0823; 1:1,000; Dako; Agilent Technologies, Inc.), LYVE-1 (cat. no. ab14917; 1:100; Abcam, Cambridge, MA, USA), VEGF (cat. no. sc-152; 1:50; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), CD204 (cat. no. KT022; 1:2,000; Trans Genic, Kobe, Japan) and E-cadherin (cat. no. 3195; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA). In each case, an evaluation was performed by two investigators independently and blindly, without the information pertaining the treatment. Positive cells from each specimen were counted from a minimum of four randomly selected fields per high power field (HPF; magnification, x400; 0.0625 μ m²) using a light microscope for Ki-67, VEGF and CD204. The number of stained microvessels was counted from a minimum of four randomly selected fields per HPF for CD31 and LYVE-1, as a marker for angiogenesis and lymphangiogenesis, respectively. E-cadherin was evaluated according to intensity level (0, none; 1, low; 2, intermediate; 3, high) (25).

Quantification of serum cytokines. Serum cytokines involved in angiogenesis, recruitment of M2 macrophages and EMT were measured by ELISA. Serum was collected and centrifuged at 10,000 \times g for 15 min at 4°C, and the supernatant was stored at -80°C in cryotubes. The profiles of eight cytokines in serum were determined using an ELISA array (cat. no. EA-1021; Signosis, Inc., Hilden, Germany), according to the manufacturer's protocol. Serum samples were thawed, mixed and then incubated in 96-well microplates coated with anti-mouse primary antibodies against eight cytokines involved in angiogenesis: Tumor necrosis factor (TNF)- α , insulin-like growth factor-1 (IGF-1), VEGF, interleukin (IL)-6, fibroblast growth factor (FGF)-b, interferon (IFN)- γ , epidermal growth factor and leptin. Samples were developed with horseradish peroxidase-conjugated secondary antibodies. After adding the substrate and stop solution, a Tecan microplate

reader (Tecan Group, Ltd., Mannedorf, Switzerland) was used to measure absorbance at 450 nm. IL-6 and VEGF were measured in serum samples of each mouse using the ELISA method (860.020.048 and SEA143Mu, respectively; Diaclone, Besancon Cedex, France and Cloud-Clone, Houston, TX, USA, respectively). In addition, transforming growth factor (TGF)- β 1 and TGF- β 2 were also measured in the serum samples of each mouse by ELISA (cat. nos. EA100357 and EA100629, respectively; OriGene Technologies, Rockville, MD, USA), according to the manufacturer's protocol. A Tecan microplate reader (Tecan Group, Ltd.) was used to measure absorbance at 450 nm.

Cell viability assays. To investigate the effect of human and mouse recombinant CSFs on proliferation ability, the cell viability assay was performed. GH-U3, UM-UC-3, MB49, and MBT2 cells were seeded in a 96-well plate at a density of 2,000 cells/well in serum-free RPMI-1640 (Nacalai Tesque, Inc.), incubated for 24 h and treated with three different concentrations of each CSF (0, 2 or 10 ng/ml) for 48 h. Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to measure viability, according to the manufacturer's protocol. The viability index was expressed relative to the untreated cells.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Semi-quantitative RT-PCR was performed to measure the RNA expression levels of G-CSFR, granulocyte macrophage colony stimulating factor receptor (GM-CSFR) and macrophage colony stimulating factor receptor (M-CSFR) in each cell line (MGH-U3, UM-UC-3 and HUVEC), as previously described (26). Briefly, cells were seeded in 6-well plates at a density of 1×10^5 cells/well in RPMI-1640 (Nacalai Tesque, Inc.) or Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA) and incubated for 24 h. RNA was extracted from these cells using the RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. Conversion to cDNA was achieved using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). Semi-quantitative PCR was performed with cDNA, 0.2 μ M each probe mix and 10 μ l AmpliTaq Gold[®] PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following conditions: Denaturation at 95°C for 10 min, 25-30 cycles of denaturation at 95°C for 15 sec, and an annealing and final extension step at 60°C for 1 min. PCR products were then electrophoresed in a 1.5% agarose gel and visualized using ethidium bromide with a transilluminator. GAPDH was used as an internal control.

Capillary tube formation assays. Each well of 96-well plates was coated with 50 μ l Matrigel and allowed to solidify for 30 min at 37°C. HUVECs were seeded on top of Matrigel in triplicate at a density of 2×10^4 cells/well in Dulbecco's modified Eagle's medium (Sigma-Aldrich; Merck KGaA) and incubated for 6 h at 37°C in an atmosphere of 5% CO₂. Similarly, HUVECs were resuspended in EBM-2 basal media with or without each human recombinant CSF (10 ng/ml) prior to seeding on top of Matrigel and incubated for 6 h at 37°C in an atmosphere of 5% CO₂. Formed tubes were stained with calcein AM (PromoCell GmbH, Heidelberg, Germany) for 15 min at room

temperature and the cells were immediately examined under a fluorescence microscope (magnification, x200; EVOS™ FL Auto Imaging System; Thermo Fisher Scientific, Inc.). The total length of tube-like structures in at least four viewed fields per well was measured using ImageJ 1.47 software (National Institute of Health, Bethesda, MD, USA).

Dual-immunofluorescence staining of human bladder cancer tissue. To investigate whether CSFRs are present at tumoral areas and cancer-free areas, human bladder cancer tissues were evaluated by dual-immunofluorescence staining as previously described (27). The protocol for the research project was approved by the Institutional Review Board for Clinical Studies (Medical Ethics Committee ID: NMU-1630; Nara Medical University, Kashihara, Japan) and all participants provided written informed consent. Two patients with newly diagnosed non-muscle invasive bladder cancer undergoing transurethral resection of bladder tumor in May 2018 at Nara Medical University were enrolled. Tissue samples were obtained at the initial treatment and each specimen of the tumor site and normal site were evaluated. The two patients were randomly selected based on being the same sex (male), a similar age (69 and 66 years old), and presenting with the same stage and grade (T1 high-grade). Dual-immunofluorescence staining was performed with antibodies specific to either CD31/G-CSFR (mouse monoclonal/goat polyclonal), CD31/GM-CSFR (mouse monoclonal/rabbit polyclonal) or CD31/M-CSFR (mouse monoclonal/rabbit polyclonal). Sections were frozen at -80°C, cut into 6-μm sections mounted on Superfrost Plus slides (Thermo Fisher Scientific, Inc.) and fixed in 10% neutral buffered formalin for 10 min at room temperature and then 3% hydrogen peroxide methanol for 10 min at room temperature, followed by blocking in donkey serum (cat. no. 017-000-001; Jackson ImmunoResearch Inc., West Grove, PA, USA) for 1 h at room temperature. The sections were incubated with either anti-CD31 (cat. no. M0823; 1:50; Dako; Agilent Technologies, Inc.) and G-CSFR (cat. no. sc-323898; 1:100; Santa Cruz Biotechnology, Inc.), GM-CSFR (cat. no. GTX51383; 1:100; GeneTex, Irvine, CA, USA) or M-CSFR (cat. no. bs-3074R; 1:100, BIOSs, Beijing, China) overnight at 4°C. The sections were then incubated with Alexa Fluor 488 anti-mouse IgG (cat. no. 715-545-150), Alexa Fluor 594 anti-rabbit IgG (cat. no. 711-585-152) and Alexa Fluor 594 anti-goat IgG (cat. no. 705-585-147) secondary antibodies (1:250; Jackson ImmunoResearch Laboratories, Inc.) for 30 min at room temperature, as appropriate, and mounted with mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The sections were immediately examined under a fluorescence microscope (magnification, x400; EVOS™ FL Auto Imaging System; Thermo Fisher Scientific, Inc.).

Statistical analysis. Statistical analyses and figure plotting were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented by bar charts or box plots and expressed as the mean ± standard error of the mean. All analyses were performed from three independent experiments. Kruskal-Wallis test followed by Dunn's multiple comparison test were applied for statistical analysis, as appropriate. Survival curves were generated using the Kaplan-Meier method and compared with a log-rank test.

$P < 0.05$ was considered to indicate a statistically significant difference.

Results

Treatment with G-CSF enhances tumor growth. Treatments were well tolerated with no body weight loss (data not shown). Fig. 1B presents representative images of mice with xenografts in each treatment group. Mice treated with G-CSF presented with the largest tumors, whereas mice treated with GM-CSF presented with the smallest tumors among the four groups. Fig. 1C presents representative H&E stained images from each treatment group, which confirmed that all mice contained tumor cells. The maximum tumor size observed in the present study was 20 mm in diameter in the control group. Fig. 1D and E presents the resected tumor weights following inoculation with MGHU-3 and UM-UC-3 cells, respectively. Significant tumor weight gain was observed in the G-CSF treatment group compared with the control in both human bladder cancer cell lines ($P < 0.0001$ and $P = 0.014$, respectively). With MGH-U3 cells, significant tumor weight loss was observed in the GM-CSF treatment group compared with the control group, which suggests that treatment with GM-CSF may exhibit an anti-tumor effect for low-grade tumor and not exhibit a tumor growth effect for high-grade tumor compared with the treatment with G-CSF ($P < 0.05$; Fig. 1D). Significant tumor weight loss was also observed in the GM-CSF and M-CSF treatment groups compared with the G-CSF treatment group in MGH-U3 ($P < 0.0001$ and $P < 0.05$, respectively; Fig. 1D) and UM-UC-3 ($P < 0.05$ and $P < 0.05$, respectively; Fig. 1E) human bladder cancer cell lines. The rate of tumor growth during the treatment was significantly higher in mice treated with G-CSF compared with the control mice for MGH-U3 ($P = 0.003$; Fig. 1F) and UM-UC-3 ($P = 0.019$; Fig. 1G) human bladder cancer cell lines.

In addition, a tumor size of ≥ 15 mm in diameter was described as an event and a tumor survival curve was generated by the Kaplan-Meier method (28). Mice treated with G-CSF were at a significantly higher risk of tumor growth compared with the control groups following inoculation with MGH-U3 ($P = 0.011$; Fig. 2A) and UM-UC-3 ($P = 0.040$; Fig. 2B) human bladder cancer cell lines.

Treatment with G-CSF results in angiogenesis, recruitment of M2 macrophages and EMT. To investigate the influence of the three CSFs on angiogenesis, lymphangiogenesis, recruitment of M2 macrophages and EMT, the present study performed IHC staining for six markers: Ki-67, CD31, LYVE-1, VEGF, CD204, and E-cadherin. Representative images of antibody-stained resected tumors are presented in Fig. 2C and the results are summarized in Table I. Treatment with G-CSF and M-CSF significantly increased the expression level of Ki-67 in MGH-U3 ($P < 0.0001$ and $P < 0.05$, respectively; Fig. S1A) and UM-UC-3 ($P < 0.05$ and $P < 0.05$, respectively; Fig. S1B) human bladder cancer cell lines compared with the control. The number of cells with CD31 was significantly enhanced by the treatment with G-CSF in MGH-U3 cells compared with the control ($P < 0.05$; Fig. S1C). With UM-UC-3 cells, the number of cells with CD31 was significantly enhanced by treatment with G-CSF and significantly suppressed by treatment with

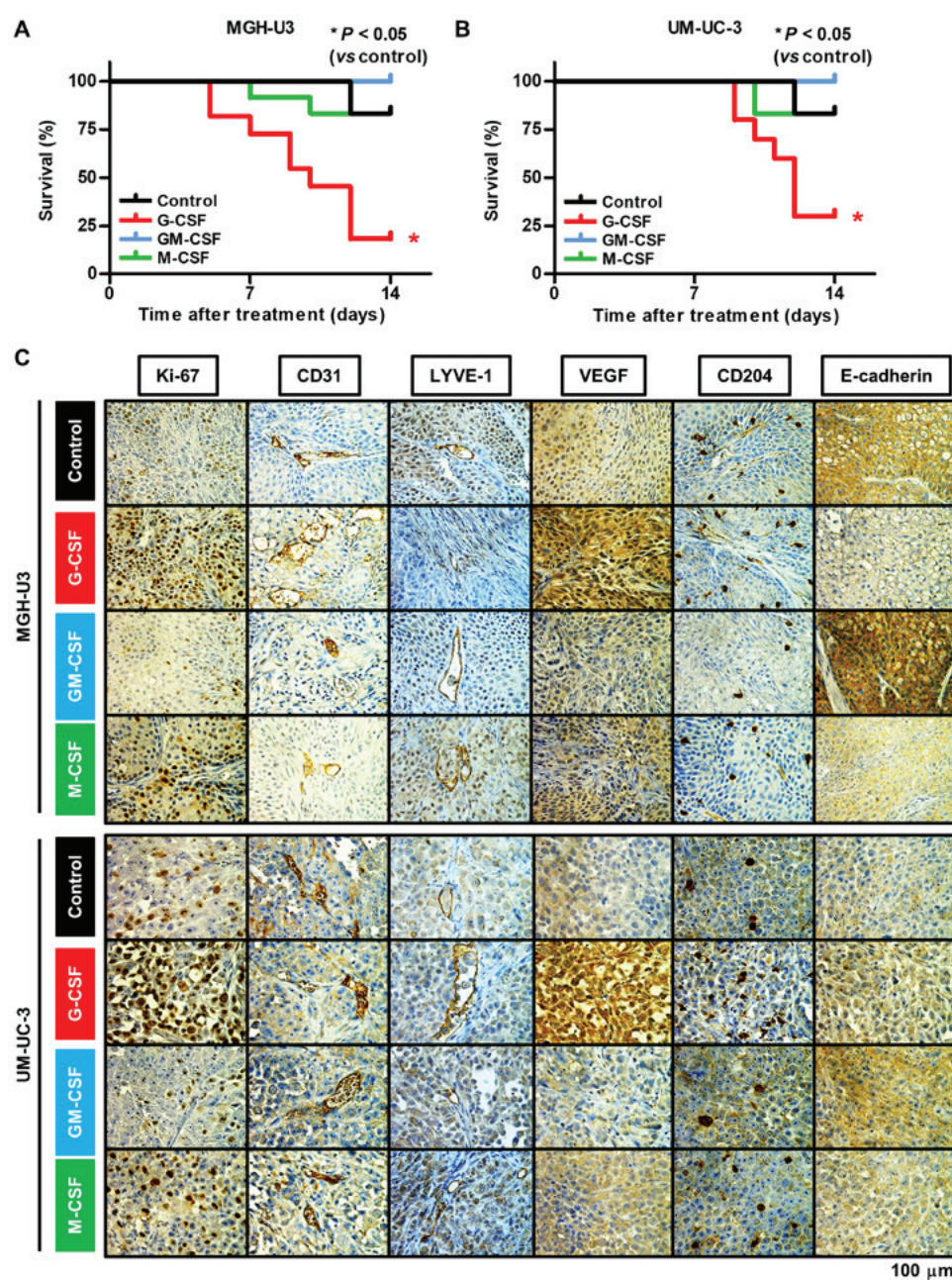


Figure 2. Antitumor activity in human bladder cancer xenograft model and immunohistochemical staining analysis in each treatment group. Tumor size of ≥ 15 mm in diameter was defined as an event. Kaplan-Meier analysis demonstrated that treatment with G-CSF was associated with a significantly higher risk of tumor growth compared with the control in (A) MGH-U3 and (B) UM-UC-3 human bladder cancer cell lines. (C) Representative images of xenografts from each treatment group stained for six immunological markers (Ki-67, CD31, LYVE-1, VEGF, CD204 and E-cadherin) in each cell line. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; LYVE1, lymphatic vessel endothelial hyaluronan receptor 1; VEGF, vascular endothelial growth factor.

GM-CSF ($P < 0.05$ and $P < 0.05$, respectively; Fig. S1D). By contrast, the number of cells with LYVE-1 was significantly inhibited by treatment with GM-CSF in MGH-U3 ($P < 0.05$; Fig. S1E). The number of M2 macrophages was significantly increased by treatment with G-CSF and significantly reduced by treatment with GM-CSF in MGH-U3 ($P < 0.05$ and $P < 0.01$, respectively; Fig. S1I). In UM-UC-3, the number of M2 macrophages was significantly induced by treatment with G-CSF and M-CSF, and significantly reduced by treatment with GM-CSF (all $P < 0.05$; Fig. S1J). The intensity level of E-cadherin was significantly reduced by treatment with G-CSF and M-CSF in MGH-U3 ($P < 0.05$ and $P < 0.01$, respectively;

Fig. S1K). These results suggest that angiogenesis is induced by treatment with G-CSF, lymphangiogenesis is suppressed by treatment with GM-CSF, M2 macrophages are induced and reduced by treatment with G-CSF and GM-CSF, respectively, and EMT is enhanced by treatment with G-CSF. In addition, the different results in the different cell lines suggest that high-grade tumors may be less affected by treatment with GM-CSF in angiogenesis, lymphangiogenesis and EMT.

Systemic changes in serum cytokines caused by treatment with CSFs. To investigate the association between treatment with CSFs and induced cytokines involved in angiogen-

Table I. Summary of immunohistochemical staining analysis and ELISA assay of murine serum.

Treatment	Immunohistochemical staining analysis of the resected tumor										ELISA of serum					
	Ki-67	CD31	LYVE-1	VEGF	CD204	E-cadherin	TNF- α	IGF-1	VEGF	IL-6	FGF-b	INF- γ	EGF	Leptin	TGF- β 1	TGF- β 2
MGH-U3 cells																
G-CSF	up ^a	up ^a	ns	up ^a	up ^a	down ^a	up ^a	up ^a	up ^a	up ^a	up ^a	up ^a	up ^a	up ^a	up ^a	up ^a
GM-CSF	ns	ns	down ^a	ns	down ^a	ns	up ^a	up ^a	up ^a	up ^a	up ^a	up ^a	up ^a	up ^a	up ^a	up ^a
M-CSF	up ^a	up ^a	ns	up ^a	down ^a	down ^a	up ^a	up ^a	up ^a	up ^a	up ^a	up ^a	ns	up ^a	up ^a	up ^a
UM-UC-3 cells																
G-CSF	up ^a	up ^a	ns	up ^a	up ^a	ns	ns	ns	ns	up ^a	up ^a	ns	up ^a	up ^a	up ^a	up ^a
GM-CSF	ns	down ^a	ns	down ^a	down ^a	ns	ns	up ^a	ns	up ^a	up ^a	up ^a	up ^a	ns	up ^a	up ^a
M-CSF	up ^a	ns	ns	up ^a	up ^a	ns	ns	up ^a	ns	up ^a	up ^a	up ^a	up ^a	up ^a	ns	up ^a

^aP<0.05 vs. PBS control. up, significantly upregulated; down, significantly downregulated; ns, not significant; ELISA, enzyme-linked immunosorbent assay; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor; IGF-1, insulin-like growth factor-1; VEGF, vascular endothelial growth factor; IL-6, interleukin-6; FGF-b, fibroblast growth factor-b; INF- γ , interferon- γ ; EGF, epidermal growth factor; TGF, transforming growth factor.

esis, the current study analyzed the serum obtained from euthanized mice by ELISA. The results are summarized in Table I. TNF- α , IGF-1, VEGF, IL-6, FGF-b, IFN- γ and leptin were significantly increased in all treatment groups compared with the control in MGH-U3-inoculated mice. In UM-UC-3-inoculated mice, IL-6 and leptin were significantly increased in all treatment groups compared with the control (Tables I and SI). Subsequently, the present study focused on IL-6 and VEGF, key cytokines involved in angiogenesis, and TGF- β 1 and TGF- β 2, cytokines involved in EMT. The concentrations of these cytokines were measured using all samples. In mice inoculated with MGHU-3, IL-6 was significantly decreased by treatment with GM-CSF (P<0.05; Fig. 3A) and VEGF was significantly increased by treatment with G-CSF (P<0.05; Fig. 3B) compared with the control. TGF- β 1 was significantly increased by treatment with G-CSF compared with the control (P<0.05; Fig. 3C). TGF- β 2 was significantly increased in all treatment groups compared with the control (all P<0.05; Fig. 3D). In mice inoculated with UM-UC-3 cells, IL-6 was significantly increased by treatment with G-CSF compared with the control (P<0.05; Fig. 3E). TGF- β 1 was significantly increased by treatment with G-CSF and GM-CSF compared with the control (all P<0.05; Fig. 3G). TGF- β 2 was significantly increased by treatment with G-CSF compared with the control (P<0.05; Fig. 3H).

Exogenous CSFs enhance the viability of human and mouse bladder cancer cell lines. To investigate the effects of treatment with exogenous CSFs on cell viability, a cell viability assay was performed with human and mouse urothelial carcinoma cell lines (human, MGH-U3 and UM-UC-3; mouse, MB49 and MBT2). With MGH-U3 cells, no significant effect of each CSF was identified (Fig. 4A). By contrast, treatment with 10 nm/ml G-CSF, GM-CSF and M-CSF significantly enhanced UM-UC-3 cell viability (all P<0.05; Fig. 4B), which suggests that high-grade tumors are more affected by treatment with CSFs directly. With regard to mouse urothelial carcinoma cell lines, treatment with G-CSF and M-CSF enhanced cancer cell viability (Fig. S2). However, a dose-dependent mechanism was not observed.

Each CSFR is not expressed in human bladder cancer cell lines. In the *in vitro* study, it was demonstrated by semi-quantitative RT-PCR analysis that there were mostly undetectable mRNA expression levels of G-CSFR, GM-CSFR or M-CSFR in MGH-U3 and UM-UC-3 cells. Whereas, HUVECs expressed mRNA of all three CSFRs examined (Fig. 4C).

Capillary tube formation capability is suppressed by treatment with GM-CSF and M-CSF. To investigate the effect of treatment with exogenous CSFs on angiogenesis, a capillary tube formation assay was performed using HUVECs. Although treatment with G-CSF had no significant effect on capillary tube formation capability compared with the control, treatment with GM-CSF significantly suppressed capillary tube formation capability compared with the control *in vitro* (P=0.0087 and P=0.041, respectively; Fig. 4D). A significant difference was also identified between treatment with G-CSF and with GM-CSF (P=0.0085; Fig. 4D). Fig. 4E presents the representative images of each treatment group.

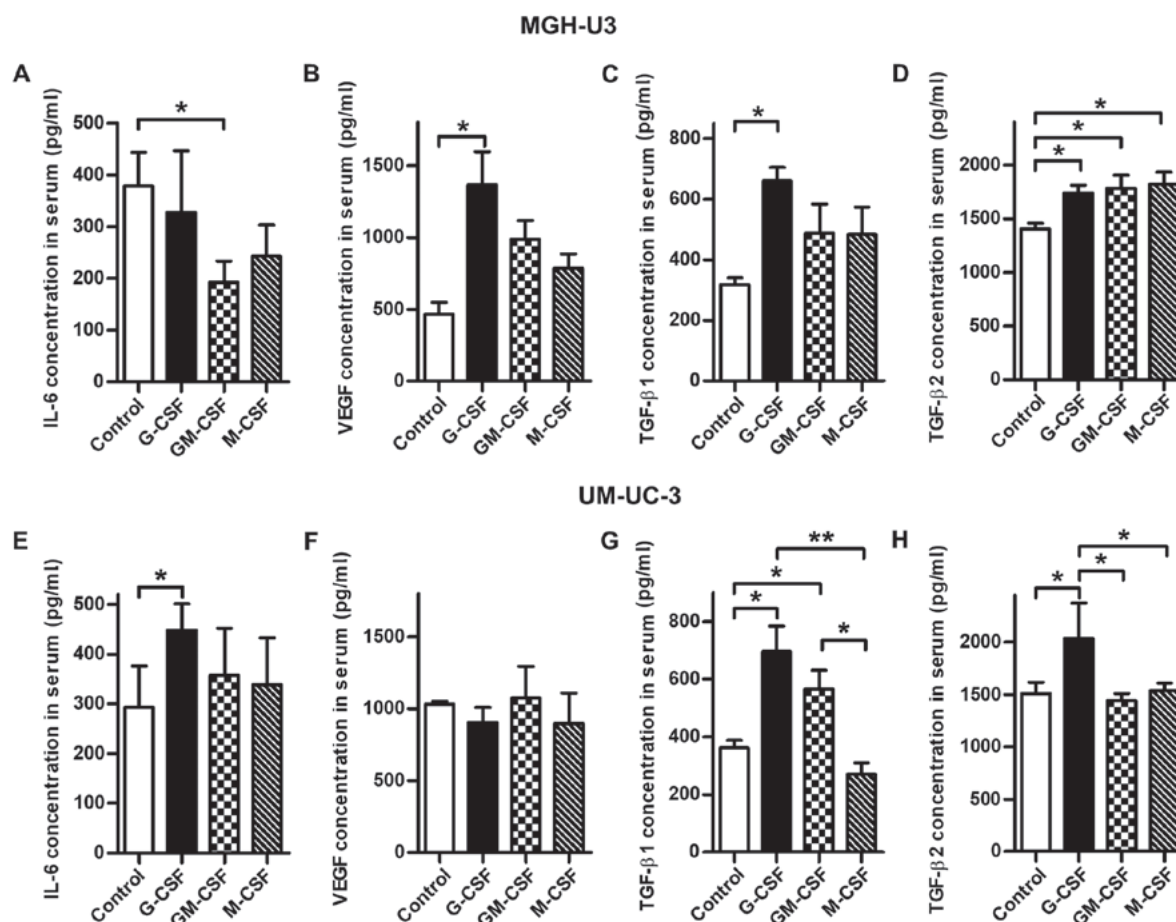


Figure 3. Comparison of each serum cytokine concentration, including IL-6, VEGF, TGF-β1 and TGF-β2, in the treatment groups and the control. (A) Treatment with GM-CSF demonstrated a significantly lower concentration of IL-6 in mice inoculated with MGH-U3 compared with the controls. (B) Treatment with G-CSF resulted in a significantly higher concentration of VEGF compared with the controls in mice inoculated with MGH-U3. (C) Treatment with G-CSF resulted in a significantly higher concentration of TGF-β1 compared with the controls in mice inoculated with MGH-U3. (D) Treatment with all three CSFs resulted in a significantly higher concentration of TGF-β2 compared with the controls in mice inoculated with MGH-U3. (E) Treatment with G-CSF demonstrated significantly higher concentration of IL-6 in mice inoculated with UM-UC-3 cells compared with the controls. (F) There was no significant difference in the concentration of VEGF following treatment with each CSF in mice inoculated with UM-UC-3 cells. (G) Treatment with G-CSF and GM-CSF resulted in significantly higher concentrations of TGF-β1 in mice inoculated with UM-UC-3 cells compared with the controls. (H) Treatment with G-CSF demonstrated a significantly higher concentration of TGF-β2 in mice inoculated with UM-UC-3 cells compared with the controls. * $P < 0.05$, ** $P < 0.01$. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; IL-6, interleukin-6; VEGF, vascular endothelial growth factor; TGF, transforming growth factor.

G-CSFR is expressed in endothelial cells but not tumor cells in the tumor microenvironment. To evaluate whether CSFRs are present in tumor and endothelial cells around the tumoral area, dual-immunofluorescence staining was performed with antibodies specific to CD31/G-CSFR, CD31/GM-CSFR and CD31/M-CSFR using tissues obtained from patients with UCB. The clinicopathological information of the patients is presented in Table SII. Representative images from fluorescence microscopy demonstrated that certain endothelial cells around the tumoral area expressed G-CSFR, GM-CSFR and M-CSFR, and these receptors were not expressed by tumor cells in human UCB tissues (Fig. 5A). By contrast, representative images of the cancer-free area revealed that endothelial cells did not express any CSFRs (Fig. S3).

Discussion

The present study demonstrated that no exogenous CSF protein had the ability to promote proliferation directly in human

bladder cancer cell lines. This can be explained by the lack of expression of their receptors, including G-CSFR, GM-CSFR and M-CSFR, in the present *in vitro* study. However, in the *in vivo* study, the administration of G-CSF and M-CSF promoted tumor growth over time, whereas the administration of GM-CSF did not promote tumor growth, which resulted in a significantly higher cancer mortality rate in mice treated with G-CSF compared with the control. Cancer mortality in mice treated with GM-CSF was almost the same as the control. The administration of CSFs could affect the acceleration or inhibition of tumor growth, angiogenesis, lymphangiogenesis, recruitment of M2 macrophages and EMT. ELISA using mouse serum samples suggested that various cytokines involved in angiogenesis and EMT serve roles in enhancing tumor growth through unknown and/or indirect mechanisms. Therefore, we hypothesize that CSF administration indirectly affects tumor growth via various mechanisms in the tumor microenvironment, resulting in progression and metastasis in patients with UCB (Fig. 5B).

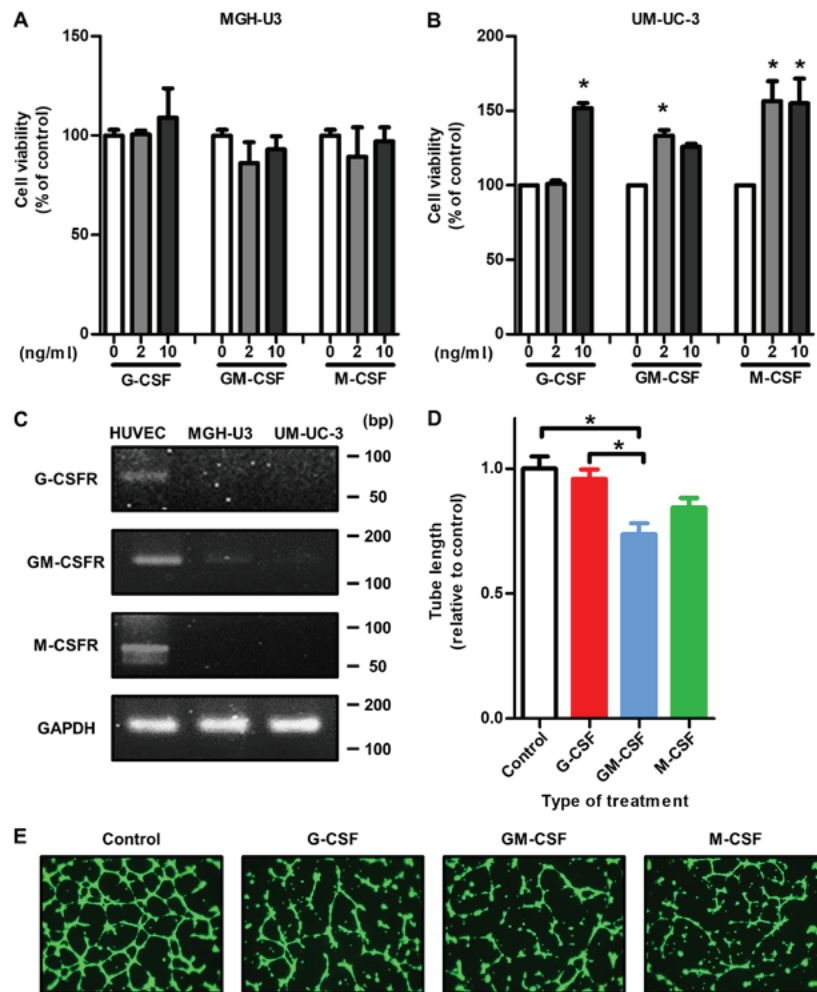


Figure 4. Treatment with each CSF indirectly affects tumor growth through stromal cells in the tumor microenvironment rather than affecting tumor growth directly. (A) There was no effect on cell viability of MGH-U3 cells following treatment with exogenous CSFs. (B) In the cell viability assay, treatment with exogenous CSFs significantly increased the proliferation ability of UM-UC-3 cells. * $P < 0.05$ vs. 0 ng/ml. (C) HUVECs expressed G-CSFR, GM-CSFR and M-CSFR. By contrast, MGH-U3 and UM-UC-3 cells did not express any CSFR. (D) In the capillary tube formation assay, treatment with GM-CSF significantly decreased the tube formation capacity of HUVECs compared with the control and cells treated with G-CSF. * $P < 0.05$. (E) Representative images of the different treatment groups stained with calcein AM in the tube formation assay. Treatment with GM-CSF and M-CSF did not promote angiogenesis to the same extent as the control and treatment with G-CSF. Magnification, x200. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; G-CSFR, granulocyte colony-stimulating factor receptor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; M-CSFR, macrophage colony-stimulating factor receptor; HUVEC, human umbilical vascular endothelial cell.

The use of CSFs, particularly G-CSF, is common for patients with chemotherapy-induced myelosuppression. The use of chemotherapy is becoming increasingly common for patients with UCB due to the success of neoadjuvant or adjuvant-based chemotherapies (4-6,8-10). Therefore, understanding of the potential effects of CSFs against cancer is important for the use of CSFs appropriately. Even though in Japan the administration of G-CSF has been approved for patients with chemotherapy-induced myelosuppression in UCB (9), the present study demonstrated that G-CSF could promote tumor growth in UCB. Segawa *et al* (29) also reported that the intraperitoneal administration of G-CSF enhanced tumor growth in animal models. In addition, although G-CSF had no effects on accelerating angiogenesis directly, endothelial progenitor cells (EPCs), which are involved in angiogenesis, were demonstrated to be induced by G-CSF (30-32). MDSCs have also been associated with tumor growth through induction of EPCs, resulting in angiogenesis. G-CSF has been demonstrated to enhance

MDSC survival and activation by signal transducer and activator of transcription 3 (STAT3) signaling (18,30). The present results suggest that G-CSF could have a stimulating effect on stromal cells in the tumor microenvironment, thereby indirectly enhancing tumor growth by angiogenesis. These effects may be induced by the interaction of cytokines and the cross-talk of cells. In a dual-immunofluorescence staining analysis using human UCB tissues, G-CSFR was identified to not be expressed at cancer-free areas; however, the expression level of G-CSFR increased in tumoral areas. G-CSFR expression may be induced by tumors and may create a favorable microenvironment for tumor survival and progression. In addition, CSFs can be ligands not only to CSFRs but also to unknown receptors (termed orphan receptors) (33). Although UM-UC-3 cells did not express any CSFRs in the present study, CSFs could promote the viability of UM-UC-3 cells. Therefore, tumor progression may be induced by unknown mechanisms including the activation of CSFs and unknown receptors.

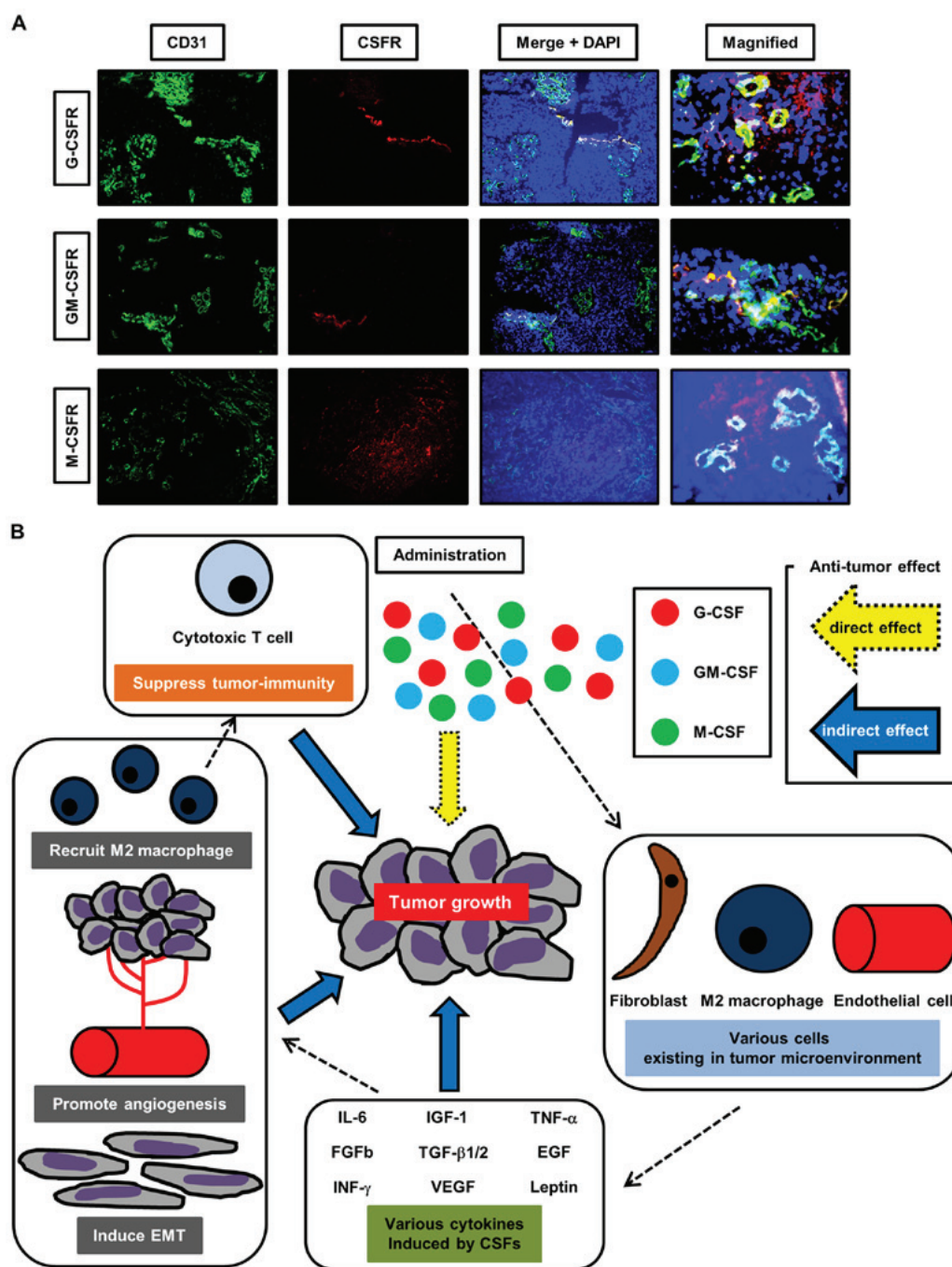


Figure 5. Dual-immunofluorescence staining analysis and a schematic diagram of the proposed mechanism of the roles of CSFs. (A) Representative dual-immunofluorescence staining images of human bladder cancer tissues stained with antibodies specific to CD31/G-CSFR, CD31/GM-CSFR or CD31/M-CSFR. At the tumoral area, certain endothelial cells expressed each CSFR. Magnification, x400. (B) Treatment with CSFs affects the degree of bladder cancer growth. Notably, the administration of G-CSF promotes tumor growth through the enhancement of angiogenesis, recruitment of M2 macrophages and induction of EMT in the tumor microenvironment. Treatment with CSFs also induces various cytokines involved in angiogenesis, recruitment of M2 macrophages and EMT. These cytokines exhibit roles of enhancing tumor growth via unknown mechanisms and activating cross-talk of stromal cells, including fibroblasts, endothelial cells and immune-associated cells, in the tumor microenvironment. Our hypothesis is that treatment with CSFs could cause tumor progression not only directly but also indirectly. Understanding of the pro- and anti-tumor effects induced by the three CSFs could lead to an improved prognosis for patients with advanced bladder cancer undergoing cytotoxic chemotherapy. G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; M-CSF, macrophage colony-stimulating factor; CSF, colony-stimulating factor; EMT, epithelial-mesenchymal transition; G-CSFR, granulocyte colony-stimulating factor receptor; GM-CSFR, granulocyte-macrophage colony-stimulating factor receptor; M-CSFR, macrophage colony-stimulating factor receptor; IL-6, interleukin-6; IGF-1, insulin-like growth factor-1; FGF-b, fibroblast growth factor-b; IFN, interferon; TGF- β 1/2, transforming growth factor- β 1/2; VEGF, vascular endothelial growth factor; TNF, tumor necrosis factor.

Treatment with G-CSF also induces lymphangiogenesis *in vitro* (34). In the current study, treatment with GM-CSF suppressed the expression level of LYVE-1, suggesting an inhibition of lymphangiogenesis in the tumor

microenvironment. Fiorentini *et al* (35) suggested an association between the induction of lymphangiogenesis and GM-CSF. However, to the best of our knowledge, in cancer, the effect of GM-CSF on lymphangiogenesis is not

clear. Therefore, the present result is a proposal for treatment with GM-CSF to suppress lymphangiogenesis in the tumor microenvironment.

M2 macrophages (also termed tumor-associated macrophages) serve multiple important roles in cancer progression through the suppression of adaptive immunity and the induction of angiogenesis (27). Van Overmeire *et al* (22) reported that a M-CSFR blockade resulted in the reduction of M2 macrophages in the tumor microenvironment. Kitoh *et al* (36) demonstrated that GM-CSF and M-CSF signals are associated with the immunosuppressive properties of M2 macrophages *in vitro*. However, the association between CSFs and M2 macrophages is unclear. The present results suggest that treatment with G-CSF has an effect on the recruitment of M2 macrophages and treatment with GM-CSF has an effect of the reduction of M2 macrophages in the tumor microenvironment among three CSFs, resulting in an alteration of immunosuppression and cancer progression. Stromal cells that express CSFRs may have positive or negative effects on tumor growth.

Although Elghonaimy *et al* (37) suggested that G-CSF is associated with the enhancement of EMT in breast cancer, the associations between CSFs and EMT in UCB remain unclear. Our results suggest that treatment with G-CSF promotes EMT by the reduction of E-cadherin expression and induction of TGF- β 1, thus enhancing tumor growth. Cui *et al* (38) reported that G-CSF promoted tumor invasion by inducing EMT in non-small-cell lung cancer when radiation therapy was performed. This previous study suggested that G-CSF binds to G-CSFR, which promotes the Janus kinase (JAK)/STAT3 signaling pathway, resulting in the induction of EMT (38). In addition, Yan *et al* (39) suggested that infiltration of M2 macrophages was associated with EMT through the TGF- β pathway. Therefore, treatment with G-CSF may be able to induce TGF- β systemically and trigger tumor growth by multiple pathways, including suppression of anti-tumor immunity and EMT in the tumor microenvironment.

The present results revealed that various cytokines involved in angiogenesis were elevated in mice treated with CSFs compared with the control. However, the elevated cytokines were similar among the three treatment groups. The current IHC and ELISA results suggest that angiogenesis is predominantly induced by the interactions between cytokines and cross-talk among cells compared with the direct effects of these cytokines on angiogenesis. In addition, TGF- β 1/2 was increased by treatment with the three CSFs. Therefore, recruitment of M2 macrophages and EMT are also induced by TGF- β 1/2 indirectly and various signal pathways, including the JAK/STAT3 pathway, may be involved. Although further studies are required to clarify the association between these cytokines and the mechanism of tumor growth, the knowledge of profiling CSF-induced cytokines could help to elucidate this in the near future.

The present study has a number of limitations. First, a single dose was tested for each treatment; therefore, dose-dependent effects were not evaluated in the current study. Second, although CSFs are often used together with cytotoxic chemotherapy (8-12), in the current study cytotoxic chemotherapeutic agents were not administered in mice. Therefore, the effects of cytotoxic chemotherapy and CSFs interaction on tumor growth could not be evaluated. Treatment with G-CSF could enhance not only the growth

of neutrophils but also tumor cells; however, the effect of G-CSF during chemotherapy needs to be further investigated. In addition, to confirm whether a combination of G-CSF and M-CSF demonstrates a similar anti-tumor effect, this combined treatment should be compared with GM-CSF alone. Although there are differences in roles, including activation of macrophages and differentiation of dendritic cells, between GM-CSF and G-CSF/M-CSF, the effect of a combination of G-CSF and M-CSF on tumor growth should be investigated. Finally, the sample size of the human bladder tissues used in the present study was small; therefore, dual staining analyses using a larger sample size are required to validate the results of the current study.

In conclusion, the present study demonstrated that treatment with CSFs has an effect of tumor growth in an UCB animal model. Notably, treatment with G-CSF exhibited the greatest effect on tumor growth among the three CSFs. Treatment with G-CSF may promote angiogenesis, recruitment of M2 macrophages and EMT in the tumor microenvironment. In addition, CSFRs, particularly G-CSFR, were demonstrated to not be expressed in cancer-free areas but were expressed by tumor cells, particularly endothelial cells, and serve a role in creating a favorable tumor microenvironment for the survival of tumor cells. An improved understanding of the association between UCB and CSFs could lead to improved outcomes and fewer side effects in patients with advanced UCB. Further studies, including clinical trials, are required to establish the appropriate use of CSFs during cytotoxic chemotherapy, including during a combination of gemcitabine and cisplatin for patients with advanced UCB.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are not publicly available due to hospital policy but are available from the corresponding author on reasonable request.

Authors' contributions

SH, MM and KF contributed to the design of the study and writing the manuscript. SH, MM, SO, YN, YT and YM performed the animal experiments and molecular biology studies. SH, YM, KO, KI, DG and YI assisted with the acquisition of patient data.

MM, YN and NT performed the statistical analysis. MM and KF assisted with writing the manuscript. MM and YT reviewed the pathological diagnosis of bladder tissues. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Committee on Animal Research of the Nara Medical University (Kashihara, Japan) approved the animal study (reference no. 11883). The Institutional Review Board of Nara Medical University (Kashihara, Japan) approved the patient study (reference no. 1630) and all participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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