

Sunitinib enhances antitumor effects against chemotherapy-resistant bladder cancer through suppression of ERK1/2 phosphorylation

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Abstract. Bladder cancer patients who are refractory to chemotherapy have a poor prognosis. Furthermore, additional chemotherapies provide little benefit to patients who have relapsed after an initial response. Recently, it was reported that several molecular pathways are implicated in bladder carcinogenesis, including the epidermal growth factor receptor (EGFR) pathway, the vascular endothelial growth factor (VEGF) pathway and the Ras-MAPK pathway. We hypothesized that sunitinib would be effective in bladder cancer as it is an oral inhibitor of multiple receptor tyrosine kinases, including VEGF receptors, platelet derived growth factor (PDGF) receptors and stem cell factor receptor (c-KIT), and is a standard first-line treatment of advanced clear cell renal carcinoma. In the present study, the antiproliferative effects of sunitinib were clearly demonstrated in KK47, KK47/DDP20 and KK47/ADR cell lines *in vitro* due to the suppression of ERK1/2 phosphorylation. In a mouse model, the antitumor effects of sunitinib were again clearly seen. Also, treatment with sunitinib decreased the abundance of regulatory T cells (Tregs). However, cytotoxic T lymphocyte (CTL) activity was not induced sufficiently as compared with an IFN- α -treated group. Our results suggested that sunitinib was effective in chemotherapy-resistant bladder cancer patients. On the other hand, these findings provided the rationale for combination therapy with sunitinib and immune-based cancer therapy for advanced malignancies to prevent the occurrence of rebound phenomena.

Introduction

Bladder cancer is one of the most common types of cancer in industrialized countries (1). Patients with primary metastatic

disease and first-line cisplatin-based combination chemotherapy with methotrexate, vinblastine, adriamycin and cisplatin (MVAC) show a long-term survival of 20% (2). However, approximately 30% of patients receiving MVAC do not respond, resulting in disease progression (3). As a result, patients who are resistant to cisplatin-based combination chemotherapy have a poor prognosis. Furthermore, additional chemotherapies provide little benefit to patients who have relapsed after an initial response (2). We have been investigating a new treatment method that is used for bladder cancer patients who are resistant to cisplatin-based chemotherapy.

Bladder cancer has been reported to develop alongside complex genetic events, which involve signal transduction, cellular proliferation, angiogenesis and apoptosis. Several molecular pathways have been implicated in bladder carcinogenesis, including the epidermal growth factor receptor (EGFR) pathway, vascular endothelial growth factor (VEGF) pathway and Ras-MAPK pathway (4). RAS proteins deliver signals from cell surface receptors, passing them from protein to protein along several different pathways (5). The RAS-RAF/MEK (mitogen extracellular kinase)/ERK (extracellular signal-related kinase) pathway, a common downstream RAS signaling pathway present in all eukaryotic cells, is upregulated in approximately 30% of all human cancers (6). Sunitinib is an oral inhibitor of multiple receptor tyrosine kinases, including VEGF receptors, platelet derived growth factor (PDGF) receptors and stem cell factor receptor (c-KIT), and is the standard first-line treatment of advanced clear cell renal carcinoma (7). The inhibition of the tyrosine kinases necessary for these growth factors to interact with their respective receptors provides a potential target for various anti-cancer therapies, inhibiting angiogenesis and tumor cell proliferation (8-10). Therefore, we postulated that sunitinib might be effective in bladder cancer.

In the present study, we evaluated the therapeutic potential and molecular mechanism of sunitinib in human and murine bladder cancers or drug-resistant human bladder cancers. Our results suggest that sunitinib is effective in MVAC-resistant bladder cancer patients accompanied with the suppression of ERK1/2 phosphorylation, overexpression of which has been recently reported to have a relation to drug resistance (11-17). In addition, treatment with sunitinib decreased the abundance

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of regulatory T cells (Tregs) in a mouse model. This might be one of the causes of the appearance of anti-tumor activity. However, because cytotoxic T lymphocyte (CTL) activity was not induced sufficiently, the necessity of combination therapy with sunitinib and immune-based cancer therapy to prevent recurrence was also postulated.

Materials and methods

Animals and reagents. Female C3H/HeN (H-2^K) mice (6-8 weeks old) and female NCr-nu/nu mice (6-8-weeks old) were obtained from Charles River Laboratories (Yokohama, Japan). Sunitinib (Sutent) was obtained from Pfizer (New York, NY). For the *in vitro* and *in vivo* studies, sunitinib was prepared as described previously (18). Mouse interferon- α (IFN- α) was supplied by Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). IFN- α was prepared as described previously (19).

Tumor cell line. KK47 cell lines, established from human transitional cell carcinoma of the urinary bladder, were used as parental cell lines. KK47 cell lines were cultured as described previously (20). Cisplatin-resistant KK47/DDP20 cells and adriamycin-resistant KK47/ADR cells derived from KK47 cell lines were cultured as described previously (20). MBT-2 cells, derived from a carcinogen-induced bladder tumor in a C3H mouse, were cultured as described previously (21,22).

Antibodies and reagents. Fluorescein isothiocyanate (FITC)-conjugated anti-CD44 mAb (IM7), anti-Foxp3 mAb (FJK-16s) and anti-CD3 mAb (145-2C11), phycoerythrin (PE)-conjugated anti-CD25 mAb (PC61.5), anti-NK (DX5) mAb and anti-CD8 mAb (53-6.7) and PerCP-Cy5.5-conjugated anti-CD4 mAb (H129.19) were purchased from BD Pharmingen (San Diego, CA). Live lymphocytes were gated by forward and side scattering. The data were analyzed with CellQuest software (BD Biosciences). Antibodies against ERK1/2 (Thr202/Tyr204) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- β actin antibody was purchased from Sigma (St. Louis, MO).

Cytotoxicity analysis. Cytotoxicity analysis was performed as previously described (23,24). Briefly, KK47, KK47/ADR and KK47/DDP20 cells (2.5×10^3) were seeded into 96-well plates. The following day the indicated concentrations of mevastatin, simvastatin, and dihydrotestosterone (DHT) were added. After 72 h, the surviving cells were stained using the Alamar Blue assay (TREK Diagnostic Systems, Cleveland, OH) for 180 min at 37°C. Absorbance in each well was measured using a plate reader (ARVOTM MX; Perkin-Elmer, Inc., Waltham, MA).

Western blotting. Whole-cell extracts were prepared as previously described (23,25,26). Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA), based on the Bradford method. Whole cell extracts (30 mg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride microporous membranes (GE Healthcare Bio-Science, Piscataway, NJ) using a semi-dry blotter. Blotted membranes were incubated for 1 h at room temperature with primary antibody. Membranes were then incubated for 40 min at room temperature with peroxidase-

conjugated secondary antibody. Bound antibody was visualized using an ECL kit (GE Healthcare Bio-Science) and membranes were exposed to high performance chemiluminescence film (GE Healthcare Bio-Science).

Intracellular FACS. Lymphocytes were harvested and surface stained in buffer containing PE-conjugated anti-CD25 mAb and PerCP-Cy5.5 anti-CD4 mAb. Cells were then subjected to intracellular staining using a BD fixation and permeabilization system (BD Biosciences) according to the manufacturer's instructions. The cells were fixed and permeabilized by incubation for 20 min at 4°C with 100 μ l of BD Cytofix/Cytoperm solution. After washing with BD perm-wash buffer, cells were stained with FITC-conjugated anti-Foxp3 mAb (FJK-16s). After intracellular staining, cellular fluorescence was analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

Challenge with tumor cells. MBT-2 cells (5×10^6) were inoculated subcutaneously (s.c.) into the shaved lateral flanks of mice. The mice were each challenged intragastrically (i.g.) with a suspension of 100 μ l sunitinib (40 mg/kg) or phosphate buffered saline (PBS) from 4 days after tumor inoculation. According to the human 4 weeks on, 2 weeks off schedule, mice were treated by sunitinib or PBS for 4 weeks before sacrificed. Sizes of primary tumors were determined every 2 or 3 days using calipers. Tumor volume was calculated using the formula $V = (A \times B^2)/2$, where V is the volume (mm³), A is the long diameter (mm) and B is the short diameter (mm).

CTL activity in the spleen. CTL activity was performed as described previously (19). Briefly, for CTL activity, splenocytes were taken from mice s.c. inoculated with MBT-2 cells on day 14 after sunitinib or PBS treatment. Subsequently, 5×10^6 cells/ml splenocytes were cultured for 5 days with 5×10^5 cells/ml mitomycin C (MMC)-treated MBT-2 cells in the presence of 25 ng/ml IL-2 (Takeda Chemical Industries, Tokyo, Japan) diluted in 2 ml of complete culture medium. Viable lymphocytes were separated by Percoll (Amersham Biosciences, Piscataway, NJ) density gradient centrifugation and the effector cells were incubated at the indicated E:T ratio with 1×10^4 ⁵¹Cr-labeled MBT-2 cells for 4 h. Cytotoxicity was calculated as follows: (experimental release - spontaneous release) / (total release - spontaneous release) x 100.

Statistical analysis. Statistical significance of the data was determined using an unpaired two-tailed Student's t-test. P<0.05 was taken as the level of significance. Analysis was carried out using Stat-View 5.0 software (Abacus Concepts, Berkeley, CA).

Results

Anti-proliferative effects of sunitinib in three bladder cancer cells lines. The inhibitory effect of statins on cell growth was examined as shown in Fig. 1. Sunitinib suppressed cell growth in KK47, KK47/DDP20 cisplatin-resistant bladder cancer cells and in KK47/ADR adriamycin-resistant bladder cancer cells in a dose-dependent manner. These data suggest that the inhibitory effect of sunitinib is effective in chemotherapy-resistant bladder cancer cells.

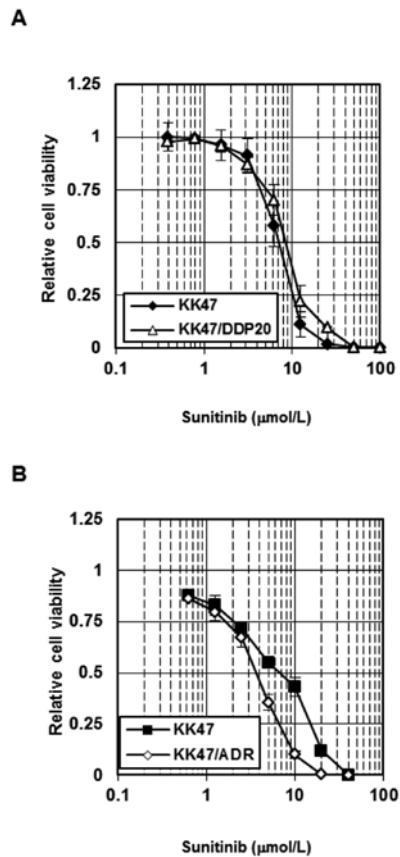


Figure 1. Growth inhibitory effect of sunitinib on bladder cancer cell lines. KK47, KK47/DDP20 and KK47/ADR cells (A) were seeded into 96-well plates. The following day, various concentrations of sunitinib were added. After 72 h, cell survival rates were evaluated using cytotoxicity analyses. Cell survival in the absence of sunitinib was set as 1. All values are representative of at least three independent experiments. Data represent the mean value of KK47, KK47/DDP20 and KK47/ADR cells subjected to cytotoxicity analyses.

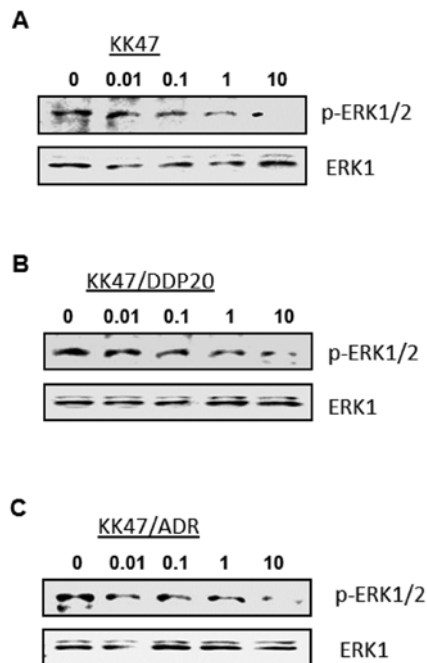


Figure 2. Downregulation of ERK1/2 phosphorylation by sunitinib. Sunitinib was added to media containing, KK47, KK47/DDP20 and KK47/ADR cells at the concentrations and durations indicated. Whole-cell extracts were subjected to SDS-PAGE. Western blotting was performed using the antibodies indicated.

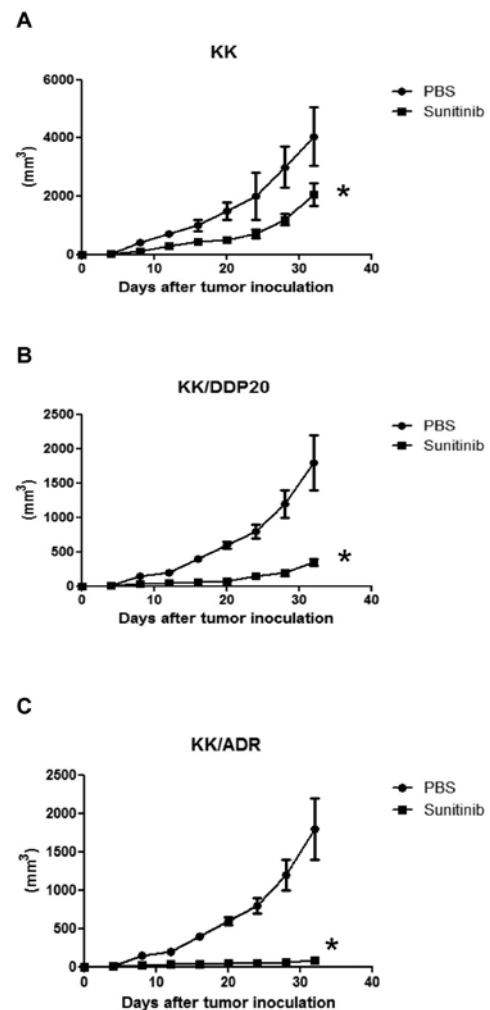


Figure 3. Antitumor effects of sunitinib in the nude mouse model. KK47 (A), KK47/DDP20 (B) or KK47/ADR (C) cells (5.0×10^7) were inoculated subcutaneously (s.c.) into the shaved lateral flanks of mice. After 4 days, 20 mice were randomly divided into two groups. These groups were treated with either sunitinib (40 mg/kg) or PBS. Sunitinib or PBS, administered orally, once a day, for 28 days. Primary tumor size was determined every 2 or 3 days using calipers. * $P < 0.05$ compared with PBS group. Each group consisted of five mice. Points, mean; bars, SD.

Western blotting for ERK1/2. ERK 1/2 has been reported to be involved in drug resistance (11-17). As shown in Fig. 2, phosphorylation of ERK1/2 was downregulated in a time- and dose-dependent manner in KK47, KK47/DDP20 and KK47/ADR cells following the addition of sunitinib.

Antitumor effects of sunitinib in the nude mouse model. Athymic nude mice were s.c. implanted with KK47, KK47/DDP20 or KK47/ADR tumors (1×10^7 cells/body) on day 0 and treatment with sunitinib (40 mg/kg) was initiated on day 4 when tumors were established. Daily treatment with sunitinib produced a more efficient inhibition of tumor growth than PBS in all three tumor cell types (Fig. 3). Namely, the anti-tumor effects of sunitinib were observed in normal bladder cancer cells, cisplatin-resistant bladder cancer cells and adriamycin-resistant bladder cancer cells.

Antitumor effects of sunitinib in the C3H/HeN mouse model. Treatment with sunitinib (40 mg/kg) was initiated on day 4

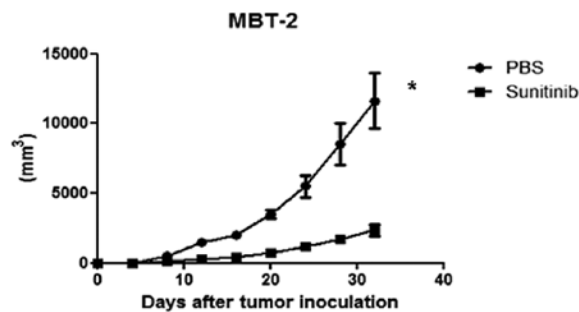


Figure 4. Anti-tumor effect of sunitinib in a mouse model. MBT-2 cells were inoculated subcutaneously (s.c.) into the shaved lateral flanks of mice. After 4 days, 20 mice were randomly divided into two groups. These groups were treated with either sunitinib (40 mg/kg) or PBS. Sunitinib or PBS, administered orally, once a day, for 28 days. Primary tumor size was determined every 2 or 3 days using calipers. *P<0.05 compared with PBS group. Each group consisted of five mice. Points, mean; bars, SD.

after MBT-2 (5.0×10^6). Tumor growth was also significantly suppressed in sunitinib treated mice as compared with those treated with PBS (Fig. 4).

Effect of sunitinib on T cells and generation of tumor-specific CTL activity in splenocytes of tumor-bearing C3H/HeN mice. We analyzed the effector cells for anti-tumor activity in the two groups described above with flow cytometry on day 21 after s.c. inoculation with MBT-2 cells. There were no significant differences in the proportions of CD3⁺NK1.1⁺ cells (NK cells) (Fig. 5B), CD8⁺ T cells (Fig. 5C), and CD4⁺ T cells (Fig. 5D). However, significant decrease in the number of splenocytes and the proportion of CD4⁺ CD25⁺ Foxp3⁺ T cells (regulatory T cells) were observed among the two groups (Fig. 5A and E).

Next, to assess the establishment of acquired immunity against MBT-2, CTL activity against MBT-2 was examined in

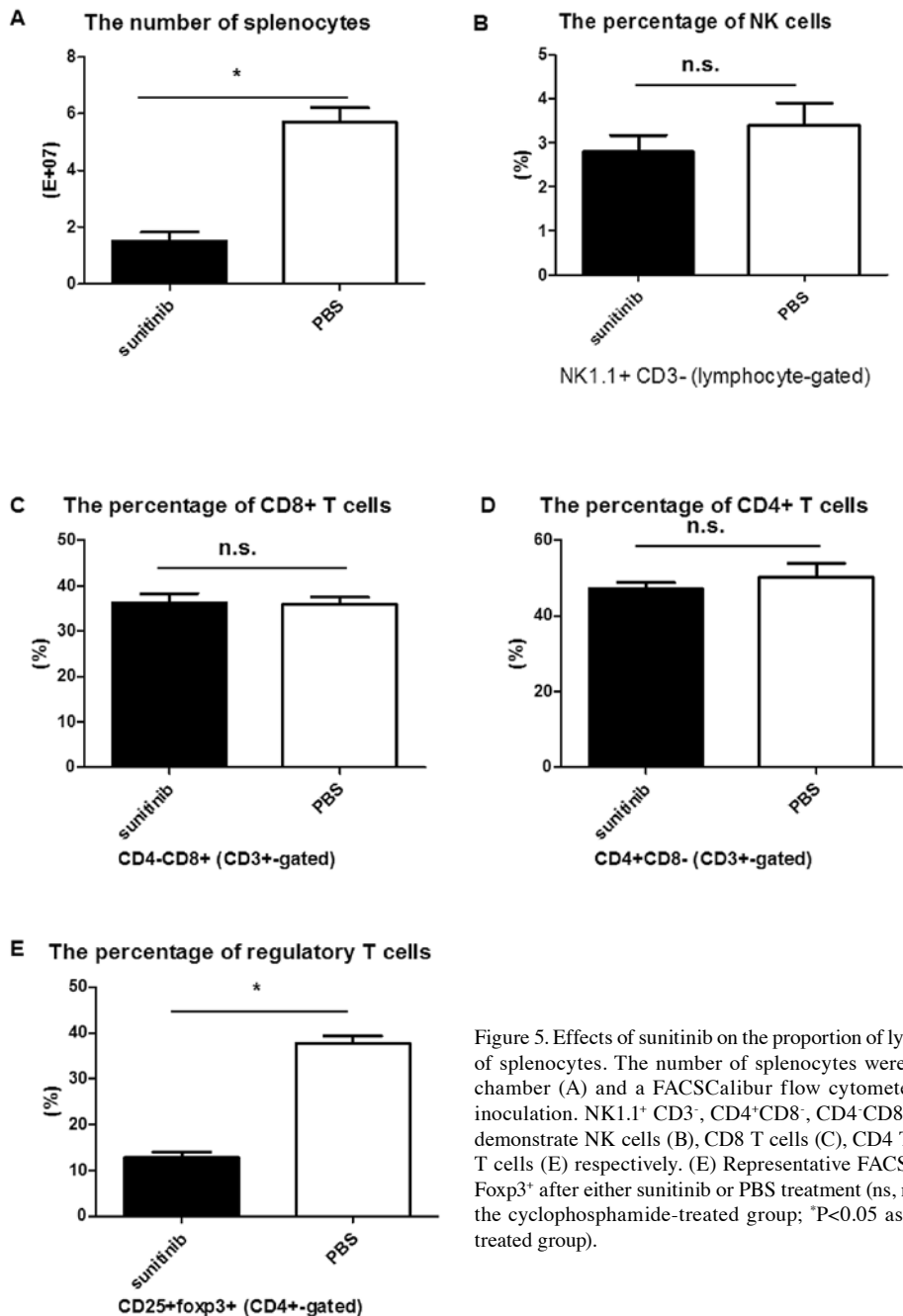


Figure 5. Effects of sunitinib on the proportion of lymphocytes and the number of splenocytes. The number of splenocytes were examined by a counting chamber (A) and a FACSCalibur flow cytometer at 32 days after tumor inoculation. NK1.1⁺ CD3⁻, CD4⁺CD8⁻, CD4⁺CD8⁺, and CD25⁺ foxp3⁺ cells demonstrate NK cells (B), CD8 T cells (C), CD4 T cells (D), and regulatory T cells (E) respectively. (E) Representative FACS patterns of CD4⁺ CD25⁺ Foxp3⁺ after either sunitinib or PBS treatment (ns, not specific compared with the cyclophosphamide-treated group; *P<0.05 as compared with the PBS-treated group).

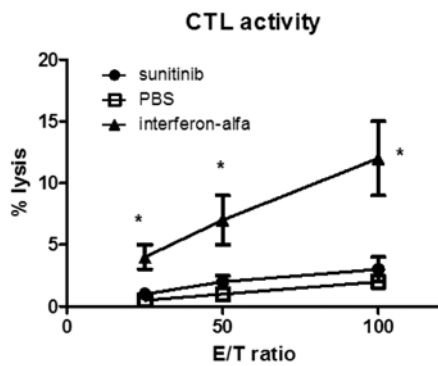


Figure 6. Effect of sunitinib on generation of tumor-specific CTL activity in spleen cells from tumor-bearing C3H/HeN mice. Spleen cells were removed 14 days after tumor inoculation, and used for the assay of CTL activity. * $P < 0.05$ compared with PBS and sunitinib groups. E:T ratio, effector cells:target cells ratio; CTL, cytotoxic T lymphocyte. Each group consisted of five mice. Points, mean; bars, SD.

the splenocytes of tumor-bearing C3H/HeN mice. In this study we used IFN- α as a positive control. In fact, CTL activity was easily induced in the mice treated with IFN- α . The mice treated with IFN- α were each challenged intraperitoneally (i.p.) with a suspension of 100 ml IFN- α (5000 U/body) 4 days after tumor inoculation. According to the sunitinib-treated mouse schedule, mice were treated once a day prior to sacrifice. As a result, IFN- α induced higher level of CTL activity as compared with control, however sunitinib did not induce significant CTL activity as compared with control (Fig. 6).

Discussion

Currently, metastatic bladder cancer is best managed by chemotherapy, although several multi-agent chemotherapy regimens have been used, no combination has demonstrated an incremental benefit to the MVAC regimen introduced >20 years ago (27). However, MVAC has been shown to be associated with significant toxicities, including leukopenia, thrombocytopenia, grade 3/4 mucositis, nausea, vomiting and sepsis-related death in 2-3% of cases (3). While, gemcitabine and cisplatin (GC) might be less toxic than MVAC as demonstrated by lower episodes of toxic death (1 versus 3%) and fewer occurrences of neutropenia, fever, sepsis, mucositis and alopecia in a recent randomized study, GC did not improve the overall survival achieved with MVAC (28). About 30% of patients receiving MVAC do not respond, resulting in disease progression (3). Identification of new treatment alternatives for patients with resistant disease remains a priority and novel molecular targets are being explored.

Recently, bladder cancer has been reported to develop alongside complex genetic events, which involve signal transduction, cellular proliferation, angiogenesis and apoptosis. Several molecular pathways have been implicated in bladder carcinogenesis, including the EGFR pathway, VEGF pathway and Ras-MAPK pathway (4). Therefore, we investigated the use of sunitinib. Sunitinib is an oral inhibitor with antiangiogenic and antitumor activities and inhibits multiple receptor tyrosine kinases, including VEGF receptors, PDGF receptors and c-KIT, which are involved in angiogenesis (7). The antitumor activity of sunitinib has been shown in preclinical and clinical

studies (7,18). Tumor regression has been shown in different xenograft models of colon cancer, breast cancer, non-small cell lung cancer, melanoma, glioblastoma and renal carcinoma (18,29-33). Sunitinib clearly inhibited cell proliferation *in vitro* and tumor growth *in vivo* in the present study (Figs. 1, 3 and 4). Moreover, we found cancer cell proliferation to be inhibited accompanied with suppression of ERK1/2 phosphorylation as shown in Fig. 2. ERK1/2 is a member of the classical MAPK cascade, which is involved in the regulation of cell growth and survival, and its upregulation leads to abnormal cell proliferation and tumorigenicity (4,6,34,35).

Recently, ERK 1/2 has been reported to be involved in drug resistance (11-17). The ERK1/2 signaling pathway could be an important therapeutic target for overcoming chemoresistance. Interestingly, in the present study sunitinib was effective in cisplatin- and adriamycin-resistant bladder cancer, as well as a cisplatin- and adriamycin-sensitive bladder cancer, accompanied with suppression of the ERK1/2 phosphorylation (Figs. 1 and 2). The results that sunitinib treatment inhibits the proliferation of drug-resistant ERK1/2-overexpressing bladder cancer cells are promising for the future treatment of chemotherapy-resistant bladder cancer.

Although anti-tumor immunity is usually suppressed in tumor-bearing mice, due to the presence of Tregs and suppressive cytokines, such as TGF- β and IL-10, IFN- α is reported to induce tumor antigen peptide-specific CTL activity in patients with chronic myelogenous leukemia (36,37). Our previous study also reported the induction of CTL activity against RENCAs (murine renal cell carcinoma cells) in the spleen cells of mice treated with IFN- α (19). An important finding of the present study was that CTL activity against MBT-2 was not induced in the spleen cells of mice treated with sunitinib, compared with mice treated with IFN- α (Fig. 6). This fact might be connected with the so-called 'rebound phenomenon', which is a rapid cancer progression soon after the treatment with molecular target drugs is stopped (19). Next, we investigated if sunitinib treatment also affected the percentage of Treg in tumor-bearing mice, using flow cytometry. Sunitinib treatment significantly decreased the percentage of CD4⁺CD25⁺Foxp3⁺ Tregs in tumor-bearing mice, whereas it did not change the percentage of other lymphocytes (Fig. 5). The correlation of molecular targeting therapy with Tregs has been recently reported. Sunitinib is reported to reduce Tregs in peripheral blood mononuclear cells of patients with MRCC and mice with colon cancer (29). Our results are compatible with these findings. The current study has implications for new therapeutic approaches based on the manipulation of Tregs. Tregs might be an important target in future therapies against cancer. It has been reported that Tregs produce immunosuppressive cytokines, such as IL-10 and TGF- β (38-40). It is well known that Tregs are responsible for inducing and maintaining peripheral tolerance and the negative regulation of immunity (21,41). The depletion of Tregs would eliminate immune-suppression mediated by Tregs leading to enhanced T-cell activity. This fact might reflect the result that higher CTL activity treated with sunitinib was induced as compared with that treated with PBS, although significant difference was not observed (Fig. 6). However, sunitinib was also reported to be associated with significant declines in total number of leucocyte (-48%), neutrophil (-62%), CD3 total T cell (-31%) and CD4 counts (-32%) (42). In fact, the number of

splenocytes was significantly decreased by sunitinib treatment in the present study (Fig. 5A). We thought sunitinib was efficient drug for various cancers and associated with inhibition of specific lymphocyte subsets which had implications for the immunological control. We should consider these methods in the future.

In conclusion, our results show that sunitinib has an anti-tumor effect in murine models of bladder cancer when used as a single agent. In addition, sunitinib also exhibits a potent effect on a chemotherapy-resistant bladder cancer. Since this patient group has few options that lead to prolonged survival, sunitinib may be a promising novel therapeutic alternative in chemotherapy-refractory bladder cancer patients. On the other hand, our data suggest that sunitinib might be used to enhance the therapeutic efficacy of existing immune-based therapies for metastatic cancer patients.

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