

Featured Article

Introduction of Midkine Gene into Human Bladder Cancer Cells Enhances Their Malignant Phenotype But Increases Their Sensitivity to Antiangiogenic Therapy

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Abstract

Purpose: Midkine (MK) is a member of a family of heparin-binding growth factors, which was reported to have an important role in angiogenesis. Although MK was reported to be associated with bladder cancer progression, the functional significance of MK expression in bladder cancer progression has not been elucidated. The objectives of this study were to determine whether overexpression of MK in bladder cancer cells enhances their malignant potential and to evaluate the inhibitory effect of the antiangiogenic agent TNP-470 on the growth of MK-overexpressing bladder cancer cells *in vivo*.

Experimental Design: We introduced the MK gene into human bladder cancer UM-UC-3 cells that do not secrete a detectable level of MK protein and generated the MK-overexpressing cell line UM-UC-3/MK. The biological activity of secreted MK was evaluated using a human umbilical vein endothelial cell proliferation assay. To investigate the *in vivo* effects of MK overexpression on tumor growth, each cell line was injected s.c. and orthotopically into nude mice. To evaluate the therapeutic effects of the antiangiogenic agent, mice were given TNP-470 after s.c. injection of each cell line. The microvessel density of tumors was quantitated by immunohistochemistry of CD31.

Results: The heparin affinity-purified conditioned media of UM-UC-3/MK cells significantly enhanced human umbilical vein endothelial cell proliferation. MK expression had no effect on *in vitro* growth but conferred a growth advantage on both s.c. and orthotopic tumors *in vivo*. Furthermore, enhanced tumor growth was closely associated with increased microvessel density. Significant inhibition of

tumor growth by TNP-470 treatment was observed only in UM-UC-3/MK tumors and not in control tumors.

Conclusions: We demonstrated that overexpression of the MK gene causes an increase in the angiogenic activity of cells through vascular endothelial cell growth, resulting in enhanced malignant potential of human bladder cancer cells. Moreover, the present findings suggest that TNP-470 could be used as a novel therapeutic adjunct to conventional agents for patients with advanced bladder cancer overexpressing MK.

Introduction

Bladder cancer is the second most common malignancy of the genitourinary tract and the fourth or fifth leading cause of cancer-related death of males in Western industrialized countries (1). From a clinical point of view, bladder cancer can be divided into superficial and deeply invasive tumors. Superficial bladder cancer has a relatively good prognosis, despite a high recurrence rate after transurethral resection. Only a small proportion of these superficial tumors progress to invasive disease, and distant metastases seldom develop (2). Invasive bladder cancer, however, has a much less favorable prognosis than superficial bladder cancer. Despite the use of aggressive therapies, invasive bladder cancer has a 5-year survival rate of $\leq 50\%$ (3). However, the molecular mechanisms involved in the initiation and progression of bladder cancer are poorly understood despite extensive studies in this field; thus, there is a pressing need for a further elucidation of these processes.

MK² is a secreted heparin-binding growth factor identified as a product of a retinoic acid response gene (4, 5). The pathophysiological effects of MK include an enhanced plasminogen activator activity (6), oncogenic transformation of fibroblasts (7), antiapoptotic activity (8), and angiogenic activity (9). MK is overexpressed in a variety of cancers such as esophageal (10), gastric (11), colon (12), pancreatic (13), lung (14), and breast cancers (15), whereas its expression is usually low or undetectable in normal adult tissue. Recently, O'Brien *et al.* (16) reported that overexpression of MK was associated with the progression of bladder cancer and could be used as a predictor for poor prognosis in patients with invasive bladder cancer. However, to our knowledge, there has been no study characterizing the functional significance of MK expression in bladder cancer progression.

It is well established that tumor growth and metastasis depend on the induction of new blood supply (17, 18). Angio-

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² The abbreviations used are: MK, midkine; bFGF, basic fibroblast growth factor; HUVEC, human umbilical vein endothelial cell; MVD, microvessel density; FBS, fetal bovine serum.

genic activity, as determined by MVD, has been shown to correlate with worse prognosis in a number of solid tumors including bladder cancer (19–21). Angiogenesis is mediated in part by the competing actions of molecules that stimulate angiogenesis such as bFGF (22), vascular endothelial growth factor (23), and interleukin 8 (24) and those that inhibit angiogenesis such as thrombospondin-1 (25) for inhibitors in bladder cancer. Interaction between these factors and vascular endothelium plays an important role in neovascularization in tumors (26). Therefore, antiangiogenic agents targeting either these factors or vascular endothelial cells are promising therapeutic modalities as tumor dormancy therapies (27).

TNP-470 is an analogue of fumagillin, which is derived from *Aspergillus fumigatus*, and strongly inhibits vascular endothelial cell proliferation and migration (28, 29). It was reported previously that TNP-470 has an inhibitory effect on the growth and metastasis of human cancers, including renal cell carcinoma (30) and gastric (31), colon (32), uterine endometrial (33), and breast and prostate cancers (34). In human transitional cell carcinoma of the urinary bladder, TNP-470 was also reported to inhibit tumor growth through inhibition of the growth of vascular endothelial cells (35, 36). However, there has been no study concerning whether the therapeutic effects of TNP-470 are affected by the angiogenic activity of target tumors.

In this study, we transfected MK cDNA into the human bladder cancer cell line UM-UC-3, which does not express a detectable level of MK protein, and investigated the significance of overexpression of this molecule in bladder cancer progression. We then compared the changes in antiangiogenic activity of TNP-470 *in vivo* according to MK expression levels in target tumors using MK-transfected and control UM-UC-3 cells.

Materials and Methods

Cell Culture. UM-UC-3, derived from human bladder cancer, was purchased from the American Type Culture Collection (Manassas, VA). It was maintained in MEM supplemented with 10% FBS. KoTCC-1, derived from human bladder cancer, was established in our laboratory (37) and maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS. HUVECs were purchased from Clonetic Corp. (San Diego, CA), cultured in MCDB 131 (Life Technologies, Inc.) supplemented with 10% FBS and 10 ng/ml bFGF, and used at passage 3 or 4.

Antiangiogenic Agent. TNP-470 was from Takeda Chemical Industries, Ltd. (Osaka, Japan). Its structure has been reported previously (28). TNP-470 was suspended a vehicle composed of 1% ethanol plus 5% gum Arabic in saline.

Expression Vector and Transfection of Tumor Cells. The gene encoding human MK was produced by reverse transcription-PCR. Briefly, total RNA was isolated from KoTCC-1, which expressed high levels of MK, with the acid-guanidinium-thiocyanate-phenol-chloroform method (38). First-strand cDNA was then synthesized from the total RNA using oligo(dT) primer and Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). The produced cDNA was amplified using primers designed to amplify full-length MK (sense primer, 5'-CGAAGCAGCGGGCAGCGA-3'; antisense primer, 5'-ATGTGACACCAGGGGCTCCTT-3'). PCR was performed with a

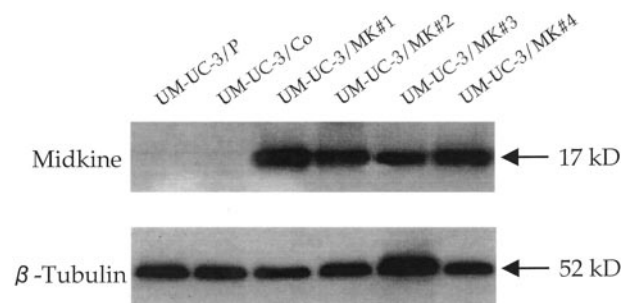


Fig. 1 Western blotting of heparin-purified conditioned medium from UM-UC-3 sublines. Ten ml of medium containing 100 μ g of heparin from 5×10^6 confluent UM-UC-3 parental cells (UM-UC-3/P), the vector-only transfected cell line (UM-UC-3/Co), and four MK transfectant clones (UM-UC-3/MK#1 to UM-UC-3/MK#4) were centrifuged with the same volume of 20% trichloroacetic acid. Analysis of MK and β -tubulin protein levels was performed by Western blotting.

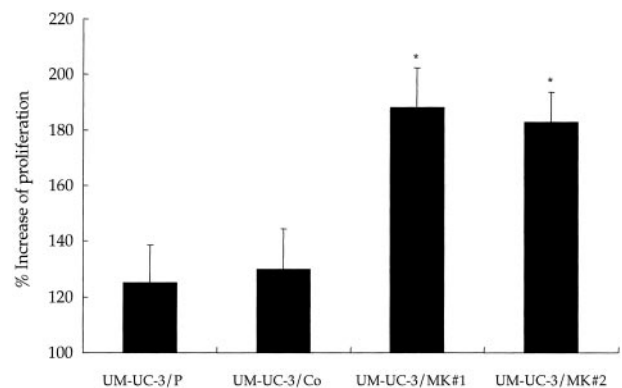


Fig. 2 Effect of heparin-agarose eluates of medium conditioned by the parental cell line UM-UC-3/P, the control vector-transfected cell line UM-UC-3/Co, and MK-transfected UM-UC-3 clones UM-UC-3/MK#1 and UM-UC-3/MK#2 on the growth of HUVECs. One ml of heparin-agarose was added to conditioned medium from UM-UC-3 sublines and incubated overnight at 4°C. Purified protein was obtained from heparin-agarose elute and added to the medium of HUVECs. Three days later, HUVECs were released by trypsin/EDTA and counted in a Coulter counter. Each point represents the mean of triplicate analyses with SD. *, differs from UM-UC-3/P and UM-UC-3/Co cells ($P < 0.01$) by Student's *t* test.

Perkin-Elmer Cetus Gene Amp PCR system 9600 (Norwalk, CT) in a 25- μ l reaction volume for 35 cycles using recombinant *Tth* DNA polymerase (Perkin-Elmer Cetus). Each cycle consisted of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 1 min. The PCR product size was 534 bp, and the PCR product was cloned directly into pcDNA3.1 (Invitrogen, Carlsbad, CA) according to the manufacturer's procedure. The expression vector was transfected into UM-UC-3 cells by the liposome-mediated gene transfer method. Briefly, 2×10^5 UM-UC-3 cells were plated in 6-cm dishes. The following day, 5 μ g of purified MK cloned pcDNA3.1 or pcDNA3.1 alone (as a control) were added to UM-UC-3 cells after preincubation for 30 min with 5 μ g of LipofectAMINE reagent and 3 ml of serum-free OPTI-MEM (Life Technologies,

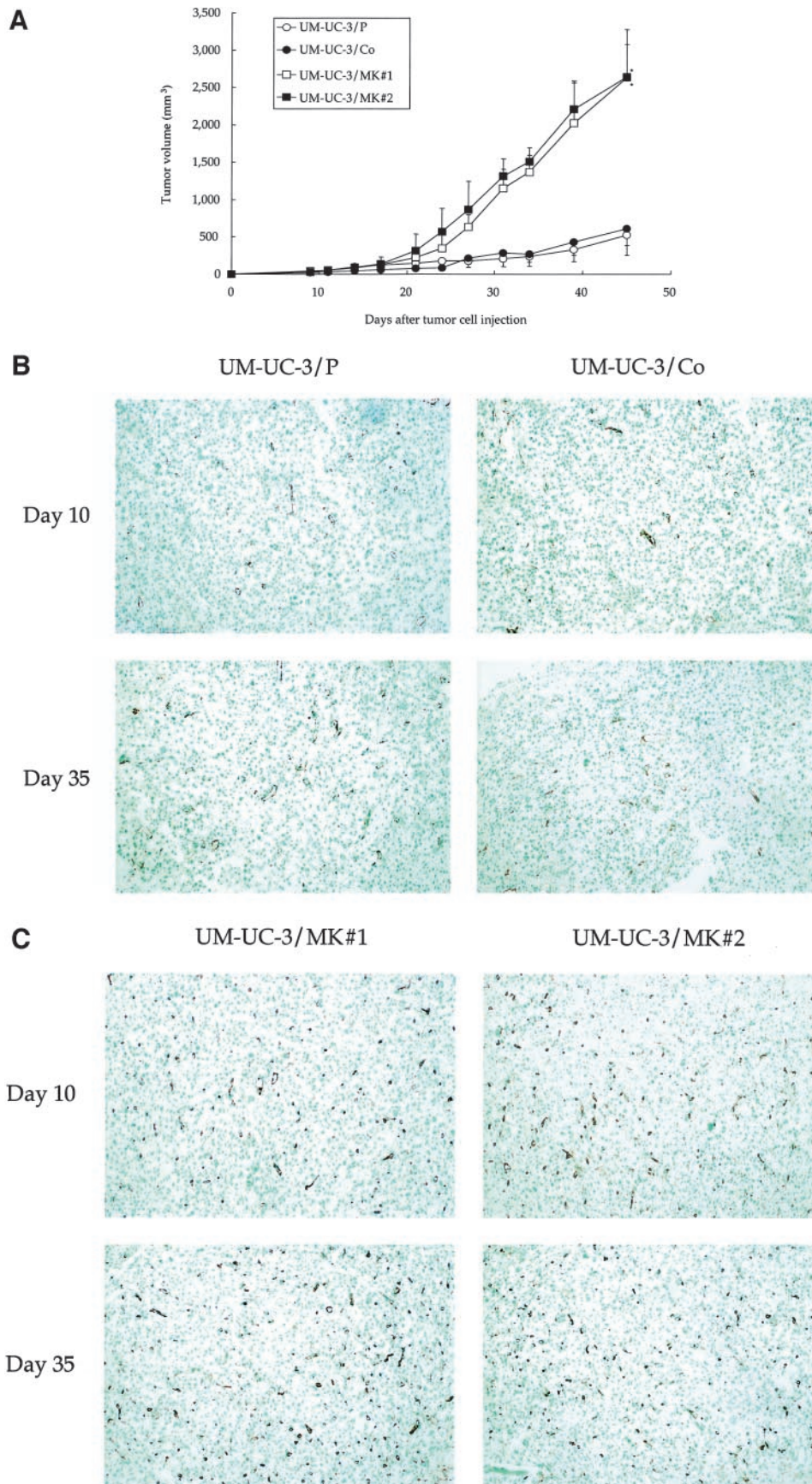


Fig. 3 Effects of MK transfection on UM-UC-3 tumor growth. In *A*, athymic nude mice received s.c. injection with 5×10^6 UM-UC-3 cells, parental UM-UC-3/P cells, control vector-transfected UM-UC-3/Co cells, and MK-transfected UM-UC-3 clone UM-UC-3/MK#1 and UM-UC-3/MK#2 cells in the right flank on day 0. Tumor volume was measured twice weekly and calculated using the formula length \times width \times depth \times 0.5236. Bars represent tumor size SD. *, differs from UM-UC-3/P and UM-UC-3/Co ($P < 0.01$) by Student's *t* test. In *B* and *C*, 10 days and 35 days after tumor cell injection, the resected tumors of each subline were zinc-fixed and embedded in paraffin. Immunohistochemical staining of CD31 was performed by the avidin-biotin complex method, and MVDs of resected tumors were determined. In *C*, representative sections from each subline at day 10 and day 35 are shown. In *D*, MVDs of each subline tumor were determined by averaging the individual microvessels in each of three different fields of the same tumor at day 10 and day 35, respectively. *, differs from UM-UC-3/P and UM-UC-3/Co ($P < 0.05$) by Student's *t* test. In *E*, central necrosis of s.c. tumors of each subline was checked macroscopically at day 10 and day 35, and the incidence of central necrosis in seven mice bearing tumors of each subline was calculated. *, differs from UM-UC-3/P and UM-UC-3/Co ($P < 0.05$) by χ^2 test.

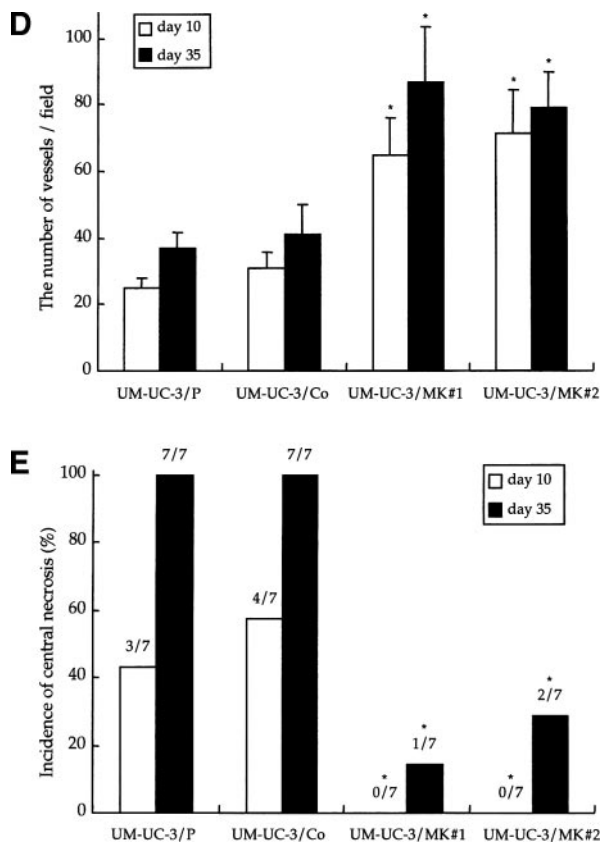


Fig. 3 Continued.

Inc.). Drug selection in 1 mg/ml Geneticin (Sigma Chemical Co., St. Louis, MO), was begun 3 days after transfection. Colonies were harvested 2 weeks after drug selection using cloning cylinders and expanded to cell lines.

Western Blotting. Medium (10 ml) containing 100 μ g of heparin from each UM-UC-3 subline was collected 2 days after feeding. The medium was then suspended in the same volume of 20% trichloroacetic acid and centrifuged for 30 min at 10,000 \times g. Samples containing equal amounts of precipitated protein (15 μ g) after centrifugation were electrophoresed on a SDS-polyacrylamide gel and transferred to a nitrocellulose filter. The filters were blocked overnight in PBS containing 5% nonfat milk powder at 4°C and then incubated for 1 h with 1:200 diluted antihuman MK goat polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in PBS containing 0.03% Tween 20. The filters were washed three times for 7 min each time with PBS containing 0.3% Tween 20 and then incubated for 30 min with horseradish peroxidase-conjugated anti-goat IgG antibody (Amersham Life Science, Arlington Heights, IL). Specific proteins were detected using an enhanced chemiluminescence Western blotting analysis system (Amersham Life Science).

Cell Proliferation Assay. To compare the *in vitro* proliferation of UM-UC-3 sublines, 1×10^3 cells of each cell line were seeded in a 6-well plate, and the number of cells in each well was counted daily by a trypan blue exclusion test in triplicate.

In Vitro Tumor Cell Invasion Assay and Migration Assay. A 6-well membrane-invasion culture system was used to measure cell invasion ability with minor modification (39). Briefly, the Matrigel coated filters were placed in Boyden Chambers (Becton Dickinson Labware, Lincoln Park, New Jersey). In the upper compartment of the chambers 1×10^5 cells per cell line were suspended in modified Eagle's medium and in the lower compartment 25 μ g/ml fibronectin was added as a chemoattractant. After 72 h incubation, cells that had migrated to the lower surface of the filters were counted manually at a $\times 200$ magnification. Similarly, the cell motility of each subline was also assessed using the Boyden chambers without Matrigel. Each assay was performed in triplicate.

Gelatin Zymography. Gelatin zymography was performed as described previously (40). Briefly, conditioned medium of each subline was collected after overnight culture in serum-free medium and concentrated 8-fold using a Centricon-30 (Amicon, Beverly, MA). Conditioned medium was loaded onto 10% gelatin zymogram gels for determination of gelatinolytic activity. Gels were incubated overnight in 50 mM Tris-HCl (pH 7.6) buffer containing 5 mM CaCl₂, 1 μ M ZnCl₂, 1% Triton X-100, and 0.5 mM aminophenylmercuric acetate and then stained with Coomassie Brilliant Blue R250 (ICN Biochemicals Inc., Aurora, OH), followed by destaining with 7% acetic acid.

Endothelial Cell Proliferation Assay. Purified MK was obtained from heparin-agarose elute as detailed previously, with minor modification (9). Briefly, 1 ml of heparin-agarose was added to conditioned medium by UM-UC-3 sublines and collected by centrifugation after overnight incubation at 4°C. The agarose was then suspended in 2 M NaCl and removed by filtration with a 0.22 μ m filter, and the flow-through was desalted using an Amicon-10 concentrator (Amicon). HUVECs were seeded at 5×10^3 cells/well in a collagen I-coated 6-well plate. The next day, cells were refed with medium lacking bFGF but containing between 10 and 50 ng of purified MK. Three days later, the cells were released by treatment with trypsin/EDTA and counted in a Coulter counter. Each experiment was performed in triplicate.

Animal Studies. Athymic nude mice (BALB/c-*nu/nu* females, 6–8 weeks old) were purchased from CLEA Japan, Inc. (Tokyo, Japan) and housed in a controlled environment at 22°C on a 12-h light/12-h dark cycle. Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Each experimental group consisted of seven mice. Each of the tumor cell lines was trypsinized and washed twice with PBS, and 5×10^6 cells were injected s.c. into the right flank or administered directly 1×10^5 cells into the bladder wall as described previously (41). s.c. tumor volume was measured twice weekly and calculated using the following formula: length \times width \times depth \times 0.5236. Six weeks after injection of tumor cells into the bladder wall, the mice were sacrificed, and the presence of metastases was examined in all abdominal organs.

Ten days after s.c. injection, mice were randomly grouped for treatment with TNP-470 or vehicle injection as a control. After randomization, mice received TNP-470 s.c. in the left flank at a dose of 30 mg/kg, 3 times/week for 3 weeks. Control animals were treated with vehicle alone, which consisted of 1%

Table 1 Changes in metastasis of UM-UC-3 parental, UM-UC-3/control, UM-UC-3/MK#1, and UM-UC-3/MK#2 cells injected into the bladder wall of nude mice

Cells	Incidence of metastasis (%) ^a			Weight of the primary tumor ^c (mg)
	Retroperitoneal lymph node metastasis	Intra-abdominal lymph node metastasis	Incidence of hemorrhagic ascites ^b (%)	
UM-UC-3/P	0/7 (0)	0/7 (0)	0/7 (0)	20 ± 10.0
UM-UC-3/Co	0/7 (0)	0/7 (0)	0/7 (0)	11 ± 10.1
UM-UC-3/MK#1	6/7 (86) ^d	4/7 (57) ^d	1/7 (14)	308 ± 71 ^e
UM-UC-3/MK#2	6/7 (86) ^d	3/7 (43) ^d	0/7 (0)	378 ± 144 ^e

^a Number of mice with tumor/number of inoculated mice.

^b Number of mice with hemorrhagic ascites/number of inoculated mice.

^c Mean ± SD.

^d The incidence of metastasis was significantly different from that in mice inoculated with UM-UC-3/P and UM-UC-3/Co ($P < 0.05$, χ^2 test).

^e The mean weight of the primary tumor was significantly different from that in mice inoculated with UM-UC-3/P and UM-UC-3/control ($P < 0.05$, Student's t test).

ethanol and 5% gum Arabic in normal saline. s.c. tumor volume was measured as described above.

Immunohistochemistry and Microvessel Quantification. For microvessel quantitation, tissues at the tumor site and skin were collected 10 days and 35 days after s.c. injection. The resected tissues were zinc-fixed and embedded in paraffin, as described previously (42). Immunohistochemical staining was performed by the avidin-biotin complex method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA). The sections were incubated with a 1:200 dilution of antimouse CD31 rat monoclonal antibody (MEC 13.3; Santa Cruz Biotechnology) for 45 min followed by biotinylated rabbit antirat IgG[H+L] (Vector Laboratories). The sections were then incubated with horseradish peroxidase-conjugated avidin for 30 min. Absorbed peroxidase was visualized by incubation with 0.05% 3,3'-diaminobenzidine (Dojindo Laboratories, Kumamoto, Japan) for 1 min and counterstained with methylgreen. Intratumoral MVD was quantified in each harvested tumor by enumerating CD31-positive vessels under a microscope in a minor modification of the method described previously (43). Briefly, the tumor sections were scanned at low power ($\times 100$), and then MVD was determined by averaging the individual microvessels in each of three different fields of the same tumor. The counting was performed by two independent observers.

Statistical Analysis. All of the data were evaluated by χ^2 test or Student's t test. Probability values of $< 5\%$ were considered significant.

Results

Stable Transfection of MK Gene into UM-UC-3 Cells.

UM-UC-3 was transfected with the MK cloned pcDNA3.1 or the pcDNA3.1 alone as a control. After drug selection, a number of Geneticin-resistant stable transfectants were isolated. A MK mRNA transcript of the expected size was readily detectable in the transfected clones by reverse transcription-PCR (data not shown). Stable transfectants were then analyzed for expression of the MK protein by Western blotting. As shown in Fig. 1, MK protein was highly expressed in four independent MK-transfected clones (UM-UC-3/MK#1 to UM-UC-3/MK#4) but was not detectable in the parental UM-UC-3 cell line (UM-UC-3/P) and the control vector-transfected cell line (UM-UC-3/Co). The four clones expressing high levels of MK protein showed almost

the same results in subsequent experiments; therefore, we henceforth report only the findings of UM-UC-3/P, UM-UC-3/Co, UM-UC-3/MK#1, and UM-UC-3/MK#2.

Characterization of UM-UC-3 Sublines by *in Vitro* Assays. The effect of heparin-agarose eluates of medium conditioned by UM-UC-3 sublines on growth of HUVECs was analyzed by endothelial cell proliferation assay. Heparin-agarose eluates from UM-UC-3/MK#1- and UM-UC-3/MK#2-conditioned media induced significant stimulation of HUVEC growth compared with those from UM-UC-3/P- and UM-UC-3/Co-conditioned media, confirming biological activity of secreted MK from UM-UC-3/MK#1 and UM-UC-3/MK#2 (Fig. 2). In contrast, no significant difference was found between the sublines in the cell proliferation assay, the cell invasion assay, the cell migration assay, and gelatin zymography (data not shown).

Overexpression of MK Stimulates *in Vivo* Tumor Growth and Intratumor Microvessel Formation.

To examine the *in vivo* effect of MK expression on tumor growth, 5×10^6 cells of each cell line were injected s.c. in the flank of athymic nude mice. The s.c. tumors formed by UM-UC-3/MK#1 and UM-UC-3/MK#2 were significantly larger than those formed by UM-UC-3/P and UM-UC-3/Co ($P < 0.01$; Fig. 3A). Moreover, when each subline were injected orthotopically, UM-UC-3/MK#1 and UM-UC-3/MK#2 cells formed tumors significantly larger than those formed by UM-UC-3/P and UM-UC-3/Co ($P < 0.05$), accompanied by a significant increase in the incidence of retroperitoneal and intra-abdominal lymph node metastases ($P < 0.05$ and $P < 0.05$, respectively; Table 1). The s.c. tumor sections were stained for the endothelial marker CD31, and vascular density was determined by the method described above. We assessed the intratumoral MVD at different times after tumor injection; that is, 10 days and 35 days after injection, respectively. At 10 days after injection, the s.c. tumor volumes of the sublines were not significantly different (Fig. 3A); however, the MVDs of UM-UC-3/MK#1 and UM-UC-3/MK#2 were significantly higher than those of UM-UC-3/P and UM-UC-3/Co ($P < 0.05$; Fig. 3, B–D). Although MVDs at 35 days after injection were slightly increased compared with those at 10 days after injection in all sublines, the MVDs of UM-UC-3/MK#1 and UM-UC-3/MK#2 were significantly higher than those of UM-UC-3/P and UM-UC-3/Co ($P < 0.05$; Fig. 3, B–D). We also evaluated the incidence of macroscopic central

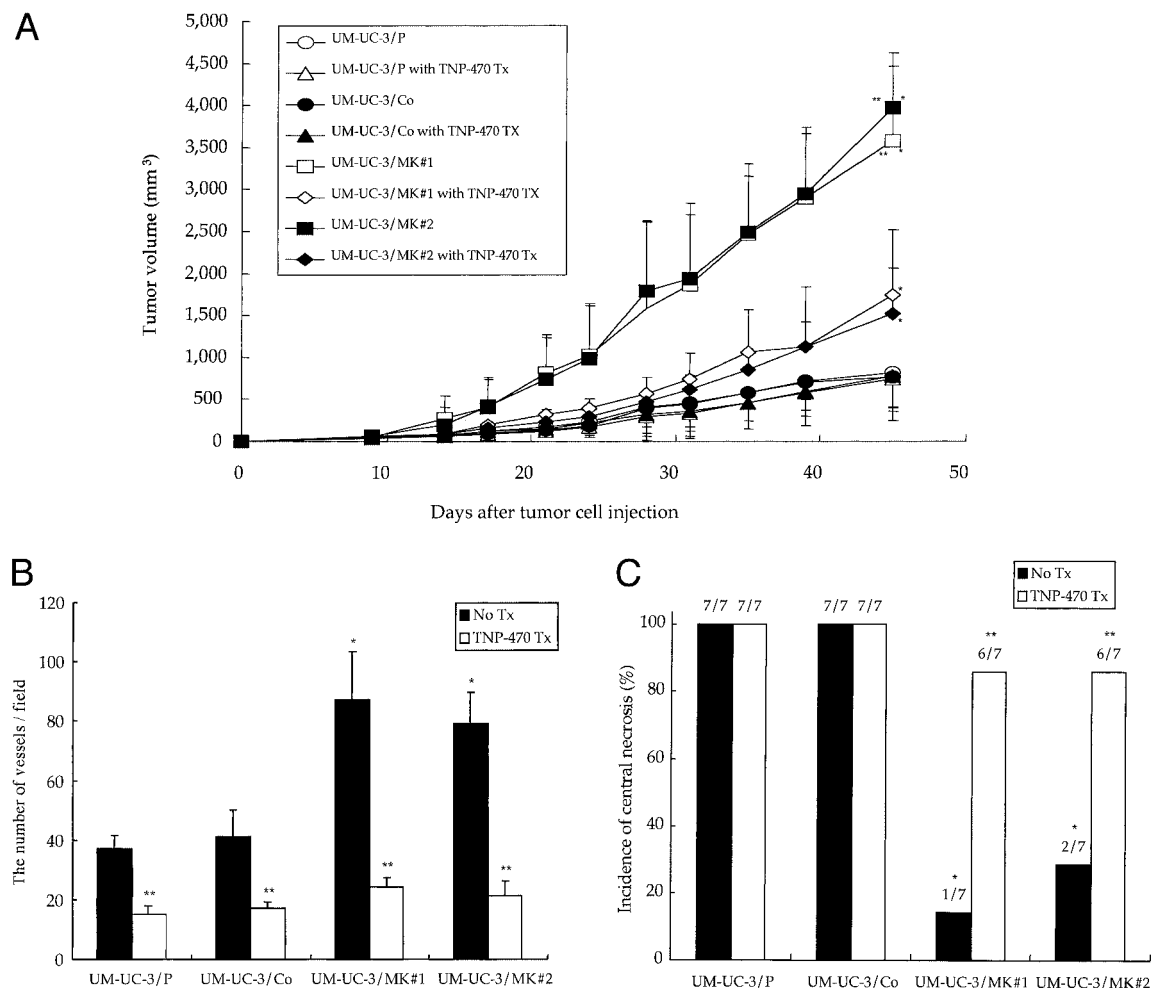


Fig. 4 Effects of TNP-470 treatment on the inhibition of tumor growth of UM-UC-3 sublines. In **A**, mice bearing tumors of UM-UC-3, the parental cell line UM-UC-3/P, the control vector-transfected cell line UM-UC-3/Co, and MK-transfected UM-UC-3 clones UM-UC-3/MK#1 and UM-UC-3/MK#2 were randomly selected for treatment with TNP-470 or vehicle alone at day 10. Seven mice from each experimental group received injection with 30 mg/kg TNP-470 or the same volume of vehicle 3 times/week for 3 weeks in the left flank. Tumor volume was measured twice weekly and calculated using the formula length \times width \times depth \times 0.5236. Bars represent tumor size SD. *, differs from UM-UC-3/P and UM-UC-3/Co ($P < 0.01$) by Student's t test. **, differs from UM-UC-3/MK#1 and UM-UC-3/MK#2 treated with TNP-470 ($P < 0.01$) by Student's t test. In **B**, 10 days and 35 days after tumor cell injection, the resected tumors of each experimental group were zinc-fixed and embedded in paraffin. Immunohistochemical staining of CD31 was performed by the avidin-biotin complex method, and MVD of resected tumors was determined. Intratumoral MVDs of each experimental group were quantified by averaging the individual microvessels in each different field of the same tumor at day 10 and day 35, respectively. Bars represent SD. *, differs from UM-UC-3/P and UM-UC-3/Co ($P < 0.01$) by Student's t test. **, differs from control group [No Tx, no treatment group ($P < 0.01$)] by Student's t test. In **C**, central necrosis of s.c. tumors of each experimental group was checked macroscopically at day 10 and day 35, and the incidence of central necrosis in seven mice bearing tumors of each experimental group was calculated. *, differs from UM-UC-3/P and UM-UC-3/Co ($P < 0.05$) by χ^2 test. **, differs from control group [No Tx, no treatment group ($P < 0.05$)] by χ^2 test.

necrosis of resected tumors when mice were sacrificed for CD31 staining. At 10 days after injection, central necrosis was detected in three of seven UM-UC-3/P tumors and four of seven UM-UC-3/Co tumors; in contrast, neither UM-UC-3/MK#1 nor UM-UC-3/MK#2 tumors had central tumor necrosis. Moreover, at 35 days after injection, central necrosis was found in all UM-UC-3/P and UM-UC-3/Co tumors; however, central necrosis was observed in only one UM-UC-3/MK#1 tumor and two UM-UC-3/MK#2 tumors (Fig. 3E).

Inhibition of Tumor Growth of UM-UC-3 Cells *in Vivo* by TNP-470 Treatment. The effect of TNP-470 treatment on inhibition of growth of s.c. tumors of UM-UC-3 sublines was

evaluated. Athymic nude mice bearing tumors of UM-UC-3 sublines less than 1 cm in diameter were randomly selected for treatment with TNP-470 or vehicle alone. TNP-470 was injected at 30 mg/kg, 3 times/week for 3 weeks. TNP-470 significantly decreased the rate of tumor growth compared with vehicle control in the UM-UC-3/MK#1 and UM-UC-3/MK#2 groups ($P < 0.01$). Forty-five days after tumor injection, the tumor volume in mice bearing UM-UC-3/MK#1 and UM-UC-3/MK#2 tumors treated with TNP-470 was 39% or 46% smaller than that in mice treated with vehicle alone, respectively. In contrast, TNP-470 did not affect the s.c. tumor volume in the UM-UC-3/P and UM-UC-3/Co groups (Fig. 4A). Intratumoral MVD of

each group was determined by immunostaining of CD31 after treatment with TNP-470. When treated with TNP-470, a significant decrease of MVD was observed in all subline groups compared with those treated with vehicle alone; however, the decreased rate of MVD in UM-UC-3/MK#1 and UM-UC-3/MK#2 tumors was significantly higher than that in UM-UC-3/P and UM-UC-3/Co tumors (Fig. 4B). The incidence of macroscopic central necrosis in UM-UC-3/P and UM-UC-3/Co tumors was 100% with or without TNP-470 treatment. On the other hand, when treated with TNP-470, central necrosis was observed in almost all UM-UC-3/MK#1 and UM-UC-3/MK#2 tumors, with significantly higher incidence compared with the no treatment group in the same subline ($P < 0.05$; Fig. 4C).

Discussion

Angiogenesis is considered an integral process for the growth and spread of solid tumors and has become a growing focus of anticancer therapy (17, 18). Recently, several studies reported that angiogenesis in bladder cancer correlated with stage (44), lymph node metastasis (45), and prognosis (21, 24, 46). It was also reported that progression of bladder cancer was associated with expression of MK (16), which was shown to have an important role in angiogenesis by activating proliferation of vascular endothelial cells (47). Taken together, these findings suggest that MK up-regulation in bladder cancers is associated with disease progression through the enhancement of angiogenic activity. However, to our knowledge, there have been no studies analyzing the functional significance of MK expression in human bladder cancer; therefore, in this study, we introduced the MK gene into human bladder cancer UM-UC-3 cells, which do not express detectable levels of MK protein, and evaluated the effect of MK overexpression on the cells' malignant potential both *in vitro* and *in vivo*.

Introduction of MK into UM-UC-3 confers growth advantages in both s.c. and orthotopic injection models, without affecting *in vitro* proliferation, migration, or invasion abilities. In addition, we demonstrated both an increased endothelial cell proliferative ability of MK-overexpressing sublines *in vitro* and an increased intratumoral MVD in MK-overexpressing tumors *in vivo*. This finding is consistent with those of previous studies showing that expression of MK enhances tumor growth and vascular density (9, 26). It is of interest that UM-UC-3/P and UM-UC-3/Co had already shown significantly lower MVD 10 days after injection, although there was no significant difference in tumor volume among all sublines. Furthermore, most s.c. UM-UC-3/P and UM-UC-3/Co tumors had macroscopic central tumor necrosis; in contrast, central necroses were barely observed in MK-overexpressing tumors. These findings suggest that the increased intratumoral MVD and the decreased incidence of central necrosis in UM-UC-3/MK#1 and UM-UC-3/MK#2 tumors may reflect the abundant new vasculature that is needed to supply the rapidly growing tumor mass.

Most cases of bladder cancer initially respond to cisplatin-based combination chemotherapy; however, the development of an acquired resistant phenotype is observed frequently with the progression of the disease (2). Targeting angiogenesis, however, offers one attractive therapeutic strategy. TNP-470 was reported to cause cell cycle slowing and/or arrest in vascular endothelial

cells (48, 49). The suppression of vascular endothelial cell proliferation by TNP-470 results in inhibition of angiogenesis, which was previously shown to cause hypoxia and trigger apoptotic death in tumor cells *in vivo*. Moreover, it was reported that the effect of TNP-470 was greater in vascular-rich tumors than that in control tumors using isogenic glioma cells differing in expression of vascular endothelial growth factor (50). Although some recent studies have shown that TNP-470 was effective for inhibition of bladder cancer cell growth *in vivo* (51, 52), there is no consensus regarding whether TNP-470 has different therapeutic effects according to the angiogenic activities in target tumor tissues. In the present study, TNP-470 significantly inhibited tumor growth only in the MK-overexpressing and vascular-rich tumor groups. The MVD in the s.c. tumors was significantly decreased not only in MK-overexpressing tumors but also in control tumors; however, the volume of control tumors did not change when treated with TNP-470. This result may be attributable to the finding that the incidence of central necrosis of the control tumors was 100% with or without TNP-470 treatment; that is, TNP-470 may have a minimal inhibitory effect on highly necrotic tumors. Another possible explanation of this issue is the protocol of TNP-470 administration. Inoue *et al.* (51) demonstrated in a human bladder cancer xenograft model that daily administration of TNP-470 at an optimal biological dose (105 mg/kg/week; 735 mg/kg/week) provided maximal antitumor and antimetastatic effects. However, a number of studies have successfully demonstrated the inhibitory effect of TNP-470 on tumor progression by administration once or three times per week at a variety of doses (30–36). To establish an efficient protocol of TNP-470 administration, further investigations will be needed.

In conclusion, the present findings suggest that the introduction of MK gene into bladder cancer cells enhances their malignant progression *in vivo* through the activation of angiogenic potential. Moreover, the present study provides evidence that TNP-470 treatment can inhibit the growth of MK-overexpressing vascular-rich tumors more effectively compared with that of vascular-poor tumors. Collectively, targeting angiogenesis in bladder cancer may have a promising role as one of the novel therapeutic modalities for patients with advanced disease.

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