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Matrix Metalloproteinase Inhibitor, Marimastat, Decreases Peritoneal Spread of Gastric Carcinoma in Nude Mice

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Marimastat, a matrix metalloproteinese inhibitor, was examined for the ability to prevent peritoneal dissemination of a human gastric cancer xenograft, TMK-1. Even with novel approaches such as molecular targeting of cancer chemotherapy, peritoneal dissemination of gastric cancer has little sensitivity to anticancer drugs, and it is impossible to inhibit its growth completely. Intraperitoneal injection of TMK-1 into nude mice at 5×10^5 cells/body resulted in carcinomatous peritonitis that mimicked clinical cases. Continuous administration of marimastat (18 mg/kg/day) from 24 h after the tumor inoculation successfully inhibited the growth of peritoneal dissemination nodules. Combined administration of marimastat (18 mg/kg/day) and mitomycin C (MMC, 2 mg/kg) showed synergistic inhibition of growth of peritoneal dissemination, being superior to MMC alone (2 mg/kg). Although marimastat alone could not increase survival time with statistical significance, combined administration of marimastat and MMC had a survival benefit with statistical significance. The combination of marimastat and MMC increased the preventive effect on peritoneal dissemination. Marimastat seems to be a candidate for the prevention of peritoneal spread of gastric carcinoma.

Key words: MMP inhibitor — Gastric cancer — Peritoneal dissemination — Marimastat

An apparent increase in incidence of early gastric cancer in Japan has resulted from enhanced detection by a well-established screening program. However, outcomes remain unsatisfactory for patients with advanced gastric cancer. In our hospital, 3724 patients underwent surgery for gastric cancer between 1960 and 1997. At initial surgery 13.6% of these patients had macroscopically evident peritoneal dissemination of cancer. Peritoneal dissemination has represented the most common recurrence site for gastric cancer at our institution, comprising over 30% of all recurrences. The 5-year survival rate of the patients with this form of recurrence was only 4.8%, and survival beyond 3 years was uncommon. The usual treatment of gastric cancer is surgical operation and chemotherapy. However, it is impossible to resect all nodules of peritoneal dissemination by surgery. Furthermore, no sensitivity of peritoneal dissemination to anticancer drugs is apparent. Therefore, development of a novel and effective therapy for peritoneal dissemination is an urgent priority.

Generally, tumor invasion and metastasis from the primary site involve multiple steps including tumor cell attachment at a new site, matrix degradation, and cell locomotion.[1)] Similar events are likely to occur in peritoneal dissemination of gastric cancer. In this process the subperitoneal extracellular matrix would be degraded by matrix metalloproteinases (MMPs). Matrix degradation by MMPs is also important in angiogenesis,[2)] especially during the formation of peritoneal dissemination nodules. Pharmacologic inhibition of MMP in a gastric cancer patient should interfere with degradation of the extracellular matrix and subperitoneal angiogenesis, thus acting against peritoneal dissemination.

Several MMP inhibitors such as R94138,[3)] Batimastat,[4–6)] AG3340,[7)] BAY12-9566,[8)] and COL-3[9, 10)] have been synthesized as anticancer drugs and some of them are undergoing clinical trials. In particular, marimastat (BB-2516) has finished a phase III clinical trial after reportedly showing anticancer activity in phase II and III trials.[11–14)] However, these trials were designed for the treatment of primary tumor and few reports have considered the action of marimastat against peritoneal dissemination.

In the present study, we examined the preventive effects of marimastat, which inhibits MMP-1, -2, -3, -7, -9, -12, using human gastric cancer xenograft administered intraperitoneally to nude mice and investigated the possibility that marimastat could be a new preventive agent or treatment for peritoneal dissemination.

MATERIALS AND METHODS

Cell lines TMK-1, a human gastric carcinoma cell line

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used for this investigation, was kindly supplied by Dr. S. Hirohashi of the Japanese National Cancer Center Research Institute. This cell line, established as a serially transplantable human tumor xenograft in nude mice by Tokuda *et al.*, was obtained from cancer tissue from a 21-year-old man with gastric cancer.[15] This cell line was also established as a cultured cell line by Ochiai *et al*.[16] The cells were cultured in RPMI-1640 medium (GIBCO, Gaithersburg, MO) supplemented with 10% bovine serum (JRH, Lenexa, KS) and 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml fungizone (GIBCO). In gelatin zymography, expression of MMP-2 and -9 was not recognized in culture medium obtained from TMK-1 soloculture, but the expression of MMP-2 was recognized in culture medium obtained from the co-culture of TMK-1 and human fibroblast cells.[3] BALB/3T3 clone A31 was established by Aaronson and Todaro in 1968 from disassociated 14- to 17-day-old BALB/c mouse embryos.[17] The BALB/3T3 clone A31 possesses many properties similar to those of 3T3 fibroblasts derived from random-bred Swiss-mouse embryos (ATCC CCL-92). The cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% calf serum and 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml fungizone.

Agents Marimastat (Fig. 1), an inhibitor of MMPs, synthesized by British Biotech (London, UK), was kindly provided by Tanabe Co., Ltd. (Osaka). Marimastat has a low molecular weight of 331.42 and a collagen-mimicking hydroxamate structure that together facilitate chelation of the zinc ion in the active sites of MMPs.[18] Mitomycin C (MMC) was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo) and dissolved in saline.

Nude mice BALB/c *nu/nu* male nude mice purchased from CLEA, Japan, Inc. (Tokyo) were maintained free of specified pathogens using an Isorack in the experimental animal center. Mice were given sterile food and water *ad libitum*. Four-to-six-week-old mice weighing 20 to 22 g were used for the experiment. In this experiment, we complied with the Guideline for the Care and Use of Laboratory Animals of Keio University School of Medicine.

MTT assay The assay method of Mosmann[19] was used, including the modifications reported previously.[20, 21] After centrifugation, tumor cells were suspended in RPMI-1640 containing 10% fetal calf serum (FCS) and counted by the trypan blue dye exclusion method. Cell suspensions were then diluted to 2×10^4 to 10^5 cells/ml. Assays were performed using 96-well microplates; 100 μl of RPMI-1640 containing 10% FCS but without cells was placed in the front row of wells as a blank. In the remaining wells of the plates, 50 μl per well of cell suspension and 50 μl per well of drug diluted in RPMI-1640 were mixed to result in 10^4 to 5×10^4 cells per well as determined in our previous study.[20] Final concentrations were 10 μM and 50 μM for marimastat, and 1.0 μM, 3.0 μM and 10.0 μM for MMC. After the plates had been incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 for 48 h, 10 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) dissolved in 5 mg/ml phosphate-buffered saline filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA) was added to each well. Following an additional 4 h of incubation, 150 μl per well of 0.04 *N* HCl in DMSO was added to dissolve the MTT-formazan product. After thorough mixing with a mechanical plate mixer (model 250, Sonifer, Branson, Danbury, CT), supernatants were transferred to other plates at a volume of 150 μl per well following centrifugation for 5 min at 3000 rpm. The absorbance was read by a model 2550 enzyme immunoassay (EIA) reader (Bio-Rad,

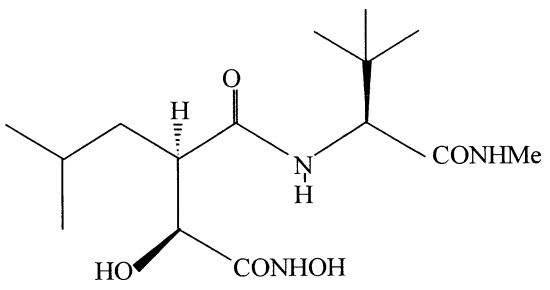

Fig. 1. Three-dimensional structural formula of marimastat, [3*R*-(2,2-dimethyl-1*S*-methylcarbamoyl-propylcarbamoyl)-2*S*-hydroxy-5-methyl hexano-hydroxamic acid], which has a molecular weight of 331.42.

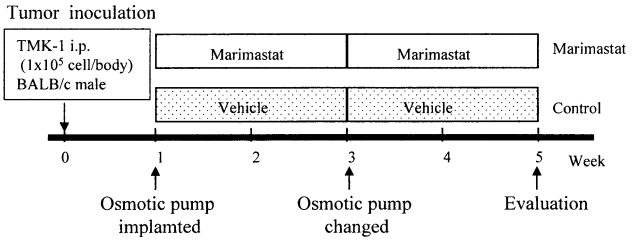

Fig. 2. Design of experiment I. TMK-1 human gastric carcinoma cells were injected intraperitoneally into 40 nude mice (5×10^5 cells/animal). Mice then were divided into treatment and control groups and underwent subcutaneous implantation of mini-osmotic pumps for continuous administration of marimastat in vehicle or of vehicle alone. Administration for 14 days began 7 days after tumor cell inoculation. At 21 days after tumor cell injection, the pumps were changed to continue administration for another 14 days. At 35 days after tumor cell injection, mice were killed and weighed. All peritoneal tumor nodules were collected and weighed. Doses of marimastat administered were 9, 18, 27, and 36 mg/kg/day (5 treated mice per dose-defined group; 5 untreated controls separately compared with each step; Table II).

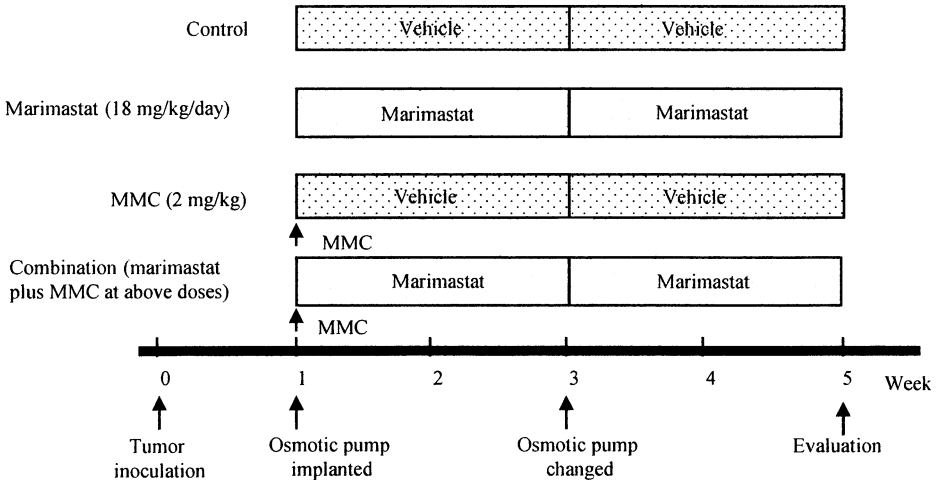

Fig. 3. Design of experiment II. TMK-1 human gastric carcinoma cells (5×10^5 cells/animal) were injected intraperitoneally into 20 nude mice four groups of 5 mice. All mice later underwent subcutaneous implantation of mini-osmotic pumps. Treatment was initiated 7 days after intraperitoneal injection of TMK-1 cells. In marimastat and combination therapy groups, pumps contained marimastat in vehicle; in control and MMC groups, pumps contained only vehicle. Pump contents were given continuously for 14 days beginning 7 days after cell injection. In MMC and combined therapy groups, MMC was injected intraperitoneally once on day 7. At 21 days after injection of TMK-1, pumps were changed to continue administration for another 14 days. At 35 days after tumor cell injection, mice were killed and weighed. All peritoneal tumor nodules were collected and weighed. Marimastat was administered at a dose of 18 mg/kg/day, while MMC was given as one dose of 2 mg/kg. The scheme for the control group followed that shown for the MMC group, without MMC injection.

Richmond, CA) at 600 nm. The inhibition rate was calculated by using the formula, inhibition rate (%)=(1−mean absorbance of treated wells/mean absorbance of control wells)×100. The assay was regarded as evaluable when the mean absorbance of control wells was equal to or greater than 0.15. An inhibition rate of 50% or more was considered to indicate positive cytotoxicity.

Tumor inoculation and drug administration In experiment I, cultured TMK-1 cells (5×10^5 cells/mouse) were injected intraperitoneally into 40 nude mice (Fig. 2). Treatment was initiated 7 days later. Mice underwent subcutaneous implantation of mini-osmotic pumps (ALZET2002; ALZA, Palo Alto, CA) containing various doses of marimastat dissolved in 50% DMSO (Fig. 3), to continuously administer marimastat for 14 days. At 21 days after injection of TMK-1, pumps were retrieved and new pumps were implanted. Thus, marimastat was administered for 28 consecutive days. A control group for each dose was given the vehicle (50% DMSO) alone in the same manner. At 35 days after injection of TMK-1, mice were killed and weighed, and all peritoneal tumor nodules were collected and weighed. Doses of marimastat administered were 9, 18, 27 and 36 mg/kg/day. Each treated and control group included 5 mice. In experiment II, TMK-1 was injected i.p. into 20 nude mice by the same method (Fig. 3). Five mice each were assigned to a control group, a marimastat group, a MMC group, or a combination therapy group. In the control group, only vehicle (50% DMSO) was administered by the subcutaneously implanted pump. Marimastat (18 mg/kg/day) was administered by pump for 28 days in marimastat group. In the MMC group, MMC (2 mg/kg) was injected i.p. at 7 days after injection of TMK-1 cells, and these animals also underwent implantation of mini-osmotic pumps containing vehicle. The

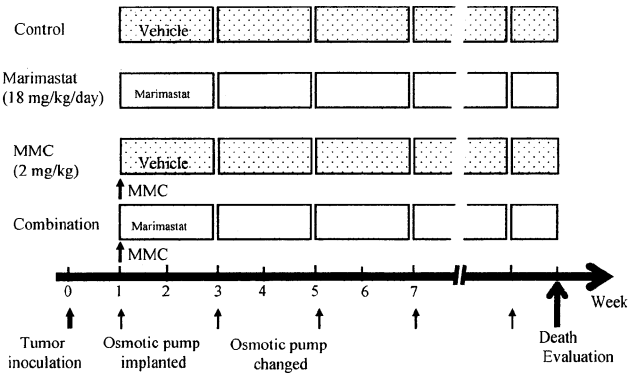

Fig. 4. Design of the survival experiment. Tumor cells and drugs were given to control, marimastat, MMC, and combination therapy groups as in experiment II, except that the mini-osmotic pump was changed every 2 weeks until mice died of peritoneal tumor dissemination.

combination therapy group received MMC i.p. as above and also received marimastat via implanted pump. At 35 days after injection of TMK-1 cells, mice were killed and weighed. All peritoneal tumor nodules then were collected and weighed.

Survival experiment TMK-1 cells were injected into 40 nude mice by the same method as above (Fig. 4), and 10 mice each were assigned to control, marimastat, MMC, and combination therapy groups. All mice underwent subcutaneous implantation of mini-osmotic pumps 7 days after cell injection. In MMC and combination therapy groups, MMC (2 mg/kg i.p.) was injected once 7 days after injection of TMK-1 cells. In control and MMC groups the pumps contained 50% DMSO. In the marimastat and combination therapy groups, marimastat (18 mg/kg/day) was administered continuously via the pumps. Pumps were reimplanted every 2 weeks until the mice

Table I. Cytotoxic Effect of Marimastat and MMC on TMK-1

Agent	Concentration (μM)	Inhibition rate (%)
Marimastat	10	2.64
Marimastat	50	1.10
MMC	1.0	0.00
MMC	3.0	43.38
MMC	10.0	50.84

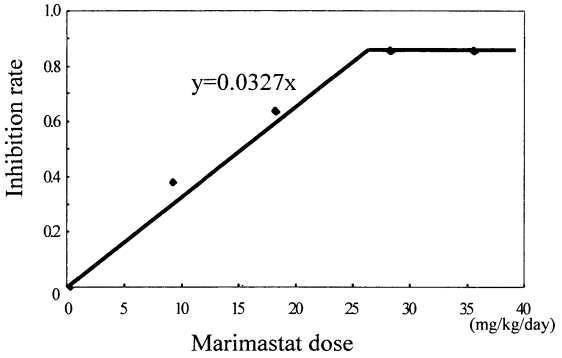

Fig. 6. Dose-dependent antitumor effect of marimastat on TMK-1 cells. Inhibition increased in a dose-dependent manner up to 26 mg/kg/day of marimastat.

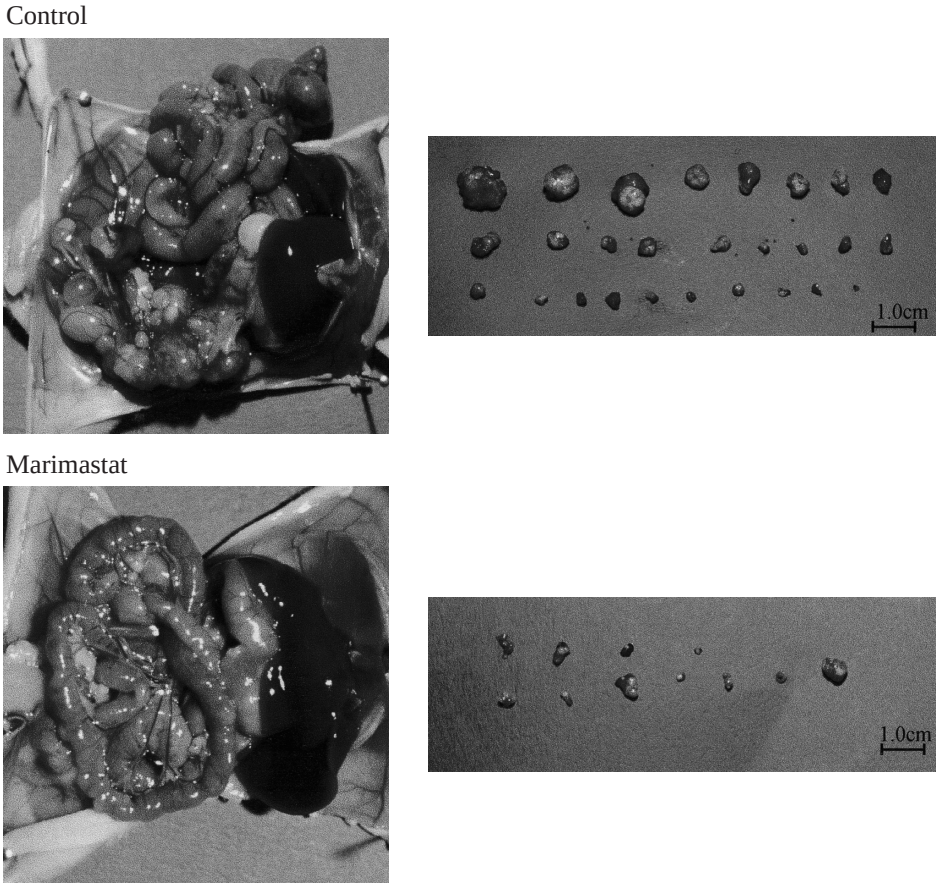

Fig. 5. Macroscopic appearance of peritoneally disseminated TMK-1 cell nodules at 5 weeks after intraperitoneal tumor cell injection (5×10^5 cells/mouse). All nodules appeared glossy and whitish. Many peritoneal dissemination nodules were observed throughout the peritoneal cavity in control group. Some peritoneal nodules were mainly observed on the mesenterium in the marimastat group. The largest nodule, 17.0 mm in diameter and 0.53 g in weight, was seen in the control group, representing confluence of many smaller nodules.

died of peritoneal dissemination. The nodules of disseminated tumor were examined postmortem.

Statistical analysis Statistical analysis was performed by Mann-Whitney *U* test (Stat View, SAS Institute, Cary, NC). $P<0.05$ was chosen as the criterion for statistical significance.

RESULTS

In the MTT assay, marimastat did not show cytotoxicity to TMK-1 cells (Table I). MMC showed cytotoxic activity in the same systems, with growth inhibition rates of 43.38% and 50.84% at 3.0 and 10.0 μM, respectively (Table I).

Table II. Side Effect of Marimastat Used to Treat Intraperitoneally Disseminated TMK-1 Cells in Nude Mice

Treatment	$n^{a)}$	Body weight (g)$^{b)}$	T/C$^{c)}$
Control$^{d)}$	5	26.05±3.60	100
Marimastat (9 mg/kg/day)$^{e)}$	5	29.47±3.60	113.1
Control	5	33.06±4.66	100
Marimastat (18 mg/kg/day)	5	29.97±2.62	90.7
Control	5	27.00±4.16	100
Marimastat (27 mg/kg/day)	5	28.30±3.30	104.8
Control	5	32.60±4.85	100
Marimastat (36 mg/kg/day)	5	29.59±2.72	90.8

a) Number of mice.
b) Body weight of nude mouse in grams as mean±SD at 5 weeks after tumor injection.
c) Treated group/control group ratio of body weights of mice (%).
d) Vehicle (50% DMSO) was administered as a control.
e) Marimastat was administered subcutaneously for 4 weeks using an osmotic pump, starting on day 7 after tumor cell injection.

Figs. 5, 6 and Table II show the results of experiment I. The weight of peritoneal tumor nodules in the marimastat group receiving 36 mg/kg/day was 1.68±0.49 g (mean± SD), while in the control group, nodules weighed 11.42± 0.92 g. Formation of peritoneal nodules as judged from nodule weight was significantly suppressed in all treated groups ($P<0.05$), except at the dose of 9 mg/kg/day. Inhibition rates were 39.7% for 9 mg/kg/day, 63.3% for 18 mg/kg/day, 89.9% for 27 mg/kg/day, and 85.3% for 36 mg/kg/day, representing dose-dependent inhibition (Fig. 6). No severe side effect was evident in terms of body weight loss (Table II). No deformity or inflammation of knee joints was evident macroscopically after the mice were killed.

In experiment II, peritoneal tumor nodules were significantly suppressed in all treatment groups ($P<0.01$). The peritoneal tumor nodules weighed 9.37±0.92 g in the con-

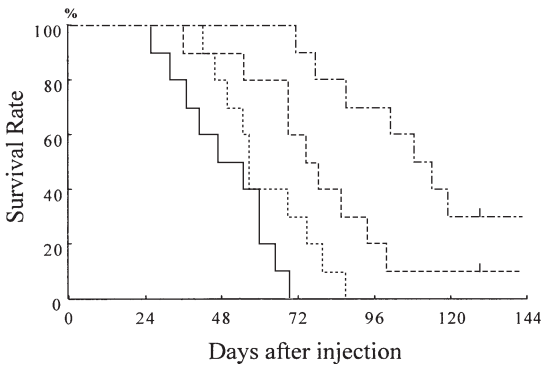

Fig. 7. Result of the survival experiment. Mice treated with marimastat alone (····) tended to survive longer than control mice (—), but the difference fell short of statistical significance. Mice treated with MMC alone (---) or with a combination of MMC plus marimastat (-·-) survived significantly longer than the control group.

Table III. Antitumor Effect of Marimastat and MMC on Intraperitoneally Disseminated TMK-1

Treatment	$n^{a)}$	Tumor weight (g)$^{b)}$	T/C$^{c)}$	Body weight (g)$^{d)}$	T/C$^{e)}$
Control$^{f)}$	5	9.37±0.92	100	32.60±4.84	100
Marimastat$^{g)}$	5	3.43±1.29*	26.6	29.58±2.72	90.7
MMC	5	0.68±0.42*	7.2	27.35±2.06	83.9
Marimastat+MMC	5	0.12±0.16*	1.3	29.02±1.01	89.1

a) Number of mice.
b) Total disseminated tumor weight in grams as mean±standard deviation at 5 weeks after tumor injection.
c) Treated group/control group ratio of tumor weights (%).
d) Body weight of nude mouse in grams as mean±SD at 5 weeks after tumor injection.
e) Treated group/control group ratio of body weights of mice (%).
f) Vehicle (50% DMSO) was administered as a control.
g) Marimastat was administered subcutaneously at a dose of 18 mg/kg/day for 4 weeks using an osmotic pump, starting on day 7.
$* P<0.01$.

trol group (mean±SD); 3.43±1.29 g (T/C 26.6%) in the marimastat group (18 mg/kg/day), 0.68±0.42 g (T/C 7.2%) in MMC group (2 mg/kg i.p.); and 0.12±0.16 g (T/C 1.3%) in the combination therapy group (marimastat 18 mg/kg/day+MMC 2 mg/kg i.p.) (Table III).

The results of the survival experiment are shown in Fig. 7. Mice treated with marimastat alone survived longer than the control group, but the difference fell short of statistical significance. Mice treated with MMC alone and also those receiving a combination of MMC and marimastat survived significantly longer than control mice ($P<0.01$). One mouse in the MMC group and two mice in the combination group survived until 6 months after tumor inoculation, when they were killed. Absence of metastatic nodules was confirmed at autopsy. At the time of their deaths, other mice had peritoneal nodules constituting more than 40% of their body weight. No distant metastasis to liver was observed in any mouse investigated.

DISCUSSION

Peritoneal dissemination of gastric cancer has little sensitivity to anticancer drugs, and it is impossible to control its growth completely. In the present study we have demonstrated that marimastat successfully inhibited the growth of peritoneal dissemination nodules in nude mice model. Furthermore, combined administration of marimastat and a conventional anticancer drug, MMC, afforded stronger inhibition of the growth of peritoneal dissemination than marimastat alone or MMC alone. Although marimastat had inhibitory activity on the enzymatic activities of MMP-1, -2, -7, -9 and -14 (MT1-MMP),[11, 14] MTT assay demonstrated that marimastat was not cytotoxic towards gastric cancer cell line, TMK-1. In experiment II, since T/C in the combination group was smaller than T/C in the marimastat group multiplied by T/C in the MMC group ($0.013<0.367\times0.073$), inhibition of nodule formation in the combination group was regarded as synergistic. MMC did not affect gelatinase activity in the present study (data not shown), so that the synergistic effect of MMC and marimastat may regarded as involving a direct cytotoxic effect of MMC and the inhibition of MMP activity by marimastat.

In the present experiments, TMK-1 tumor cells were injected into the peritoneal cavity, where they became attached to the surface of the peritoneum. Beneath the peritoneal lining, extracellular matrix (ECM) constituents, especially collagens, are abundant. MMP-1, -2, -9 and -14 (gelatinase A and B and MT1-MMP) originating from cancer cells and other cells may degrade the ECM after fenestration of mesothelial cells, permitting the cancer cells to enter the subperitoneal layer.[22, 23] Also, basement membrane which mainly consists of type IV collagen can be degraded by these gelatinases.[24] Marimastat inhibits gelatinase A, gelatinase B, MT1-MMP and other MMPs, and thus could prevent the steps of ECM degradation and cancer cell invasion. Since all MMP activities cannot be completely inhibited *in vivo*, some cancer cells formed tiny nodules, representing peritoneal dissemination, in our experiments despite the treatment with MMP inhibitor.

Metastatic nodules require establishment of a blood supply from the host in order to grow beyond minimal size. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are known to be important in such tumor angiogenesis. VEGF expression and MMP activity are closely correlated in lung cancer[25] and breast cancer.[26] In MMP-2 knockout mouse, tumor-induced angiogenesis was reduced and implanted tumor growth was suppressed in B16-BL16 melanoma and Lewis lung carcinoma.[27] MMPs are required in angiogenesis to assure unimpeded migration of vascular endothelial cells.[28–30] These data support an important role of MMP in tumor angiogenesis. Several MMP inhibitors have been demonstrated to have antiangiogenic activity,[31, 32] mainly because the degradation of ECM by endothelial cells is essential to the process of angiogenesis.

Although marimastat alone could not contribute survival benefit with statistical significance, combined administration (marimastat and MMC) contributed survival benefit with statistical significance. This benefit would be clinically quite important. Because the dose of cytotoxic drugs could be reduced without loss of effect, the adverse effect of the cytotoxic drugs would be reduced, and, this might improve patient compliance. Furthermore, it might afford a better quality of life for gastric cancer patients.

We have recently demonstrated that a citrus flavonoid, nobiletin, suppressed the peritoneal dissemination of gastric cancer in the SCID mice model.[33] Not only inhibitory activity towards MMP-9, but also antiproliferative activity on TMK-1 was demonstrated by gelatin zymography and by MTT assay. It was also reported that nobiletin suppressed the production of PGE2 and the expression of COX-2 protein.[34] Combination of marimastat with these drugs, which have different mechanisms, may provide enhanced effects in cancer treatment.

In conclusion, marimastat inhibited peritoneal dissemination of human gastric cancer in nude mice when given by controlled subcutaneous administration, and this MMP inhibition showed a synergistic effect with MMC, which significantly prolonged the survival of mice. Marimastat is believed to be suitable for long-term administration without producing severe adverse effects. A clinical study of marimastat and MMC in combination appears warranted for patients with peritoneal dissemination of gastric cancer.

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