Gabexate Mesilate Inhibits Colon Cancer Growth, Invasion, and Metastasis by Reducing Matrix Metalloproteinases and Angiogenesis

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ABSTRACT

Gabexate mesilate (GM), a synthetic protease inhibitor, has an antiproteinase activity on various types of plasma serine proteases. However, its role on matrix metalloproteinases (MMPs) has not been identified. In this study, we investigated the effect of GM on MMPs and on the invasion and metastasis of human colon cancer cell lines and neoangiogenesis. The activities of MMPs secreted from these cells were significantly reduced by GM but unaffected by the serine protease inhibitor aprotinin. GM directly inhibited purified progelatinase A derived from T98G human gliogenesis. The activities of MMPs secreted from these cells were significantly reduced by GM but unaffected by the serine protease inhibitor aprotinin. GM directly inhibited purified progelatinase A derived from T98G human glioblastoma cells. In vitro, GM significantly reduced the invasive ability of colon cancer cells but not cellular motility, whereas aprotinin affected neither. Liver metastatic ability and tumorigenic potential in nude mice were remarkably reduced on treatment with GM. Immunohistochemical analysis of GM-treated tumors in mice showed a marked increase in apoptosis and a significant reduction in tumor angiogenesis. Human umbilical vein endothelial cell proliferation, tube formation, and neoangiogenesis in the rabbit cornea and Matrigel implanted in mice were significantly inhibited by GM. These results suggest that GM is a novel inhibitor of MMPs and that it may inhibit the invasion and metastasis of human colon cancer cells by blocking MMPs and neoangiogenesis.

INTRODUCTION

Despite advances in multimodality therapies, 5-year survival in colorectal cancer is approximately 55% and has remained essentially unchanged over the last 40 years (1, 2). Because metastatic disease is the major cause of treatment failure, a new therapeutic strategy is essential.

Tumor cell invasion and metastasis are regarded as multistep phenomena involving the proteolytic degradation of the basement membrane (BM) and extracellular matrix (ECM), altered cell adhesion, and physical movement of tumor cells. Among the many steps in invasion and metastasis, excessive degradation of the matrix is one of the hallmarks of this process (3, 4). Many proteinases are capable of degrading ECM components, but two families of enzymes that appear to be particularly important for ECM degradation, namely, the matrix metalloproteinases [MMPs (MMP-2 and MMP-9)] and urokinase-type plasminogen activator [uPA (3, 5–10)]. These proteinases have been closely linked with the invasive and metastatic phenotype of cancer cells (7, 9). Inhibitors of MMPs and uPA can block these enzymes and subsequently inhibit invasion and metastasis (11–19). The MMPs and uPA-plasmin system are also implicated in tumor angiogenesis, and their inhibitors, both synthetic and endogenous, inhibit angiogenic responses in vitro and in vivo (17–23).

Of the fibrinolytic components, thrombin has been identified as an activator of pro-MMP-2 in human umbilical vein endothelial cells (HUVECs) via a membrane-type 1-MMP-independent pathway (24). Moreover, plasmin converts pro-MMP-3 to active MMP-3, which becomes a potent activator of MMP-9 (25, 26). Therefore, activation pathways of some MMPs are closely related to serine proteases. These findings suggest that a multifunctional antiprotease strategy, if available, could provide a useful antimetastatic and antiangiogenic therapy.

Gabexate mesilate (GM) is the generic name given to [ethyl p-(6-guanidinoethyl)benzoate] methanesulfonate, a synthetic serine protease inhibitor that inhibits various kinds of plasma proteinases, such as thrombin, plasmin, kallikrein, trypsin, C1 esterase in the complement system, and factor Xa in the coagulation cascade (27, 28). GM has been used for disseminated intravascular coagulation and acute pancreatitis in the clinical field in Japan and Korea; however, its effects on MMP-2 and MMP-9 have not been identified. The present study demonstrates that GM is a novel inhibitor of MMPs; thus, it could inhibit the invasion and metastasis of colon cancer cells. In addition, we show that the antitumorigenic effect of GM is related in part to the antiangiogenic effect of GM.
MATERIALS AND METHODS

Cell Lines and Culture Conditions. Human colon cancer cell lines SW480 and LoVo were obtained from American Type Culture Collection (Manassas, VA), and HM7 cells were kindly donated by Prof. Young S. Kim (Gastrointestinal Research Laboratory, University of California, San Francisco, CA). These colon cancer cells were expanded as a monolayer culture by serial passage in DMEM (GIBCO BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO BRL), penicillin (100 units/ml), and streptomycin (100 μg/ml) in a 5% CO2 environment.

HUVECs were isolated from fresh umbilical cords obtained by caesarean section using a modification of the technique of Jaffe et al. (29). Briefly, the umbilical vein was cannulated, and 0.1% type IV collagenase (Sigma Chemical Co., St. Louis, MO) in PBS was introduced and incubated for 20 min. The endothelial cells liberated by the collagenase were obtained by rinsing the umbilical vein with medium 199 (GIBCO BRL). The cells obtained were washed with medium 199 three times and cultured in 75-cm² tissue culture flasks (Nunc A/S, Roskilde, Denmark) coated with 0.2% gelatin (Sigma Chemical Co.). The growth medium consisted of medium 199 supplemented with 20% fetal bovine serum, 50 μg/ml endothelial cell growth factor (ECGF; Sigma Chemical Co.), 50 μg/ml heparin (Sigma Chemical Co.), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml amphotericin B (Sigma Chemical Co.). HUVECs were used at passages 2–4.

Colon Cancer Cell Proliferation Assay. The effect of GM on the growth of colon cancer cells was evaluated using 10⁵ cells seeded on 24-well plates (Nunc A/S), which were treated with GM 12 h after cell plating. To evaluate the toxicity of GM at concentrations of 0.01, 0.1, and 1 mM, culture media were maintained for up to 72 h, and the number of cells was counted using a hemocytometer after a brief trypsinization.

In Vitro Invasion and Motility Assay. Transwell cell culture chambers containing 6.5-mm-diameter polycarbonate filters with 8-μm pores (Costar, Cambridge, MA) were used for the assay using a previously described method (30), with some modification. For the invasion assay, filters coated with BM Matrigel (160 μg/filter) were used. To investigate the effect of GM on invasion, 0.01 or 0.1 mM GM was added to the culture medium. After 72 h of incubation, cells on the top of the filter, which were generated by noninvasive cells, were removed using cotton swabs. The filters were then removed, and the invasive cells beneath the filters were stained with hematoxylin and counted under a microscope. For the motility assay, the same system was used, but without the Matrigel.

Protease Analysis by Substrate-Embedded Gel. For the zymographic assay (14), 80% confluent cells were washed three times with calcium-magnesium-free PBS and cultured in DMEM without fetal bovine serum. Conditioned media (CM) were obtained after 24 h of culture and centrifuged at 3000 × g for 10 min to remove cells and debris. Cell-free CM was concentrated about 10-fold using a Centricon-10 device (Amicon, Beverly, MA), and aliquots of the concentrated CM were normalized for cell number. Proteins in the normalized CM were then separated by electrophoresis in 10% polyacrylamide gel impregnated with 1 mg/ml gelatin (Fisher Chemical Co., Fair Lawn, NJ) or 1 mg/ml casein (Sigma Chemical Co.) containing 13 μg/ml plasminogen (Sigma Chemical Co.) under nonreducing conditions. After electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min, proteolyzed with reaction buffer [50 mM Tris-HCl, 5 mM CaCl2, and 0.02% NaN3 (pH 8.0)] for 72 h at 37°C, and stained with Coomassie Blue G-250. To further characterize the proteases, 1,10-phenanthroline (5 mM; Sigma Chemical Co.) as a metalloproteinase inhibitor or aprotinin (5 μg/ml; Sigma Chemical Co.) as a serine protease inhibitor was added to the incubation buffer. To investigate the role of GM on MMPs, various concentrations of GM (Dong-A Pharmaceutical Co., Seoul, Korea) or aprotinin were used. The purified tissue inhibitor of metalloproteinase (TIMP)-2-free gelatinase A, which originated from T98G human glioblastoma cells, was kindly donated by Dr. Yung-Hee Kho (Korea Research Institute of Bioscience and Biotechnology), and single chain uPA was purchased from American Diagnostica Inc. (Greenwich, CT). Both enzymes were used to further confirm the inhibitory activities of GM on MMP-2 and uPA.

Nude Mice Tumorigenicity. Confluent colon cancer cell cultures were harvested by a brief trypsinization (0.05% trypsin-0.02% EDTA in HBSS without calcium or magnesium), washed three times with calcium and magnesium-free PBS, and resuspended at a final concentration of 10⁸ cells/ml in serum-free DMEM. Single-cell suspensions were confirmed by phase-contrast microscopy, and cell viability was determined by trypsin blue exclusion; only single-cell suspensions with a viability of >90% were used. Pathogen-free female BALB/cAnNCrj-nu athymic nude mice (4 weeks old; Charles River Laboratories, Kanazawa, Japan) were anesthetized with diethyl ether by inhalation, and 10⁷ HM7 colon cancer cells in 100 μl of serum-free DMEM were inoculated s.c. into the right flank. From the day of tumor cell inoculation, mice received an i.p. injection of GM (100 μg/100 μl saline/mouse, twice a day) or the same amount of physiological saline for 14 days after tumor cell implantation. Mice were surveyed regularly, tumors were measured with a caliper, and tumor volumes were determined using the following formula: volume = 0.5 × (width² × length). Each experimental group consisted of eight animals, and P < 0.05 was considered statistically significant (31).

All experiments were performed in accordance with institutional ethical procedures for animal experimentation.

Immunohistochemistry. After the mice had been sacrificed, tumors were removed and bisected. One part of the tumor was placed in neutral buffered formalin for paraffin block preparation, and the other part was frozen for cryocut sections. The degree of apoptosis was evaluated using an ApopTag apoptosis detection kit (S7101; Intergen, Norcross, GA) according to the manufacturer’s recommendations. The apoptotic index was calculated as the percentage of nuclei stained by peroxidase and showing nuclear halo or apoptotic bodies. Positive cells were counted among a minimum of 400 cells/histological section at a magnification of ×400. To evaluate the proliferation index, paraffin sections were incubated with a monoclonal mouse Ki-67 antibody (MIB-1; Dako, Carpinteria, CA) at a dilution of 1:100. Staining was carried out with a universal labeled streptavidin-biotin kit (Dako), using the standard protocol. The proliferation index was determined by counting stained cells at ×400. To immunolocalize tumor blood vessels, cryosections were
stained with a monoclonal rat antimouse CD31 antibody (PECAM-1; BD PharMingen, San Diego, CA) at a dilution of 1:50. Visualization of the antigen-antibody reaction was carried out using an antirat immunoglobulin horseradish peroxidase detection kit (BD PharMingen), according to the manufacturer’s recommendations. Vessel density was determined by counting the stained vessels at ×200.

In Vivo Liver Colonization Assay. The inhibitory effect of GM on the ability of colon cancer cells to colonize the liver after entry into the hepatic-portal system was tested in a splenic injection model (30). Tumor cells were resuspended at a final concentration of 10^7 cells/ml in serum-free DMEM. Athymic nude mice were then anesthetized with diethyl ether by inhalation, and the spleen was exteriorized through a flank incision. One million cells in 100 μl were then slowly injected into the lower polar side of the splenic pulp through a 27-gauge needle, and this was followed by splenectomy 1 min later. GM (100 μg/mouse) was injected i.p. twice a day for 12 days from the day of operation. Animals were sacrificed 4 weeks later, livers were removed and weighed, and tumor metastatic nodules in the liver were counted (30). All experiments were performed in 6 animals/group. P < 0.05 was considered statistically significant.

HUVEC Proliferation Assay. Growth assays of HUVECs were carried out according to the procedure described by Bae et al. (31), with some modifications. Briefly, HUVECs were seeded in the 0.2% gelatin-coated wells of a 96-well culture plate (Costar) at an initial density of 5 × 10^3 cells/well in 200 μl of medium 199 and then grown under standard conditions at 37°C in 5% CO_2. On the following day, 0.01, 0.1, and 1 mM GM were added to each well for 3 days, and then cell numbers were quantitated by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-based colorimetric assay (31); absorbance was read at 540 nm.

Capillary Tube Formation on Matrigel. To further assess the antiangiogenic effect of GM, vascular tube formation experiments were performed. HUVECs were seeded at a density of 10^4 cells/well in Matrigel-coated 24-well plates and incubated at final GM concentrations of 0.01, 0.1, and 0.5 mM. During these incubations, HUVEC morphological changes were monitored using an inverted phase-contrast microscope (model IX 70; Olympus, Tokyo, Japan) and photographed.

In Vivo Matrigel Angiogenesis Assay. Liquid Matrigel maintained at 4°C was used as a vehicle to inject angiogenic factors s.c. into mice. C57BL/6 mice (Charles River Laboratories) each received s.c. injection with a Matrigel mixture containing 600 μl of Matrigel, 200 μl of serum-free DMEM, 50 ng of basic fibroblast growth factor (Boehringer Mannheim GmbH, Mannheim, Germany), and 40 units of heparin (Sigma Chemical Co.) with or without 33.4 μg of GM near the abdominal midline using a 25-gauge needle. The injected Matrigel rapidly formed a single, solid gel that persisted for 10 days (32). The mice were subsequently killed at 6 days. The overlying skin was removed, the gels were exposed for photography, and, subsequently, the gels were excised for hemoglobin measurement. Hemoglobin was measured using a Drabkin’s reagent kit (Sigma Chemical Co.), as instructed by the manufacturer (33). The concentration of hemoglobin was calculated from a standard amount of hemoglobin, which was assayed in parallel.

Rabbit Cornea Neovascularization Assay. Male New Zealand White rabbits (Charles River Laboratories) weighing 3 kg were used for the corneal neovascularization experiment. Ketamine anesthesia (44 mg/kg, i.m.) was supplemented by retrobulbar infiltration with 2% lidocaine. The eye was propoised with fingers, and a small superficial incision was made at the apex of the cornea using a #11 blade. The incision was then continued down into, but not through, the cornea. A #15 blade was inserted, and an oblong pocket was fashioned within the corneal stroma. The pocket was enlarged toward the limbus, placing its base at 3 mm from the corneal-limbal junction. Recombinant human vascular endothelial growth factor 165 (10 ng; R&D Systems, Minneapolis, MN), with or without 33.4 μg of GM, was added on a sterile 3.0-mm-diameter Thermoxan disk (Nunc, Naperville, IL) and dried overnight. A disk was deposited in the bottom of each pocket, which then sealed spontaneously (31). All procedures were performed under sterile conditions. Sixteen days later, when neovascularization was prominent, the corneal vessels were photographed with a photo slit lamp (FS-2; Nikon, Tokyo, Japan). Five animals were used in each experimental group.

Statistical Analysis. Statistical analyses were performed as recommended by an independent analyst. These included unpaired Student’s t test (cell proliferation, in vitro invasion and motility, liver weight, size of s.c. tumors, immunohistochemistry, and hemoglobin contents) and the Mann-Whitney test (number of tumor nodules after splenic-portal injection). For the purpose of the latter analysis, if a liver contained >500 nodules (too numerous to count), an arbitrary value of 500 nodules was used. All values were expressed as means ± SD. Statistical significance was assigned when P was <0.05.

RESULTS

Effect of GM on Colon Cancer Cell Proliferation. To evaluate the inhibitory effect of GM on cell proliferation, human
colon cancer cells were cultured with various concentrations of GM for up to 3 days, and cell numbers were counted daily. At concentrations below 0.1 mM, cell numbers were similar to the control, whereas at a concentration of 1 mM, the number of cells was markedly reduced after 3 days of culture (Fig. 1A). We also tested the effect of aprotinin at concentrations of 0.5, 1 and 2 μM, and we found that cell numbers were unaffected (Fig. 1B).

**Reduced Activities of MMP-2, MMP-9, and uPA by GM.** SW480, LoVo, and HM7 colon cancer cells secreted MMPs, which include 92-, 72-, and 54-kDa gelatinolytic enzymes by zymography (Fig. 2A, control lanes). Of these colon cancer cells, the CM of HM7 cells showed additional gelatinolytic activity at 160, 110, and 30 kDa. These activities were not inhibited by the MMP inhibitor 1,10-phenanthroline, but they were completely inhibited by the serine protease inhibitor aprotinin (data not shown). Therefore, these enzymes might be serine proteases. We also tested the activity of plasminogen activator of these cells. According to casein and plasminogen impregnated zymography, the CM of SW480 and HM7 colon cancer cells showed single chain uPA activity, and the CM of LoVo cells showed double chain uPA activity. These caseinolytic bands, including the 55-kDa single chain uPA, were completely inhibited with the uPA inhibitor amiloride (1 mM; data not shown).

To evaluate the inhibitory effect of GM on MMPs derived from human colon cancer cells, the CM of SW480, LoVo, and HM7 cells were evaluated zymographically. With concentrations of GM of 0.1 and 1 mM in the reaction buffer, the MMP-2 and MMP-9 activities of these cells were significantly reduced (Fig. 2A). Moreover, serine proteases from HM7 cells, shown in gelatin gel, were also inhibited by GM. Because there was a possibility that the CM of these cells contained contaminating serum components and remaining plasminogen activator-activated MMPs and, subsequently, that GM inhibited uPA activity,
we evaluated whether the down-regulation of MMP activity by GM was due to the inhibition of plasminogen activator. To evaluate this possibility, adequate inhibitory concentrations of aprotinin (i.e., 5 μg/ml; 0.77 μM) in combination with GM (1 mM) were added to reaction buffer to compare the inhibitory effect of GM alone. We reasoned that if plasminogen activator down-regulated MMP activity, then reaction buffer containing aprotinin and GM should inhibit MMPs even more than reaction buffer containing GM alone. In this experiment, no difference in the activity of either MMP-2 or MMP-9 was found, regardless of the addition of aprotinin to GM (Fig. 2B).

For further confirmation of the inhibitory effects of GM on MMPs and the uPA system, purified TIMP-2-free progelatinesis A and purified single chain uPA were used in zymographic experiments. As shown in Fig. 3A (left panel), treatment of the gel with GM for 3 h significantly inhibited the activity of TIMP-2-free progelatinesis A in a dose-dependent manner (0.1–2 mM). To evaluate the pattern of inhibition of MMPs by GM, we performed Lineweaver-Burk plot analysis using purified MMP-2 (Boehringer Mannheim GmbH) and human type IV collagen (N-[propionate-2,3-3H]-propionylated; New England Nuclear, Boston, MA). The $K_m$ value for type IV collagen was 0.32 μg/ml, and the $K_i$ value for GM was 80 μM. The $V_{max}$ was constant, and the $V_{max}$ was reduced on increasing the GM concentration (Fig. 3B). Therefore, the inhibitory pattern of GM on MMP-2 might be noncompetitive. Moreover, the MMP-2 gene was unaffected in HM7 cells by reverse transcription-PCR analysis after treatment with GM (0.01–0.1 mM; 24–72 h treatment; data not shown). These results indicate that GM has a novel direct inhibitory activity on MMP-2 and MMP-9, although the inhibitory mechanism is unknown. In addition, GM, markedly reduced purified uPA activity in a casein and plasminogen-impregnated gel system (Fig. 3A, right panel). These findings suggest that the inhibitory effect of MMPs and uPA by GM may provide a broad spectrum antimetastatic and antiangiogenic strategy in cancer.

**Antinvasive Activity of GM on Colon Cancer Cells in Vitro.** To determine whether GM affects the invasive ability of human colon cancer cells in vitro, we performed invasion and motility assays using 0.01 and 0.1 mM GM. At a concentration of 0.1 mM, SW480, LoVo, and HM7 cells showed marked invasion inhibition (79.3%, 91.1%, and 83.6%, respectively; $P < 0.05$), and the inhibitory ratio of invasion was concentration dependent in these cells (0.01–0.1 mM GM), whereas their motile activities were unaffected at these GM concentrations (Fig. 4A). To elucidate the effects of MMPs on colon cancer cell invasion versus serine proteases, we performed invasion and motility assays with aprotinin at concentrations of 1 and 2 μM. However, these were found to be similar to the control (Fig. 4B).

**Effect of GM on Human Colon Cancer Cell Growth in Vitro.** Tumorigenesis is a complex process and requires the coordinated regulation of multiple events, including tumor vascularization and matrix degradation (5, 6). At some point in tumor progression, a critical stage is reached where further growth is prevented unless angiogenesis occurs. There is experimental evidence that MMPs are involved in the early stages of tumor growth and development (34, 35) and that neovascularization is crucial for sustained tumor growth because it facilitates the oxygenation and nutrition of the tumor as well as the removal of waste products. We investigated the effect of GM on the growth of HM7 human colon cancer cells that were transplanted into nude mice. Daily treatment with GM (100 μg/mouse, twice a day) for 2 weeks resulted in a significant reduction in HM7 tumor volume; volume was reduced by as much as 41.3% and 63.6% compared with saline-treated control mice, 1 and 2 weeks postinoculation, respectively ($P < 0.05$; Fig. 5, top panel).

**Immunohistochemical Analysis of Proliferation, Apoptosis, and Angiogenesis in HM7 Tumors.** Histological sections of the HM7 tumors grown in nude mice for 2 weeks were
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of invading or motile control cells were normalized to 100%. A, and the number of stained cells was counted under a microscope. For the motility assay, the same system was used without Matrigel. The percentages were removed with cotton swabs. Cells beneath the filter, which were generated by invasive or motile cells, were stained with Gill’s hematoxylin, and the number of stained cells was counted under a microscope. For the motility assay, the same system was used without Matrigel. The percentages of invading or motile control cells were normalized to 100%. A, effect of GM (−0.1 mM) on in vitro invasion and motility. B, effect of aprotinin (−2 μM) on in vitro invasion and motility. The data shown represent the means ± SD of three replicates from three independent experiments (*, *P < 0.05 versus control; **, *P < 0.01 versus control).

Effect of GM on Liver Colonization of Human Colon Cancer Cells. The ability of GM to inhibit the development of liver metastasis by HM7 colon cancer cells injected into the spleen of nude mice was examined. Animals were treated with repeated i.p. injections of GM (100 μg/mouse, twice a day) over a 12-day period, as described above. Under these experimental conditions, massive hepatic tumor burden, ascites, and severe cachexia were evident 4 weeks after the inoculation of HM7 cells into the portal system in 100% of the saline-treated control mice. Cancer cells into the portal system in 100% of the saline-treated control mice. Moreover, the mean liver weight of the control mice was 4.7 g, whereas the GM-treated mice appeared normal (Fig. 6A). The number of HUVECs cultured on gelatin was examined under a low-power light microscope, they had a “honeycomb” appearance (Fig. 7B; control). However, this interconnected network formation, which resembled a vessel-like structure, progressively disappeared with increasing GM concentrations (Fig. 7B; 0.01–0.5 mM GM).

Inhibition of Matrigel Angiogenesis in Vivo. In this study, we examined whether GM is antiangiogenic in a mouse model. Each mouse was given a s.c. injection of a Matrigel

Effect of GM on HUVEC Growth and Capillary Tube Formation. Angiogenesis is both complex and dynamic and requires proliferation of endothelial cells from preexisting blood vessels, breakdown of the ECM, and migration of endothelial cells (5, 34). Thus, the growth and development of blood vessels within tumors require the same factors that are crucial to tumor invasion, and the MMPs play a central role in all of these processes (34). To elucidate the antiangiogenic effect of GM, HUVEC proliferation and tube formation were investigated.

The number of HUVECs cultured on gelatin was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to determine the cytotoxic effect of GM at concentrations of 0.01, 0.1, 0.5, and 1 mM. In this experiment, GM was found to have a significant dose-dependent cytotoxic effect on HUVECs. The percentage of growth inhibition was 20.3%, 57.2%, 91.2%, and 91.2% at 0.01, 0.1, 0.5, and 1 mM GM, respectively (Fig. 7A).

We also compared the morphological features of HUVECs on Matrigel-coated plates after treatment with various concentrations of GM. Eighteen h posttreatment, the control HUVECs had formed an interconnected network of anastomosing cells; under a low-power light microscope, they had a “honeycomb” appearance (Fig. 7B; control). However, this interconnected network formation, which resembled a vessel-like structure, progressively disappeared with increasing GM concentrations (Fig. 7B; 0.01–0.5 mM GM).

analyzed for proliferation, apoptosis, and neovascularization. Immunohistochemical analysis of cell proliferation (Ki-67) showed no difference between the control and GM-treated HM7 tumors (69.4 ± 6.7% and 69.9 ± 5.5%, respectively; *P = 0.7737; Fig. 5A). However, the apoptotic index was 3.4-fold higher in GM-treated tumors (1.4 ± 0.3% versus 4.7 ± 0.6%; **P < 0.0001; Fig. 5B). Furthermore, the neoangiogenesis in tumors, as determined by the number of CD31-stained microvessels, was significantly lower (29.4% of control tumors) in GM-treated tumors (46.6 ± 10.3 versus 13.7 ± 3.9 vessels/high-powered field; **P < 0.0001; Fig. 5C). Thus, the suppression of tumor neoangiogenesis and the increase in apoptosis induced by GM may have important roles in the growth retardation of HM7 tumors.

In this study, we examined whether GM is antiangiogenic in a mouse model. Each mouse was given a s.c. injection of a Matrigel
Inhibition of Rabbit Cornea Neovascularization. To corroborate the results obtained by the Matrigel plug assay, the effect of GM on rabbit corneal angiogenesis in vivo was examined by determining the ability of GM to block vascular endothelial growth factor-induced angiogenesis. A vascular endothelial growth factor-containing Thermox disk induced angiogenesis in 100% of the implanted rabbit corneas. In contrast, GM (33.4 μg) completely inhibited this vascular endothelial growth factor-induced angiogenic response in five of five animals (Fig. 7D). These results indicate that GM potently inhibits angiogenesis both in vitro and in vivo.

DISCUSSION

To successfully complete the complex invasion and metastatic process, tumor cells must attach and traverse BM barriers and penetrate vascular structures of both primary and distant organs. In this process, the invasion of BMs by tumor cells is thought to be one of the most critical steps. This requires changes in proteolysis, an activity representing a balance between the local concentrations of activated enzymes and their endogenous inhibitors (3, 5, 34). Angiogenesis, the formation of new blood vessels, is essentially a requirement for tumor growth and successful tumor invasion and metastasis. Angiogenesis is a complex and dynamic process and requires the proliferation of endothelial cells from preexisting blood vessels, the breakdown of the ECM, and the migration of endothelial cells (5, 34). Thus, the growth and development of blood vessels within tumors require factors crucial for tumor invasion, and the MMPs play a central role in all of these processes (34). Important enzymes that have been shown to be closely associated with invasive and metastatic potential are MMP-2 (gelatinase A), MMP-9 (gelatinase B), and uPA (3, 5–10, 36). These enzymes are also believed to participate in angiogenesis (20–23, 36).

Recent experimental evidence shows that recombinant TIMP is a potent inhibitor of a MMP present in a highly metastatic rat embryo cell line (4R) transfected with c-Ha-ras 1 and that recombinant TIMP can inhibit the metastatic potential of this cell line to the lungs by as much as 83% in vivo (14). Moreover, synthetic MMP inhibitors have been reported to inhibit the liver and lung metastasis of human colon cancer cells (11) and the primary tumor growth of HT1080 fibrosarcoma cells, which overproduce MMPs (12). On the other hand, the MMP inhibitor BE16627B did not inhibit the growth of HCT116 human colon cancer cells, which barely secrete MMPs (12), express uPA, and display uPA receptors (16). Because many types of tumor cells secrete or use various proteolytic enzymes to degrade BMs, a multispecific protease inhibitor may be more useful for an anti-invasive and antimetastatic strategy.

GM is a synthetic serine protease inhibitor that inhibits various kinds of plasma proteinases. However, its effect on MMPs has not been previously identified. Thus, we report a novel bifunctional inhibitory effect of GM on the MMPs and uPA system. In addition, we describe the inhibitory effect of GM on the invasiveness and metastasis of human colon cancer cells and neoangiogenesis.

In this study, we found that GM reduces colon cancer cell-derived MMP-2 and MMP-9. Because the MMPs, like the gelatinases A and B, can be activated by uPA, such inhibi-
tory action of GM on MMPs may be due to the inhibition of serine protease activity by GM. To elucidate the role of serine protease inhibition, the effect of the serine protease inhibitor aprotinin was compared with GM with respect to MMP inhibition. Aprotinin was found not to inhibit the activities of MMPs in colon cancer cells. However, GM attenuated the activities of MMPs in a dose-dependent manner. Furthermore, GM inhibited purified TIMP-2-free progelatinase A activity by gelatin zymography.

The inhibitory pattern of GM on MMP-2 was noncompetitive, based on $K_m$ and $V_{max}$ values obtained from Lineweaver-Burk plot analysis. According to reverse transcription-PCR analysis, GM did not affect MMP-2 gene regulation in HM7 cells. These results indicate that GM has a novel inhibitory activity on both MMP-2 and MMP-9, although the inhibitory mechanism is unknown.

To assess the inhibitory effect of GM on human colon cancer cell invasion, we performed an in vitro invasion assay. This experiment showed that GM significantly inhibited the invasion of SW480 cells (by 79.3%), LoVo cells (by 91.1%), and HM7 cells (by 83.6%) at 0.1 mM, a concentration that did not affect either the proliferation or motility of these cells. Therefore, we conclude that the observed reduction in the invasiveness of colon cancer cells was due to inhibition of MMPs by GM.

We further investigated the ability of GM to inhibit metastasis to the liver and primary tumor growth of HM7 cells. GM was found to markedly reduce liver colonization as much as 99.2% at a dose of 200 µg/mouse/day after a 12-day treatment. Moreover, this dosage was very low compared with the doses of other MMP inhibitors required to obtain a similar response, i.e., batimastat or BE16627B, which required 0.8 and 2 mg/mouse, respectively (11, 12). Surprisingly, GM reduced tumor volume by 63.6% at the end of a 2-week treatment, and the antitumor effect seemed to be slowly diminished after treatment discontinuance. Several studies have been undertaken to determine the effects of MMP or uPA inhibitors on solid tumor growth reduction in vivo (12, 37–40). Possible explanations for the observed antitumor effects of MMP inhibitors include the following: (a) inhibition of MMPs is essential for the degradation of connective tissue proteins specifically localized in the interface between tumor cells and normal tissue for the expansion of tumor mass (41, 42); (b) inhibition of MMPs is required for the angiogenesis required to support tumor growth (30, 43, 44); or (c) the antitumor effect might be due to a direct cytotoxic effect. It is not possible to directly compare the concentrations of GM used in in vivo and in vitro experiments. However, a direct cytolytic effect of GM on tumor growth inhibition seems to be unlikely because in our exper-

**Fig. 6** Gabexate mesilate (GM) inhibits metastasis to the liver of HM7 human colon cancer cells in nude mice. HM7 cells (10⁶ cells/100 µl) were injected into the spleen, and this was followed by splenectomy 1 min later. GM (100 µg/100 µl) was administered i.p. twice a day for 12 days from the day of splenic injection. Animals were sacrificed 4 weeks later, livers were removed and weighed, and tumor nodules in livers were counted. All experiments were performed with 6 animals/group. A, massive liver metastasis was evident 4 weeks after inoculation of HM7 cells into the portal system in the 100% saline control group, whereas the GM-treated group showed a normally shaped liver. B, quantitation of liver weight: control versus GM treatment, $5.1 \pm 2.2$ versus $1.0 \pm 1.0$ g ($\ast$, $P < 0.05$). C, quantitation of the number of liver nodules: control versus GM treatment, 425.0 ± 129.9 versus 3.5 ± 2.9 ($\ast\ast$, $P = 0.001$).
iment, we used a low dose of GM. Nevertheless, GM showed a direct toxic effect on HUVEC growth and on the formation of an interconnected vascular network, that is, on so-called tube formation, which progressively disappeared with increasing GM concentration. In addition, GM significantly reduced neoangiogenesis in Matrigel plug and rabbit cornea assay models in vivo. Furthermore, the findings of a marked increase of apoptosis and a profound suppression of angiogenesis in GM-treated HM7 tumors suggest that the antitumor effect of GM is due, at least in part, to an antiangiogenic effect.

The expression of MMP-2 has been previously correlated with tumor progression (45) and with reduced angiogenesis and tumor progression in MMP-2-deficient mice (35). Although the mechanism of the antitumor effect of GM remains to be solved, it could be an additional benefit of blocking serine proteases in addition to MMP inhibition, especially if one considers that many fibrinolytic components such as thrombin, plasmin, and kallikrein are linked to MMP-2 or MMP-9 activation (24–26, 46). Moreover, a number of points of intersection have been identified between the coagulation and fibrinolytic pathways and the MMP activation cascade (47).

Taken together, our results indicate that the serine protease inhibitor GM inhibits both MMPs and the uPA system and has anti-invasive, antimitastic, and antiangiogenic properties. Clinically, GM has been used for over 20 years as a drug treatment for disseminated intravascular coagulation and pancreatitis in the clinical field in Korea and Japan without report of major toxicity. Therefore, we believe it could be a useful therapeutic modality for antimetastatic and antiangiogenic treatment of colon cancers. Additional clinical studies to examine these effects are warranted.

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