involved in S1P production in our study, hepatocytes represented one source of S1P, as the S1P elevation by AdASM was inhibited in both SphK1-deficient livers and SphK1-deficient primary cultured rat hepatocytes (data not shown). Previous studies have shown that the generated S1P can be secreted and act as a ligand for S1P receptors (S1PRs; refs. 27, 36, 37), while the S1P/S1PR1 axis controls the trafficking and migration of immune cells, including macrophages (16, 17, 36). In accordance with these prior reports, S1P increased the migration of peritoneal macrophages in vitro, with less and more macrophage accumulation, respectively, in the tumors of $Asm^{-/-}$ and AdASM-infected mice compared with controls. These findings indicate that ASM in hepatocytes leads to macrophage accumulation in tumors via S1P production and that this process exerts an antitumor effect, in contrast to macrophage depletion. Although it has been reported that TAMs, which resemble regulatory M2 macrophages (13, 14), function as promoters of tumor progression (7–9), the macrophages in the tumors inoculated with SL4 colon cancer cells in this study were M1 dominant. It has been proposed that a gradual shift of TAM polarization from the M1 to the M2 subtype occurs during different stages of tumor progression, due to dynamic changes in the tumor microenvironment (38), and is paralleled by the gradual inhibition of NF-κB (39). In this study, we observed immunofluorescent staining of NF-κB p65 in the nuclei of F4/80$^+$ cells in the tumors (data not shown), which suggests that NF-κB is activated in these macrophages. This finding may indicate that in metastatic liver tumors characterized by rapid growth, such as by increased numbers of SL4 cells (40), the accumulated TAMs may retain an M1 phenotype that displays a cytotoxic proinflammatory phenotype. Thus, the number of macrophages present in the tumor is inversely correlated with tumor growth.

Myofibroblasts represent another important component of the tumor stroma; these cells are driven by migration and proliferation-stimulating factors released from cancer cells. Whereas some studies have reported a supportive role for myofibroblasts in cancer cell development (41) and a direct correlation between their presence and poor prognosis (42), others have reported a protective role of myofibroblasts against cancer cells (43, 44), with p53 in stromal fibroblasts demonstrated to inhibit tumor growth and malignant progression (45). In the current study using SL4 cell inoculation, hMF accumulation was found to be dependent on macrophage accumulation and inversely correlated with tumor growth. Specifically, hMF accumulation was increased only around the invasive margins of the tumors, and no hMF infiltration or collagen deposition was observed in the central part of tumors (data not shown). These findings indicate that the supportive effects of hMFs in tumor growth may be limited. However, the observation of enhanced tumor growth in $Timp1^{-/-}$ mice indicates that TIMP1 produced from hMFs, stimulated by S1P, might inhibit tumor growth through inhibition of ECM degradation. Although previous studies reported a protumor role for TIMP1, TIMP1 staining was observed only around the tumor invasive margin in the current study, which indicates that the supportive effects of TIMP1 in tumor growth may also be limited.

Whereas ceramide acts as a signaling molecule in the induction of cell responses, such as apoptosis and growth arrest (3, 46, 47), S1P acts as a potent mitogen for a variety of cell types (48), with SphK1/S1P contributing to colon carcinogenesis (49). Ceramide levels in colon carcinoma tissue are significantly lower than those in normal tissue (50), reflecting the fact that the dynamic balance between intracellular levels of ceramide and S1P (the "ceramide/S1P rheostat") determines cell growth and cell death in colon cancer. In the present study, adenoviral overexpression of SphK1 in the liver inhibited tumor growth when colon cancer cells were inoculated after infection. In contrast to AdASM, AdSphK did not exert an inhibitory effect when administered after cell inoculation, which suggests that its inhibitory effect on existing tumors is less than that of AdASM. The observation that AdSphK increased the proliferation of cultured SL4 cells suggests that it decreased the ceramide/S1P ratio in the cancer cells, which may promote their proliferation. In contrast, AdASM might increase the ceramide/S1P ratio, which may inhibit SL4 cell proliferation. We therefore conclude that AdASM, but not AdSphK, may be a useful candidate for gene therapy against metastatic liver tumors of colon cancer.

In $Asm^{-/-}$ mice, B16C2M melanoma cells formed small metastatic lesions in the liver (4 of 9, 44.4%) whereas none of the $Asm^{+/+}$ mice developed metastases (0 of 11, 0%; Supplemental Figure 7), which suggests that the antitumor effect of ASM is not specific for colon cancer cells. However, use of the *Asm* knockout may have secondary effects due to the severe lipid storage defect in these mice, particularly in macrophages. $Asm^{-/-}$ mice are known to have severe immune defects (51, 52) and display severe neurocutaneous disease by 8 weeks of age. Thus, $Asm^{-/-}$ mice may have various abnormalities in the liver as a normal pathology. In our study, the livers of $Asm^{-/-}$ mice without injection of SL4 cells did not show the nodular appearance. In addition, liver weight of $Asm^{-/-}$ mice without injection of SL4 cells was comparable to that of $Asm^{+/+}$ mice. Thus, it seems unlikely that the changes are due to normal $Asm^{-/-}$ mouse pathology. In addition, besides ASM, ceramide synthesized by the de novo pathway can be involved in TIMP1 regulation. These data do not provide direct evidence that S1P production in hepatocytes recruits macrophages, and experiments using mice with conditional knockout of SphK are also required to confirm the involvement of SphK and S1P in macrophage recruitment and TIMP1 regulation. Moreover, the mechanism by which tumor cells stimulate ASM expression in hepatocytes remains unclear, and there is no direct evidence of a positive correlation between ASM activity in hepatocytes and TIMP1 expression in hMFs; further studies are needed to resolve these uncertainties. In conclusion, we found here that ASM in hepatocytes inhibited tumor growth via S1P formation and subsequent cytotoxic macrophage accumulation. This S1P formation in turn stimulated TIMP1 production by hMFs, leading to tumor suppression. Thus, targeting ASM may represent a new therapeutic strategy for treating liver metastasis of colon cancer.

Methods

Animals. $Asm^{-/-}$ mice (C57BL/6 background; ref. 47) were bred for use in this study. GFP^+ mice (C57BL/6 background), which express EGFP ubiquitously under the CAG promoter (chicken β-actin promoter, rabbit β-globin poly A, CMV-IE enhancer), were obtained from Riken Bio Resource Center. $Timp1^{-/-}$ mice (C57BL/6 background) were obtained from Jackson Laboratory. Male Wistar rats and female C57BL/6J wild-type mice were obtained from Japan SLC. $GFP^+Asm^{-/-}$ mice were generated for this study.

Cell culture. A red fluorescent protein–expressing mouse colon adenocarcinoma cell line (SL4; Anti-Cancer Japan, Osaka, Japan) was maintained as a monolayer culture in RPMI-1640 (Invitrogen) containing 10% FBS supplemented with penicillin and streptomycin (Invitrogen). Cells were harvested using trypsin and EDTA, washed with PBS, and then resuspended in serum-free RPMI-1640 (2×10^5 or 5×10^6 cells/ml).

Liver metastasis model. Female wild-type (C57BL/6J) mice aged 8–10 weeks were divided into 2 groups. After a small incision was made under anesthesia to expose the spleen, 0.1 ml of a viable cell suspension containing 5×10^5 cells/mouse was injected into the spleen of group 1, and the same suspension containing 2×10^4 cells/mouse was injected into group 2. Mice were sacrificed on day 7 (group 1) and day 14 (group 2) after cell inoculation. The livers of group 1 were immediately removed, washed in ice-cold PBS, and weighed, after which a part of the dissected liver tissue was frozen in liquid nitrogen. For assessment of liver metastatic tumors in group 2, the intrahepatic tumor was scored as the hepatic replacement area and maximum diameter of tumor, based on examination of 3 nonsequential H&E-stained sections using ImageJ software (NIH). For detection of GFP fluorescence, livers were first perfused in situ with 0.5 mM EGTA containing salt solution to remove peripheral blood cells. The livers were then perfused with 4% paraformaldehyde, fixed with 4% paraformaldehyde for 24 hours, soaked in 15% sucrose in PBS for 12 hours, soaked in 30% sucrose for 24 hours at 4°C under constant agitation, and then embedded in OCT compound to cut sections on a cryostat. The GFP$^+$ area was determined using ImageJ software and shown as a percentage of the total section area.

Histological analysis. F4/80, desmin, α-SMA, and TIMP1 were stained with anti-F4/80 (Santa Cruz Biotechnology), anti-desmin (Lab Vision), anti–α-SMA (clone 1A4; Sigma-Aldrich), and anti-TIMP1 (R&D Systems) antibodies, respectively, using Vectastain Elite ABC Kit or M.O.M Immunodetection Kit (Vector Laboratories). Diaminobenzidine tetrahydrochloride was used as peroxidase substrate, and sections were counterstained with hematoxylin. The immunostain-positive area was determined using ImageJ software and shown as a percentage of the total section area. In some experiments, fluorescent dye–labeled secondary antibodies (Alexa Fluor 350 anti-rat for F4/80, Alexa Fluor 488 anti-rabbit for desmin, and Alexa Fluor 594 anti-goat for TIMP1) were used for detection of primary antibodies.

Depletion of macrophages. Liposome-encapsulated alendronate (Sigma-Aldrich) was generated as previously reported (53). The alendronate was injected in the mice 4 times: 1 day before cell inoculation and 4, 7, and 10 days after. Liposome-encapsulated PBS was injected into the mice as a control.

Recombinant adenoviruses. Mice were infected with 2 adenoviruses (5×10^8 pfu/mouse) by intravenous injection 1 day before or 5 days after cell inoculation. Of the 2 adenovirus-5 variants, AdGFP expresses GFP, whereas AdASM expresses both ASM and GFP, because it contains a GFP sequence (27).

Mass spectrometric (MS) analysis of S1P. Electrospray ionization MS/MS analysis was performed using a Thermo-Finnigan TSQ 7000 Triple Quadrupole Mass Spectrometer (GenTEch Scientific Inc.) in multiple-reaction monitoring positive ionization mode, as previously reported (54). S1P level was normalized by total protein per sample.

Macrophage migration assay. For isolation of peritoneal macrophages, wild-type mice were injected intraperitoneally with cold normal saline. Peritoneal macrophages were purified using MACS Cell Separation Columns with CD11b microbeads (Miltenyi Biotec) from the lavage fluid. The cell migration assay was performed using 24-well transwell plates (5-μm pore size; Costar). The peritoneal macrophages (1×10^5 cells) in DMEM containing 1% BSA were loaded into the upper chambers, while the lower chambers were filled with medium with or without 1 μM S1P (Matreya LLC). Cells were incubated for 4 hours, then the migrated macrophages on the underside of the chambers were fixed and stained with Giemsa solution (Wako). Migration was expressed as the number of cells per field.

Quantitative real-time RT-PCR. Rat primary liver hMFs were isolated as previously described (25). The cells were cultured in DMEM with 10% FBS on plastic dishes for 4 hours and administered S1P or vehicle (1 μM), and RNA was extracted 0 or 72 hours after S1P stimulation. The RNeasy and DNase Kits (Qiagen) was used for RNA extraction from liver tissue and cultured cells, and the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for reverse transcription. Quantitative real-time RT-PCR was performed using the SYBR Premix Ex Taq (Takara) for *Timp1* (forward, TGGGGAACCCATGAATTTAG; reverse, TCTGGCATCCTCTTGTTGC) and probe and primer sets (Applied Biosystems) for 18S with LightCycler 480 (Roche Applied Science). The changes were normalized based on 18S rRNA values.

Statistics. Data are expressed as mean ± SD of data collected from 5 independent experiments. Data between groups were analyzed by 2-tailed Student's *t* test. A *P* value less than 0.05 was considered significant.

Study approval. All experiments were conducted in accordance with the institutional guidelines of Gifu University and were approved by the animal research committee and the committee on living modified organisms of Gifu University.

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