Stromal Fibroblasts Activated by Tumor Cells Promote Angiogenesis in Mouse Gastric Cancer*

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Myofibroblasts, also known as activated fibroblasts, constitute an important niche for tumor development through the promotion of angiogenesis. However, the mechanism of stromal fibroblast activation in tumor tissues has not been fully understood. A gastric cancer mouse model (Gan mice) was recently constructed by simultaneous activation of prostaglandin (PG) E₂ and Wnt signaling in the gastric mucosa. Because both the PGE₂ and Wnt pathways play a role in human gastric tumorigenesis, the Gan mouse model therefore recapitulates the molecular etiology of human gastric cancer. Microvessel density increased significantly in Gan mouse tumors. Moreover, the expression of vascular endothelial growth factor A (VEGFA) was predominantly induced in the stromal cells of gastric tumors. Immunohistochemistry suggested that VEGFA-expressing cells in the stroma were α-smooth muscle actin-positive myofibroblasts. Bone marrow transplantation experiments indicated that a subset of gastric myofibroblasts is derived from bone marrow. Importantly, the α-smooth muscle actin index in cultured fibroblasts increased significantly when stimulated with the conditioned medium of Gan mouse tumor cells, indicating that gastric tumor cells activate stromal fibroblasts. Furthermore, conditioned medium of Gan mouse tumor cells induced VEGFA expression both in embryonic and gastric fibroblasts, which further accelerated the tube formation of human umbilical vein endothelial cells in vitro. Notably, stimulation of fibroblasts with PGE₂ and/or Wnt1 did not induce VEGFA expression, thus suggesting that factors secondarily induced by PGE₂ and Wnt signaling in the tumor cells are responsible for activation of stromal fibroblasts. Such tumor cell-derived factors may therefore be an effective target for chemoprevention against gastric cancer.

Accumulating evidence has indicated that cancer development is regulated by interactions between tumor cells and activated stromal cells (1). Experiments in mouse models have shown that fibroblasts in the stromal microenvironment play an important role in tumor formation (2–4). Moreover, carcinoma-associated fibroblasts (CAFs) stimulate tumor progression of the initiated epithelial cells (5). Stromal fibroblasts isolated from human breast cancer show distinctive gene expression profiles, although no genetic alteration is found (6). These results indicate that the stromal fibroblasts in tumor tissues possess biological characteristics distinct from those of the normal fibroblasts, which contribute to tumor development. Immunohistochemical studies have shown that a large number of myofibroblasts, which express α-smooth muscle actin (α-SMA), are present in the stromal component of human breast cancer, intestinal polyps, and gastric cancer (7–9). These myofibroblasts, which are also known as “activated fibroblasts,” therefore contribute to tumor formation.

The promotion of angiogenesis is an important function of myofibroblasts during tumorigenesis. Angiogenesis is a key mechanism that supports tumor development by providing nutrients and oxygen (10). The CAFs extracted from human breast cancer, which exhibit the traits of myofibroblasts, promote angiogenesis through the expression of stromal cell-derived factor 1 (11). Moreover, prostaglandin E₂ (PGE₂) stimulates intestinal myofibroblasts to express vascular endothelial growth factor A (VEGFA), epidermal growth factor-like growth factor, and amphiregulin, thus leading to the promotion of angiogenesis as well as epithelial proliferation (12). In addition, stromal cell-derived PGE₂ plays an important role in angiogenesis in intestinal tumorigenesis through the induction of VEGF and basic fibroblast growth factor (13, 14). Although it has been shown that the conversion of fibroblasts constitutes the major source of myofibroblasts (15), the mechanisms underlying stromal activation and promotion of angiogenesis are still not fully understood. It is important to examine the in vivo tumor mouse model to understand the interaction between tumor cells, stromal fibroblasts, and angiogenesis.

This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Health, Labour and Welfare of Japan, and Takeda Science Foundation, Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–3.

2 The abbreviations used are: CAF, carcinoma-associated fibroblast; α-SMA, α-smooth muscle actin; CM, conditioned medium; COX-2, cyclooxygenase-2; GF, gastric fibroblasts; HGF, hepatocyte growth factor; HUVEC, Human umbilical vein endothelial cell; LMD, laser microdissection; MEF, mouse embryonic fibroblast; mPGES-1, microsomal prostaglandin E synthase-1; MVD, microvessel density; PGE₂, prostaglandin E₂; RT, reverse transcription; TNF-α, tumor necrosis factor-α; VEGFA, vascular endothelial growth factor-A; vWF, vonWillebrand factor; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; GFP, green fluorescent protein; EGFP, enhanced GFP; GM-CSF, granulocyte-macrophage colony-stimulating factor; DMEM, Dulbecco’s modified Eagle’s medium.
A gastric cancer mouse model (K19-Wnt1/C2mE mice) was recently established by the transgenic expression of Wnt1, cyclooxygenase-2 (COX-2), and microsomal PGE synthase-1 (mPGES-1) in the gastric mucosa (16). COX-2, a rate-limiting enzyme for prostaglandin biosynthesis, is induced in more than 70% of gastric cancer and plays an important role in gastric tumorigenesis (17). An inducible PGE2-converting enzyme, mPGES-1, appears to be functionally coupled with COX-2 (18), and mPGES-1 expression is also induced in gastric cancer, thus suggesting an increased PGE2 level in gastric cancer tissues (19). On the other hand, the canonical Wnt signaling is a critical pathway for gastrointestinal tumorigenesis (20). β-Catenin nuclear localization, a hallmark of Wnt pathway activation, is found in 29% of gastric cancer (21), suggesting that the activation of the Wnt pathway is one of the major causes for gastric carcinogenesis. Importantly, K19-Wnt1/C2mE mice, in which both the PGE2 and Wnt pathways are activated simultaneously, develop intestinal-type gastric adenocarcinomas (16). Therefore, K19-Wnt1/C2mE mice (hereafter Gan mice for Gastric neoplasia) recapitulate a subpopulation of human gastric cancers not only in molecular mechanism but also in tumor pathology.

This study examined the epithelial-stromal interaction in tumor angiogenesis using the Gan mouse model. Although it has been established that PGE2 signaling is important for angiogenesis in intestinal tumors (12–14, 22), the mechanisms underlying the induction of angiogenic factors remain to be elucidated. We show that Gan mouse gastric tumor cells activate stromal fibroblasts to become myofibroblasts, and that these myofibroblasts stimulated by gastric tumor cells express VEGFA and other angiogenic factors, which may lead to the promotion of angiogenesis.

**EXPERIMENTAL PROCEDURES**

**Transgenic Mice**—The establishment of the K19-C2mE, K19-Wnt1, and Gan mice (K19-Wnt1/C2mE) has been described previously (16, 23). Briefly, COX-2 and mPGES-1 are expressed in the stomach of K19-C2mE mice, whereas Wnt1 is expressed in the stomach of K19-Wnt1 mice. The expression of these genes is regulated by the cytokeratin 19 gene promoter. For the inhibition of COX-2, Gan mice from 28 to 30 weeks of age were injected subcutaneously with 10 mg/kg/day of NS-398 (Sigma) for 3 weeks (n = 3). All animal experiments were carried out according to the protocol approved by the Committee on Animal Experimentation of Kanazawa University.

**Histology and Immunohistochemistry**—Mouse stomach tissues were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned at 4-μm thickness. These sections were stained with hematoxylin and eosin and processed for immunostaining. To detect capillary vessels, polyclonal anti-von Willebrand factor (vWF) antibody was used (DakoCytomation, Carpinteria, CA) as the primary antibody of immunohistochemistry. To detect VEGFA-expressing cells, monoclonal anti-VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used. To detect lymphatic vessels, polyclonal anti-Lyve-1 antibody (Acris Antibodies, Hiddenhausen, Germany) was used. To detect macrophages, rat monoclonal antibody for F4/80 (Serotec, Oxford, UK) was used. To detect myofibroblasts, polyclonal anti-α-smooth muscle actin (Sigma) and monoclonal anti-tenasin-c antibodies (Abcam) were used. To detect smooth muscle cells, anti-calponin-1 monoclonal antibody (Epitomax) was used. To detect GFP, polyclonal anti-GFP antibody (Molecular Probes) was used. The MOM kit (Vector Laboratories, Burlingame, CA) was used to minimize the background. Staining signals were then visualized using the Vectorstain Elite kit (Vector Laboratories). For immunofluorescence, Alexa Fluor 594 or Alexa Fluor 488 antibody (Molecular Probes, Eugene, OR) was used as the secondary antibody.

**Immunocytochemistry**—Mouse embryonic fibroblasts (MEFs) and gastric fibroblasts (GFs) grown on coverslips were fixed with 10% neutral buffered formalin and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline. Anti-α-SMA monoclonal antibody (Sigma) was used as the primary antibody, and anti-mouse IgG Alexa Fluor 488 (Molecular Probes) was used as the secondary antibody. Next, the coverslips were mounted using Vectashield mounting medium (Vector Laboratories) that contained 4′,6-diamidino-2-phenylindole for nuclear staining. The number of α-SMA-positive cells was counted using five microscopic fields for each sample, and the mean α-SMA positive index was calculated.

**Scoring Microvessel Density**—Microvessel density (MVD) was scored using histological sections immunostained with anti-vWF antibody. The number of vessels was then counted in high magnification fields (×400) for each group (n = 3). At least five microscopic fields per mouse were scored for each genotype, and then the mean relative MVD to the wild-type MVD was calculated.

**Reverse Transcription (RT)-PCR**—Total RNA was extracted from the tissues or cells using ISOGEN (Nippon Gene, Tokyo, Japan). Epithelial and stromal tissues were isolated from cryosections, respectively, using a laser microdissection (LMD) system (Leica Microsystems, Wetzlar, Germany). Extracted RNA was reverse-transcribed and PCR-amplified by GeneAmp PCR System 9700 (Applied Biosystems). RT-PCR was carried out using the following primer set: VEGFA (F-5′-CTTCTCTACACACACGAGATGTGGA-3′; R-5′-TGGTGACATGGTTATCGGTCTTTC-3′) and hepatocyte growth factor (HGF) (F-5′-TTCCCCAGCTGTTCTATGTC-3′; R-5′-TGGTGCTGACTGCAATTTCTC-3′). Specific β-actin primers were used as an internal control. For real time RT-PCR, total RNA was reverse-transcribed using the PrimeScript RT reagent kit (Takara, Japan), and then it was PCR-amplified by ABI Prism 7900HT (Applied Biosystems) using SYBR Premix Ex TaqII (Takara, Japan). The primer sets used in real time RT-PCR to detect VEGFA, HGF, Tie2, and CXCR4 were purchased (Takara, Japan).

**Bone Marrow Transplantation**—Bone marrow cells were isolated from EGFP transgenic mice (24). Bone marrow cells were suspended in phosphate-buffered saline at 1 × 107 cells. The 6-week-old recipient K19-C2mE mice and wild-type mice were irradiated with 8 Gy γ-ray and transplanted with 1 × 106 GFP+ donor cells intravenously. K19-C2mE mice that received a bone marrow transplant were autopsied 20 weeks later.

**Cell Culture Experiments**—MEFs were prepared from 12.5 days post-coitum embryos and cultured with DMEM (Invitro-gen) containing 10% fetal bovine serum. Human umbilical vein
endothelial cells (HUVECs) were purchased from American Type Culture Collection and maintained using the EGM-2 bullet kit (Lonza, Basel, Switzerland). The primary gastric epithelial cells were prepared as described previously (23) and cultured on collagen-coated 6-well plates. When the cell density reached 80% confluence, the medium was replaced with DMEM/F-12, and the conditioned medium was collected after a culture of 48 h. For the isolation of GFs, mouse stomach tissues or gastric tumors were digested with collagenase (1 mg/ml at 37 °C for 30 min), washed with DMEM, seeded in collagen-coating dishes, and washed with medium after 30 min to remove epithelial cells. After two rounds of passages, epithelial cells were absent in the culture, and fast growing GFs were enriched. For direct co-culture experiments, gastric epithelial cells were absent in the culture, and fast growing GFs were enriched. For direct co-culture experiments, gastric epithelial cells and MEFs were mixed at a ratio of 1:1 and plated in 6-well plates. For indirect co-culture experiments, 1 × 10^5 MEFs or GFs were plated in 48-well plates, and the medium was replaced with mixture of conditioned medium from the primary culture (250 µl) and fresh DMEM (250 µl). At 48 h after stimulation with conditioned medium, medium and cell lysates were collected and used for the following assays. To analyze VEGFA induction by cytokines, MEFs were stimulated with IL-1α, IL-6, IL-17α, GM-CSF (R & D Systems) or tumor necrosis factor (TNF)α (Calbiochem) at 10 and 100 ng/ml for 24 h, and the VEGFA expression in the medium was examined by ELISA.

Cytokine Expression Analysis—Conditioned media obtained from the primary cultured Gan mouse tumor cells and wild-type mouse gastric epithelial cells were used for the expression analysis of cytokines. Expression of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17α, interferon-γ, TNF-α, granulocyte-CSF, and GM-CSF in each medium was examined using the Multi-Analyte Profiler ELISArray kit (Super Array) according to the manufacturer’s protocol.

ELISA—The concentration of VEGFA in the culture medium was measured using an ELISA kit (BioSource International, Camarillo, CA), and the total protein of cultured cells was measured using a BCA protein detection kit. For the primary culture of epithelial cells, VEGFA concentration was calculated as medium VEGFA (pg) per total protein (mg) of cells or medium volume (ml) because of difficulty in counting the number of cells. For the MEF culture, medium VEGFA (pg) per 10^5 cells was calculated.

HUVEC Tube Formation Assay—HUVECs (5 × 10^4 cells) were suspended in mixture of conditioned medium (125 µl) and EGM-2 medium (125 µl) in the presence or absence of PGE2 at 1 µM with 0.5% fetal bovine serum, and seeded on 150 µl of Matrigel in 48-well culture plates. After incubation for 18–24 h, photographs of each well were taken, and the number of tubular structures and the length of the tubes in each field were measured using Image J application software (National Institutes of Health).

Statistical Analysis—Statistical analyses were carried out by Student’s t test, and p values <0.05 were considered to be statistically significant.

RESULTS

Gan mice (K19-Wnt1/C2mE mice) develop gastric adenocarcinomas in the glandular stomach caused by the simultaneous activation of Wnt and PGE2 pathways (16). Notably, gastric tumors developed in Gan mice showed hyperemia that was not found in the wild-type mouse stomach (Fig. 1, A and B). Histologically, numerous capillary vessels were detected in the Gan mouse gastric tumors by immunostaining using anti-vWF antibody, whereas vessels were sparsely found in the normal gastric mucosa (Fig. 1, D and E). The MVD in the Gan mouse tumors increased significantly to more than five times that of the wild-type level (Fig. 1F). PGE2 signaling plays an important role in
Angiogenesis in gastric cancer. We confirmed the abundant expression of VEGFA in the stromal cells of K19-C2mE mice and K19-Wnt1 mice (Fig. 1, A–D). These results indicate that the activation of the COX-2/PGE2 and Wnt pathways is essential for angiogenesis in gastric cancer. On the other hand, the number of capillary vessels and MVD did not increase in the gastric mucosa of K19-C2mE mice or K19-Wnt1 mice (Fig. 1, F–I), which express PGE2 or Wnt1 in the stomach, respectively (16, 23). Accordingly, it is possible that both the PGE2 and Wnt pathways are important for angiogenesis in gastric tumors. However, further experiments demonstrated that secondarily induced factors in the tumor cells are responsible for angiogenesis (see below and see Fig. 4, C and D), although activation of both PGE2 and Wnt pathways is essential for gastrointestinal tumorigenesis (16, 25).

The COX-2 pathway is also important for lymphangiogenesis (26). However, lymphatic vessels were rarely detected in the gastric mucosa of the wild-type, K19-C2mE, and K19-Wnt1 mice as well as Gan mouse gastric tumors (supplemental Fig. 1). In the stomach of these mouse models, lymphatic vessels were predominantly found in the smooth muscle layers. Accordingly, it is conceivable that activation of PGE2 and Wnt signaling does not promote lymphangiogenesis in gastric cancer.

Consistent with the results of the MVD analyses, the VEGFA mRNA level determined by real time RT-PCR significantly increased in the Gan mouse gastric tumors in comparison with that in the wild-type, K19-C2mE, or K19-Wnt1 mouse stomach (Fig. 2A). We also examined expression of HGF, Tie2, and CXCX4 by real time RT-PCR, because they have been reported to play a role in angiogenesis (27–29). Interestingly, the expression of all these genes significantly increased in the Gan mouse tumors in comparison with that in the gastric mucosa of other genotype mice except CXCR4 in K19-C2mE mice (Fig. 2A). The expression of CXCR4 is induced in both K19-C2mE and Gan mice, thus suggesting the PGE2-dependent induction. These results indicate that several angiogenic pathways are activated simultaneously in the gastric tumor tissues, although the underlying molecular mechanisms remain to be investigated. Next, epithelial cells and stromal cells were isolated from Gan gastric tumor specimens using the LMD system. The expression of VEGFA and HGF was predominately detected in the stromal cells but not in the epithelial cells (Fig. 2B). Consistently, VEGFA mRNA was at the wild-type level in the primary cultured Gan tumor epithelial cells, in which stromal cells were removed (Fig. 2C). These results indicate that stromal cells are the major source for VEGFA and HGF in gastric tumors. Further analyses focused on the expression of VEGFA because of its significant role in angiogenesis. We confirmed the abundant expression of VEGFA in the stromal cells of Gan mouse gastric tumors by immunostaining (Fig. 2D, asterisks). In contrast, VEGFA-expressing cells were rarely found in the stroma of the wild-type, K19-C2mE, or K19-Wnt1 mouse gastric mucosa. We confirmed the immunostaining specificity by a negative control of nonprimary antibody using a Gan tumor section (Fig. 2D, right).

It has been shown that myofibroblasts play a key role in tumor angiogenesis (9, 10). Therefore, the expression of α-SMA was examined in the Gan mouse tumors, which was a defining characteristic of the myofibroblasts (30). Importantly, a substantial number of α-SMA-positive cells were found in Gan mouse tumor stroma, whereas α-SMA-positive cells were sparsely detected in the nontumorous gastric mucosa (Fig. 3A). Moreover, most α-SMA-positive cells expressed tenasin-c, another marker for myofibroblasts (Fig. 3B, left). In contrast, the majority of α-SMA-positive cells were negative for smooth muscle specific marker calponin-1, although small number of cells expressed both α-SMA and calponin-1 (Fig. 3B, right). These results strongly suggest the major cell type expressing VEGFA in the Gan mouse tumor stroma are myofibroblasts (compare Fig. 2D and Fig. 3A). On the other hand, immunostaining analyses revealed that VEGFA expression was rarely found in the macrophages or capillary vessels in the Gan mouse tumors (Fig. 3C).

It has been reported that bone marrow cells contribute to stromal myofibroblasts of tumor tissues (31). Moreover, inflammatory responses significantly increase the contribution...
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FIGURE 3. Bone marrow-derived myofibroblasts in gastric lesions. A, immunostaining for α-SMA-expressing cells (brown) in nontumorous (left) and tumor region (right) of Gan mouse stomach. B, double immunostaining for tenascin-c (green) and α-SMA (red), and calponin-1 (green) and α-SMA (red). The arrowheads indicate tenascin-c and α-SMA double-positive cells (left), and calponin-1-negative and α-SMA-positive cells (right). C, double immunostaining for F4/80 (green) and VEGFA (red), and vWF (green) and VEGFA (red). The arrowheads indicate macrophages (left) and capillary vessels (right). D, immunostaining for α-SMA (left) and GFP (right) in gastric hyperplasia of K19-C2mE mice that received a bone marrow transplantation from EGFP transgenic mice. E, double immunostaining for GFP (green) and α-SMA (red), and GFP (green) and F4/80 (red) in gastric hyperplasia of K19-C2mE mice that received a bone marrow transplantation from EGFP transgenic mice. The arrowheads indicate double-positive cells for GFP and α-SMA (left) and GFP and F4/80 (right). F, immunostaining for GFP (left) and α-SMA (right) in the normal gastric mucosa of a wild-type mouse that received a bone marrow transplant from EGFP transgenic mice. The arrow indicates infiltrated bone marrow-derived mononuclear cells. The arrowheads indicate the α-SMA-positive pericytes of capillary vessels. Bars in A–F indicate 100 μm.

of bone marrow cells to myofibroblasts (32). Therefore, the contribution of bone marrow cells to gastric myofibroblasts was examined using the K19-C2mE mouse that develop inflammation-associated gastric hyperplasia caused by increased PGE₂ signaling (23). As in the Gan mouse tumor tissues, α-SMA-positive cells were increased in the stroma of K19-C2mE mouse stomach (Fig. 3D, left). Bone marrow transplantation to γ-ray irradiated K19-C2mE mice from EGFP transgenic mice revealed that the number of GFP-positive bone marrow-derived cells infiltrated to the gastric stroma (Fig. 3D, right). Notably, the subset of α-SMA-positive cells in the gastric stroma also showed GFP expression (Fig. 3E, left). The mean percentage of GFP-positive cells in the α-SMA-expressing cells was 12.1%, thus indicating that part of the stromal myofibroblasts is derived from bone marrow cells. We also found 58% of the GFP-positive cells in the gastric stroma to be macrophages (Fig. 3E, right). In contrast, bone marrow cells rarely contributed to normal gastric mucosa of the transplanted wild-type mice except with infiltrated mononuclear cells (Fig. 3F).

To examine the activation of stromal cells by tumor cells, MEFs and gastric fibroblasts (GFs) were treated with a conditioned medium (CM) of the primary cultured Gan tumor epithelial cells (supplemental Fig. 2). Importantly, the α-SMA index increased significantly in both MEFs and GFs when treated with Gan tumor CM. These results suggest that tumor epithelial cells activate stromal fibroblasts, thus resulting in conversion to myofibroblasts. It is thus conceivable that both bone marrow cells and preexisting fibroblasts therefore contribute to gastric myofibroblasts.

We next examined whether gastric tumor epithelial cells induce VEGFA expression in stromal fibroblasts. We performed direct or indirect co-culture experiments using the primary cultures of gastric epithelial cells and fibroblasts (Fig. 4A). MEFs as well as GFs were used for the experiments, because MEFs were activated by CM of Gan tumor cells (supplemental Fig. 2). In the direct co-culture of MEFs and Gan tumor cells, the VEGFA mRNA level increased significantly in comparison with that in either of MEF or tumor epithelial cell monoculture (Fig. 4B, left). Consistently, the VEGFA concentration in the media increased more than 10-fold in the direct co-culture of MEFs and Gan tumor cells than that in either monoculture. Interestingly, VEGFA mRNA level also increased in the direct co-culture of MEFs and wild-type epithelial cells (Fig. 4B, right), suggesting that factor(s) inducing VEGFA are also expressed in the cultured normal epithelial cells at a basal level. However, VEGFA concentration in the medium was significantly higher in the co-culture of MEFs with Gan tumor cells (14,100 pg/ml) in comparison with that with normal gastric epithelial cells (5,200 pg/ml). We confirmed by real time RT-PCR that the VEGFA expression level increased in the co-culture of Gan tumor epithelial cells and MEFs. These results suggest that fibroblast-stimulating factors for VEGFA expression are significantly induced in the gastric tumor epithelial cells.

We next examined the VEGFA induction in MEFs by indirect co-culture experiments. Conditioned media of the primary cultured epithelial cells were prepared from the wild-type, K19-C2mE, and K19-Wnt1 mouse stomach and Gan mouse tumors. Before the experiments, the VEGFA concentration in the respective CM was confirmed to be at the same basal level (supplemental Fig. 3). The treatment of MEFs with CM of the wild-type epithelial cells resulted in increased VEGFA expression, which may be caused by activating factors nonspecifically expressed in the cultured normal epithelial cells, therefore con-
sidered as background (Fig. 4C). The VEGFA expression level of MEFs treated with CM of the K19-C2mE or K19-Wnt1 epithelial cells was also at the background level, thus suggesting that either PGE2 or Wnt signaling alone cannot induce VEGFA in the fibroblasts. Consistently, treatment of MEFs with PGE2 alone resulted in no increase of the VEGFA level in the medium. In contrast, CM of Gan tumor cells increased VEGFA expression significantly beyond the background level (Fig. 4C), indicating that Gan tumor cells express soluble factors that induce VEGFA in fibroblasts. Importantly, however, the VEGFA level was not increased when MEFs were stimulated with CM of K19-Wnt1 cells supplemented with PGE2, which contained both PGE2 and Wnt1 (Fig. 4C). These results rule out the possibility that direct stimulation by PGE2 and Wnt ligand induces VEGFA expression in fibroblasts. Therefore, it is conceivable that secondary factors induced by PGE2 and Wnt signaling in the tumor cells stimulate fibroblasts, although both the PGE2 and Wnt pathways are required for tumor formation (16).

We next tested whether gastric tumor cells also stimulate GFs to express VEGFA. Notably, treatment of Gan tumor GFs with CM of the tumor epithelial cells significantly increased the VEGFA concentration in the medium (Fig. 4D). Consistent with the results in MEF experiments (Fig. 4C), the CM of K19-Wnt1 cells supplemented with PGE2 did not increase the VEGFA level in Gan tumor GFs, thus indicating that factor(s) other than Wnt and PGE2 are responsible for the VEGFA induction in GFs. Although VEGFA in the wild-type GFs also increased after treatment with CM, the increase was not significant. Accordingly it is possible that gastric tumor fibroblasts possess higher responsiveness to stimulation by the tumor cells than normal stomach fibroblasts.

Inflammatory responses are associated with the development of gastric hyperplasia in the K19-C2mE mice (23). We thus examined whether tumor cell-derived cytokines play a role in VEGFA induction in fibroblasts. Cytokine ELISAs revealed the level of IL-1α, IL-6, IL-17α, G-CSF, and GM-CSF in the medium of Gan tumor cell cultures to increase significantly in comparison with that in the wild-type epithelial cells (Fig. 5). However, the treatment of MEFs with IL-1α, IL-6, IL-17α, GM-CSF as well as TNF-α did not induce VEGFA expression, whereas the CM of Gan mouse tumor cells did (data not shown and Fig. 4C). These results suggest that other factors than inflammatory cytokines expressed by tumor cells may play a role in the VEGFA induction in stromal fibroblasts.

We finally examined whether fibroblasts activated by tumor cells induce angiogenesis in vitro using the HUVEC tube formation assay. Secondary CM was prepared from MEF cultures after stimulation with CM of wild-type or gastric tumor epithelial cells. HUVECs formed a network of capillary-like structures after stimulation with CM of wild-type or Gan tumor cell cultures (Fig. 6A, left). Significantly, CM of MEFs stimulated with Gan tumor cells dramatically accelerated tube formation (Fig. 6A, right), in comparison with those treated with CM of MEFs treated with wild-type epithelium (Fig. 6A, center). The number and length of HUVEC tubes increased by 14 and 10 times, respectively, by treatment with CM of tumor cell-stimulated MEFs (Fig. 6, B and C). Consistent with the results of co-culture experiments (Fig. 4, C and D), CM of MEFs stimulated with CM of K19-Wnt1 gastric epithelial cells with PGE2 did not increase HUVEC tube formation (Fig. 6, B and C). Although the VEGFA level increased in the culture medium of MEFs stimulated with CM of wild-type or K19-Wnt1 mouse epithelium (Fig. 5C), HUVEC tube formation was enhanced significantly by CM of MEFs treated with Gan mouse...
tumor cells in comparison with that of wild-type cells or K19-Wnt1 epithelial cells with PGE2. These results, taken together, suggest that gastric tumor cells activate fibroblasts to promote angiogenesis through the induction of not only VEGFA but also other angiogenic factors as shown in Fig. 2A.

**DISCUSSION**

We have previously shown using genetic mouse models that the COX-2/PGE2 pathway is important for angiogenesis in intestinal polyps (13, 14). The disruption of the COX-2 gene (Ptgs2) or PGE2 receptor EP2 gene (Ptger2) causes suppression of VEGF expression and decrease of MVD in tumor tissues. On the other hand, the VEGF expression has been reported to be regulated by Wnt signaling (33). However, the present results indicate that activation of either PGE2 or Wnt signaling alone does not induce VEGF expression in the stomach in vivo as well as in the fibroblasts in vitro. Importantly, simultaneous stimulation by PGE2 and Wnt1 also failed to induce VEGF expression in fibroblast cultures. Accordingly, it is possible that promotion of angiogenesis in Gan mouse gastric tumors is not simply caused by activation of the PGE2 and Wnt pathways. Previously, we have shown that simultaneous activation of the PGE2 and Wnt pathways is responsible for development of gastric adenocarcinoma (16). Therefore, it is possible that tumor cells transformed by PGE2 and Wnt pathways secondarily express responsible factors other than PGE2 or Wnt ligands, which activate stromal fibroblasts and bone marrow-derived cells to convert to myofibroblasts and stimulate the expression of angiogenic factors. TNF-α has been reported to induce angiogenesis, and this induction is dependent on IL-8 and basic fibroblast growth factor signaling (34). Moreover, an increased PGE2 level in the K19-C2mE mouse stomach induces inflammatory responses that lead to induction of TNF-α (35). However, the present results suggest that other tumor cell-derived factors than inflammatory cytokines are responsible for the induction of VEGFA in stromal fibroblasts.

It has been shown that CAFs extracted from human breast cancer tissues promote the angiogenesis and growth of cancer cells more significantly than normal mammary fibroblasts (11). Moreover, the α-SMA positive index of CAFs is constitutively higher in culture than that of the counterpart normal fibroblasts, suggesting that CAFs are reversibly activated by the tumor environment. In this study, a substantial number of α-SMA and tenascin-c positive myofibroblasts were observed in the gastric tumor stroma. However, the α-SMA index of cultured gastric tumor fibroblasts

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**FIGURE 5. Expression of inflammatory cytokines from the primary cultured gastric tumor epithelial cells.** A, photograph of a representative ELISA plate. Three independent culture medium samples each for wild-type gastric epithelial cells and Gan tumor epithelial cells were used. A yellow color indicates positive reaction. B, mean OD values of ELISA results for indicated cytokines are shown. OD >1.0 is indicated as 1.0. *, p < 0.05 versus wild-type level.

**FIGURE 6. HUVEC tube formation assay.** A, representative photographs of HUVEC culture in Matrigel under a dissection microscope (18 h). HUVECs were treated with the secondary CM of MEFs that were stimulated with the primary CM of wild-type epithelial cells (center) or Gan tumor epithelial cells (right). CM of unstimulated MEFs was used as a control (left). Bars indicate 0.5 mm. B and C, number of HUVEC tubes and their relative lengths are shown, respectively (mean ± S.D.). Wnt1-CM+PGE2 indicates CM of MEFs treated with the primary CM of K19-Wnt1 epithelial cells and PGE2 at 1 μM. *, p < 0.05.
was at the same level as that from the normal stomach, suggesting that activation of *Gan* mouse tumor fibroblasts is reversible. It is therefore possible that long term interaction between fibroblasts and tumor cells in human malignancies is required for irreversible education of CAFs.

Interestingly, the responsiveness to conditioned medium of gastric tumor cells is higher in the tumor-derived gastric fibroblasts (Fig. 4D and supplemental Fig. 2C). These results suggest that gastric tumor fibroblasts have characteristics that are distinct from normal gastric fibroblasts, even though they are not irreversibly activated. It has been shown that bone-marrow derived cells contribute to myofibroblasts (30, 36) and that inflammatory responses enhance this contribution in tumor stroma (31). Moreover, it has been established that chronic gastritis caused by *Helicobacter pylori* infection is closely associated with gastric cancer development (37), and that the COX-2 pathway is induced in most cases of gastric cancer (17). The present study using *K19-C2mE* mice demonstrated that a subset of gastric myofibroblasts originated from bone marrow cells. Accordingly, it is conceivable that chronic gastritis caused by *H. pylori* infection and subsequent COX-2/PGE2 induction are involved in the contribution of bone marrow cells to myofibroblasts, which may contribute to distinct characteristics of tumor fibroblasts.

The specific molecules secreted by tumor cells that activate gastric fibroblasts to induce angiogenesis remain to be identified. However, the present results suggest that tumor fibroblasts require stimulation by tumor cells to express angiogenic factors, including VEGFA. Therefore, the suppression of fibroblast activation by the inhibition of tumor cell-derived factors might thus be an effective strategy for the chemoprevention of gastric cancer in combination with the eradication of *H. pylori*. Accordingly, an analysis of gene expression profiles using *Gan* mouse tumors is thus considered to provide important evidence to make it possible to determine the responsible factors.

**Acknowledgment—We thank Manami Watanabe for valuable technical assistance.**

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