

# Effects of Vitamin D<sub>3</sub>-Binding Protein-Derived Macrophage Activating Factor (GcMAF) on Angiogenesis

Shigeru Kanda, Yasushi Mochizuki, Yasuyoshi Miyata, Hiroshi Kanetake, Nobuto Yamamoto

**Background:** The vitamin D<sub>3</sub>-binding protein (Gc protein)-derived macrophage activating factor (GcMAF) activates tumoricidal macrophages against a variety of cancers indiscriminately. We investigated whether GcMAF also acts as an antiangiogenic factor on endothelial cells. **Methods:** The effects of GcMAF on angiogenic growth factor-induced cell proliferation, chemotaxis, and tube formation were examined *in vitro* by using cultured endothelial cells (murine IBE cells, porcine PAE cells, and human umbilical vein endothelial cells [HUVECs]) and *in vivo* by using a mouse cornea micropocket assay. Blocking monoclonal antibodies to CD36, a receptor for the antiangiogenic factor thrombospondin-1, which is also a possible receptor for GcMAF, were used to investigate the mechanism of GcMAF action. **Results:** GcMAF inhibited the endothelial cell proliferation, chemotaxis, and tube formation that were all stimulated by fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor-A, or angiotensin 2. FGF-2-induced neovascularization in murine cornea was also inhibited by GcMAF. Monoclonal antibodies against murine and human CD36 receptor blocked the antiangiogenic action of GcMAF on the angiogenic factor stimulation of endothelial cell chemotaxis. **Conclusions:** In addition to its ability to activate tumoricidal macrophages, GcMAF has direct antiangiogenic effects on endothelial cells independent of tissue origin. The antiangiogenic effects of GcMAF may be mediated through the CD36 receptor. [J Natl Cancer Inst 2002;94:1311–19]

Macrophages in cancer-bearing hosts can be divided into two groups: tumoricidal macrophages (1,2) and angiogenic macrophages (3–6). In cancer-bearing hosts, inflammation induced by

bacille Calmette-Guérin (BCG) or inflammatory lipid metabolites (i.e., alkylglycerols and lysophospholipids) results in the activation of highly cytotoxic macrophages, which leads to tumor regression (7,8). By contrast, certain conditions such as hypoxia (9) result in the activation of angiogenic macrophages and are characterized by the expression of heme oxygenase-1 (5). Because activated macrophages produce angiogenic cytokines such as interleukin-8, fibroblast growth factor-2 (FGF-2), and vascular endothelial growth factor (VEGF) (6), it is therefore possible that tumor-associated macrophages may induce angiogenesis, which supports tumor growth and progression (10–12).

Inflammation-primed activation of tumoricidal macrophages requires the vitamin D<sub>3</sub>-binding protein (known as Gc protein) and the participation of B and T lymphocytes. The stepwise modification of Gc protein by membrane-bound  $\beta$ -galactosidase on inflammation-primed (i.e., lysophospholipid-treated) B cells and then by membrane-bound Neu-1 sialidase on T cells results in a potent macrophage activating factor, GcMAF (13–16). Mac-

*Affiliations of authors:* S. Kanda, Department of Molecular Microbiology and Immunology, Division of Endothelial Cell Biology, and Department of Urology, Nagasaki University Graduate School of Medicine, Nagasaki, Japan; Y. Mochizuki, Y. Miyata, H. Kanetake, Department of Urology, Nagasaki University Graduate School of Medicine; N. Yamamoto, Socrates Institute for Therapeutic Immunology, Philadelphia, PA.

*Correspondence to:* Shigeru Kanda, M.D., Ph.D., Department of Molecular Microbiology and Immunology, Division of Endothelial Cell Biology, Nagasaki University Graduate School of Medicine, 1-7-1, Sakamoto, Nagasaki 852-8501, Japan (e-mail: shigeruk@net.nagasaki-u.ac.jp).

See "Notes" following "References."

© Oxford University Press

rophages activated with GcMAF are highly tumoricidal against a variety of malignancies (1,17,18).

It has been reported that  $1\alpha,25$ -dihydroxyvitamin  $D_3$ , the active metabolite of vitamin  $D_3$ , has antiangiogenic activity against endothelial cells (19). Whether vitamin  $D_3$ -binding protein derivatives such as GcMAF also have antiangiogenic activity is unknown. Because macrophages activated by GcMAF are highly tumoricidal, we hypothesized that GcMAF may also have direct antiangiogenic activity. In the present study, we determined the effects of GcMAF on various angiogenic responses of endothelial cells *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Materials

Human recombinant FGF-2 was obtained from Roche Diagnostics (Tokyo, Japan). Human recombinant VEGF-A and angiopoietin 2 (Ang2) were purchased from R&D Systems (Minneapolis, MN). Human platelet thrombospondin-1 (TSP-1) was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). Anti-human CD36 monoclonal antibody (subclass, immunoglobulin M [IgM]) and anti-murine CD36 monoclonal antibody (subclass, IgA) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO) and Cascade BioScience (Winchester, MA), respectively.

### Preparation of GcMAF

Gc protein, the precursor of GcMAF, was purified from sera of healthy volunteer donors by 25-hydroxyvitamin  $D_3$ -linked affinity chromatography, as previously described (20). GcMAF was prepared by a stepwise treatment of the purified Gc protein with immobilized galactosidase and sialidase, as previously described (16). GcMAF prepared by this protocol was greater than 99.5% pure. Purified GcMAF administered to humans with or without cancer or administered to mice did not lead to any apparent toxic or negative inflammatory side effects (1,18).

### Cell Culture

The "Immortomouse" brain endothelial cell line IBE was established from brain capillaries of mice transgenic for a temperature-sensitive mutant simian virus 40 (SV40) large T antigen (21). IBE cells do not express VEGF receptor-2 (receptor for VEGF-A) but do express FGF receptor-1 (receptor for FGF-2) and Tie 2 (receptor for Ang2) (21,22). When cultured at 33 °C, FGF-2-treated IBE cells proliferate, migrate, and form lumen-containing tube-like structures (21,23). All experiments with IBE cells were performed at 33 °C. IBE cells were grown in Ham's F-12 medium supplemented with 20% fetal bovine serum (FBS), bovine pituitary gland endothelial cell growth supplement (75  $\mu$ g/mL), insulin (5  $\mu$ g/mL), and epidermal growth factor (10 ng/mL) (21).

The porcine aortic endothelial cell line PAE was kindly provided by Dr. Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). PAE cells were cultured in Ham's F-12 medium containing 10% FBS. Primary human umbilical vein endothelial cells (HUVECs) were obtained from BioWhittaker, Inc. (Walkersville, MD) and cultured in endothelial cell basal medium (EBM; BioWhittaker, Inc.) supplemented with 2% FBS, bovine pituitary gland endothelial cell growth supplement (75  $\mu$ g/mL), insulin (5  $\mu$ g/mL), dexamethasone ( $10^{-7}$ M), and epidermal growth factor (10 ng/mL).

For the various angiogenesis studies, IBE cells were used in proliferation, chemotaxis, and tube formation assays, whereas PAE cells and HUVECs were used only in chemotaxis assays. In our laboratory, PAE cells and HUVECs require FBS in the culture medium to survive. Growth factor treatment of these cells in serum-containing media did not stimulate growth substantially (data not shown). PAE cells do not form tube-like structures, even after stimulation with VEGF-A. Although HUVECs can form tube-like structures on Matrigel, their ability to do so is independent of growth factor stimulation.

### Uptake of Acetylated Low-Density Lipoprotein

The uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein (DiI-Ac-LDL; Biomedical Technologies, Inc., Stoughton, MA) by cultured endothelial cells has been described (21). After endothelial cells have taken up DiI-Ac-LDL, the lipoprotein is degraded within lysosomes, liberating the fluorescent tag (DiI), which then accumulates in lysosomal membranes. Endothelial cells were cultured in the presence or absence of 10  $\mu$ g/mL of DiI-Ac-LDL for 4 hours. Cells were washed, fixed with 10% buffered formalin, and observed by fluorescence microscopy, according the protocol recommended by Biomedical Technologies, Inc. BALB/c 3T3 fibroblasts, which do not take up DiI-Ac-LDL, were used as a negative control.

### Cell Proliferation Assay

The IBE cell proliferation assay was described previously (24). Briefly, cells were suspended in Ham's F-12 medium containing 10% FBS and seeded into fibronectin-coated (20  $\mu$ g/mL) wells of 24-well plates at a density of  $2 \times 10^4$  cells/well. The next day, the culture medium was replaced with Ham's F-12 medium containing 0.25% bovine serum albumin (BSA) and supplemented (or not) with FGF-2 (5 ng/mL) and/or GcMAF (50 or 100 pg/mL). This concentration of FGF-2 (5 ng/mL) was previously shown to provide maximal proliferative stimulation (24). After 3 days, the cells were detached with trypsin and counted with the use of a hemocytometer. No dead floating cells were observed with any of the treatment conditions. Data are presented in absolute numbers of the means of triplicate wells from a single experiment with 95% confidence intervals. The experiment was repeated twice, with similar results.

### Chemotaxis Assay

To assess cell migration, we performed chemotaxis assays (24). Briefly, IBE cells were suspended in Ham's F-12 medium containing 0.25% BSA at a density of  $1.5 \times 10^5$  cells/mL, and 100  $\mu$ L/membrane of cell suspension was put onto the upper surface of fibronectin (20  $\mu$ g/mL)-coated Transwell inserts (6.7 mm in diameter). The inserts were then placed in wells containing Ham's F-12 medium supplemented with 0.25% BSA with or without FGF-2 (100 ng/mL) or Ang2 (1  $\mu$ g/mL) and/or GcMAF (100 pg/mL). The cells were incubated for 4 hours and then fixed with 100% methanol. The cells remaining on the upper surface of the insert membrane were removed with cotton swabs. Cells that migrated to the lower surface of the insert membrane were stained with Giemsa and counted with the use of a microscope. Cells treated with FGF-2 (100 ng/mL) and Ang2 (1  $\mu$ g/mL) were used as positive controls because these concentrations were previously determined to provide maximal chemotactic stimulation (22,24).

To measure PAE cell chemotaxis, cells were suspended in Ham's F-12 medium containing 2% FBS and seeded onto the upper surface of type I collagen (0.3 mg/mL)-coated Transwell inserts ( $1.5 \times 10^4$  cells/membrane). The inserts were placed in wells that contained FGF-2 (20 ng/mL) and/or GcMAF (100 pg/mL) and incubated for 4 hours. The cells were fixed and processed as described above. FGF-2 (20 ng/mL) maximally stimulated PAE cell chemotaxis (Kanda S: unpublished observations). For measuring HUVEC chemotaxis, cells were suspended in EBM containing 0.5% FBS and seeded onto the upper surface of gelatin (0.1%)-coated Transwell inserts ( $1.5 \times 10^4$  cells/membrane). The inserts were placed in wells that contained EBM supplemented with 0.5% FBS with or without VEGF-A (20 ng/mL) and/or GcMAF (100 pg/mL) and incubated for 4 hours. The cells were fixed and processed as described above. VEGF-A (20 ng/mL) maximally stimulated HUVEC chemotaxis (data not shown).

To test the effect of DiI-Ac-LDL on GcMAF-mediated inhibition of chemotaxis by endothelial cells, IBE cells, PAE cells, and HUVECs were seeded onto the upper surface of Transwell inserts with or without DiI-Ac-LDL (5  $\mu$ g/mL) and then placed in wells that contained culture medium in the presence or absence of FGF-2 or VEGF-A at concentrations that maximally stimulate chemotaxis for each cell line. Cells were cultured for 4 hours, fixed, and processed as described above.

To determine the effect of blocking the TSP-1 receptor CD36 on endothelial cell chemotaxis, we included monoclonal antibodies against murine CD36 or against human CD36 (10 mg/mL and 12.5 mg/mL, respectively) in the upper chamber of the Transwell insert and in the lower well. The antibodies included in the upper chamber were added to the cell suspension medium, and the cells were incubated and processed as described above. Because the anti-human CD36 antibody solution contained sodium azide, it was dialyzed against HEPES-buffered saline overnight before use. Monoclonal antibodies against murine CD36 at a concentration of 10 mg/mL or against human CD36 at a concentration of 12.5 mg/mL blocked the inhibitory activity of TSP-1 on VEGF-A-mediated chemotaxis of HUVECs and FGF-2-mediated chemotaxis of IBE cells. All chemotaxis data are expressed in absolute numbers of migrated cells with 95% confidence intervals.

### Tube Formation Assay

The tube formation assay was performed as described (21). Aliquots (0.5 mL/well) of a mixture containing eight volumes of bovine type I collagen (3.0 mg/mL), one volume of 10 $\times$  Ham's F-12 medium and one volume of concentrated buffer (260 mM NaHCO<sub>3</sub>, 200 mM HEPES, and 50 mM NaOH) were placed in each well of 12-well plates. After gelation, IBE cells suspended in Ham's F-12 medium containing 0.25% BSA with or without FGF-2 (10 ng/mL) and/or GcMAF (100 pg/mL) were seeded onto the collagen gels. After 4 hours, the medium was gently removed by aspiration, and the cells were covered with the collagen mixture (0.5 mL/well) and then incubated at 33 °C until gelation. After the collagen mixture had solidified, Ham's F-12 medium containing 0.25% BSA was overlaid to reduce evaporation. After 18 hours, tube formation was examined by phase-contrast microscopy. FGF-2 at 5–10 ng/mL gave the maximal stimulation of tube formation of IBE cells (21).

To quantify tube length, three random phase-contrast photomicrographs ( $\times 10$  objective) from the center of the wells were taken per well. The images were scanned, and the tube length was measured by using NIH Image software (version 1.62; <http://rsb.info.nih.gov/nih-image/>). Tube length obtained from FGF-2-stimulated cells was set to 100 arbitrary units. Untreated cells were used as negative controls. The experiments were repeated three times. Data are expressed in arbitrary units as means of the three experiments with 95% confidence intervals relative to the tube length of the positive control (FGF-2-stimulated cells).

### Mouse Cornea Neovascularization Assay

The animal experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Nagasaki University School of Medicine. The mouse cornea assay for *in vivo* angiogenesis was performed as described (25). Sucralfate (BM Research a/s, Vaelose, Denmark) and BSA with or without FGF-2 and GcMAF were dissolved in sterile water and mixed with hydron polymer type NCC in ethanol (IFN Science, New Brunswick, NJ). After centrifugation under vacuum, the viscous mixture was pasted onto sterile nylon mesh and air-dried. The nylon strings were removed from the mesh by forceps to obtain the dried pellets made in each pore of the nylon mesh.

A micropocket was made with a small surgical knife 1 mm from the lymbus in one cornea of each 6-week-old male Std ddY mouse (Charles River Japan, Shizuoka, Japan). Hydron pellets containing 80 ng of FGF-2 and 0.5% BSA with or without 100 pg of GcMAF or a sucralfate pellet containing 0.5% BSA alone was implanted into the corneal micropocket. Six mice received pellets containing BSA only, and eight mice received pellets containing FGF-2 with or without GcMAF. After 5–6 days, the corneas were examined for evidence of neovascularization by light microscopy. Three or four mice per group were anesthetized, and then the abdominal cavity was opened. Saline was infused into the aorta to remove the blood from the anesthetized mice, which were then arterially perfused with water-proof drawing ink (Rotring, Hamburg, Germany) to visualize blood vessels within the eyes. Eyes were excised and fixed in 10% buffered formalin. The irises were then removed, examined microscopically, and photographed by using a camera directly connected to a light microscope with a  $\times 4$  objective.

### Immunoblot Analyses for Mitogen-Activated Protein Kinase (MAPK) Activity

IBE cells were incubated overnight with serum- and growth factor-starved Ham's F-12 medium containing 0.25% BSA, whereas PAE cells were incubated for 60 minutes with medium supplemented with 0.5% FBS before 100 pg/mL of GcMAF was added to the cells for 60 minutes. Cells were then stimulated with FGF-2 (100 ng/mL) or left unstimulated for 5 minutes and 60 minutes. After this last incubation, the culture medium was removed, and the cells were immediately lysed in sodium dodecyl sulfate (SDS)-sample buffer and boiled. Two hundred micrograms of total protein per lysate was separated by electrophoresis on 9% SDS-polyacrylamide gels. Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Membranes were incubated with antiphosphorylated (activated) MAPK polyclonal antibody (New England BioLabs, Beverly, MA) or anti-Erk1/2 antibody (Upstate Biotechnologies, Inc., Lake Placid, NY) and then with

a species-specific peroxidase-conjugated secondary antibody. Proteins were visualized using enhanced chemiluminescence reagents (Amersham Biosciences UK Limited, Buckinghamshire, U.K.). The membranes were stripped between each probing, as described (22).

### **In Vitro Kinase Assay for c-Fyn Activity**

IBE cells, grown in 6-cm dishes, were serum- and growth supplement-starved overnight. The culture medium was replaced with Ham's F-12 medium containing 0.25% BSA with or without GcMAF (100 pg/mL), and the cells were incubated for 60 minutes. Some cell cultures then were left unstimulated or were stimulated with FGF-2 (100 ng/mL) for 10 minutes. Cells were washed with ice-cold Tris-buffered saline (pH 7.5) and lysed with Nonidet P-40 (NP40) lysis buffer (50 mM HEPES [pH 7.5], 150 mM NaCl, 10% glycerin, 1% NP40, 100 U/mL aprotinin, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM orthovanadate) on ice. After the lysates were clarified by centrifugation, they were immunoprecipitated with a c-Fyn antibody (Santa Cruz Biotechnology, Santa Cruz, CA), according to the manufacturer's recommended concentration and protocol, and then incubated with protein-A agarose (Pierce, Rockford, IL) beads. The antigen-antibody complexes bound to the beads were washed four times with NP40 lysis buffer, twice with Tris-buffered saline, and twice with kinase buffer (25 mM Tris-HCl [pH 7.4], containing 10 mM MnCl<sub>2</sub> and 2 mM MgCl<sub>2</sub>).

To measure the c-Fyn kinase activity, the kinase buffer was supplemented with 0.37 MBq/sample of [ $\gamma$ -<sup>32</sup>P]ATP and 0.5  $\mu$ g/sample of acid-denatured rabbit muscle enolase (Roche Diagnostics) and incubated with immunoprecipitates for 10 minutes at room temperature. The reaction was stopped by the addition of 2 $\times$  sample buffer. The samples were boiled, and the eluted proteins were then separated by electrophoresis on 9% SDS-polyacrylamide gels. Gels were incubated with destain (10% methanol and 7% glacial acetic acid) for 30 minutes, fixed with 2.5% glutaraldehyde, rinsed with water, treated with 1 M KOH for 30 minutes at 55 °C to remove phosphorylated serine residues, and then incubated with destain for 30 minutes at room temperature. Gels were dried and analyzed for radioactivity of particular proteins by using a Bio Imager BAS 5000 (Fuji, Tokyo, Japan).

### **Statistical Analysis**

For the cell proliferation assays, data were analyzed with Friedman's test (26) to confirm their suitability for Dunnett's test and then further examined by Dunnett's test (27). Data from the chemotaxis assays were analyzed by the Kruskal-Wallis test (28) and then Scheffé's test (29). Data from the mouse cornea assay were analyzed by Fisher's exact test. All statistical tests were two-sided. All statistical analyses were done with the statistical package StatView for Windows (version 5.0; Abacus Concepts, Inc., Berkeley, CA).

## **RESULTS**

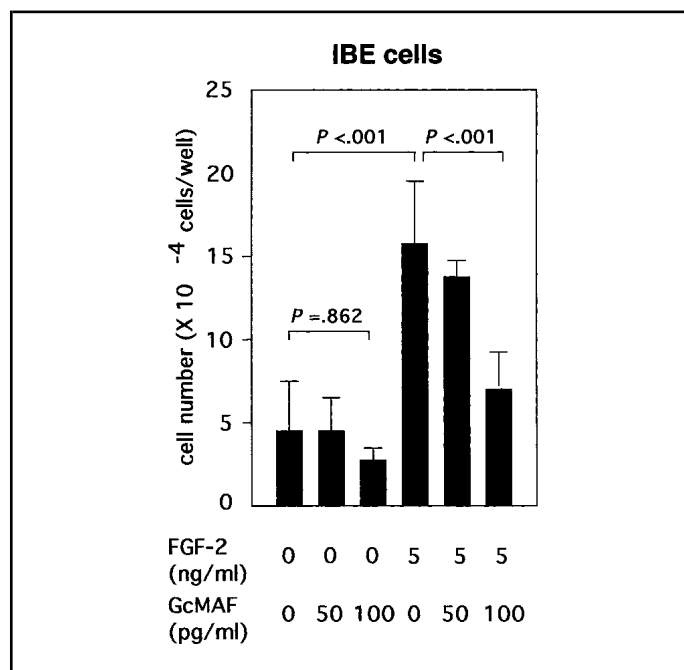
### **Effects of GcMAF on Angiogenic Activities *In Vitro***

During tumor angiogenesis, endothelial cells proliferate, migrate, and form new vessels or tubes. Although macrophages activated by GcMAF are highly tumoricidal, we hypothesized that GcMAF may itself have direct antiangiogenic activity. We examined whether GcMAF could directly inhibit endothelial cell

proliferation, migration (chemotaxis), or tube formation. IBE cells cultured in the presence of FGF-2 (5 ng/mL) for 3 days proliferated 3.6-fold (95% CI = 3.0-fold to 4.2-fold) more than untreated control cells (Fig. 1). Although IBE cell proliferation was not statistically significantly affected by GcMAF alone, FGF-2-stimulated IBE cell proliferation was dose-dependently reduced by GcMAF (Fig. 1). The proliferation of FGF-2-stimulated IBE cells treated with GcMAF (100 pg/mL) was inhibited to 73.1% (95% CI = 67.3% to 78.9%) that of FGF-2-stimulated IBE cells. The data suggest that GcMAF inhibited the FGF-2-mediated IBE cell proliferation.

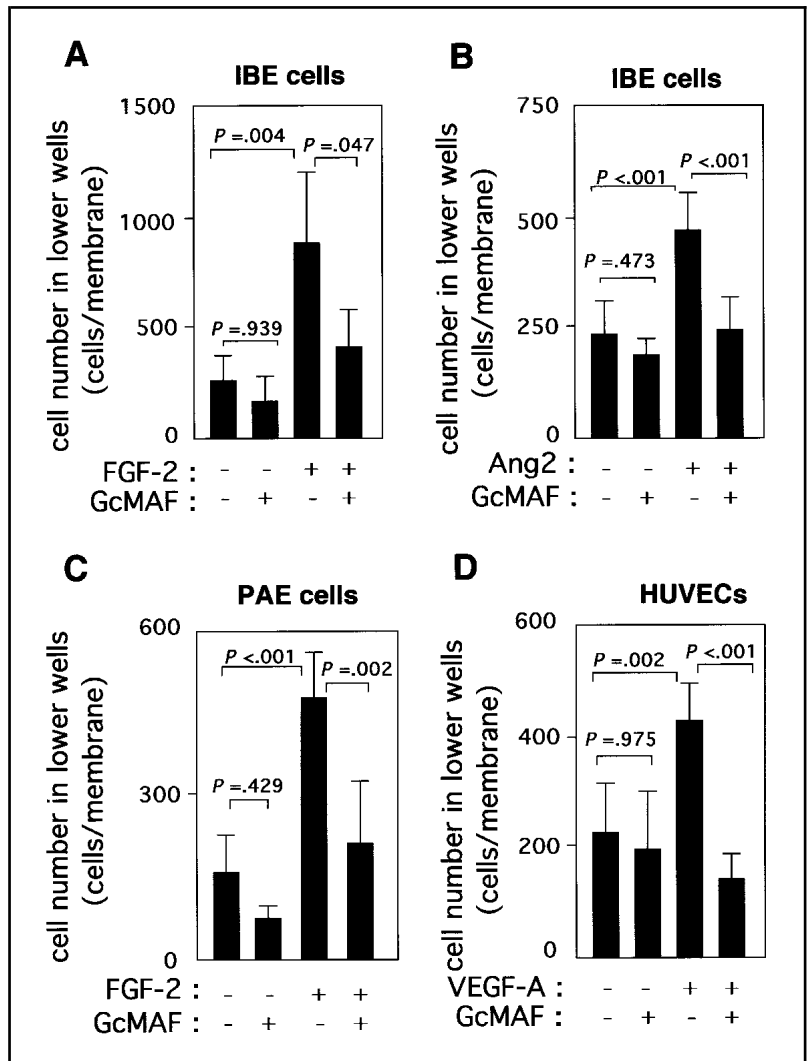
FGF-2 and Ang2 induce chemotaxis of IBE cells (22,24). We therefore next examined whether GcMAF could affect IBE cell chemotaxis. FGF-2 stimulated chemotaxis 3.7-fold (95% CI = 2.8-fold to 4.5-fold) and Ang2 stimulated chemotaxis approximately 2.1-fold (95% CI = 1.8-fold to 2.3-fold) that in untreated IBE cells (Fig. 2, A and B). GcMAF alone did not statistically significantly affect chemotaxis of IBE cells ( $P = .862$ ). FGF-2-stimulated or Ang2-stimulated IBE cell chemotaxis was reduced to almost basal levels (i.e., the level of untreated cells) when IBE cells were cultured in the presence of GcMAF (Fig. 2, A and B).

To determine whether the effects of GcMAF were specific to IBE cells, we assessed the chemotactic response of two other endothelial cell types, PAE cells and HUVECs. FGF-2 and VEGF-A stimulate chemotaxis of PAE cells and HUVECs, respectively. Compared with untreated cells, FGF-2 stimulated chemotaxis of PAE cells 3.2-fold (95% CI = 2.4-fold to 4.0-fold) (Fig. 2, C). GcMAF inhibited chemotaxis of unstimulated PAE cells by 50.0% (95% CI = 40.1% to 59.1%) and inhibited chemotaxis of FGF-2-stimulated PAE cells by 90.4% (95% CI



**Fig. 1.** Effect of Gc protein macrophage activating factor (GcMAF) on fibroblast growth factor-2 (FGF-2)-mediated proliferation of murine IBE cells. IBE cells were cultured with or without FGF-2 (5 ng/mL) in the presence or absence of GcMAF (50 pg/mL or 100 pg/mL) at 33 °C for 3 days. Cell number was counted by a hemocytometer. Bars represent mean absolute cell number  $\pm$  95% confidence intervals for triplicate wells from a single experiment. The experiment was repeated twice, with similar results.

**Fig. 2.** Effect of Gc protein macrophage activating factor (GcMAF) on endothelial cell chemotaxis toward fibroblast growth factor-2 (FGF-2), angiopoietin 2 (Ang2), and vascular endothelial growth factor (VEGF)-A. Cells were seeded onto coated Transwell membranes and then inserted into wells containing the chemoattractant in the presence or absence of GcMAF (100 pg/mL). Murine IBE cells were seeded onto fibronectin-coated Transwell membranes, and the chemoattractants were FGF-2 (100 ng/mL) (A) or Ang2 (1  $\mu$ g/mL) (B); porcine PAE cells were seeded onto type 1 collagen-coated Transwell membranes, and the chemoattractant was FGF-2 (100 ng/mL) (C); and human umbilical vein endothelial cells (HUVECs) were seeded onto gelatin-coated Transwell membranes, and the chemoattractant was VEGF-A (20 ng/mL) (D). All cells were cultured for 4 hours, and the number of cells that had migrated onto the lower surface of Transwell inserts was then counted. Bars represent the mean value of migrated cells from quadruplicate wells  $\pm$  95% confidence intervals. The experiment was performed three times, with similar results. Statistical significance of the differences between groups was determined by the Kruskal–Wallis test, followed by Scheffé’s test.



= 72.5% to 106.7%) (Fig. 2, C). VEGF-A stimulated primary HUVEC chemotaxis by 2.1-fold (95% CI = 1.8-fold to 2.4-fold). GcMAF had marginal effects on untreated HUVECs and inhibited VEGF-A-stimulated HUVEC chemotaxis by 77.0% (95% CI = 74.2% to 81.9%). These results indicate that inhibition of chemotaxis toward angiogenic factors is not limited to IBE cells.

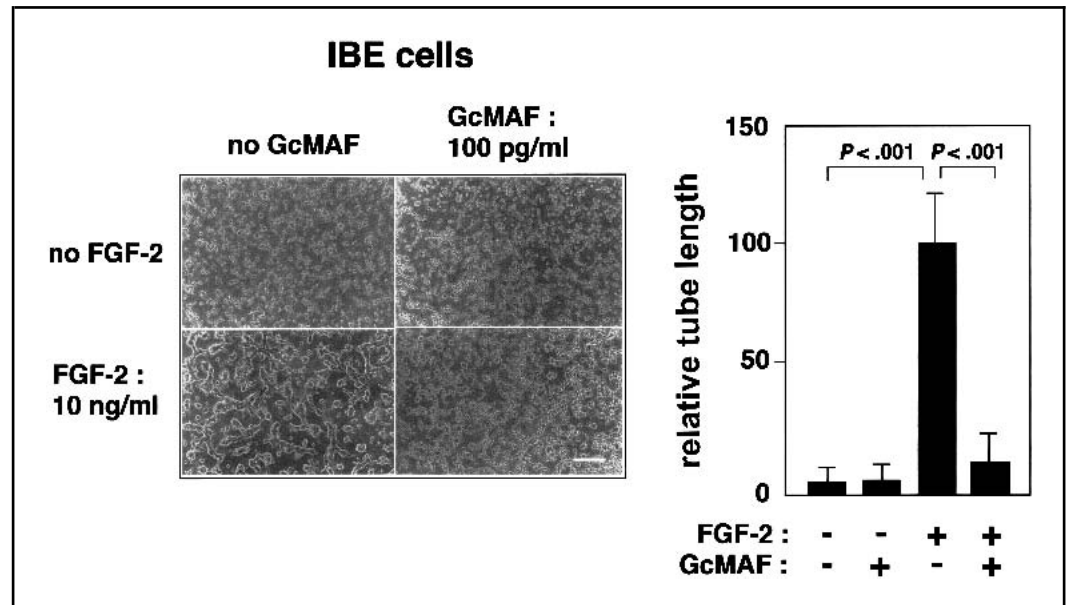
We next assessed the *in vitro* angiogenic property of tube formation. When cultured between two layers of collagen gel in the presence of FGF-2, IBE cells can form lumen-containing tube-like structures (21,23). After FGF-2 treatment for 2 hours, the cells begin to form aggregates, and then aggregated cells start to sprout and fuse to form tube-like structures (30). We cultured IBE cells in the presence and absence of FGF-2 with and without GcMAF for 18 hours and then quantified the relative tube length in each culture. IBE cells cultured in the presence of FGF-2 formed tube-like structures that had an approximate relative tube length that was 13.0-fold (95% CI = 11.4-fold to 14.5-fold) longer than that of untreated cells (Fig. 3). Although GcMAF treatment did not affect FGF-2-mediated cell survival, GcMAF treatment did affect aggregate formation and subsequent sprouting and tube-like structure formation, reducing the relative tube length by 87.3% (95% CI = 84.6% to 90.0%) (Fig. 3). From the results of these *in vitro* experiments, we conclude that GcMAF exhibits a spectrum of inhibitory ac-

tions on the angiogenic responses of cultured endothelial cells. During these assays, and in agreement with previous observations (1,14,16), GcMAF had no apparent cytotoxic effects on cultured endothelial cells.

#### Effects of GcMAF on Angiogenic Activities *In Vivo*

To test the effect of GcMAF on *in vivo* angiogenesis, we performed a mouse cornea micropocket assay (25). Because the mouse cornea is typically without visible signs of vascularization, this assay provides a means to monitor and measure new vessel formation. After 5–6 days, the corneas of mice that received control pellets containing BSA without FGF-2 had no evidence of induced neovascularization (Fig. 4), whereas the corneas of mice that received FGF-2-containing pellets had areas of neovascularization, with newly formed blood vessels infiltrating toward the pellet (Fig. 4 and Table 1). By contrast, the corneas of mice treated with pellets containing both FGF-2 and GcMAF had little evidence of neovascularization (Fig. 4 and Table 1). After quantifying the number of infiltrating vessels as a measure of neovascularization and comparing the differences among the groups (Table 1), we found a statistically significant difference between the number of vessels observed in mice that received FGF-2 alone compared with those that received FGF-2 and GcMAF ( $P = .007$ ). These results suggest that GcMAF has antiangiogenic activity *in vivo*.

**Fig. 3.** Effect of Gc protein macrophage activating factor (GcMAF) on fibroblast growth factor-2 (FGF-2)-induced murine IBE cell tube formation. IBE cells were grown between two collagen gel layers in the presence or absence of FGF-2 (10 ng/mL) and GcMAF (100 pg/mL) in each well of a 12-well plate. The tube length was measured after 18 hours. To quantify the tube length, three random phase-contrast photomicrographs per well were taken, and the length of the tube was measured by using NIH Image software (version 1.64). Tube length obtained from FGF-2-stimulated cells was set to 100 arbitrary units. **Bar** = 100  $\mu$ m. A representative experiment is shown. The data were similar in three independent experiments.

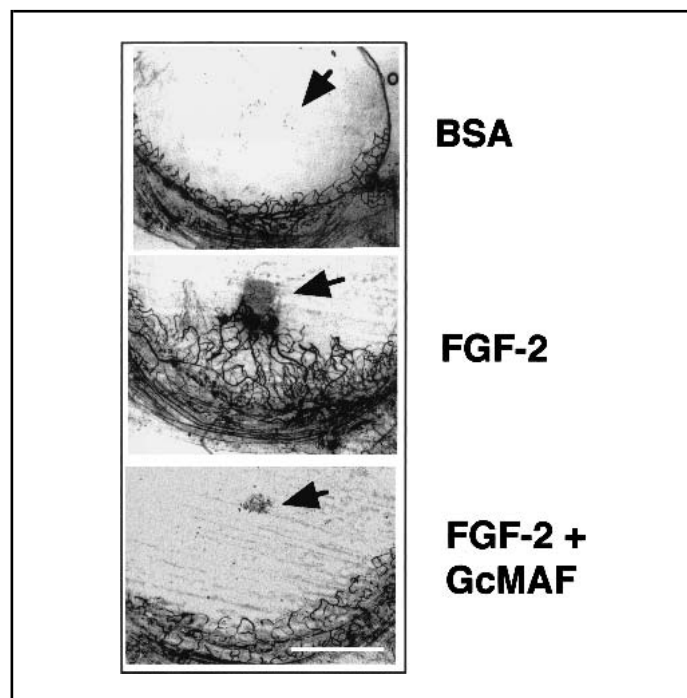


### Effect of Antibodies Against the CD36 Receptor and GcMAF Activity

GcMAF activates macrophages and inhibits the angiogenic properties of endothelial cells. One of the common features of macrophages and endothelial cells is the uptake of DiI-Ac-LDL *via* scavenger receptors, including CD36, which are expressed by both macrophages and endothelial cells (31–33). Uptake of DiI-Ac-LDL could be detected in IBE cells, PAE cells, and HUVECs (data not shown), suggesting that these cells express

scavenger receptors. We therefore examined the effect of DiI-Ac-LDL on GcMAF-mediated inhibition of endothelial cell chemotaxis. Although DiI-Ac-LDL (5  $\mu$ g/mL) alone had no effect on the growth factor-stimulated chemotaxis of IBE cells, PAE cells, and HUVECs, it completely blocked GcMAF-mediated inhibition of chemotaxis by these cells (data not shown). These results suggest that GcMAF may use scavenger receptors for entry into endothelial cells.

CD36, a receptor for the antiangiogenic factor TSP-1, is one of the scavenger receptors expressed commonly by macrophages and endothelial cells (34). We first examined the effect of monoclonal antibodies against CD36 on FGF-2- and VEGF-A-mediated endothelial cell chemotaxis (Fig. 5, A and B). The presence of control or specific anti-CD36 antibodies did not affect the chemotaxis of unstimulated or stimulated IBE cells or HUVECs. Compared with stimulated cells, TSP-1 inhibited chemotaxis of FGF-2-stimulated IBE cells by 137.6% (95% CI = 106.2% to 148.0%) and of VEGF-A-stimulated HUVECs by 118.0% (95% CI = 100.9% to 130.9%). However, monoclonal antibodies against murine and human CD36 blocked the inhibitory action of TSP-1 on the chemotaxis toward FGF-2 by IBE cells and toward VEGF-A by HUVECs.



**Fig. 4.** The effect of Gc protein macrophage activating factor (GcMAF) on fibroblast growth factor-2 (FGF-2)-mediated neovascularization in mouse corneas. Hydon pellets containing either bovine serum albumin (BSA) alone, FGF-2 alone (80 ng), or FGF-2 (80 ng) and GcMAF (100 pg/mL) were implanted into the micropockets of mouse corneas. The images were taken after 6 days. The **arrows** indicate hydon pellets. **Bar** = 1 mm.

**Table 1.** Effect of GcMAF on neovascularization in a mouse cornea micropocket assay\*

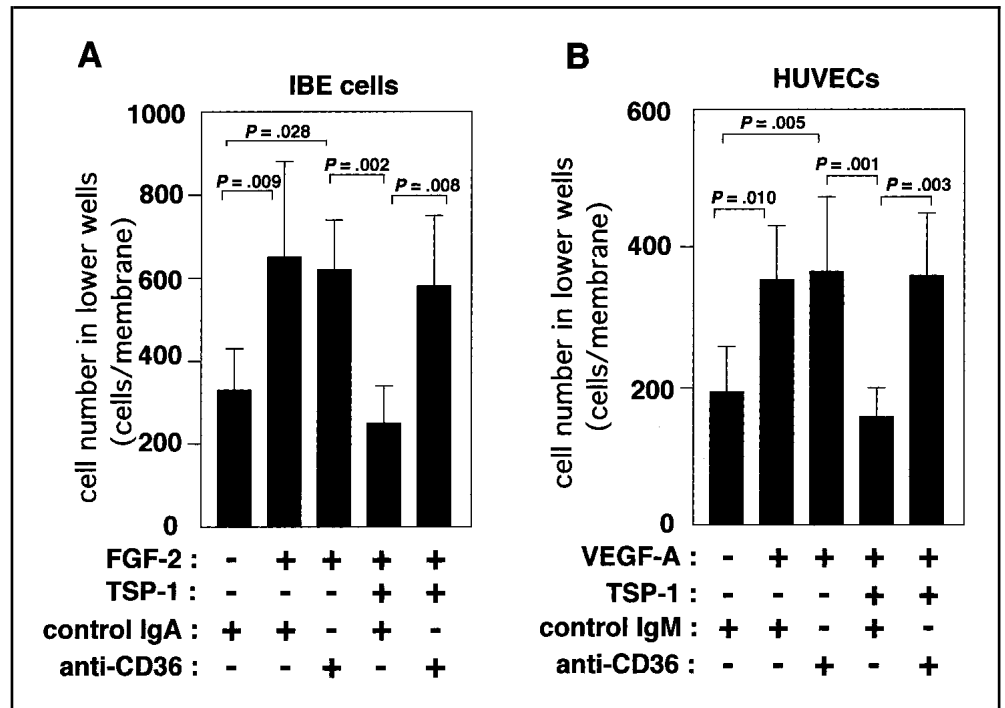
Pellet (concentration)	No. of corneas†		P value‡
	±	+	
BSA (0.5%)	6	0	
FGF-2 (80 ng)	2	6	
FGF-2/GcMAF (80 ng/100 pg)	8	0	.007

\*GcMAF = Gc protein macrophage activating factor; BSA = bovine serum albumin; FGF-2 = fibroblast growth factor 2.

†One cornea per mouse was used. ± = includes no neovascularization or minimal neovascularization (<5 blood vessels invaded pellets); + = prominent neovascularization (>20 blood vessels invaded pellets). In no case was the number of invading blood vessels between 5 and 20.

‡The P value was assessed by Fisher's exact test comparing the neovascularization of corneas from mice that received FGF-2 with those from mice that received FGF-2 and GcMAF.

**Fig. 5.** Effect of monoclonal antibodies against CD36 on the chemotaxis of angiogenic factor-stimulated endothelial cells exposed to thrombospondin-1 (TSP-1). Murine endothelial cells (IBE) or human umbilical vein endothelial cells (HUVECs) were seeded onto Transwell membranes and placed in wells that contained the angiogenic chemoattractant fibroblast growth factor-2 (FGF-2; 10 ng/mL) (A) or vascular endothelial growth factor (VEGF)-A (20 ng/mL) (B) and the antiangiogenic factor TSP-1 (10  $\mu$ g/mL). The indicated wells contained monoclonal antibodies against murine or human CD36 (10  $\mu$ g/mL or 12.5  $\mu$ g/mL, respectively) or isotype control antibodies. **Bars** represent the mean number of migrating cells  $\pm$  95% confidence intervals from quadruplicate wells.



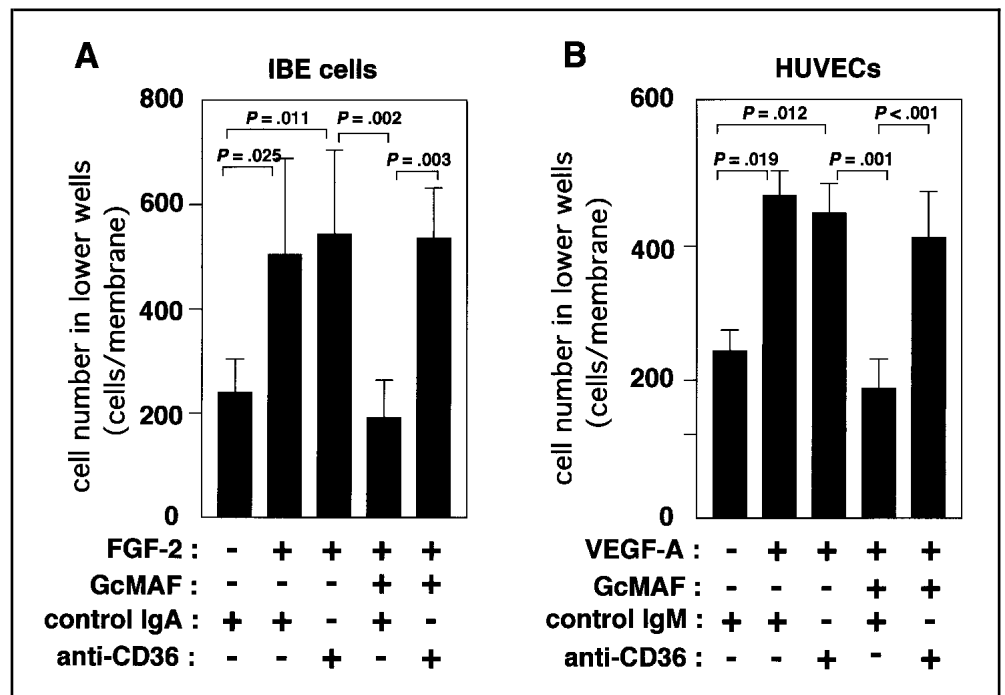
We then performed a similar experiment, replacing TSP-1 with GcMAF. The inhibition of FGF-2-stimulated IBE cell chemotaxis or VEGF-A-stimulated HUVEC chemotaxis by GcMAF was not affected by the presence of control antibodies (Fig. 6). However, anti-CD36 antibodies blocked the inhibitory action of GcMAF on growth factor-induced chemotaxis of IBE cells and HUVECs (Fig. 6). These data suggest that the antiangiogenic action of GcMAF is mediated *via* CD36.

To further investigate the mechanism underlying GcMAF-mediated antiangiogenic activity on endothelial cells, we examined the signal transduction pathways. The antiangiogenic effects of GcMAF were demonstrated against a spectrum of

angiogenic cellular responses and signals. Because the Ras/MAPK pathway is activated by many extracellular stimuli and may play an important role in angiogenic cellular responses (35–38), by western blot analysis we examined the effect of GcMAF on the FGF-2-induced MAPK activation in IBE cells and PAE cells and found that it had no effect (data not shown).

Recently, it has been demonstrated that TSP-1 binding to CD36 leads to sequential activation of the tyrosine kinase c-Fyn and activation of caspase 3-like protease and p38 MAPK, resulting in endothelial cell apoptosis (39). Although GcMAF appears to require CD36 signaling, we were unable to detect activated c-Fyn in GcMAF-treated IBE cells (data not shown).

**Fig. 6.** Effect of monoclonal antibodies against CD36 on the chemotaxis of angiogenic factor-stimulated endothelial cells exposed to Gc protein macrophage activation factor (GcMAF). Murine endothelial cells (IBE) or human umbilical vein endothelial cells (HUVECs) were seeded onto Transwell membranes and placed in wells that contained the angiogenic chemoattractant fibroblast growth factor-2 (FGF-2; 10 ng/mL) (A) or vascular endothelial growth factor (VEGF)-A (20 ng/mL) (B) and GcMAF (100  $\mu$ g/mL). Some wells contained monoclonal antibodies against murine or human CD36 (10  $\mu$ g/mL or 12.5  $\mu$ g/mL, respectively) or isotype control antibodies. **Bars** represent the mean number of migrating cells from quadruplicate wells  $\pm$  95% confidence intervals. The results were similar in two independent experiments.



These results indicate that GcMAF transduced signals *via* CD36 by an as yet unknown mechanism.

## DISCUSSION

Our accumulated evidence suggests that GcMAF is a potent factor that activates tumoricidal macrophages, resulting in tumor regression (1,2,18,19). However, Ono et al. (4) reported that tumor-associated macrophages may stimulate angiogenesis, resulting in tumor progression. Whether GcMAF may stimulate angiogenic macrophages is unknown. Thus, we began to investigate effects of GcMAF on endothelial cells. In the present study, we found that GcMAF has direct antiangiogenic effects on endothelial cells *in vitro* and *in vivo*. We found that human GcMAF was effective against different endothelial cell types, including different species (human, porcine, and murine) and tissue origin (brain, aorta, umbilical cord, and cornea), against different chemotactic stimuli (FGF-2, Ang2, and VEGF-A), and in different *in vitro* and *in vivo* models. Because tumor cells can produce a variety of angiogenic growth factors, such as FGF-2, VEGF-A, and Ang2, that promote tumor growth and progression (10–12,22), and the angiogenic signals initiated through the receptors for these signals were all inhibited by GcMAF, the antiangiogenic activity of GcMAF is complementary to the potent tumoricidal activity of GcMAF-primed macrophages.

The antiangiogenic effects of GcMAF were demonstrated in a spectrum of angiogenic cellular responses and signals. Because the Ras/MAPK pathway is induced by many extracellular stimuli and is an important component of angiogenic cellular responses (35–38), we assessed the effects of GcMAF on MAPK induction in the endothelial cell lines (IBE cells and PAE cells). GcMAF treatment showed no effect on MAPK induction in growth factor-treated endothelial cells, as determined by western blot analyses (data not shown). Recently, it has been demonstrated that the binding of TSP-1 to CD36 leads to the sequential activation of the tyrosine kinase c-Fyn, the caspase 3-like protease, and p38 MAPK, resulting in endothelial cell apoptosis (39). Although GcMAF appears to signal through the CD36 receptor, we were unable to detect c-Fyn kinase activation in GcMAF-treated IBE cells (data not shown). In fact, GcMAF-treated endothelial cells neither showed morphologic changes nor detached from the plastic surface, suggesting that GcMAF did not cause apoptosis in these endothelial cells. It is therefore possible that GcMAF uses a signaling pathway that is distinct from the Ras/MAPK and c-Fyn/p38 MAPK signaling pathways.

Although Mantell et al. (19) reported that  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> has antiangiogenic activity in VEGF-treated bovine aortic endothelial cells, it is unlikely that contamination with this vitamin D<sub>3</sub> metabolite explains our observations. The GcMAF precursor, Gc protein, was purified by the use of 25-hydroxyvitamin D<sub>3</sub> affinity chromatography (which specifically binds vitamin D binding sites of the protein) (20), which should eliminate any of the vitamin D<sub>3</sub> derivatives that contaminate the GcMAF preparation. Indeed, our established purification protocol results in a preparation that is more than 99% pure GcMAF. Furthermore, we found that  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> had no inhibitory effect on FGF-2-induced proliferation and tube formation of the IBE cells used in the present study (data not shown), indicating that the antiangiogenic activity of GcMAF was unlikely to be the result of contamination from vitamin D<sub>3</sub> derivatives in the preparation.

In the present study, we found that GcMAF has antiangiogenic activity in endothelial cells that appears to be mediated through the CD36 receptor. This scavenger receptor is used by TSP-1 to inhibit angiogenesis (34). However, although both of these antiangiogenic factors appear to act on endothelial cells *via* the CD36 receptor, they appear to do so through different signaling pathways. Because angiogenesis is essential for solid tumor growth, the scavenger receptor CD36 may be a potential target for antiangiogenic therapies.

## REFERENCES

- (1) Yamamoto N, Naraparaju VR. Immunotherapy of BALB/c mice bearing Ehrlich ascites tumor with vitamin D-binding protein-derived macrophage activating factor. *Cancer Res* 1997;57:2187–92.
- (2) Korbek M, Naraparaju VR, Yamamoto N. Macrophage-directed immunotherapy as adjuvant to photodynamic therapy of cancer. *Br J Cancer* 1997;75:202–7.
- (3) Polverini PJ. Role of macrophages in tumor angiogenesis. In: Voetst EE, D'Amore PA, editors. *Tumor angiogenesis and microcirculation*. 1<sup>st</sup> ed. New York (NY): Marcel Dekker; 2001. p. 335–47.
- (4) Ono M, Torisu H, Fukushi J, Nishie A, Kuwano M. Biological implications of macrophage infiltration in human tumor angiogenesis. *Cancer Chemother Pharmacol* 1999;43:S69–71.
- (5) Nishie A, Ono M, Shono T, Fukushi J, Otsubo M, Onoue H, et al. Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human glioma. *Clin Cancer Res* 1999;5:1107–13.
- (6) Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj R, Sorg C. Macrophages and angiogenesis. *J Leukoc Biol* 1994;55:410–22.
- (7) Zbar B, Tanaka T. Immunotherapy of cancer: regression of tumors after intralesional injection of living *Mycobacterium bovis*. *Science* 1971;172:271–3.
- (8) Yamamoto N, St. Clare DA Jr, Homma S, Ngwenya BZ. Activation of mouse macrophages by alkylglycerols, inflammation products of cancerous tissues. *Cancer Res* 1988;48:6044–9.
- (9) Knight DR, Hunt TK, Scheuenstuhl H, Halliday BJ, Werb Z, Banda MJ. Oxygen tension regulates the expression of angiogenesis factor by macrophages. *Science* 1983;221:1283–5.
- (10) Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other diseases. *Nature Med* 1995;1:27–31.
- (11) Ferrara N, Alitalo K. Clinical applications of angiogenic growth factors and their inhibitors. *Nature Med* 1999;5:1359–64.
- (12) Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature* 2000;407:249–57.
- (13) Homma S, Millman I, Yamamoto N. A serum factor for macrophage activation after *in vitro* dodecylglycerol treatment of mouse lymphocytes. *Immunol Cell Biol* 1990;68:137–42.
- (14) Yamamoto N, Homma S. Vitamin D3 binding protein (group-specific component) is a precursor for the macrophage-activating signal factor from lysophosphatidylcholine-treated lymphocytes. *Proc Natl Acad Sci U S A* 1991;88:8539–43.
- (15) Yamamoto N, Kumashiro R. Conversion of vitamin D3 binding protein (group-specific component) to a macrophage activating factor by the stepwise action of beta-galactosidase of B cells and sialidase of T cells. *J Immunol* 1993;151:2794–802.
- (16) Yamamoto N. Structural definition of a potent macrophage activating factor derived from vitamin D3-binding protein with adjuvant activity for antibody production. *Mol Immunol* 1996;33:1157–64.
- (17) Yamamoto N, Naraparaju VR, Neil RN, Suyama H, Nakazato H. Therapeutic efficiency of vitamin D3-binding protein-derived macrophage activating factor for prostate, breast, and colon cancers. *Proc Am Assoc Cancer Res* 1997;38:31.
- (18) Yamamoto N, Urade M. Therapeutic efficacy of Gc protein derived macrophage activating factor for adenocarcinoma. *Proc Am Assoc Cancer Res* 2002;43:914.
- (19) Mantell DJ, Owens PE, Bundred NJ, Mawer EB, Canfield AE.  $1\alpha,25$ -dihydroxyvitamin D(3) inhibits angiogenesis *in vitro* and *in vivo*. *Circ Res* 2000;87:214–20.

- (20) Link RP, Perlman KL, Pierce EA, Schnoes HK, DeLuca HF. Purification of human serum vitamin D-binding protein by 25-hydroxyvitamin D3-Sepharose chromatography. *Anal Biochem* 1986;157:262–9.
- (21) Kanda S, Landgren E, Ljungstrom M, Claesson-Welsh L. Fibroblast growth factor receptor 1-induced differentiation of an endothelial cell line established from tsA58 large T transgenic mice. *Cell Growth Differ* 1996; 7:383–95.
- (22) Mochizuki Y, Nakamura T, Kanetake H, Kanda S. Angiopoietin-2 stimulates migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn. *J Cell Sci* 2002;115:175–83.
- (23) Rahmanian M, Pertoft H, Kanda S, Christofferson R, Claesson-Welsh L, Heldin P. Hyaluronan oligosaccharides induce tube formation of a brain endothelial cell line *in vitro*. *Exp Cell Res* 1997;237:223–30.
- (24) Kanda S, Lerner EC, Tsuda S, Shono T, Kanetake H, Smithgall TE. The non-receptor protein-tyrosine kinase c-Fes is involved in FGF-2-induced chemotaxis of murine brain capillary endothelial cells. *J Biol Chem* 2000; 275:10105–11.
- (25) Kanda S, Shono T, Tomasini-Johanson B, Klint P, Saito Y. Role of thrombospondin-1-derived peptide, 4N1K, in FGF-2-induced angiogenesis. *Exp Cell Res* 1999;252:262–72.
- (26) Campbell RA. A comparison of the Quade and Friedman tests to the unbalanced two-way analysis of variance with biomedical data. *Comput Biol Med* 1988;18:441–7.
- (27) Dunnett CW, Gent M. An alternative to the use of two-sided tests in clinical trials. *Stat Med* 1996;15:1729–38.
- (28) Scheirer CJ, Ray WS, Hare N. The analysis of ranked data derived from completely randomized factorial designs. *Biometrics* 1976;32:429–34.
- (29) Edwards D, Berry JJ. The efficiency of simulation-based multiple comparisons. *Biometrics* 1987;43:913–28.
- (30) Kanda S, Tomasini-Johanson B, Klint P, Dixelius J, Rubin K, Claesson-Welsh L. Signaling via fibroblast growth factor receptor-1 is dependent on extracellular matrix in capillary endothelial cell differentiation. *Exp Cell Res* 1999;248:203–13.
- (31) Platt N, Gordon S. Is the class A macrophage scavenger receptor (SR-A) multifunctional?—The mouse's tale. *J Clin Invest* 2001;108:649–54.
- (32) Febbraio M, Hajjar DP, Silverstein RL. CD36: a class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. *J Clin Invest* 2001;108:785–91.
- (33) Krieger M. Scavenger receptor class B type I is a multiligand HDL receptor that influences diverse physiologic systems. *J Clin Invest* 2001;108: 793–7.
- (34) Dawson DW, Pearce SF, Zhong R, Silverstein RL, Frazier WA, Bouck NP. CD36 mediates the *in vitro* inhibitory effects of thrombospondin-1 on endothelial cells. *J Cell Biol* 1997;138:707–17.
- (35) Klint P, Kanda S, Kloog Y, Claesson-Welsh L. Contribution of Src and Ras pathways in FGF-2-induced endothelial differentiation. *Oncogene* 1999;18: 3354–64.
- (36) Tanaka K, Abe M, Sato Y. Roles of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in the signal transduction of basic fibroblast growth factor in endothelial cells during angiogenesis. *Jpn J Cancer Res* 1999;90:647–54.
- (37) Giuliani R, Bastaki M, Coltrini D, Presta M. Role of endothelial cell extracellular signal-regulated kinase1/2 in urokinase-type plasminogen activator upregulation and *in vitro* angiogenesis by fibroblast growth factor-2. *J Cell Sci* 1999;112:2597–606.
- (38) Takahashi T, Ueno H, Shibuya M. VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. *Oncogene* 1999;18:2221–30.
- (39) Jimenez B, Volpert OV, Crawford SE, Febbraio M, Silverstein RL, Bouck N. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. *Nat Med* 2000;6:41–8.

## NOTES

Supported by a grant-in-aid from the Japan Society for the Promotion of Science.

We are grateful to Dr. C.-H. Heldin for a kind gift of PAE cells. We also appreciate T. Shimogama and M. Yoshimoto for their outstanding and skillful help.

Manuscript received January 24, 2002; revised June 25, 2002; accepted July 8, 2002.