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Human Pituitary Tumor-Transforming Gene Induces Angiogenesis*

HIROKI ISHIKAWA, ANTHONY P. HEANEY, RUN YU, GREGORY A. HORWITZ, AND SHLOMO MELMED

Cedars-Sinai Research Institute, University of California School of Medicine, Los Angeles, California 90048

ABSTRACT

Angiogenesis is a key determinant and rate-limiting step in tumor progression and metastatic spread. As pituitary tumor-transforming gene (PTTG) induces basic fibroblast growth factor (bFGF), we tested angiogenesis induced by conditioned medium (CM) derived from NIH-3T3 transfectants overexpressing wild-type human PTTG (WT-hPTTG-CM). We also examined the relationship between PTTG expression and tumor vascularity in a series of human tumors. CM from Wt-hPTTG transfectants induced proliferation, migration, and tube formation of human umbilical vein endothelial cells *in vitro*. The bFGF concentration in WT-hPTTG-CM was elevated (10.5 ± 0.56) compared with CM from nontransfected NIH-3T3 cells (3.3 ± 0.56 pg/mL), and addition of anti-bFGF antibody to CM abrogated these angiogenesis markers ($P < 0.01$). *In vivo*, concentrated WT-hPTTG-CM induced chick chorioallantoic membrane spoke-wheel-like appearances. Moreover, CM derived from hPTTG transfectants harboring a point mutation on the C-terminus proline-rich region of PTTG induced weaker angiogenic activity than WT-hPTTG-CM ($P < 0.01$). Thus, human PTTG induces an angiogenic phenotype in both *in vitro* and *in vivo* angiogenesis models, and high PTTG messenger ribonucleic acid is associated with an angiogenic phenotype in human tumors. These PTTG-directed angiogenic actions may be mediated through bFGF, which also contributes to tumor growth. (*J Clin Endocrinol Metab* **86:** 867–874, 2001)

THE DEVELOPMENT of new blood vessel growth has been implicated as a mechanism mediating pituitary tumorigenesis (1–4). Pituitary tumor-transforming gene (PTTG), originally isolated from rat pituitary GH_4 cells (5), encodes a 202-amino acid protein and is located on chromosome 5q33. NIH-3T3 cells overexpressing PTTG form colonies in soft agar and tumors in nude mice. These transformed fibroblasts also exhibit higher basic fibroblast growth factor (bFGF) messenger ribonucleic acid (mRNA) expression and secrete higher levels of bFGF than nontransfected cells (6). A proline-rich P-X-X-P potential SH3 binding motif appears critical to confer PTTG-mediated cell transformation and increased bFGF expression. PTTG-mediated transformation leads to increased bFGF expression, and bFGF, in turn, also regulates PTTG (7). We and others have demonstrated abundant PTTG expression in several human tumor types (8, 9), cancer cell lines, and colorectal carcinoma, where highest PTTG mRNA expression was observed in invasive colorectal tumors (10). Estrogen, a known inducer of angiogenesis, also induces pituitary and thyroid PTTG levels (7, 11). As we have reported concordant PTTG and bFGF expression in human pituitary tumors (10) and in animal pituitary tumor models (7), we sought to determine the interrelationship of new blood vessel growth with PTTG functions. We therefore investigated hPTTG involvement in the angiogenic process by testing the proliferation, migration, and tube formation of human umbilical vein endothelial cells (HUVEC) *in vitro* and *in vivo* chick chorioallantoic membrane new vessel formation after treatment with conditioned medium (CM) derived from wild-type human (h) PTTG-transfected NIH-3T3 cells.

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Address all correspondence and requests for reprints to: Shlomo Melmed, M.D., Cedars-Sinai Medical Center, 8700 Beverly Boulevard, Room 2015, Los Angeles, California 90048. E-mail: melmed@csmc.edu.

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Materials and Methods

Materials

Rat tail collagen type I was obtained from Sigma (St. Louis, MO), and the modified Boyden chamber and Transwells were obtained from Corning, Inc.-Costar (Cambridge, MA). Human recombinant, anti-bFGF antibody, and preimmune goat IgG were purchased from R&D Systems, Inc. (Minneapolis, MN), growth factor-reduced Matrigel basement membrane matrix (GFR Matrigel) was obtained from Becton Dickinson and Co. (Bedford, MA), and fertilized White Leghorn chicken eggs were purchased from Chino Valley Ranchers (Arcadia, CA). All standard chemicals used were of the highest available commercial grade.

Cell culture

NIH-3T3 cells were cultured in low glucose DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% bovine calf serum and antibiotics. HUVECs (Clonetics, San Diego, CA) were grown in endothelial growth medium according to the vendor's instructions and were grown to less than 10 passages for all experiments.

Stably transfected NIH-3T3 cells (2×10^6) expressing wild-type (WT), mutant hPTTG, and vector alone, as previously described (6), were plated in 100-mm gelatinized dishes. Western blot analysis confirmed that equivalent amounts of PTTG protein were expressed in both WT and mutant PTTG-transfected cells. After 24 h, the maintenance medium was replaced with 10 mL serum-free DMEM, and the cells were incubated an additional 48 h. This CM was then harvested wild-type hPTTG (WT-hPTTG-CM)-, mutant hPTTG (M-hPTTG-CM)-, and vector alone (C-CM)-transfected NIH-3T3 cells and from nontransfected NIH-3T3 cells (N-CM), respectively; filtered through a 0.2-μm pore size filter to remove debris; and stored until further study.

bFGF enzyme-linked immunosorbent assay

CM (1 mL) was lyophilized with Speed-Vac (Savant, Farmingdale, NY) and resuspended in 100 μL PBS, and the bFGF concentration was

assayed (Quantikine HS Human FGF Basic Immunoassay Kit, R&D Systems, Inc.).

Endothelial cell proliferation assay

HUVECs were plated onto 48-well gelatinized culture plates at approximately 5000 cells/well for 24 h. Medium was then replaced with equal aliquots of CM derived from cultures of transfected or nontransfected NIH-3T3 cells as described previously. As a positive control, DMEM was enriched with 1 ng/mL recombinant human bFGF, and as a negative control, serum-free DMEM was used. To investigate the activity of bFGF in each CM, 100 ng/mL anti-bFGF antibody or preimmune goat IgG was first added to each CM. After 48 h, HUVEC cells were trypsinized and counted with a Coulter counter (Coulter Electronics, Hialeah, FL). All experiments were performed in triplicate.

Wound migration assay

The wound assay was performed as previously described with some modifications (12). Confluent monolayers of HUVECs in 35-mm gelatinized culture dishes were wounded by pressing a razor blade to cut the cell sheets and mark the plate. The blade was gently moved to one side to remove part of the sheet. Cells were then washed twice with PBS. The transfected NIH-3T3 cell-derived CM described above was applied, and the HUVECs were incubated in CM for an additional 16 h. Cells were then fixed with absolute methanol, stained with Giemsa, and photographed. Migration was quantified by counting cells in 100 × 2500-μm sections from the cut edge under microscopy with an ocular grid. The values represent the mean derived from three random fields. All experiments were repeated in triplicate.

Modified Boyden chamber migration assay

Migration was also measured with 6.5-mm, 8.0-μm Transwells as previously described with some modifications (13). The polycarbonate membrane was coated with 0.1% gelatin (1 h at 37 C). Six hundred microliters of each CM sample were added to the lower chamber and incubated at 37 C for 30 min. Subconfluent HUVECs that had been cultured in the growth factor-free medium for 16 h were harvested, washed, resuspended in serum-free DMEM (100 μL), and added to the upper chamber. After 24-h incubation, all nonmigrant cells were removed from the upper face of the membrane with a cotton swab, and migrant cells on the lower face were fixed with absolute methanol, stained with Giemsa, and photographed. For quantitative analysis, stained cells were subsequently extracted with 10% acetic acid, and absorbance was determined at 595 nm.

Tube forming assay

Assay of the capillary tube-like structure formation of HUVEC was performed with commercial GFR Matrigel. Twenty-four-well plates were thickly coated with 300 μL GFR Matrigel (11 mg/mL) and incubated at 37 C for 30 min to promote gelling. HUVECs suspended in 500-μL aliquots of sample CM were added to each well to bring the final culture to approximately 5×10^4 cells/well. After 24-h incubation, tube formation was evaluated by phase contrast microscopy and photographed by spot color digital camera (W. Nuhsbaum, Inc., McHenry, IL). Digital images were skeletonized with NIH Image software, and pixel numbers were counted as previously described (14). All experiments were repeated in triplicate.

Chrorio-allantoic membrane (CAM) assay

To investigate angiogenic activity of each CM *in vivo*, fertilized White Leghorn chicken eggs were incubated at 37 C without CO_2 in a humidified incubator (15). After 3-day incubation, a round window was opened in the shell, and 3 mL albumin were removed to detach the CAM from the shell. Ten milliliters of CM from WT-hPTTG, M-hPTTG, vector-transfected, and untransfected 3T3 cells were lyophilized with (Speed-Vac) and resuspended in 100 μL PBS. One microgram of bFGF in 5 μL PBS was used as a positive control, and similarly concentrated serum-free DMEM was used as a negative control. On day 9, 5 μL of either concentrated CM or the positive or negative controls were applied to 0.5 mg rat tail collagen type I sponge. Sample-soaked sponges were then placed onto the CAM. On day 13, shell windows were carefully extended, and the sponge and surrounding CAM area were photographed. For quantitative analysis, the number of blood vessels entering the collagen sponges was counted under stereomicroscopy at ×25 magnification. Three eggs were used for each sample, and experiments were repeated in triplicate.

Statistical analysis

Statistical analyses were performed using Student's *t* test. All *P* values were two-tailed, and those less than 0.05 were considered significant.

Results

bFGF concentration in CM

As hPTTG regulates bFGF secretion (6), we measured the bFGF concentration in CM derived from stably transfected cells ($\sim 2 \times 10^6$) after 48-h culture in serum-free medium (Fig. 1). The bFGF concentration in CM harvested from WT-hPTTG transfectants was 10.5 ± 0.56 pg/mL, markedly higher than bFGF levels in CM derived from other transfected cell lines (Mut-hPTTG, 3.3 ± 0.27; control vector (Ctr-vector), 2.3 ± 0.72; normal 3T3 cells, 3.3 ± 0.56 pg/mL; $P < 0.01$). The bFGF concentration in CM from Mut-hPTTG, Ctr-vector-transfected and normal 3T3 cells did not differ. Total cell number and protein concentration at the time of CM collection were similar for each independent cell line or transfectant.

Endothelial cell proliferation assay

HUVECs were cultured in each CM for 48 h, after which cell number was determined (Fig. 2). As expected CM derived from all cell lines exhibited proliferative activity compared with serum-free DMEM. CM from WT-hPTTG-transfected cells induced significantly higher cell proliferation

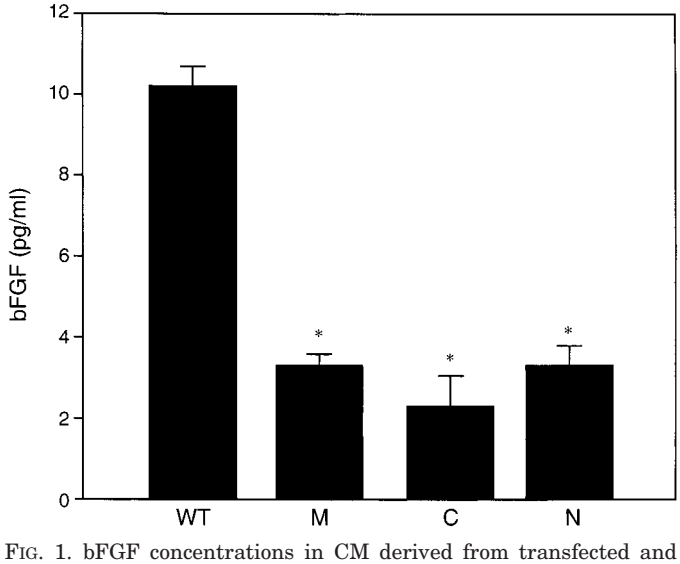

FIG. 1. bFGF concentrations in CM derived from transfected and nontransfected NIH-3T3 cells. WT-PTTG, Mut-hPTTG, or vector-transfected or nontransfected NIH-3T3 cells were incubated in serum-free DMEM for 48 h, and aliquots of the CM were collected. The bFGF concentration was measured by enzyme-linked immunosorbent assay. WT, WT-hPTTG-CM; M, Mut-hPTTG-CM; C, C-CM; N, N-CM. The data shown are the mean ± SD of three separate experiments. *, $P < 0.01$ *vs.* WT-hPTTG-CM.

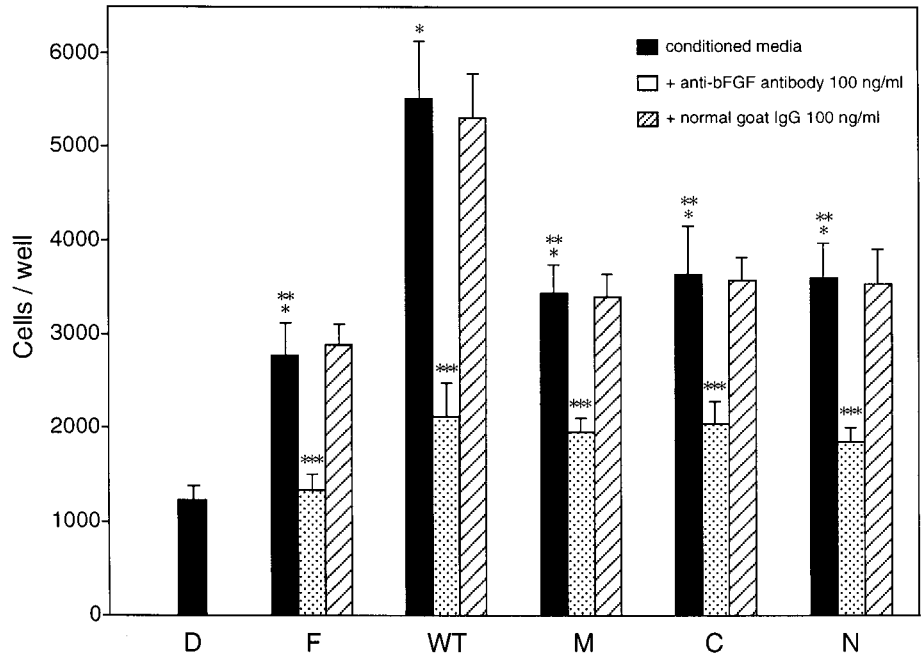

FIG. 2. Endothelial cell proliferation. HUVECs were cultured on 48-well culture plates in 500 μL CM derived from transfected or nontransfected NIH-3T3 cells. Cell numbers were determined after 48-h incubation. The depicted results are the mean ± SD of three separate experiments. D, Serum-free DMEM; F, 1 ng/mL bFGF in DMEM; WT, WT-hPTTG-CM; M, M-hPTTG-CM; C, C-CM; N, N-CM. $P < 0.01$ *vs.* serum-free DMEM (*),WT-hPTTG-CM (**), and respective CM alone (***).

than CM derived from Mut-hPTTG, vector-transfected, and normal 3T3 cells ($P < 0.01$). Addition of anti-bFGF antibody to each CM suppressed proliferation activity by 62% in WT-hPTTG-CM, 43% in M-hPTTG-CM, 44% in C-CM, and 49% in N-CM cells. However, cell proliferation after adding anti-bFGF antibody was still higher than that in serum-free DMEM alone. Proliferation of HUVECs was not altered by adding preimmune goat IgG to each CM, confirming that the induced proliferation was mediated by bFGF.

Endothelial migration in wound assay and Boyden chamber assay

In the wound assay (Fig. 3), migration was quantified by counting the number of HUVECs that migrated into the nonwounded region with a grid marked in 100-μm increments. HUVECs that had been incubated (48 h) in WT-hPTTG-CM migrated further and in greater numbers than HUVECs that had been incubated in CM from the other cell lines harboring Mut-PTTG, vector alone, or untransfected 3T3 cells. Anti-bFGF antibody suppressed activity in all cell lines, but preimmune goat IgG had no effect. Using the modified Boyden chamber assay CM from Wt-hPTTG transfectants induced HUVEC cell migration through membrane pores (Fig. 4; $P < 0.01$). Similar results were obtained when transfected or nontransfected NIH-3T3 cells were plated in the lower chambers and HUVECs plated in the upper chamber in a coculture manner (data not shown). Anti-bFGF antibody suppressed migration activity in all cell lines similarly to that observed in the wound assay. Suppressive effects of bFGF of WT-hPTTG-CM, M-hPTTG-CM, C-CM, and N-CM were 55%, 40%, 43%, and 39% respectively.

Tube forming assay

Matrigel is useful for studying HUVEC attachment and differentiation. As Matrigel itself induces HUVEC differential activity, we used GFR Matrigel to reduce the effect of growth factors from the Matrigel itself. As shown in Fig. 5A, when HUVECs adhered on GFR Matrigel, they aligned with one another and formed tubes resembling a capillary plexus under the influence of differential activity in the CM. Quantitative analysis of HUVEC tube formation (16) revealed that WT-hPTTG-CM enhanced HUVEC tube formation compared with that observed when HUVECs were incubated in CM derived from other cell lines ($P < 0.01$). The morphological changes resembling capillary formation were suppressed by adding anti-bFGF antibody to each CM. Suppressive effects of anti-bFGF antibody on WT-hPTTG-CM, M-hPTTG-CM, C-CM, and N-CM were 74%, 58%, 57%, and 62%, respectively.

CAM assay

Vessel growth of CAM was tested *in vivo* using 9-day-old chick egg embryos. Sample-soaked collagen sponges were loaded on CAM, and neovascularization of surrounding collagen sponges was evaluated after 4-day incubation. As shown in Fig. 6A, application of sponges presoaked in WT-hPTTG-CM induced a spoke-wheel-like appearance that was more evident than CAM vessel formation after application of sponges immersed in the other CMs. The number of detectable blood vessels entering the collagen sponges were counted under stereomicroscopy, and as predicted, all CM samples derived from both transfected and nontransfected NIH-3T3 cells induced stronger angiogenic responses than did serum-free DMEM alone ($P < 0.01$). Application of sponges containing WT-hPTTG-CM to the CAM induced highest angiogenic activity ($P < 0.01$), although higher angiogenic activity was observed when recombinant bFGF (1 μg/egg) was added to the CAM.

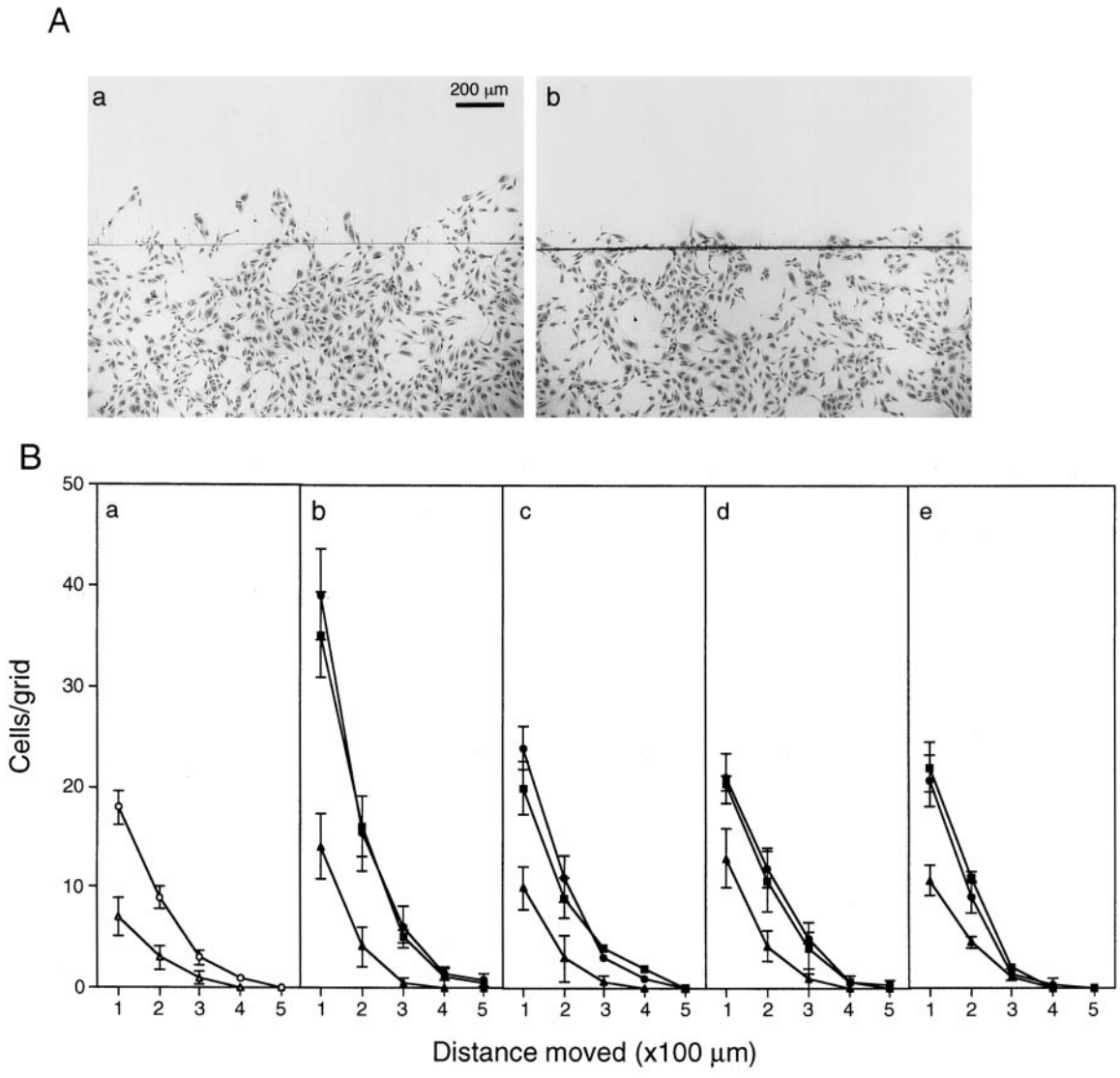

FIG. 3. Migration of HUVECs in wound assay. The wounded monolayer of HUVEC was exposed for 16 h to respective aliquots of CM. Migrated cells were then fixed, stained, and photographed. A, Representative micrographs of migrated HUVECs in WT-hPTTG-CM are shown. a, CM alone; b, CM plus 100 ng/mL anti-bFGF antibody. B, Quantification of migrated HUVECs. The number of cells within a 0.1 × 2.5-mm area in three fields was counted using the original mark made by the razor blade as the origin. The results shown are the average number of cells ± SD per field of three separate experiments. a, Control; b, WT-hPTTG-CM; c, M-hPTTG-CM; d, C-CM; e, N-CM. ○, 1 ng/mL bFGF in DMEM; △, serum-free DMEM; ●, CM alone; ▲, CM plus 100 ng/mL anti-bFGF antibody; ■, CM plus 100 ng/mL preimmune goat IgG.

Discussion

The sequence of events leading to the formation of new from preexisting vessels is highly regulated (17, 18) and involves dissolution of vessel basement membranes and formation of new lumen and pericytes by vascular endothelial cells. During tumor-associated angiogenesis, sustained production of angiogenic factors by cancer cells or indirect macrophage stimulation causes dysregulated immature vessel growth (19). Several assays are useful for studying angiogenesis (17, 20). Although *in vitro* assays allow for control of experimental variables, cellular events contributing to angiogenesis do not occur in isolation, and these assays do not always reflect the totality of *in vivo* angiogenesis. In this study we tested endothelial cell proliferation and migration by using the wound healing assay and modified Boyden chamber assay, and we also examined tube formation of HUVEC cells. WT-hPTTG-CM induced strong angiogenic activity in all of these assays. As similar angiogenic activity was observed using M-hPTTG-CM to C-CM and N-CM, and these were all significantly lower than angiogenic activity mediated by Wt-hPTTG CM, it appears that the proline-rich domains of PTTG are important not only for transforming action, but also for PTTG-mediated angiogenic properties.

We previously reported that wild-type hPTTG-transfected NIH-3T3 cells induce bFGF mRNA expression and secretion in CM (6). bFGF is angiogenic, inducing endothelial cell proliferation and migration (21–23). Vascular endothelial cell growth factor (VEGF) plays a key role in tumor angiogenesis, and although hPTTG transfected NIH-3T3 do not express VEGF efficiently *in vitro* (6), we have shown *in vivo* pituitary

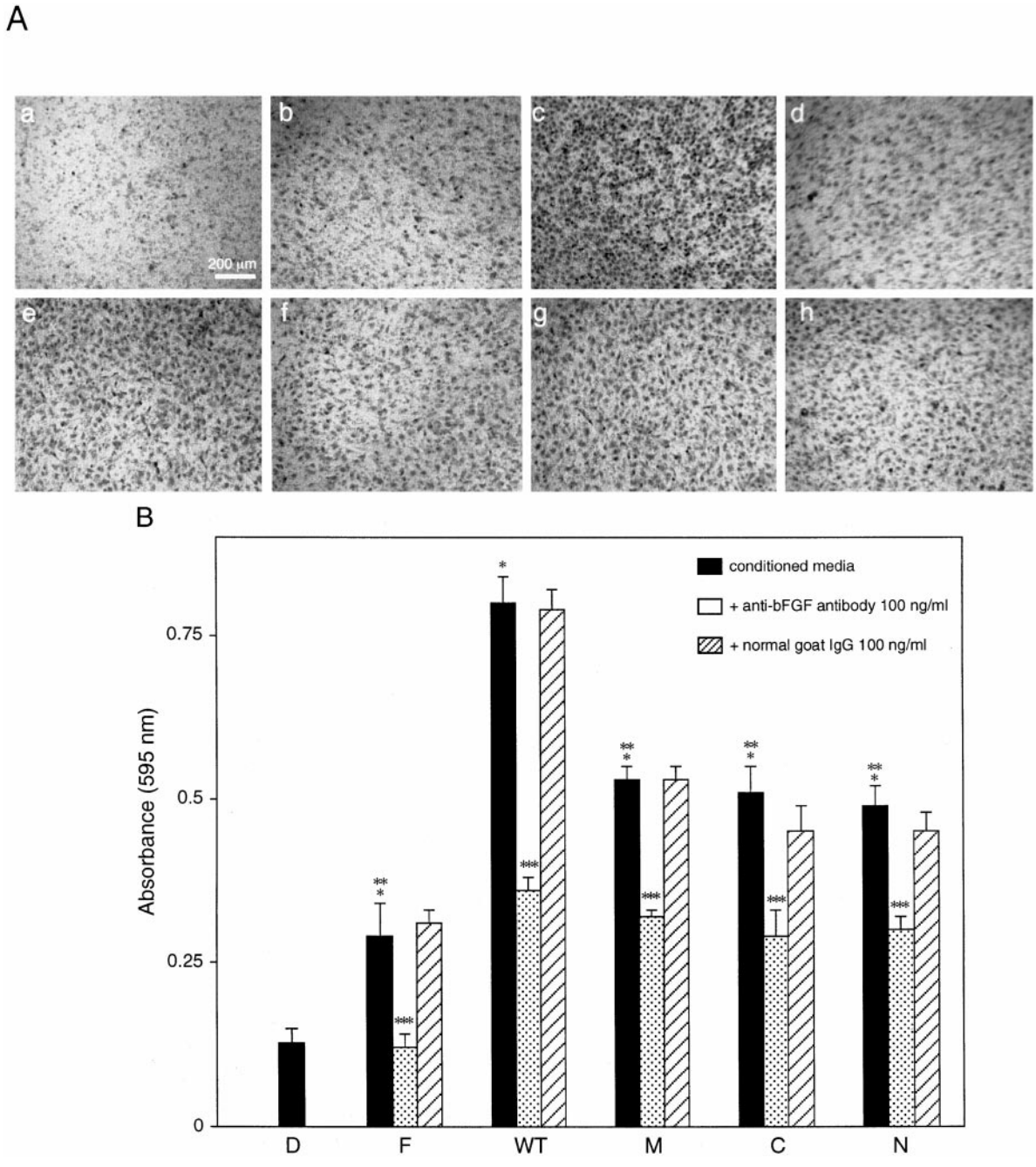

FIG. 4. Migration of HUVECs in a modified Boyden chamber assay. The sample CM was placed in the lower chamber, and HUVECs were added to the upper chamber of a modified Boyden chamber. After 24-h incubation, nonmigrating cells were removed, and cells that migrated through membrane pores (8 μm) were stained with Giemsa and photographed. A, Micrographs of migrated HUVECs from representative membranes under each experimental condition. a, Serum-free DMEM; b, 1 ng/mL bFGF in DMEM; c, WT-hPTTG-CM; d, WT-hPTTG-CM plus 100 ng/mL anti-bFGF antibody; e, WT-hPTTG-CM plus 100 ng/mL preimmune goat IgG; f, M-hPTTG-CM; g, C-CM; h, N-CM. B, Quantification of migrated cells. Stained cells were extracted with 10% acetic acid, and absorbance of extracted solution was determined. The data shown are the mean ± SD of three separate experiments. D, Serum-free DMEM; F, 1 ng/mL bFGF in DMEM; WT, WT-hPTTG-CM; M, M-hPTTG-CM; C, C-CM; N, N-CM. $P < 0.01$ *vs.* DMEM (*), WT-hPTTG-CM (**), and respective CM alone (***).

bFGF and VEGF induction coincident with estrogen-stimulated PTTG expression and marked pituitary angiogenesis (10). This raised the possibility that bFGF might be involved in PTTG-mediated angiogenesis actions, so we tested the effects of specific bFGF antibodies on activity of CM. Inhibitory effects of the anti-bFGF antibody on CM-mediated angiogenesis were more evident in WT-hPTTG-CM than in CM derived from the other cell lines. Thus, angiogenic activity of WT-hPTTG-CM is abrogated by neutralizing bFGF antibody. Addition of neutralizing bFGF antibody did not completely reverse the angiogenic effects of CM from WT-PTTG-transfected cells, suggesting that some PTTG-directed angiogenesis is probably due to other CM-derived factors. Although bFGF does not possess a secretory signal peptide, and the

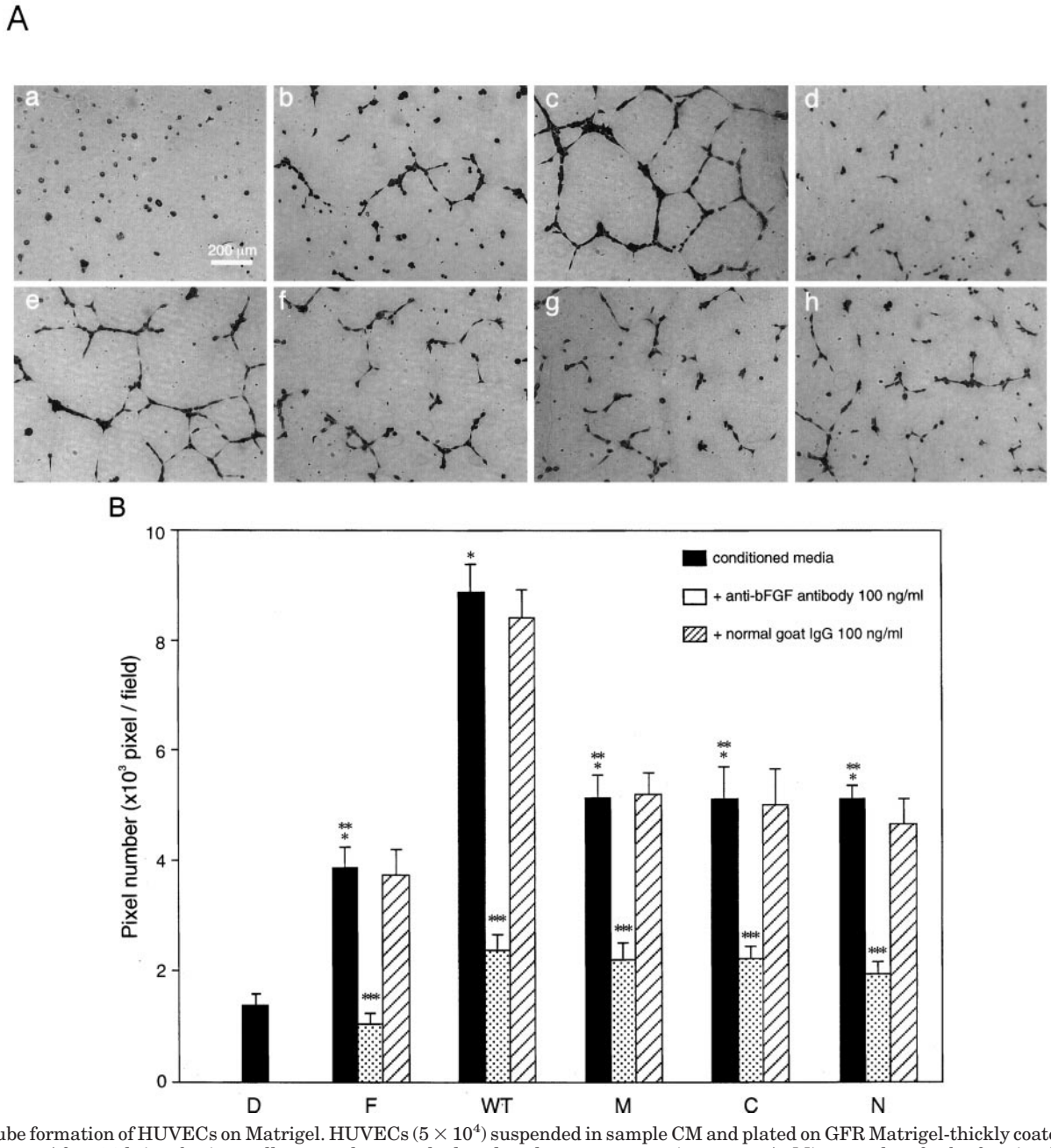

FIG. 5. Tube formation of HUVECs on Matrigel. HUVECs (5×10^4) suspended in sample CM and plated on GFR Matrigel-thickly coated 24-well culture plates. After 24-h incubation, cells were photographed under phase contrast microscopy. A, Micrographs of tube-forming HUVECs. Representative photographs for each experimental condition are shown. a, Serum-free DMEM; b, 1 ng/mL bFGF in DMEM; c, WT-hPTTG-CM; d, WT-hPTTG-CM plus 100 ng/mL anti-bFGF antibody; e, WT-hPTTG-CM plus 100 ng/mL preimmune goat IgG; f, M-hPTTG-CM; g, C-CM; h, N-CM. B, Quantification of tube formation. Tube length was quantified as described in *Materials and Methods*. The mean pixel number ± SD of three separate experiments are expressed. D, Serum-free DMEM; F, 1 ng/mL bFGF in DMEM; WT, WT-hPTTG-CM; M, M-hPTTG-CM; C, C-CM; N, N-CM. $P < 0.01$ *vs.* DMEM (*), WT-hPTTG-CM (**), and respective CM alone (***).

precise mechanism of its secretion has not been clarified (24), we detected high concentrations of bFGF in WT-hPTTG-CM and postulate that this soluble bFGF is involved in angiogenic activity in *vitro* and *in vivo*. As expected, CM derived from all transfectants conferred some angiogenic activity compared with serum-free DMEM.

We also examined PTTG-mediated angiogenic activity *in vivo* by CAM assay. Chick CAM provides an ideal microenvironment to induce new vessel development from preexisting vessels, although the quantification of angiogenesis can be complex, and several quantitation methods have been suggested (25). We observed that CM from WT-hPTTG-transfected cells induced a spoke-wheel-like appearance on the CAM, and this effect was more marked than that observed with CM derived from other cell lines. In contrast to the *in vitro* assays, quantitative angiogenic activity of sponges soaked in WT-hPTTG-derived CM was weaker than that observed after application of a sponge soaked in bFGF

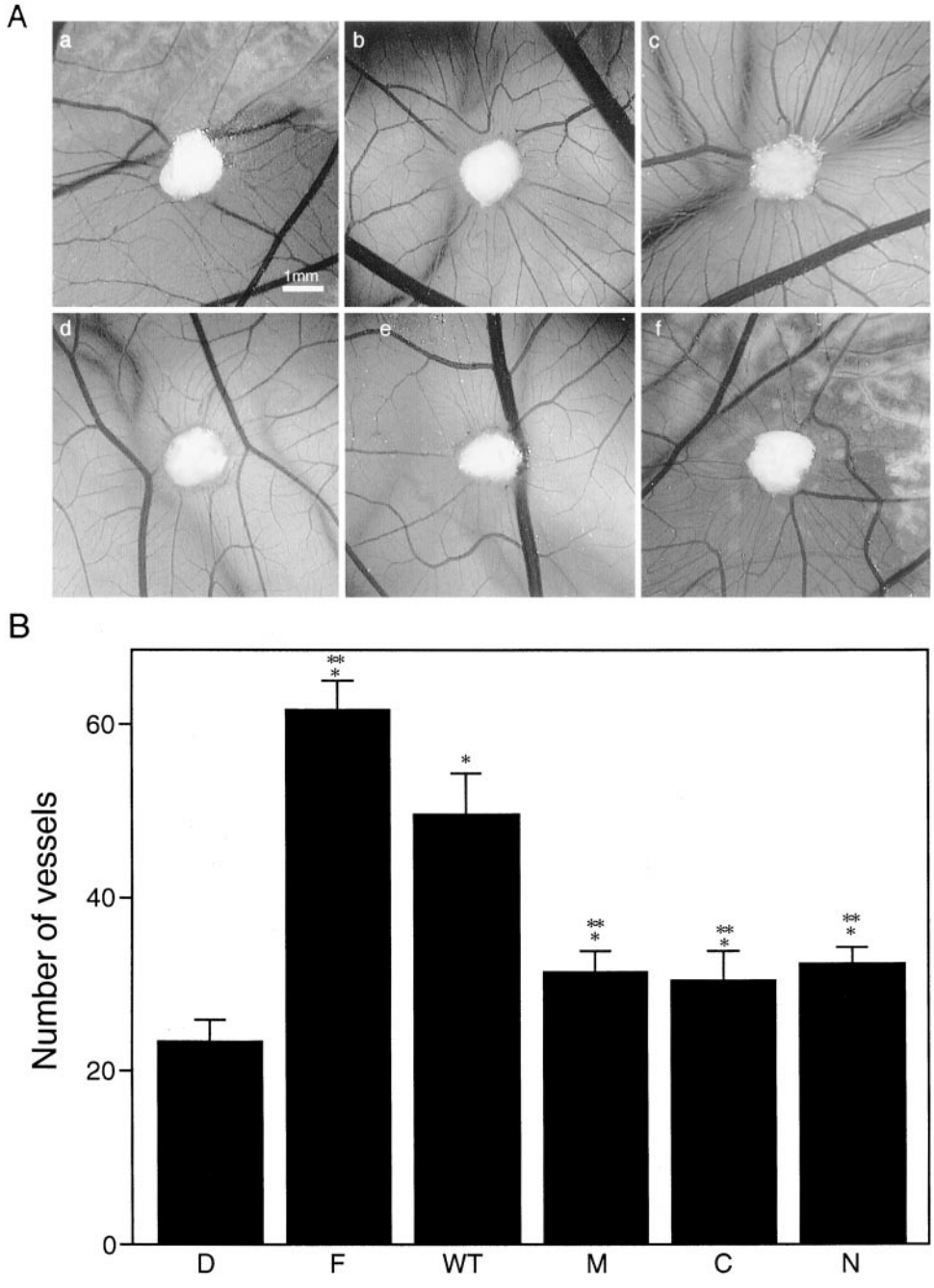

FIG. 6. Vascular reactions of CAM to CM. Test samples and the positive or negative control in collagen sponges were loaded on CAM of 9-day-old chick embryos. After 4 days, CAMs were photographed. A, Photographs of representative CAMs of 13-day-old chick embryo. a, Serum-free DMEM; b, 1 ng/mL bFGF in DMEM; c, WT- hPTTG-CM; d, M-hPTTG-CM; e, C-CM; f, N-CM. B, Quantification of induced vessels. The number of blood vessels entering the collagen sponges was counted under stereomicroscopy. The data shown are the mean ± SD of three separate experiments. D, Serum-free DMEM; F, 1 ng/mL bFGF in PBS; WT, WT-hPTTG-CM; M, M-hPTTG-CM; C, C-CM; N, N-CM. $P < 0.01$ *vs.* PBS (*) and WT-hPTTG-CM (**).

(1 μg/egg). However, bFGF bioavailability in the recombinant peptide-soaked sponge and the CM-soaked sponge may differ, accounting for this discrepancy.

The mechanisms for bFGF induction by hPTTG are as yet undefined. Some oncogenes up-regulate angiogenesis-promoting factors from tumor cells (26). Mutant H- or K-*ras* oncogenes as well as v-*src* and v-*raf* induce VEGF. On the other hand, p53, a tumor suppressor gene, regulates the expression of the angiogenesis inhibitor, thrombospondin, and inactivated p53 results in a loss of angiogenesis inhibition (27). PTTG is expressed at low levels in most normal tissues, but is abundantly expressed in cancer cell lines (6) and human cancers (10, 11). In colorectal tumors the highest PTTG mRNA expression was seen with lymph node invasion or metastases (10). In many solid tumors, tumor vascularity may inversely correlate with prognosis (28), and both bFGF and VEGF expression have been reported to predict prognosis (29). We previously correlated PTTG expression with tumor invasiveness and vascularity in colorectal tumors (10). Our description here of PTTG-mediated angiogenesis supports this observation in a variety of *in vitro* and *in vivo* models. The mechanism of PTTG-mediated angiogenesis is unclear, although our studies here and prior work, defining the requirements of an intact proline-rich potential SH3 binding motif, suggest that bFGF may be the effector for PTTG-driven angiogenesis. As estrogen induces pituitary PTTG

levels (10), new vessel formation (1), and adenoma growth (2), antiangiogenic factors may be important antineoplastic therapeutic options for these tumors (16, 30, 31).

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