Expression of the Antiangiogenic Factor 16K hPRL in Human HCT116 Colon Cancer Cells Inhibits Tumor Growth in *Rag1*$^{-/-}$ Mice

Frauke Bentzien, Ingrid Struman, Jean-François Martini, Joseph Martial, and Richard Weiner

Center for Reproductive Sciences, University of California School of Medicine, San Francisco, California 94143 [F. B., J-F. M., R. W.], and Laboratoire de Biologie Moléculaire et de Génie Génétique, Université de Liège, B-4000 Sart Tilman Belgium [I. S., J. M.]

ABSTRACT

The M_r 16,000 NH_2-terminal fragment of human prolactin (16K hPRL) is a potent antiangiogenic factor inhibiting endothelial cell function *in vitro* and neovascularization *in vivo*. The present study was undertaken to test the ability of 16K hPRL to inhibit the growth of human HCT116 colon cancer cells transplanted s.c. into *Rag1*$^{-/-}$ mice. For this purpose, HCT116 cells were stably transfected with an expression vector encoding a peptide that included the signal peptide and first 139 amino acid residues of human prolactin (HCT116^{16K}). Stable clones of HCT116^{16K} cells secreted large amounts of biologically active 16K hPRL into the culture medium. Growth of HCT116^{16K} cells *in vitro* was not different from wild-type HCT116 (HCT116^{wt}) or vector-transfected HCT116 (HCT116^{vector}) cells. Addition of recombinant 16K hPRL had no effect on the proliferation of HCT116^{wt} cells *in vitro*. Tumor growth of HCT116^{16K} cells implanted into *Rag1*$^{-/-}$ mice was inhibited 63% in four separate experiments compared with tumors formed from HCT116^{wt} or HCT116^{vector} cells. Inhibition of tumor growth of HCT116^{16K} cells was correlated with a decrease in microvascular density by 44%. These data demonstrate that biologically active 16K hPRL can be expressed and secreted from human colon cancer cells using a gene transfer approach and that production of 16K hPRL by these cells was capable of inhibiting tumor growth and neovascularization. These findings support the potential of 16K hPRL as a therapeutic agent for the treatment of colorectal cancer.

The abbreviations used are: bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; 16K hPRL, 16 kilodalton NH_2-terminal fragment of human prolactin; CAM, chorioallantoic membrane; *Ragl*, recombination activation gene 1 ; BBE, bovine brain capillary endothelial; BCS, bovine calf serum; PAI-1, plasminogen activator inhibitor 1; vWF, von Willebrand factor; PMB, polymyxin B

INTRODUCTION

Angiogenesis, the formation of new blood vessels from a preexisting endothelium, is an essential component of tumor growth (1). Increasing evidence supports the concept that angiogenesis is controlled both by stimulatory angiogenic factors and inhibitory antiangiogenic factors. Angiogenic factors include: bFGF[3] (2), VEGF (3), and angiogenin (4). Known antiangiogenic factors include: the 16K hPRL (5, 6), thrombospondin (7), angiostatin (8), endostatin (9), and platelet factor 4 (10). It is the balance between angiogenic and antiangiogenic factors that determines endothelial quiescence or activation. Tumor progression appears to involve a proangiogenic component, which is essential for neovascularization of the tumor and may correlate with rapid tumor growth and metastasis (11).

In colon cancer, as well as in other tumors, progression correlates with increased vascularity and VEGF expression. Vessel counts are greater in metastatic tumors than in nonmetastatic tumors (12), and the proportion and intensity of VEGF expression are associated with progression from a benign adenoma to colon cancer (13). Vessel counts were highest in tumors with high VEGF expression and greater in metastatic than nonmetastatic tumors (14). Increased VEGF expression in metastatic tumors correlated with expression of the VEGF receptor, KDR, in colon cancer (12). A causal relationship between VEGF expression and colon cancer growth and metastasis was established in animal models. Treatment with a monoclonal antibody against VEGF inhibited tumor growth by up to 90% of Ls Lim6 and FΓM7 human colon carcinoma cell lines implanted s.c. in athymic mice (15). An inhibitor of the KDR receptor, SU5416, inhibited liver metastases, microvessel formation, and increased tumor and endothelial cell apoptosis after intrasplenic injection of CT-26 colon cancer cells (16).

Several antiangiogenic factors have been tested in their ability to inhibit tumor growth and metastasis in colon cancer animal models. The injection of the fumagillol (TNP-470) into nude mice inhibited the growth and metastasis to the liver of orthotopic implants of several human colon cancer cell lines (17, 18). Injection of platelet factor 4 fragments inhibited growth of HCT116 colon cancer cells implanted s.c. (19). Stable transfection

of SW620 colon cancer cells with an endostatin-expressing vector resulted in inhibition of tumor growth and metastasis to the liver (20).

For this study, we asked whether 16K hPRL, a potent antiangiogenic factor *in vitro,* was capable of inhibiting tumor growth *in vivo*. 16K hPRL negatively regulates multiple cellular processes necessary for the development of new capillaries, *i.e.,* it inhibits VEGF and bFGF-induced capillary endothelial cell growth (6), signaling (21, 22), migration, and organization (6) and activates apoptosis (23). In addition, recombinant 16K hPRL inhibited development of capillaries in the chick CAM assay (6, 24) and corneal vasculogenesis model (25). The effects of 16K hPRL are specific for nontransformed vascular endothelial cells and have not been observed in numerous other cell types, *e.g.,* COS, HCT116, Y-1, HEK293, and BHK-21, and Ishikawa cells and human primary endometrial stromal cells (5, 23). The actions of 16K hPRL are mediated via a novel, high-affinity, saturable binding site, distinct from the PRL receptor, and found only on vascular endothelial cells (26).

Taking into consideration that *in vitro* 16K hPRL inhibits activation of two angiogenic pathways, we undertook this study to determine the ability of 16K hPRL to inhibit the growth of HCT116 colon cancer cells transplanted s.c. into *Rag1*$^{-/-}$ mice, which are Ragl homozygous knockout mice. We show for the first time that 16K hPRL stably expressed by HCT116 cells reduces tumor growth *in vivo.*

MATERIALS AND METHODS

Reagents. Cell culture medium and supplements for HCT116 cells and BBE cells were purchased from Life Technologies, Inc. (Grand Island, NY) unless otherwise noted, and bovine fetal and calf serum were purchased from Hyclone (Logan, UT). bFGF was purchased from Promega Corp. (Madison, WI). [^{3}H]Thymidine (6.7 Ci/mmol) was purchased from NEN (Boston, MA). PMB sulfate and E-TOXATE, a kit for detection and semiquantification of endotoxin levels, were obtained from Sigma Chemical Co.-Aldrich (St. Louis, MO).

Cell Culture. The human colon cancer cell line HCT116, used for expression of 16K hPRL, was purchased from the American Type Culture Collection (Rockville, MD) and grown in McCoy's 5A medium without tricine. The medium was supplemented with 10% (vol/vol) FCS and 100 units/ml penicillin and streptomycin. BBE cells used for testing the bioactivity of expressed 16K hPRL were isolated as described previously (27). BBE cells were cultured in low-glucose DMEM (DMEM H16), supplemented with 10% (vol/vol) BCS, 2 mM glutamine and antibiotics (100 units/ml penicillin, 100 units/ml streptomycin, and 2.5 μg/ml fungizone). Human recombinant bFGF was added to the cell medium at a concentration of 1 ng/ml every other day. Experiments were initiated from confluent cell cultures between passage 7 and 13.

Construction of 16K hPRL Expression Vector. For expression of 16K hPRL in mammalian cells, a Kozak consensus sequence (GCCGCCACC) was inserted 5' to the translational start site of the full length PRL cDNA (28) including the signal peptide. To produce a peptide consisting of the signal peptide and the first 139 amino acids of human prolactin, codon 140 was replaced by a stop codon by site-directed mutagenesis (16K hPRL-139; Ref 6). Cysteine 58 (TGG) was replaced with serine (TCC) to avoid the formation of normative disulfide bridges. The rabbit *β*-globin intron (29) was inserted at the 5' end of the 16K hPRL cDNA, which was then subcloned into *Bam*HI and *Hind*III sites of the pRc/CMV vector (Invitrogen, Carlsbad, CA). The construct was verified by sequencing.

Stable Transfection of HCT116 Cells. HCT116 cells were plated at a density of 300,000 cells/10-cm tissue culture plate 1 day prior to transfection. Ten μg of pRC/CMV vector or vector containing the 16K hPRL insert were transfected into HCT116 cells by the calcium phosphate-DNA method. Calcium-DNA precipitates were left on the cells for 6 h, cells were then treated with 20% glycerol in unsupplemented medium for 2 min, after which 10% supplemented growth medium was added. Stably transfected cells were selected 72 h after transfection by neomycin resistance at a G418 sulfate (Life Technologies, Inc.) concentration of 0.3 mg/ml. G418-resistant colonies were cloned by limiting dilution, individual clones were grown to confluence, and conditioned medium was assayed for 16K hPRL expression by Western Blot analysis and RIA .

RNA Extraction and Northern Analysis. Total cytoplasmic RNA was prepared by the guanidine thiocyanate extraction method and was purified by centrifugation through a CsCl solution. Ten μg of total RNA were then electrophoresed through 1.2% agarose-formaldehyde gel, transferred by capillary blotting onto a nylon-based membrane Hybond N (Amersham Pharmacia Biotech, Piscataway, NJ), and hybridized with 3×10^6 cpm of the ^{32}P randomly primed 16K hPRL cDNA fragment (Oligolabeling kit; Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization was followed by low stringency washes in 2×SCC and 0.1% SDS at room temperature, followed by high stringency washes in 0.2×SCC and 0.1% SDS at 68°C Filters were then exposed for 6 to 24 h

at -70°C.

Collection of Conditioned Medium and Western Blot Analysis. Conditioned medium from stably transfected, subconfluent HCT116 cells was collected 48 h after addition of the medium, centrifuged at 12,000 rpm for 15 min at 4°C to remove cellular debris, filtered through 0.2 µm filters (Gelman Sciences, Ann Arbor, MI), and stored at -80°C Conditioned medium was analyzed by electrophoresis through 15% polyacrylamide gels, followed by semidry transfer onto polyvinylidene difluoride membranes (Immobilon P; Millipore, Bedford, MA) and Western blot analysis. Western blots were probed with either a polyclonal anti-PRL antibody (1:500; provided by Genzyme) or a polyclonal anti-16K hPRL antibody (1:100; J. Martial, Liege, Belgium). Antigen-antibody complexes were visualized by using l:10,000-diluted goat antirabbit antiserum (Amersham) coupled to horseradish peroxidase and ECL detection system (Amersham).

RIA for 16K hPRL. Recombinant 16K hPRL was iodinated using a milder variation (30) of the chloramine T method (31). We used a rabbit anti-hPRL polyclonal antibody (Genzyme) that recognizes 16K hPRL as first antibody. Mouse and human PRL were not detected by this antibody. Bound from free complexes were precipitated with a goat antirabbit immunoglobulin antibody. The sensitivity of the assay was 10-20 ng/ml, and the intraassay coefficient of variation was 5-10%.

BBE Cell Proliferation Assay by Determination of DNA Synthesis. DNA synthesis was assayed by measuring [^{3}H]thymidine incorporation into trichloroacetic acid-insoluble material. BBE cells were plated for 24 h at a density of 10,000 cells/well (six-well plates) in 1 ml of culture medium containing 10% BCS, serum-starved for 24 h in 0.5% BCS in antibiotic and glutamine supplemented DMEM, and then incubated for 24 h with 50 pM bFGF in the presence of increasing amounts of conditioned medium from either HCT116^{wt}, HCT116^{vector}, or HCT116^{16K} cells. Four h prior to harvesting, cells were pulsed with 500,000 cpm/well of [^{3}H]thymidine (NEN). The reactions were terminated by addition of ice-cold 5% trichloroacetic acid for 20 min at 4°C, followed by three washes in 5% trichloroacetic acid, solubilization in 0.25 N NaOH, and scintillation counting.

PAI-1 Western Blot Analysis. Stimulation of PAI-1 secretion into conditioned medium of BBE cells was assayed as described previously (32). BBE cells were treated for 18 h with conditioned medium from HCT116^{wt}. HCT116^{vector}, or medium from two different HCT116^{16K} clones. Ten µl of BBE-conditioned medium were resolved by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Millipore) and immunoblotted with an anti-bovine PAI-1 mouse monoclonal antibody (Life Technologies, Inc.) at a dilution of 1:2,000. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated secondary antibody (Amersham) and ECL (Amersham).

DNA Fragmentation Assay. BBE cells were cultured as described previously above and, after stimulation with conditioned medium, lysed for analysis with the Cell Death Detection Elisa^{plus} (Roche, Indianapolis, IN) according to manufacturer's instructions. Levels of DNA fragmentation in BBE cells were expressed as a factor of enrichment, calculated by dividing the absorbance of a given sample by the absorbance of the corresponding 10% BCS control. Three separate spectrophotometric measurements (A_{405nm}/A_{490nm}) were averaged, and the background value for the assay was subtracted from each of these averages.

Purification of 16K hPRL Out of Conditioned Media. Conditioned medium from cloned HCT116^{16K} cells grown in McCoys' 5A containing 2.5% FCS was collected, brought to 850 mM $(NH_4)_2SO_4$, and incubated overnight at 4°C with phenyl Sepharose 6 Fast Flow low sub media (Pharmacia), 4 ml of resin/liter of medium. Resin was packed and washed with 850 mM $(NH_4)_2SO_4$-20 mM ethanolamine (pH 8.3) at 1 ml/min. Chromatography was performed in 20 mM ethanolamine (pH 8.3), and 16K hPRL was eluted within a gradient of $(NH_4)_2SO_4$ from 850 mM to 0. Fractions containing 16K hPRL were collected, dialyzed against 20 mM ethanolamine (pH 8.5) and loaded onto an anion exchange Hitrap Q column (Pharmacia). The 16K hPRL was eluted within a gradient of NaCl from 0 to 1 M. Fractions containing purified 16K hPRL were pooled, dialyzed against 20 mM ethanolamine (pH 8.5), and stored at -20°C

Chick CAM Assay. On day 3 of development, fertilized chick embryos were removed from their shells and placed in plastic Petri dishes. On day 6, 5-mm discs of methylcellulose (0.5%; Sigma Chemical Co.) containing 10 µg of recombinant purified protein and 2 µg of BSA were laid on the advancing edge of the chick CAM as described previously (33). After a 48-h exposure period to test substances, white India ink was injected into the chorioallantoic sac to enhance photographic visualization. Results were considered positive only if an avascular zone of 5 mm in diameter or greater was observed.

HCT116 Cell Proliferation Assay. Cell proliferation studies were performed in 12-well culture plates (Falcon) seeded with 10,000 wild-type, plasmid, or 16K hPRL transfected HCT116 cells/well. Cells were grown in 10% BCS-supplemented medium for 6 days. Cell number was assayed after 2, 4, and 6 days using a hemocytometer.

Tumor Studies. Male *Rag1*$^{-/-}$ mice (10-12 weeks of age) were housed in a barrier facility, and cell injections were performed under a laminar flow cabinet. The T- and B-cell-deficient *Rag1*$^{-/-}$ mouse used in our experiments was created by a targeted disruption of the V(D)J *Rag1*. This mutation resulted in loss of all of the cellular components of the immune system, making it less leaky than that of the *scid* or *nude* mouse (34). For inoculation, HCT116^{wt}, HCT116^{vector}, or HCT116^{16K} cell suspensions were prepared in serum-free culture medium. Mice were anesthetized by Methoxyflurane (Schering-Plough Animal Health Corp., Union, NJ) inhalation, and 5×10^6 cells in 100 µl of medium were injected s.c. into the dorsal region. Four experiments with four separate HCT116^{16K} clones were undertaken. In each separate, experiment mice were sacrificed on the same day. The mean length of the tumor studies was 28 days, varying by 9 days in the four experiments [experiment 1 (clone 4) 20 days, experiment 2 (clone 10) 24 days, experiment 3 (clone 10.2) 27 days, and experiment 4 (clone 22) 41 days]. The tumors were excised, weighed, and fixed in 4% paraformaldehyde (EMS, Fort Washington, PA). The liver, lungs, and kidneys were examined for metastases macroscopically, and microscopically after fixation and staining by H&E.

Immunohistochemistry. Assessment of tumor vascularity was done on paraffin-embedded, 5-µm tissue sections using a primary polyclonal antihu-man antibody recognizing vWF (Dako, Carpinteria, CA) at a dilution of 1:2000 in blocking solution containing TBS (pH 7.3) and 3% horse serum. Incubation with primary antibody was carried out at 4°C overnight, followed by incubation with a biotinylated goat antirabbit secondary antibody (Vector Laboratories), at a dilution of 1:200 for 30 min at room temperature. Positive staining for respective antigens was detected by substrate reaction with 3,3-diamino-benzidine substrate (Zymed Laboratories, Inc., South San Francisco, CA). Sections were counterstained with hematoxylin for 10 s, dehydrated, and mounted in Cytoseal 60 (Stephens Scientific, Riverdale, NJ). Intratumor mi-crovessel density was determined by first selecting vWF-positive vascular hot spots at low magnification (x20), systematically scanning the whole section. Number of individually stained microvessels in a defined area were counted as reported previously (35) at x100. Immunolocalization experiments were repeated three times on multiple sections and at different tumor depths (serially sectioning to every 10th section). Experiments included negative controls for determination of background staining, which was negligible.

Statistical Analysis. All values are expressed as mean ± SD. Multiple comparisons between treatment conditions were assessed by one-way ANOVA, followed by a post hoc analysis with the Student-Newman-Keuls test. Statistical significance was defined as a value of $P < 0.05$.

RESULTS

Expression of 16K hPRL in HCT116 Colon Cancer Cells.

We first evaluated expression of the antiangiogenic factor 16K hPRL after stable gene transfer into the human colon cancer cell line HCT116. The cells were transfected with the 16K hPRL expression vector (HCT116^{16K}) or the expression vector alone (HCT116^{vector}). All constructs conferred neomycin resistance, and stable clones were selected by resistance to G418. Expression of 16K hPRL by cloned HCT116^{16K} cells was confirmed by Northern and Western analysis. Four different stable clones of HCT116^{16K} cells were shown to contain a transcript of expected size (624 bp; Fig. 1A), whereas the controls, HCT116^{wt} and HCT116^{vector} cells, contained no 16K hPRL transcript. The level of rnRNA expression varied among the different clones of HCT116^{16K} cells.

The Western blot analysis with a polyclonal anti-PRL antibody recognizing 16K hPRL in conditioned medium from HCT116^{16K} cells demonstrated the presence of proteins of expected M_r 16,000 size (Fig. 1*B*). In addition to confirming the correct size of 16K hPRL expressed by HCT116^{16K} cells, we used a RIA specific for 16K hPRL to determine the amount of 16K hPRL secreted into the conditioned medium. The magnitude of 16K hPRL secreted in 24 h into the medium by four different clones of HCT116^{16K} cells ranged from 0.2 to 1 µg/ml. Our previous data showed that recombinant 16K hPRL inhibits mitogen-stimulated proliferation of BBE cells with an EC_{50} of 1-2 nM (6). The amount of 16K hPRL secreted into the conditioned medium by the lowest producing HCT116^{16K} clone (c4) therefore exceeded 1 nM, whereas the highest producing clone (c10) secreted 16K hPRL at a rate 50 times greater than necessary for biological activity.

Biological Activity of HCT116^{16K} Cell Conditioned Medium.

We next asked whether the conditioned medium obtained from stably transfected HCT116^{16K} cells contained biologically active 16K hPRL. Three different assays established in our laboratory for measuring 16K hPRL activity were used to test this biological activity: inhibition of BBE cell proliferation (5); stimulation of PAI-1 expression (28); and activation of apoptosis in both BBE and human umbilical vein endothelial cells by measuring DNA fragmentation (21).

As shown in Fig. 24, conditioned medium from HCT116^{16K} cells inhibited bFGF-stimulated BBE cell proliferation in a dose-dependent fashion with an EC_{50} of 2.5 µl of conditioned medium from the highest 16K hPRL-producing clone (c10). Because the EC_{50} for recombinant 16K hPRL in this assay is 1 ΠM, there appears to be ~6.4 µg/ml of 16K hPRL in the conditioned medium, indicating that large amounts of biologically active 16K hPRL were secreted by clone 10 of the HCT116^{16K} cells. Conditioned medium from HCT116^{wt} cells had no effect on BBE cell growth, whereas conditioned medium from HCT116^{vector} cells appeared to stimulate endothelial growth.

Conditioned medium from two different HCT116^{16K} clones (clones c4 and c10) stimulated production of PAI-1 in BBE cells as measured by Western blot analysis (Fig. 2B*).* In contrast, conditioned medium from HCT116^{wt} and HCT116^{vector} cells (data not shown) had no effect on PAI-1 production. Because endotoxin is a known stimulator of PAI-1 (36), we ascertained that conditioned medium contained no significant endotoxin activity by preincubating the conditioned medium with PMB, an inhibitor of endotoxin activity. This had no effect on the stimulation of PAI-1 production by conditioned medium from HCT116^{16K} cells. In addition, no endotoxin could be measured in the conditioned medium using the E-TOXATE (*Limulus* amebocyte ly-sate) assay.

The level of DNA fragmentation, an early indicator of cells undergoing apoptosis, was determined after exposure to conditioned medium by measuring the cytosolic level of mono- and oligonucleosomes with an ELISA assay (21). Experiments were performed in BBE cells grown in low serum conditions (Control BBE, 0.5%) for 2 days, a treatment that caused a 2-fold increase in nucleosome formation compared with cells cultured in 10% BCS (Control BBE, 10%; Fig. 2C). Addition of 5 µl of conditioned medium from HCT116^{16K} clones 4 and 10 to medium containing 0.5% BCS increased DNA fragmentation 4.7- and 3.2-fold compared with control medium (0.5% BCS), respectively. Conditioned medium from HCT116^{wt} or HCT116^{vector} cells had no effect. A 10 nM concentration of recombinant 16K hPRL increased DNA fragmentation 7.4-fold in BBE cells. Preincubation with PMB had no effect on the stimulation of DNA fragmentation by conditioned medium from HCT116^{16K} cells or on the stimulation by 10 nM recombinant 16K hPRL. This demonstrated further that the conditioned medium contained no significant endotoxin activity. In contrast, heat denaturation of 16K hPRL by boiling the sample for 2 min completely inhibited the stimulation of DNA fragmentation by both the recombinant 16K hPRL as well as conditioned medium from HCT116^{16K} clones 4 and 10. In addition, 20 µl of conditioned medium from HCT116^{16K} cells caused a 4-fold increase in nucleosome formation in human umbilical vein endothelial cells. A similar level of stimulation was observed with 5 nM recombinant 16K hPRL, confirming previously published results (23). No effect was observed with conditioned medium from HCT116^{wt} or HCT116^{vector} cells.

Activity of Purified 16K hPRL from HCT116^{16K} Cell Conditioned Medium.

To further characterize the 16K hPRL secreted into the conditioned medium by the HCT116^{16K} cells, it was purified by hydrophobic interaction on a phenyl Sepharose column. The eluted product comigrated with recombinant 16K hPRL *made in. Escherichia coli* when examined by Western blot analysis (data not shown). To assess the integrity of the purified 16K hPRL, we determined its antiangiogenic activity *in vitro* with the BBE proliferation assay and *in vivo* with the CAM assay. 16K hPRL purified from conditioned medium of clone c4 inhibited bFGF-stimulated BBE cell proliferation in a dose-dependent manner with an EC_{50} of 2 nM (Fig. *3A*). Purified 16K hPRL was also active *in vivo*, because it inhibited capillary formation in the chick CAM assay (Fig. *3B*). Ten µg of 16K hPRL were able to inhibit capillary formation in the area of the CAM surrounding the disc containing the 16K hPRL, whereas no effect was observed in the BSA control (*arrows* indicate the edge of the disc). These findings were in agreement with earlier work with recombinant 16K hPRL made in *E. coli* (6, 22), and they further strengthen the conclusion that HCT116^{16K} cells secrete biologically active 16K hPRL.

Antitumoral Action of 16K hPRL.

To use the HCT116^{16K} cells in xenograph studies using immunodeficient *Rag-1*$^{-/-}$ mice, we first needed to

determine whether 16K hPRL had any direct effect on the proliferation rate of HCT116 cells *in vitro.* We showed that $HCT116^{16K}$ cells grew at the same rate *in vitro* as $HCT116^{wt}$ and $HCT116^{vector}$ cells (Fig. 44). Also, the addition of increasing concentrations of recombinant 16K hPRL to cultures of $HCT116^{wt}$ cells had no effect on the rate of cell proliferation (Fig. *4B*). Therefore, 16K hPRL had no detectable direct effect on the growth of these tumor cells. This finding agrees with previous observations that 16K hPRL only affected the growth of vascular endothelial cells (5) and validated the use of these cells in xenograph studies in *Rag1*$^{-/-}$ mice.

To study the growth of $HCT116^{16K}$ colon cancer cells in *Rag1*$^{-/-}$mice, we injected five million cells s.c. in the scapular region under methoxyflurane anesthesia. *In vivo* growth of four different stable clones of $HCT116^{16K}$ cells were compared with control $HCT116^{wt}$ and $HCT116^{vector}$ cells. The size of tumors formed by $HCT116^{16K}$ cells was dramatically inhibited by a mean of 63% in four separate experiments ($P < 0.01$) compared with tumors formed from $HCT116^{wt}$ and $HCT116^{vector}$ cells (Fig. 5). Each of the four experiments shown was performed with a different subclone of the $HCT116^{16K}$ cells to exclude the possibility that clonal variability contributed to the observed tumor growth inhibition. All established $HCT116^{16K}$ clones inhibited the growth of s.c. tumors, and no tumor metastasis to the liver or other tissues was observed in these experiments with any of the cells.

After removal of the tumors, increased peripheral vascularity could be observed around the periphery of tumors formed from $HCT116^{vector}$ cells but not in tumors formed from the $HCT116^{16K}$ cells (Fig. 6/4). In addition, tumor surfaces viewed during the sectioning process showed an even greater macroscopic difference in vascularity. To assess the vessel density in hotspots of tumors formed from $HCT116^{16K}$ (clone 22) and $HCT116^{vector}$ cells, tumor sections were analyzed for microvessel density by immunostaining with an antibody to vWF. Ten random fields of vessel hot spots were evaluated per tumor at three different depths through each tumor. Fig. *6B* shows data from one level in which intratumoral microvessel density was decreased by 46% by the expression of 16K hPRL. The mean level of inhibition at all three levels was 44%. These data support the conclusion that 16K hPRL inhibited tumor growth by decreasing the level of angiogenesis in the tumors.

DISCUSSION

These studies demonstrate clearly that the antiangiogenic factor, 16K hPRL, is capable of inhibiting tumor formation from HCT116 cells implanted in *Rag1*$^{-/-}$ mice. HCT116 cells were stably transfected with an expression vector expressing 16K hPRL. Conditioned medium from $HCT116^{16K}$ cells inhibited the stimulation of BBE cell proliferation by bFGF, increased PAI-1 expression, and increased DNA fragmentation in BBE cells. Furthermore, 16K hPRL purified from the conditioned medium was capable of inhibiting angiogenesis in the CAM assay.

Therefore, a stably transfected human colon cancer cell line was capable of producing and secreting large amounts of biologically active 16K hPRL. Tumor growth from $HCT116^{16K}$ cells implanted in *Rag1*$^{-/-}$ mice was significantly inhibited. This antitumoral action of expressed 16K hPRL appeared to be a result of its antiangiogenic action, because microvascular density was inhibited in these tumors.

Problems arising from the production of recombinant proteins in *E. coli* were circumvented by stably transfecting HCT116 cells with an expression vector for 16K hPRL. The creation of stably transfected cells was successfully used for the expression of other antiangiogenic factors including thrombospondin-2 in human A431 squamous cell carcinoma cells (37) and angiostatin in murine T241 fibrosarcoma cells (38).

Large amounts of biologically active 16K hPRL were secreted into the medium by $HCT116^{16K}$ cells (0.2-1 μg/ml in 24 h). These levels were sufficient to have strong antiangiogenic effects in all of the assays studied. To increase the rate of translation of the 16K hPRL mRNA, a Kozak consensus sequence was inserted prior to the translational start site. The signal peptide targeting the peptide to the secreted pathway was also included in the construct. 16K hPRL was predominantly secreted into the culture medium (>95%), which is consistent with the efficient secretion of endogenous prolactin into the circulation from the anterior pituitary.

A major issue in studying antiangiogenic activity *in vitro* has been contamination of recombinant protein preparations made in *E. coli* with endotoxin. Endotoxin has a potent antiangiogenic activity in *in vitro* assays with endothelial cells. Recombinant proteins made in *E. coli* are contaminated to varying degrees with endotoxin that is present in large amounts in these Gram-negative bacteria. Endotoxin levels can be decreased to levels where the activity is negligible; however, the techniques used for the removal of the endotoxin can lead to

inactivation of the biological activity of the peptide. The problem with endotoxin contamination was circumvented by the use of a gene transfer technique. We found no evidence that the activity of 16K hPRL secreted from $HCT116^{16K}$ cells was caused by contamination with endotoxin. In addition, we have shown that even with recombinant 16K hPRL preparations made in *E. coli* that the antiangiogenic activity is not dependent on endotoxin contamination but is dependent on the presence of the 16K hPRL molecule (21).

That the antitumoral action of 16K hPRL expression was attributable to the antiangiogenic activity of the molecule is supported by several observations. The 16K hPRL secreted by the $HCT116^{16K}$ cells was antiangiogenic both *in vitro* and *in vivo*. More importantly, the inhibition of tumor growth from $HCT116^{16K}$ cells was correlated with a 44% decrease in microvascular density. This is similar to the level of inhibition of tumor growth and vascular density with that observed with angiostatin expression in T241 fibrosarcoma cells (33). The antitumoral action of 16K hPRL did not appear to involve a direct effect on growth of the HCT116 cells, because recombinant 16K hPRL had no effect on the growth of cultured $HCT116^{wt}$ cells. Furthermore, $HCT116^{16K}$ cells grew *in vitro* at the same rate as $HCT116^{wt}$ and $HCT116^{vector}$ cells.

These findings provide the rationale for performing further studies on the ability of 16K hPRL to inhibit the growth and metastasis of tumors formed from human colorectal cell lines in appropriate models. The current studies do not address the role of angiogenesis in metastasis, because metastasis is infrequently observed after s.c. implantation of tumor cells (39).

In conclusion, these data demonstrate clearly that stably transfected HCT116 cells are capable of secreting biologically active 16K hPRL that inhibits growth of tumors formed from a human colorectal cell line. That human colon cancer cells can produce large amounts of biologically active 16K hPRL supports the use of gene transfer approaches with viral vectors to inhibit colorectal cancer growth and metastasis.

FIGURES

Fig. 1: *Expression of 16K hPRL-139 in HCT116 colon cancer cells.* A, Northern blot analysis of mRNA extracted from $HCT116^{wt}$ and stable clones of $HCT116^{vector}$ and $HCT116^{16K}$ cells. 16K hPRL mRNA was detected as 624-bp transcripts in four $HCT116^{16K}$ subclones not present in mRNA of $HCT116^{wt}$ or $HCT116^{vector}$ cells. B, Western blot analysis with polyclonal anti-hPRL antibody recognizing 16K hPRL protein in conditioned medium from HCT11616K cells but not $HCT116^{wt}$ or $HCT116^{vector}$ cells

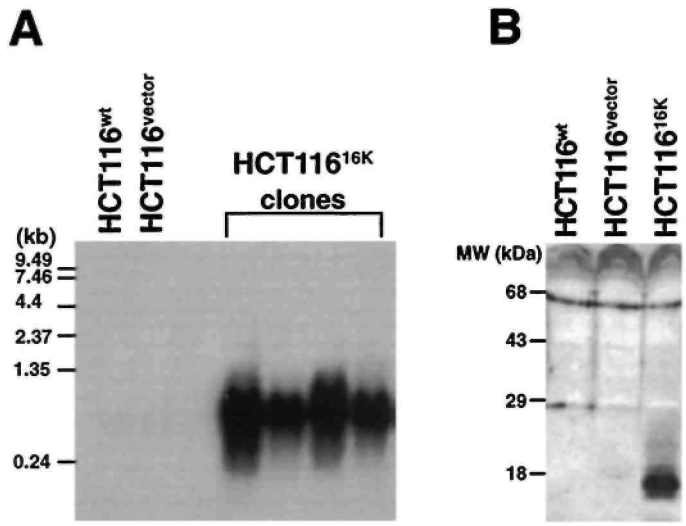


Fig. 2: *Biological activity of 16K hPRL expressed by HCT116^{16K} cells.* A, conditioned medium from HCT116^{wt}, HCT116^{vector}, or HCT116^{16K} cells were tested for the ability to inhibit the mitogenic action of bFGF in the BBE cell proliferation assay. Cell proliferation was estimated by the rate of incorporation of [^{3}H]thymidine, as described in "Materials and Methods." Results are means of triplicate wells for one representative experiment; bars, SD. B, Western blot analysis for PAI-1 levels in BBE cells treated with conditioned medium ± PMB from HCT116^{wt} and clones c4 and c10 of HCT116^{16K} cells. BBE cells were treated for 18 h with conditioned medium followed by collection of culture medium, which was subjected to Western blot analysis using an anti-bovine PAI-1 monoclonal antibody. C, conditioned medium from HCT116^{wt}, HCT116^{vector}, and HCT116^{16K} clones c4 and c10 were tested for effects on DNA fragmentation in BBE cells. Quiescent BBE cells were left untreated (Control BBE 10%), serum deprived (Control BBE 0.5%), or stimulated in 0.5% BCS with 5 µl of conditioned medium from HCT116^{wt}, HCT116^{vector} or HCT116^{16K} cells. Ten nM recombinant 16K hPRL was used as a positive control. The same conditioned medium or recombinant 16K hPRL pretreated with PMB or boiled for 2 min was also tested. Data are means of three independent experiments; bars, SD

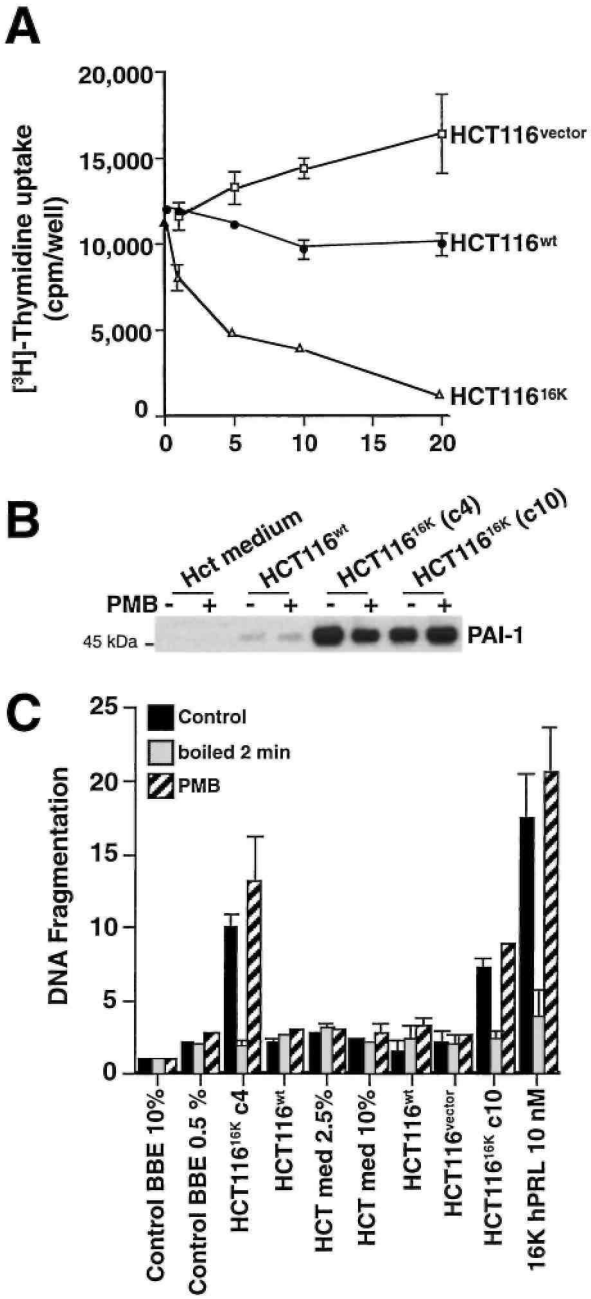


Fig. 3: Biological activity *in vitro* and *in vivo* of purified 16K hPRL from conditioned medium of HCT116^{16K} cells. *A*, purified 16K hPRL from HCT116^{16K} clone c4 inhibited the stimulation of BBE cell proliferation by bFGF in a dose-dependent fashion. Data are means of triplicate wells for a representative experiment; *bars*, SD. *B*, the same preparation of purified 16K hPRL inhibited capillary formation in the chick CAM assay, as described in "Materials and Methods." An area >5 mm in diameter devoid of capillaries was observed in three of four membranes surrounding the 16K hPRL (10 µg)-impregnated disc *(arrow)*. No effect can be observed in the control in which the disc contained BSA *(arrow)*

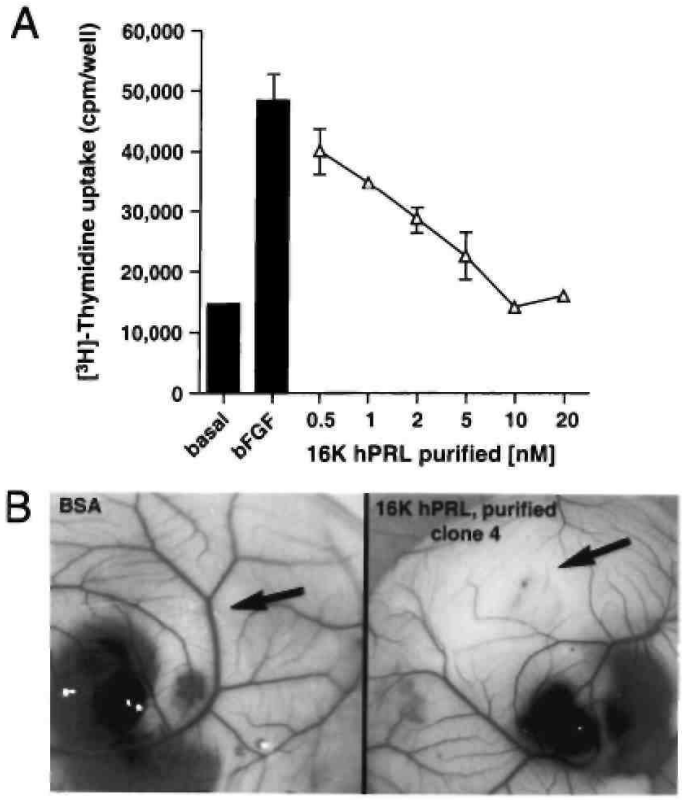


Fig. 4: *HCT116 cells are not affected by 16K hPRL expression. A,* HCT116^{16K}, HCT116^{wt} and HCT116^{vector} cells all grew at the same rate in culture. Growth rate of cells was monitored over a 6-day period by determining cell number of triplicate wells by hemocytometer (means; *bars,* SD). *B,* the addition of increasing concentrations of recombinant 16K hPRL had no effect on the rate of proliferation of HCT116^{wt} cells as quantitated by [^{3}H]thymidine incorporation. Data are means of triplicate wells; *bars,* SD

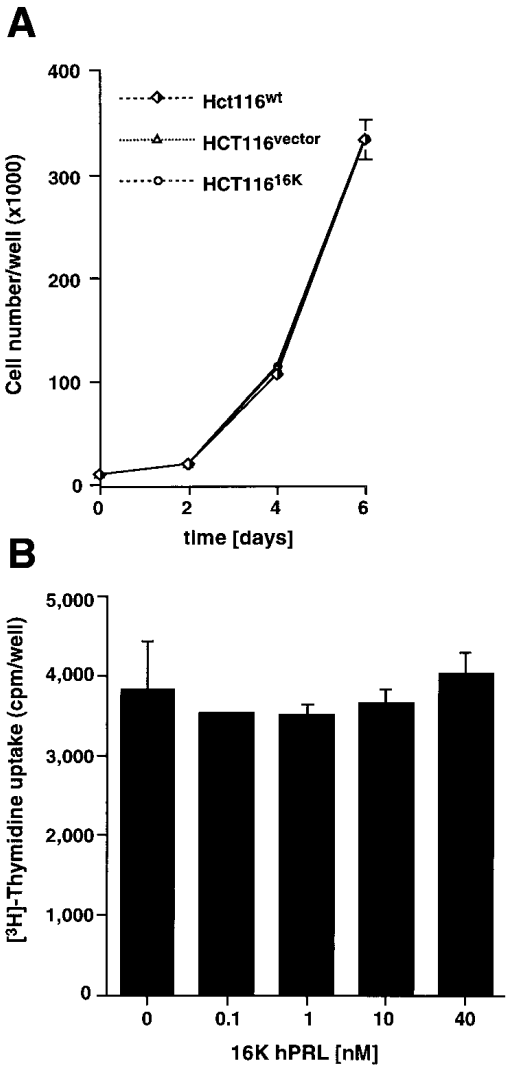


Fig. 5: Size of tumors formed from HCT116^{16K}, HCT116^{wt}, and HCT116^{vector} cells injected s.c. in *Rag1*$^{-/-}$ mice, as described in "Materials and Methods." Significant decrease in weight of HCT116^{16K} tumors compared with HCT116^{wt} and HCT116^{vector} tumors (*, $P < 0.01$). Four separate experiments with different subclones of stable HCT116^{16k} cells are shown *(upper left corner*, clone number). Data are means; *bars*, SD The number of animals per group is indicated in *parentheses* at the *bottom* of each *column*

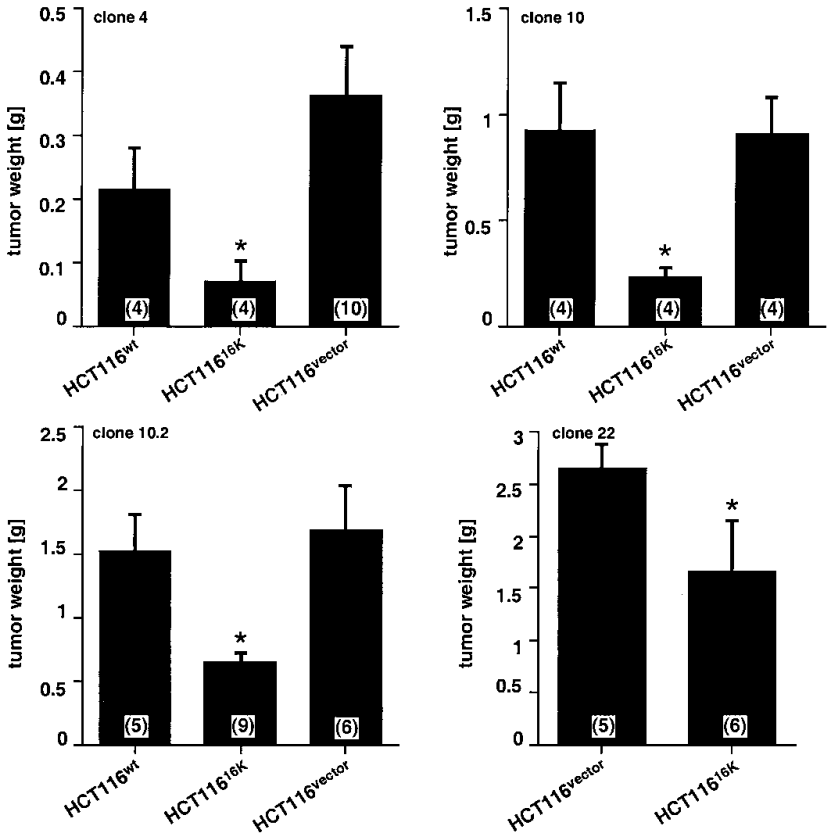
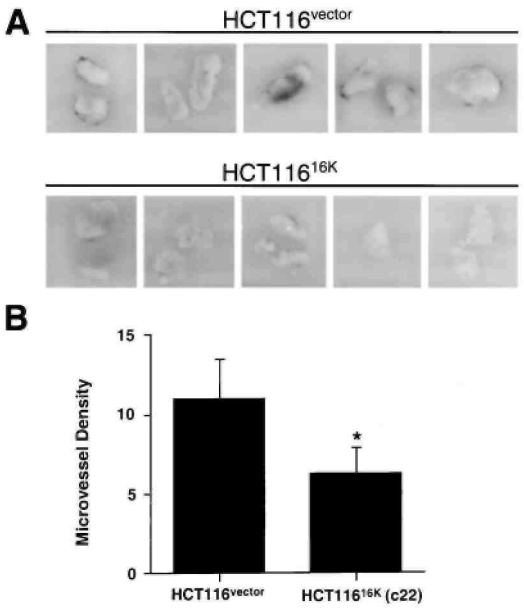


Fig. 6: *A*, digital scanning of tumor surfaces from tumors formed by s.c. injection of HCT116^{16K} *(clone 22)* and HCT116^{vector} cells in *Rag1*$^{-/-}$ mice *(lower right panel*, respective tumor sizes). Increased peripheral vascularity and hemorrhagic areas can be observed in the tumors formed from HCT116^{vector} cells but not from tumors formed from HCT116^{16K} clone c22. *B*, decrease in mean microvessel density in tumors formed by HCT116^{16K} compared with HCT116^{vector} cells (*, $P < 0.001$). Number of tumor vessels immunostained with an antibody to vWF were counted in 10 random fields of vascular hotspots in each tumor section at x100. Data are means of vWF-positive vessel in tumor sections from HCT116^{vector} and HCT116^{16K} cells (n = 50); *bars*, SD



REFERENCES

1. Folkman, J. What is the evidence that tumors are angiogenesis dependent? J. Natl. Cancer Inst, *82:* 4-6, 1990.

2. Gross, J. L., Herblin, W. F., Dusak, B. A., Czerniak, P., Diamond, M. D., Sun, T., Eidsvoog, K., Dexter, D. L., and Yayon, A. Effects of modulation of basic fibroblast growth factor on tumor growth *in vivo*. J. Natl. Cancer Inst., *85:* 121-131, 1993.

3. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N, Phillips, H. S., and Ferrara, N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth *in vivo.* Nature (Lond.), *362:* 841-844, 1993.

4. Olson, K. A., Fett, J. W., French, T. C, Key, M. E., and Vallée, B. L. Angiogenm antagonists prevent tumor growth *in vivo*. Proc. Natl. Acad. Sci. USA, *92:* 442-446, 1995.

5. Ferrara, N, Clapp, C, and Weiner, R. The 16K fragment of prolactin specifically inhibits basal or fibroblast growth factor stimulated growth of capillary endothelial cells. Endocrinology, *129:* 896-900, 1991.

6. Clapp, C, Martial, J. A., Guzman, R. C, Rentier-Delure, F., and Weiner, R. I. The 16-kilodalton N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis. Endocrinology, *133:* 1292-1299, 1993.

7. Good, D. L, Polverini, P. J., Rastinejad, F., Le Beau, M. M., Lemons, R. S., Frazier, W. A., and Bouck, N. P. A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of throm-bospondm. Proc. Natl. Acad. Sci. USA, *87:* 6624-6628, 1990.

8. O'Reilly, M. S., Holmgren, L., Shmg, Y., Chen, C, Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma [see comments]. Cell, *79:* 315-328, 1994.

9. O'Reilly, M. S., Boehm, T., Shmg, Y., Fukai, N, Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, *88:* 277-285, 1997.

10. Sharpe, R. L, Byers, H. R., Scott, C. F., Bauer, S. I, and Maione, T. E. Growth inhibition of murine melanoma and human colon carcinoma by recombinant human platelet factor 4. J. Natl. Cancer Inst., *82:* 848-853, 1990.

11. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, *86:* 353-364, 1996.

12. Takahashi, Y., Kitadai, Y., Bucana, C. D., Cleary, K. R., and Ellis, L. M. Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. Cancer Res., *55:* 3964-3968, 1995.

13. Lee, J. C, Chow, N. H., Wang, S. T., and Huang, S. M. Prognostic value of vascular endothelial growth factor expression in colorectal cancer patients. Eur. J. Cancer, *36:* 748-753, 2000.

14. Takahashi, Y., Bucana, C. D., Cleary, K. R., and Ellis, L. M. p53, vessel count, and vascular endothelial growth factor expression in human colon cancer. Int. J. Cancer, *79:* 34-38, 1998.

15. Warren, R. S., Yuan, H., Math, M. R., Gillett, N. A., and Ferrara, N. Regulation by vascular endothelial growth factor of human colon cancer tumorigenesis in a mouse model of experimental liver metastasis. J. Clin. Investig., *95:* 1789-1797, 1995.

16. Shaheen, R. M., Davis, D. W., Liu, W., Zebrowski, B. K., Wilson, M. R., Bucana, C. D., McConkey, D. J., McMahon, G., and Ellis, L. M. Antiangiogenic therapy targeting the tyrosine kinase receptor for vascular endothelial growth factor receptor inhibits the growth of colon cancer liver metastasis and induces tumor and endothelial cell apoptosis. Cancer Res., *59:* 5412-5416, 1999.

17. Tanaka, T., Konno, H., Matsuda, I, Nakamura, S., and Baba, S. Prevention of hepatic metastasis of human colon cancer by angiogenesis inhibitor TNP-470. Cancer Res., *55:* 836-839, 1995.

18. Takatsuka, S., Yamada, N., Sawada, T., Ogawa, Y., Maeda, K., Ohira, M., Ishikawa, T., Nishino, H., Seki, S., and Hirakawa, Y. S. C. K. Contribution of angiogenesis to the progression of colon cancer: possible inhibitory effect of angiogenesis inhibitor TNP-470 on tumor growth and hepatic metastasis. Int. J. Oncol., *17:* 253-258, 2000.

19. Maione, T. E., Gray, G. S., Hunt, A. J., and Sharpe, R. J. Inhibition of tumor growth in mice by an analogue of platelet factor 4 that lacks affinity for heparin and retains potent angiostatic activity. Cancer Res., *51:* 2077-2083, 1991.

20. Yoon, S. S., Eto, H., Lin, C. M., Nakamura, H., Pawhk, T. M., Song, S. U, and Tanabe, K. K. Mouse endostatin inhibits the formation of lung and liver metastases. Cancer Res., *59:* 6251-6256, 1999.

21. D'Angelo, G., Struman, I, Martial, J., and Weiner, R. I. Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16-kDa N-termmal fragment of prolactin. Proc. Natl. Acad. Sci. USA, *92:* 6374-6378, 1995.

22. D'Angelo, G., Martini, J-F., lin, T., Fantl, W. J., Martial, J., and Weiner, R. I. 16K human prolactin inhibits vascular endothelial growth factor-induced activation of Ras m capillary endothelial cells. Mol. Endocrinol., *13:* 692-704, 1999.

23. Martini, J. F., Plot, C, Humeau, L. M., Struman, I, Martial, J. A., and Weiner, R. I. The antiangiogenic factor 16K PRL induces programmed cell death in endothelial cells by caspase activation. Mol. Endocrinol., *14:* 1536-1549, 2000.

24. Struman, I, Bentzien, F., Lee, H., Mainfroid, V., D'Angelo, G., Goffin, V., Weiner, R. I, and Martial, J. A. Opposing actions of intact and N-terminal fragments of the human prolactin/growth hormone family members on angiogenesis: an efficient mechanism for the regulation of angiogenesis. Proc. Natl. Acad. Sci. USA, *96:* 1246-1251, 1999.

25. Duenas, Z., Torner, L., Corbacho, A. M., Ochoa, A., Gutierrez-Ospina, G., Lopez-Barrera, F., Barrios, F. A., Berger, P., Martinez de la Escalera, G., and Clapp, C. Inhibition of rat corneal angiogenesis by 16-kDa prolactin and by endogenous prolactm-like molecules. Investig. Ophthalmol. Vis. Sci., *40:* 2498-2505, 1999.

26. Clapp, C, and Weiner, R. I. A specific, high affinity, saturable binding site for the 16-kilodalton fragment of prolactin on capillary endothelial cells. Endocrinology, *130:* 1380-1386, 1992.

27. Gospodarowicz, D., Cheng, L, and Lirette, M. Bovine brain and pituitary fibroblast growth factors: comparison of their abilities to support the proliferation of human and bovine vascular endothelial cells. J. Cell Biol., *97:* 1677-1685, 1983.

28. Cooke, N. E., Coit, D., Shine, J., Baxter, J. D., and Martial, J. A. Human prolactin. cDNA structural analysis and evolutionary comparisons. J. Biol. Chem., *256:* 4007-4016, 1981.

29. Werner, S., Weinberg, W., Liao, X., Peters, K. G., Blessing, M., Yuspa, S. H., Weiner, R. L., and Williams, L. T. Targeted expression of a dominant-negative FGF receptor mutant in the epidermis of transgenic mice reveals a role of FGF in keratinocyte organization and differentiation. EMBO J., *12:* 2635-2643, 1993.

30. Barkey, R. L, Shani, J., Goldhaber, G., and Barzilai, D. Binding of iodinated rat and ovine prolactins to prolactin receptors and to its antibodies. Mol. Cell. Endocrinol., *23:* 105-113, 1981.

31. Greenwood, F. C, Hunter, W. M., and Glover, J. S. The preparation of ^{131}I-labelled growth hormone of high specific radioactivity. Biochem. J., *89:* 114-123, 1963.

32. Lee, H., Struman, I, Clapp, C, Martial, L, and Weiner, R. I. Inhibition of urokinase activity by the antiangiogenic factor 16K prolactin: activation of plasminogen activator inhibitor 1 expression. Endocrinology, *139:* 3696-3703, 1998.

33. Taylor, S., and Folkman, J. Protamine is an inhibitor of angiogenesis. Nature (Lond.), *297:* 307-312, 1982.

34. Mombaerts, P., Iacomini, L, Johnson, R. S., Herrup, K., Tonegawa, S., and Papaioannou, V. E. Rag-1-deficient mice have no mature B and T lymphocytes. Cell, *68:* 869-877, 1992.

35. Weidner, N, Semple, J. P., Welch, W. R., and Folkman, J. Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. N. Engl. J. Med., *324:* 1-8, 1991.

36. Gallicchio, M., Hufnagl, P., Wojta, J., and Tipping, P. IFN-gamma inhibits thrombin-and endotoxin-induced plasminogen activator inhibitor type 1 in human endothelial cells. J. Immunol., *157:* 2610-2617, 1996.

37. Streit, M., Riccardi, L., Velasco, P., Brown, L. F., Hawighorst, T., Bornstein, P., and Detmar, M. Thrombospondin-2: a potent endogenous inhibitor of tumor growth and angiogenesis. Proc. Natl. Acad. Sci. USA, *96:* 14888-14893, 1999.

38. Cao, Y., O'Reilly, M. S., Marshall, B., Flynn, E., Ji, R. W., and Folkman, J. Expression of angiostatin cDNA in a murine fibrosarcoma suppresses primary tumor growth and produces long-term dormancy of metastases [published erratum appears m J. Clin. Investig. *102:* 2031, 1998]. J. Clin Investig., *101:* 1055-1063, 1998.

39. Fidler, I. J. Rationale and methods for the use of nude mice to study the biology and therapy of human cancer metastasis. Cancer Metastasis Rev., *5:* 29-49, 1986.