Antiangiogenic Activity of Genistein in Pancreatic Carcinoma Cells Is Mediated by the Inhibition of Hypoxia-Inducible Factor-1 and the Down-Regulation of *VEGF* Gene Expression

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BACKGROUND. Previous reports indicate that Genistein, a naturally occurring isoflavonoid, exhibits strong antiangiogenic activity. The underlying mechanism of inhibition, however, remains unclear. Among the biologic effects of Genistein are the inhibition of tyrosine kinases and the inhibition of hypoxic activation of hypoxia-inducible factor-1 (HIF-1), one of the main regulators of *VEGF* gene expression.

METHODS. Hypoxic cell culture was performed in a modular incubator chamber. Vascular endothelial growth factor (VEGF) protein secretion was measured using the enzyme-linked immunosorbent assay, binding of DNA by HIF-1 was measured using the electrophoretic mobility shift assay, and mRNA quantification was performed using Northern blot analysis. Pancreatic carcinoma was studied in an orthotopic murine model. Angiogenesis in vivo was quantified by staining xenograft tumors for endothelial cell markers.

RESULTS. VEGF protein secretion was dose-dependently suppressed with increasing doses of Genistein. Furthermore, treatment of pancreatic carcinoma cells with Genistein led to impaired activation of HIF-1 under hypoxic culture conditions. Northern blot analysis indicated that *VEGF* mRNA expression decreased upon treatment with Genistein, both under normoxic and hypoxic culture conditions. In vivo, Genistein inhibited tumor growth for xenograft pancreatic carcinoma cells, whereas extensive hypoxia was observed in xenograft tumors and was not influenced by Genistein therapy. Similarly, decreased *VEGF* mRNA levels were observed in Genistein-treated Capan-1 xenograft tumors.

CONCLUSIONS. The current study indicates that the previously reported antiangiogenic activity of Genistein probably is mediated by the inhibition of HIF-1, an important regulator of *VEGF* gene homeostasis, particularly under low-oxygen conditions. Therefore, this bioactive compound may well be beneficial to patients with pancreatic carcinoma. ***Cancer* 2004;100:201–10.**

KEYWORDS: Genistein, pancreatic carcinoma, angiogenesis, hypoxia-inducible factor-1, vascular endothelial growth factor, pancreatic carcinoma model.

Cancer of the exocrine pancreas, mostly involving ductal differentiation, accounts for more than 90% of pancreatic malignancies.[1,2] With nearly 28,000 newly diagnosed cases per year and almost the same number of fatalities, it is one of the deadliest diseases in humans.[2] At present, pancreatic carcinoma is the fourth leading cause of death due to malignant disease among both men and women in Western countries.[2] Most patients present with advanced disease,

which disqualifies them from receiving operative therapy; palliative therapy, however, does not control tumor growth.[3] Therefore, new therapies are urgently required to better treat these patients. The recent development of the concept of antiangiogenic therapy may provide new hope in this regard.

Aggressive local and early systemic tumor growth are hallmarks of pancreatic carcinoma and distinguish this disease from other types of adenocarcinoma.[4–6] Pancreatic carcinoma is further characterized by extensive desmoplastic tissue remodeling and by a hypoxic microenvironment within the solid tumor mass.[7–9] In fact, we and others have demonstrated that hypoxia specifically activates a transcription factor called hypoxia-inducible factor-1 (HIF-1), which, upon hypoxic activation, transcriptionally up-regulates vascular endothelial growth factor (VEGF), a protein responsible for many of the vascular responses observed in human tumors, including the formation of new blood vessels and the permeabilization of existing ones.[10–13] Stimulation of tumor angiogenesis (e.g., by hypoxia) induces the up-regulation of angiogenic factors, the most important of which is VEGF.[10,14–16] HIF-1α, the hypoxia-specific subunit of HIF-1, and VEGF have been shown to be overexpressed in patients with pancreatic carcinoma.[10,17]

Previous studies reported that Genistein, a bioactive substance found in soybeans, possesses strong antiangiogenic activity.[18–20] To date, the mechanisms underlying these previous observations have not been investigated. Genistein exhibits multiple effects on the growth of human malignant cells via the inhibition of tyrosine kinases.[20–23] Of particular interest is Genistein's antiangiogenic activity, which we speculate is mediated by the inhibition of transcriptional activation of HIF-1 under low-oxygen conditions.

In the current study, we investigated one possible mechanism by which Genistein may exert its previously reported antiangiogenic activity. We have demonstrated that Genistein potently inhibits angiogenesis and tumor growth. Neoangiogenesis probably is inhibited via inhibition of hypoxic activation of HIF-1, which in turn reduces *VEGF* gene expression. In vitro, hypoxia was found to be a potent stimulus for VEGF production in all pancreatic carcinoma cell lines tested, and Genistein was found to inhibit hypoxic activation of HIF-1 in a dose-dependent manner; this inhibition was accompanied by the dose-dependent down-regulation of VEGF in vitro. In vivo testing of Genistein took place in an orthotopic murine pancreatic carcinoma model that recently was developed by our group.[24,25] Using this model, we demonstrated that human pancreatic carcinoma xenografts grow in a hypoxic environment and that Genistein reduces tumor growth via the inhibition of tumor neoangiogenesis, most likely through down-regulation of VEGF expression. These results provide further evidence that the bioactive flavone Genistein inhibits tumor growth by modulation of various tumor growth–suppressive pathways, including inhibition of cell cycle progression, induction of apoptosis, and inhibition of angiogenesis.[20,21,26]

MATERIALS AND METHODS

Cell Culture

Five different human pancreatic carcinoma cell lines were used in the current study. The moderately differentiated human pancreatic adenocarcinoma cell lines Capan-1 and Capan-2 and the less differentiated human pancreatic carcinoma cell lines AsPc-1, PANC-1, and Mia PaCa-2 were obtained from the American Tissue Type Culture Collection (Rockville, MD). Cell lines were cultured in Dulbecco Modified Eagle Medium (DMEM; Life Technology, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technology), penicillin G (100 units/mL), and streptomycin (100 μg/mL). Cells were grown as a monolayer culture at 37 °C in humidified air with 5% (for Capan-1, Capan-2, and AsPc-1) or 10% (for Mia PaCa-2 and PANC-1) CO_2. Unless otherwise indicated, all chemicals were purchased from Sigma Chemicals (St. Louis, MO).

RNA Extraction and Northern Blot Analysis

Total RNA was extracted using the guanidine isothiocyanate method, and 20 μg total RNA was size-fractionated on a denaturing 1.2% agarose gel containing 1.8 M formaldehyde and stained with ethidium bromide for verification of RNA integrity and evenness of loading.[27] RNA was electrotransferred onto a nylon membrane (Hybond+; Amersham Pharmacia Biotech, Piscataway, NJ) and cross-linked with ultraviolet irradiation. The blots then were prehybridized, hybridized, and washed under appropriate conditions for cDNA probes. Membranes were prehybridized for 6 hours at 42 °C in a buffer containing 50% formamide, 1% sodium dodecyl sulfate (SDS), 0.75 M sodium chloride, 5 mM ethylenediamine tetraacetic acid (EDTA), 5X Denhardt solution (1X Denhardt solution, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 100 μg/mL salmon sperm DNA, 10% dextran sulfate, and 50 mM Na_2PO_4 (pH 6.5). Hybridization was performed at 42 °C overnight with the labeled cDNA probe, which had a specific activity of $> 1 \times 10^5$ counts per minute (cpm) per mL. The blots were rinsed twice with 2X sodium chloride/sodium citrate (SSC) and then washed 3 times at 55 °C with 0.2X SSC and 2% SDS. Blots then were autoradio-

graphed at −80 °C with intensifying screens to Kodak BioMax Films (Kodak, Rochester, NY) for the indicated period. VEGF hybridization signals and 7S cDNA probe control signals were quantified by scanning densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). *VEGF* mRNA expression was quantified by calculating the specific signal intensity of the *VEGF* band in relation to the signal intensity of the 7S control band to compensate for loading inhomogeneity. In the cell culture experiments, the hybridization signals of untreated normoxic control cells were set equal to 1.0. In the experiments involving animal tissue, the median hybridization signal for untreated control animals was set equal to 1.0.

Generation of cDNA Probes

Total RNA extracted from normal human pancreatic tissue was used to synthesize cDNA by reverse transcription (RT) in a 20 μL reaction containing 0.5 μg random primers and 200 units of SuperScript RNase H^- reverse transcriptase (Life Technology), 1 μg total RNA, 4 mL 5X RT buffer, 5 mM dithiothreitol (DTT), each deoxynucleotide at a concentration of 0.1 mM, 20 units of RNAsin (Promega, Madison, WI), and diethyl pyrocarbonate–treated water. Each reaction mixture was incubated at 42 °C for 1 hour and then chilled on ice. RT products were amplified by the polymerase chain reaction (PCR), using gene-specific primers based on the published human cDNA sequence for *VEGF* (M32977). The oligonucleotide primers used for amplification of a 531–base pair (bp) fragment of the human *VEGF* gene were 5′-GGAGTACCCTGATGAGATCGA-3′ and 5′-GGACTGTTCTGTCGATGGTGA-3′. The PCR product was cloned into a pGEM Easy vector, which was amplified in JM109 *Escherichia coli* cells (Promega). The DNA sequence and cloning orientation were determined by sequencing. Isolated *VEGF* cDNA fragments were labeled via the incorporation of [α-^{32}P]deoxycytidine triphosphate (ICN Radiochemicals, Irvine, CA) through random priming.

Determination of VEGF Protein Levels in Cell Supernatant

To determine VEGF protein expression levels, equal densities of cells were analyzed. AsPc-1, Mia PaCa-2, and PANC-1 cells were seeded at 2.5×10^6 cells per 100 mm tissue culture plate, whereas Capan-1 and Capan-2 cells were seeded at 3.7×10^6 cells per plate to compensate for their slower growth. Cells were grown for 24 hours in DMEM supplemented with 10% FBS, washed 3 times with phosphate-buffered saline (PBS), and transferred into 10 mL DMEM supplemented with 1% FBS. Identical preparations for normoxic and hypoxic culture then were incubated for 24 hours and harvested at the same time point for protein analysis and cell counting. The amount of VEGF protein found in the supernatant from the cells was determined using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. VEGF protein levels were calculated in units of pg per 10^6 cells.

Preparation of Nuclear Extracts

For harvesting, cells were washed twice with cold Dulbecco PBS, scraped into ice-cold PBS, and pelleted by centrifugation at 1300 revolutions per minute (rpm) for 5 minutes at 4 °C. Nuclear extracts were prepared as described elsewhere.[11,28] Buffers A and C contained 0.5 mM DTT, 0.4 mM phenylmethylsulfonyl fluoride, 2 μg/mL pepstatin, 2 μg/mL leupeptin, and 1 mM sodium vanadate (all from Sigma Chemicals). The cell pellet was washed with 4 packed-cell volumes (pcv) of Buffer A (10 mM Tris-HCl [pH 7.8], 1.5 mM $MgCl_2$, and 10 mM KCl), resuspended in 4 pcv of Buffer A, and incubated on ice for 10 minutes. The cell suspension was Dounce-homogenized with a type B pestle, and the nuclei were pelleted by centrifugation at 3000 rpm for 5 minutes. The pellet was resuspended in 3 pcv of Buffer C (0.42 M KCl, 20 mM Tris-HCl [pH 7.8], 1.5 mM $MgCl_2$, and 20% glycerol) and mixed on a rotator at 4 °C for 30 minutes. Nuclear debris was pelleted by centrifugation for 30 minutes at 14,000 rpm and 4 °C. The supernatant was dialyzed against 1 change of Buffer Z (20 mM Tris-HCl [pH 7.8], 0.1 M KCl, 0.2 mM EDTA, and 20% glycerol) for at least 3 hours at 4 °C. The dialysate was centrifuged for 10 minutes at 14,000 rpm and 4 °C, and aliquots were frozen in liquid N_2 and stored at −80 °C for further analysis. Protein concentration was measured using a Bio-Rad (Hercules, CA) assay, with serum albumin as a calibration standard.

Electrophoretic Mobility Shift Assay (EMSA)

Oligonucleotide probes containing the hypoxia response element (HRE) within the *VEGF* promoter sequence were purchased from Life Technology. The binding site–specific sequence (coding strand) of the wild-type probe was 5′-AGCTTGCCCTACGTGCTGTCTCAG-3′. The sequence of the mutant probe was 5′-GAGCTTGCCCTAAAAGCTGTCTCAG-3′. Radioactive oligonucleotides were generated by 5′-end labeling of the sense strand with [γ-^{32}P]adenosine triphosphate (ICN Radiochemicals) using T4 polynucleotide kinase (Amersham Pharmacia Biotech), annealing to an excess of the antisense strand. Binding reactions were performed in an overall volume of 20 μL, containing 5 μg nuclear extracts and 0.1 μg denatured calf thymus DNA in 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT,

and 5% glycerol. After preincubation for 5 minutes on ice, 1 ng of the radiolabeled probe (10,000 cpm) was added. Incubation on ice was continued for 10 minutes; in the case of a supershift, an additional 20-minute incubation on ice was carried out. In competition experiments, aliquots of competing DNA were added 5 minutes before the addition of the labeled probe.

The reaction mixture then was loaded onto a 5% nondenaturing polyacrylamide gel, which was prerun at 185 volts (V) for 2 hours. Electrophoresis was performed at 185 V in 0.3X TBE (1X TBE: 89 mM Tris-HCl, 89 mM boric acid, and 5 mM EDTA) at 4 °C. Gels were vacuum-dried and autoradiographed with intensifying screens to Kodak BioMax Films at −80 °C for 1–2 days.

Immunohistochemistry

At sacrifice, tumors were harvested snap-frozen or fixed in formalin for further analysis. For assessment of vascularity, we randomly selected 3 frozen tumor samples and cut 2 frozen sections, each 5 μm thick, from different regions within each sample. Thus, six slides were processed to determine the microvessel density in each xenograft tumor. The sections were fixed in acetone for 10 minutes. Upon fixation, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide and 0.1% sodium azide in PBS. After the slides were blocked in normal goat serum for 20 minutes, biotinylated antimouse CD31 antibody (BD Pharmingen, San Diego, CA) was added at a dilution of 1:40 and incubated overnight at 4 °C. After thorough rinsing with Tris-buffered saline–Tween washing solution, slides were incubated with streptavidin-biotin for 25 minutes and then with streptavidin peroxidase for 15 minutes. For color development, 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemicals) served as a substrate for peroxidase for 5 minutes. In control experiments, either the first or second antibody was omitted; in these cases, no immunopositivity was detectable. Unless otherwise indicated, all immunohistochemical reagents were purchased from Kirkegaard & Perry (Gaithersburg, MD). Stained tissue specimens were analyzed by two independent observers who were blinded to the animal's status. Microvessel density was determined as described by Weidner and colleagues.[29,30] The area of greatest vascularization was determined by scanning the slide at low-power magnification. Microvessel density was determined by counting the number of stained vessels in ten 200× fields (0.74 mm^2 per field).

Polarographic Oxygen Measurement—Eppendorf Histograph

At sacrifice, mice were anesthetized with sodium pentobarbital, and a laparotomy was performed to ensure complete access to the abdominal cavity. Without further mobilization of the pancreatic tumor mass, three individual oxygen measurements were made from different directions in each tumor. The Eppendorf pO_2 Histograph Model KIMOC-6650 (Eppendorf, Hamburg, Germany), along with a sterile polarographic needle microelectrode, was used to measure tumor oxygen tension (pO_2). The electrode consisted of a gold wire (0.012 mm in diameter) contained within a 0.3 mm steel probe casing and covered with a Teflon (DuPont, Wilmington, DE) membrane at its tip. The electrode was biased to a negative potential of 700 mV against a reference Ag/AgCl anode. Oxygen readings were corrected for tissue temperature before the measurements were made. Additional technical characteristics of the probe are well documented elsewhere.[31] Immediately before each measurement, the probe was calibrated in a sterile, room-temperature 0.9% NaCl solution through which sterile, pure nitrogen and room air alternately flowed. Only probes with fast response times and a drift of less than 0.05% per minute at equilibration were used to make measurements. The probe initially was inserted 0.5 mm into the tumor under direct vision and was allowed to adapt to the tissue environment, as indicated by a series of steady readings; subsequently, the probe automatically was advanced into the tumor under computer control in 1.0 mm steps. Each forward step of 1.0 mm was followed by a 0.3 mm retraction, resulting in a net step length of 0.7 mm between individual measurements. Artifacts due to vessel compression and the O_2 consumption of the cathode were minimized by the selection of probes with fast response times and by the special movement pattern of the probes, as described elsewhere.[31,32]

The track length was set according to the size of each individual xenograft tumor. Thirty to 60 measurements (10–20 measurements per track over 3 tracks) were performed in each tumor xenograft, but the number of measurements was strictly limited to avoid excessive track length, which would increase the risk that the probe would pass completely through the tumor, resulting in measurements being made inadvertently in normal tissue or in injury to neighboring organs or peripancreatic blood vessels. The probe was continuously observed during measurement. When probe bending occurred, without actual advancement of the probe into the tumor, the measurement was halted immediately and the data obtained during

bending were excluded. As part of the computerized measurement process, at the end of each track measurement, the probe automatically was removed from the tumor and repositioned for another measurement. The exact location of each measurement was recorded according to position on an imaginary clock face, with the head of the mouse at 12 o'clock. All data were stored automatically in the Eppendorf Histograph. At the end of each assay, the probe was recalibrated as described above. Recalibrated pO_2 measurements and pO_2 histograms were obtained for each track, with a class width of 2.5 mm Hg.

Statistical Analysis

Results are presented as mean values ± standard errors. Statistical significance was determined by the Student *t* test. A two-tailed *P* value less than 0.05 indicated statistical significance.

RESULTS

Suppression of Hypoxic Induction of *VEGF* Gene Expression in Pancreatic Carcinoma Cell Lines by Genistein

When cultured for 24 hours in a hypoxic microenvironment, all human pancreatic carcinoma cell lines that were tested responded with a strongly significant increase in VEGF protein secretion.[10] Treatment of these cell lines with Genistein at a concentration of 10, 50, 100, or 250 μM resulted in dose-dependent and time-dependent suppression of VEGF protein secretion (Fig. 1). Low-dose therapy with 10 μM Genistein reduced VEGF levels in certain cell lines; however, treatment with 50 μM Genistein resulted in significant suppression of VEGF secretion in all cell lines tested. Genistein treatment at a dose of 250 μM resulted in nearly complete reversion of hypoxic VEGF protein secretion to the levels observed in normoxically cultured cells (Fig. 1).

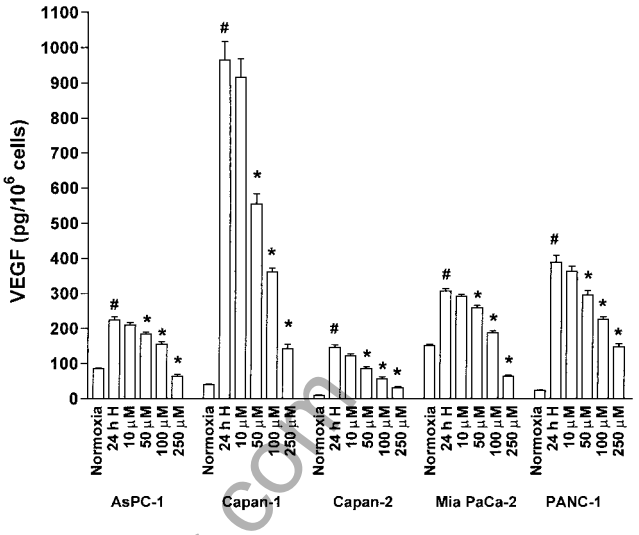

FIGURE 1. Effect of Genistein treatment on experimentally induced hypoxic vascular endothelial growth factor (VEGF) protein secretion. Five different cell lines (AsPc-1, Capan-1, Capan-2, Mia PaCa-2, and PANC-1) were cultured for 24 hours under normoxic or hypoxic conditions, either without Genistein or in the presence of the indicated dose of Genistein (10, 50, 100, or 250 μM). VEGF protein levels were measured in conditioned cell medium using an enzyme-linked immunosorbent assay. Individual cell counts were made using a hemocytometer, and the amount of secreted VEGF protein was calculated in units of pg per 10^6 pancreatic carcinoma cells. Values are expressed as mean values ± standard errors. #: $P < 0.05$ in comparison with normoxic cells; *: $P < 0.05$ in comparison with untreated cells; 24 h H: 24 hours under hypoxic conditions.

Activation and Inhibition of Hypoxic Induction of HIF-1

We previously demonstrated that hypoxia activates the transcription factor HIF-1, which regulates VEGF production in human pancreatic carcinoma.[10] Genistein is an inhibitor of protein tyrosine kinases and reportedly inhibits hypoxic activation of HIF-1.[33,34] After a 4-hour period of hypoxia, Genistein treatment dose-dependently suppressed the DNA-binding activity of HIF-1. The lower doses of Genistein (10 and 50 μM) did not exhibit a significant inhibitory effect as measured by EMSA, whereas the higher doses (100 and 250 μM) blocked the hypoxia-induced DNA-binding activity of HIF-1 (Fig. 2).

Reduction of *VEGF* mRNA Expression Due to HIF-1 Inhibition

Inhibition of HIF-1 and its effect on *VEGF* gene expression were further analyzed using the human pancreatic carcinoma cell lines Capan-1 and Mia PaCa-2. Capan-1 is a moderately differentiated cell line, whereas Mia PaCa-2 is an undifferentiated cell line. To test whether HIF-1 inhibition resulted in transcriptional suppression of *VEGF* gene expression, mRNA levels were quantified with Northern blot analysis after 24 hours of either normal or hypoxic culture conditions (Fig. 3A). Under hypoxic conditions, *VEGF* mRNA expression sharply increased in both cell lines tested (Fig. 3). The culturing of pancreatic carcinoma cells under hypoxic conditions in the presence of various doses of Genistein reduced *VEGF* mRNA expression as measured after 24 hours of hypoxia. Genistein doses of 100 and 250 μM, which are comparable to the doses at which the DNA-binding activity of HIF-1 was inhibited, led to a significant reduction in *VEGF* mRNA expression in both cell lines. The inhibitory effect of Genistein was more pronounced in the undifferentiated Mia PaCa-2 cell line (Fig. 3B). Under normoxic culture conditions, *VEGF* mRNA was detectable in

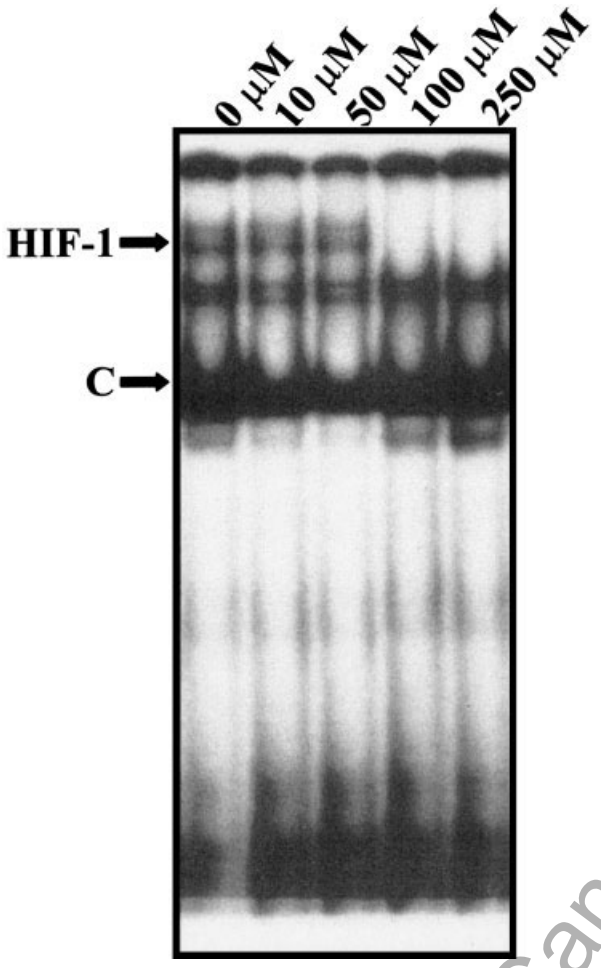

FIGURE 2. Suppression of binding to the vascular endothelial growth factor (*VEGF*) promoter by hypoxia-inducible factor-1 (HIF-1) with increasing doses of Genistein in Mia PaCa-2 cells. Electrophoretic mobility shift assays were used to investigate the binding of HIF-1 to the *VEGF* promoter in Mia PaCa-2 cells. Cells were cultured for 4 hours under hypoxic conditions in 0–250 μM Genistein, which was preincubated for 30 minutes before hypoxic exposure. Nuclear extracts were prepared as described in Materials and Methods. Dose-dependent suppression of HIF-1 DNA-binding activity was observed, with no detectable DNA binding at Genistein doses of 100 and 250 μM. HIF-1: specific inducible HIF-1 band; C: constitutive band.

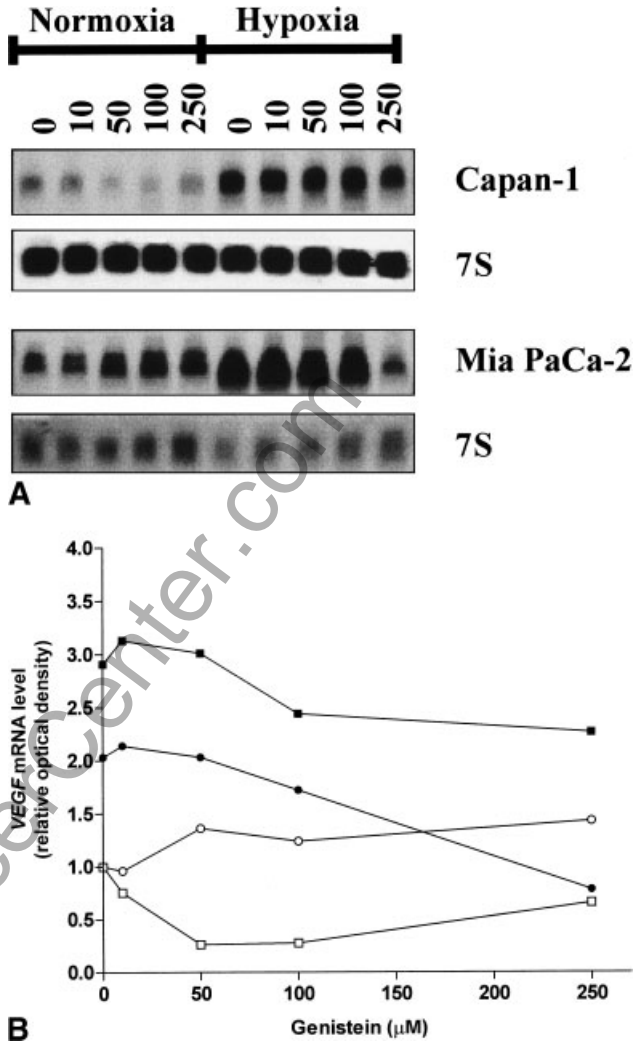

FIGURE 3. Effect of Genistein on vascular endothelial growth factor (*VEGF*) mRNA expression in human pancreatic carcinoma cells. (A) The human pancreatic carcinoma cell lines Mia PaCa-2 and Capan-1 were cultured for 24 hours under either normal or hypoxic conditions. Total RNA was isolated, size-fractionated, and transferred to a membrane, where hybridization with a cDNA probe containing a 500–base pair fragment of the human *VEGF* gene took place. The 7S probe served as a control against unequal loading. (B) Densitometry was performed as described in Materials and Methods. The optical density of untreated normoxic control cells was measured and defined as a relative optical density of 1.0. Individual measurements were made to determine the increase or decrease in optical density relative to untreated normoxic cells. Empty squares: Capan-1 cells under normoxic conditions; filled squares: Capan-1 cells under hypoxic conditions; empty circles: Mia PaCa-2 cells under normoxic conditions; filled circles: Mia PaCa-2 cells under hypoxic conditions.

both cell lines, although the Capan-1 cell line expressed only constitutively low levels (Fig. 3A).

Suppression of Tumor Growth In Vivo in Nude Mice by Genistein

To test the effects of Genistein in vivo, the undifferentiated Mia PaCa-2 cell line and early passages of the more differentiated Capan-1 cell line were used. To optimize the microenvironment for pancreatic tumor growth and to better emulate human pancreatic carcinoma, we used an orthotopic xenograft model.[35,36] This model closely reproduced many of the typical symptoms found in humans, including metastasis and tumor ascites.[6,26,37] Animals were injected daily with 1.3 mg Genistein intraperitoneally; this amount was comparable to the 100 μM dose used in vitro. In the

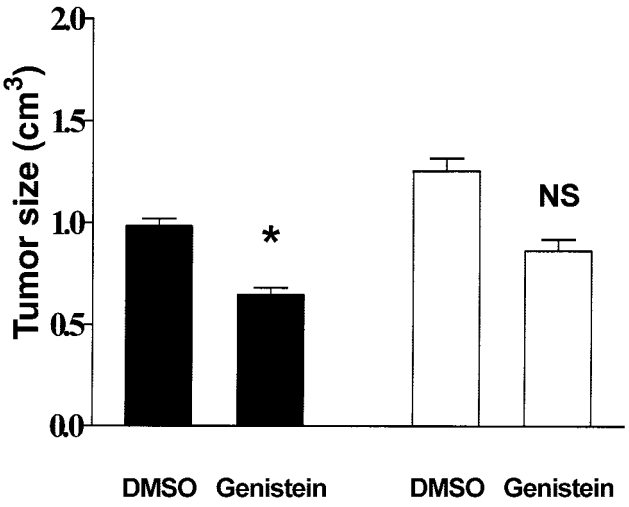

FIGURE 4. Survival of animals bearing orthotopic pancreatic carcinoma xenografts. Pancreatic carcinoma was induced by transplantation of a small xenograft tumor piece derived from a subcutaneous tumor from either the Capan-1 (black bars; $n = 16$) or Mia PaCa-2 cell line (clear bars; $n = 16$). One week after tumor transplantation, Genistein treatment was initiated. Each animal received either 1.3 mg Genistein per day intraperitoneally or an equivalent volume of diluted dimethylsulfoxide (DMSO). After 8 weeks of therapy, animals were sacrificed and the tumor was measured in 3 perpendicular dimensions. *: $P < 0.05$; NS: not significant.

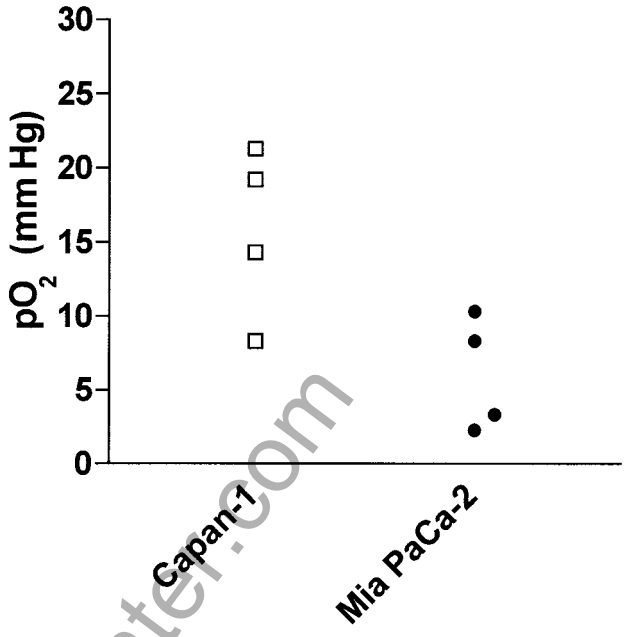

FIGURE 5. Tissue oxygenation of human pancreatic carcinoma xenografts grown in nude mice. Tumor xenografts derived from the Mia PaCa-2 and Capan-1 cell lines grew orthotopically within the pancreatic parenchyma of nude mice. Before sacrifice, mice were anesthetized and tumor oxygenation was measured using the Eppendorf Histograph (Eppendorf, Hamburg, Germany). Data points represent the median oxygenation levels of different xenograft tumors, as described in Materials and Methods.

sham-treated group, animals received an equal volume of diluted dimethylsulfoxide (DMSO), which was the solvent for Genistein. Animals were treated over a period of 8 weeks. In animals treated with Genistein, tumor growth was slower than in animals treated with DMSO (Fig. 4). At sacrifice, no significant difference was noted with regard to animal weight peripancreatic tumor infiltration, or the amount of ascites produced (data not shown).

Growth of Human Tumor Xenografts in a Hypoxic Microenvironment

Whether and to what extent hypoxia is present during the growth of human xenograft tumors is unknown. Because HIF-1 reportedly is activated only at lower oxygen concentrations, tumor oxygenation was quantified in four untreated Mia PaCa-2 tumor xenografts and four untreated Capan-1 tumor xenografts (Fig. 5). A polarographic measurement method was used to quantify tumor oxygenation.[38] Undifferentiated Mia PaCa-2 xenograft tumors had a median oxygenation level of 6.1 mm Hg, whereas the better-differentiated Capan-1 xenografts had a median oxygenation level of 16.3 mm Hg (Fig. 5). Oxygenation levels in both sets of xenograft tumors were found to be within the range necessary for HIF-1 activation, which occurs at levels < 35 mm Hg ($\approx 5\%$ O_2).[39,40]

Down-Regulation of *VEGF* mRNA in Tumor Specimens

Using Northern blot analysis, expression of *VEGF* mRNA was quantified in both treated and untreated animals. Because Genistein inhibits hypoxic activation of HIF-1 DNA-binding activity, *VEGF* mRNA levels in Genistein-treated Capan-1 xenograft tumors were lower than in the untreated controls; however, no significant difference in *VEGF* mRNA levels was detectable in Mia PaCa-2 xenograft tumors (Fig. 6).

Microvessel Density

Because the combination of HIF-1 and VEGF may play an important role in the formation of new tumor blood vessels, we attempted to determine whether reduced expression of the angiogenic cytokine VEGF and retardation of tumor growth were associated with differences in the numbers of blood vessels in treated animals. For specific vessel staining, we used frozen tumor specimens from both groups along with a monoclonal antimouse CD31 antibody. Both types of xenograft tumors exhibited fewer CD31-stained blood vessels; however, statistical significance was observed only in the Capan-1 xenografts. Nonetheless, a clear trend also was noted in Mia PaCa-2 xenografts (Table 1).

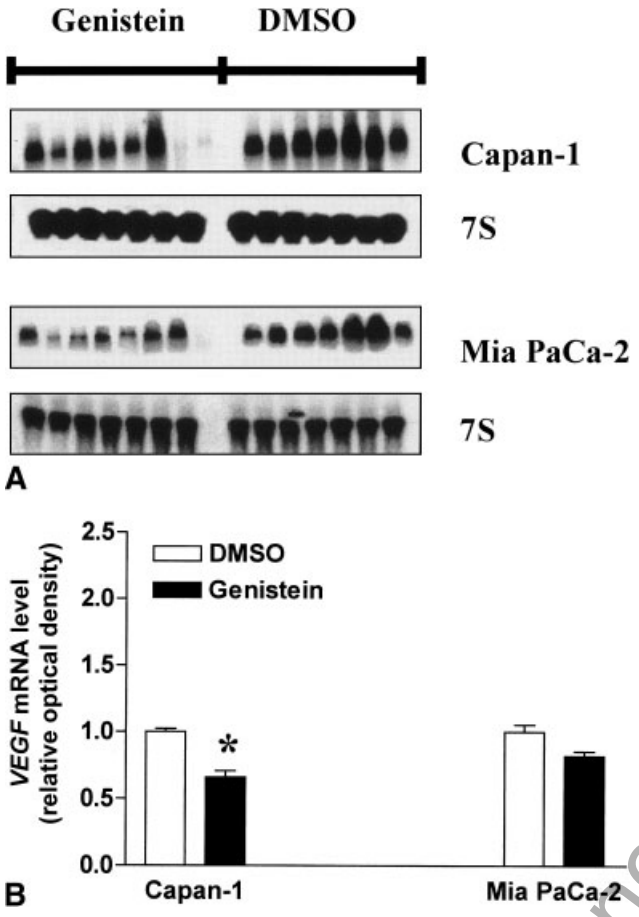

FIGURE 6. Expression of the angiogenic cytokine vascular endothelial growth factor (VEGF) in human xenograft tumors. (A) Mia PaCa-2 and Capan-1 xenograft tumors were grown orthotopically in nude mice. At sacrifice, tumors were resected and immediately frozen in liquid nitrogen. Total mRNA was extracted using the guanidium isothiocyanate method and size-fractionated on a denaturating 1.2% agarose gel. Radioactively labeled cDNA probes were used for the hybridization reaction. Membranes were exposed for 12 hours (VEGF). A 7S cDNA probe was used to control against unequal loading. (B) Densitometry was performed as described in Materials and Methods. For each cell line, the density readings for control-treated animals were pooled and compared with the pooled readings for experimentally treated animals. The optical density for the experimental group was calculated relative to the optical density for the control group. DMSO: dimethylsulfoxide.

DISCUSSION

Hypoxia is a microenvironmental condition that has been shown to influence many physiologic and pathologic processes.[41] Because low-oxygen conditions not only select for *p53*-mutated cells but also induce tumor neoangiogenesis, these conditions are believed to play a more active role during the growth and progression of human malignancies.[33,41–45] Among several other factors, tumor hypoxia is a key pathophysiologic stimulus for *VEGF* gene expression, a peptide that is closely linked to tumor neoangiogenesis.[15,44,46] It is not known to what extent tumor hypoxia influences the growth of human pancreatic carcinoma, but it is well known that this scirrhous malignancy grows in an environment of excessive hypoxia.[9] Both HIF-1 and VEGF are overexpressed in human pancreatic carcinoma, and HIF-1 is detectable in the nuclei of human pancreatic carcinoma cells but only barely detectable in normal pancreatic tissue.[10]

Genistein is a naturally occurring isoflavonoid that reportedly has multiple effects on malignant cell growth, including inhibition of cell proliferation and induction of apoptosis.[20–23,26,47,48] Furthermore, it has been reported that Genistein potently inhibits tumor angiogenesis.[18,19,49] In the current study, we have identified one possible mechanism by which Genistein inhibits angiogenesis; this mechanism involves the inhibition of hypoxic activation of HIF-1, which leads to down-regulation of *VEGF* gene expression under hypoxic growth conditions. Because of the importance of tumor angiogenesis in tumor formation, diet-derived phytochemicals with antiangiogenic activity may be beneficial to humans.[18–20,47,49–51]

Genistein is a nonspecific inhibitor of HIF-1, but to date, there are no known selective inhibitors of HIF-1. Treatment of pancreatic carcinoma cells with various doses of Genistein led to a dose-dependent and time-dependent down-regulation of *VEGF* gene expression in vitro. In DNA binding experiments, inhibition of HIF-1 DNA-binding activity was observed in the same dose range in which *VEGF* gene expression was inhibited. The dose required to suppress *VEGF* mRNA expression was higher than the dose required to suppress VEGF protein secretion. This observation may be related to the internal stabilization of *VEGF* mRNA under low-oxygen conditions, as is described elsewhere.[52–54] Stabilization of mRNA moieties also may be the reason for the discrepancy between secreted VEGF protein levels under low-oxygen con-

TABLE 1
Microvessel Density in Xenograft Tumors Stained with an Anti-CD-31 Antibody[a]

	Capan-1	Mia PaCa-2
Sham-treated group (DMSO)	94.8 ± 23.1	125.9 ± 31.9
Genistein-treated group (100 μM)	73.2 ± 21.5[b]	94.8 ± 44.6[c]

DMSO: dimethylsulfoxide.

[a] Orthotopically grown xenograft tumors were snap-frozen and stored at −80 °C. Frozen sections measuring 6 μm in thickness were stained with a purified monoclonal mouse anti-CD-31 antibody. Two different sections from each tumor specimen were analyzed. Mean values ± standard errors per microscopic field are displayed.

[b] $P < 0.05$.

[c] $P = 0.0897$.

ditions and the amount of *VEGF* mRNA that is detectable under these conditions. Alternatively, alterations in protein secretion under hypoxic conditions may be the reason why, for example, Capan-1 cells secreted more VEGF protein than did Mia PaCa-2 cells, despite the finding that Mia PaCa-2 cells had higher mRNA levels under these conditions. In addition, the observation of reduced *VEGF* mRNA levels under normoxic conditions upon treatment with Genistein, particularly in Capan-1 cells, indicates that Genistein may also influence *VEGF* mRNA homeostasis through other mechanisms that are independent of HIF-1.

The value of these in vitro observations was assessed in vivo in an orthotopic murine pancreatic carcinoma model. Animals were treated daily with 1.3 mg Genistein, which was comparable to the 100 μM dose used in vitro. Because HIF-1 is activated specifically at low oxygen levels, we measured tumor oxygenation in eight xenograft tumors to ensure that oxygen levels were sufficiently low for HIF-1 activation. The results from these measurements strongly suggest that the angiogenic pathway of HIF-1 and VEGF regulation is activated during tumor formation. Despite the finding that Genistein treatment did not completely prevent xenograft tumor growth, tumors in treated animals grew more slowly than did tumors in sham-treated animals. The growth-suppressive effect of Genistein treatment was at least partially related to its antiangiogenic activity, as tumor specimens from treated animals had lower microvessel counts than did tumor specimens from untreated animals. Furthermore, Genistein treatment resulted in the inhibition of VEGF expression, as lower *VEGF* mRNA levels were observed in tumor specimens from Genistein-treated animals. The observation that the growth-suppressive effect of Genistein was more pronounced in the moderately differentiated Capan-1 cell line than in the undifferentiated Mia PaCa-2 cell line may indicate that especially well-differentiated tumors are a target for Genistein. It is noteworthy that the bioactivity of Genistein, with its tumor suppressive effects, may well target several cellular pathways, including apoptosis and cell cycle regulation, that are important in tumor growth and metastasis.[19–21,26,35,49]

In summary, in the current study, we have demonstrated that the previously reported antiangiogenic activity of the naturally occurring isoflavonoid Genistein may be mediated by the inhibition of hypoxic activation of the transcription factor HIF-1 and by the resultant inhibition of hypoxic up-regulation of *VEGF* gene expression.

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