Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

Research Article

Molecular Cancer Therapeutics

Antiangiogenic Activities of 2,5-Dimethyl-Celecoxib on the Tumor Vasculature

Jenilyn J. Virrey[1], Zhi Liu[1], Hee-Yeon Cho[2], Adel Kardosh[3], Encouse B. Golden[1], Stan G. Louie[4], Kevin J. Gaffney[5], Nicos A. Petasis[5], Axel H. Schönthal[3], Thomas C. Chen[1,2], and Florence M. Hofman[1]

Abstract

Our laboratory has previously shown that a novel compound, 2,5-dimethyl-celecoxib (DMC), which is structurally similar to the cyclooxygenase-2 (COX-2) inhibitor celecoxib but lacks the COX-2–inhibitory function, mimics the antitumor effects of celecoxib. Most studies on DMC, however, focused on its effects on tumor cells. Here, we investigated the activities of DMC as an antiangiogenic agent in both *in vitro* and *in vivo* systems. Using primary cultures of human glioma specimens, we found that DMC treatment was cytotoxic to tumor-associated brain endothelial cells (TuBEC), which was mediated through the endoplasmic reticulum stress pathway. In contrast, confluent cultures of quiescent human BEC did not undergo cell death. DMC potently suppressed the proliferation and migration of the TuBEC. DMC caused no apparent effects on the secretion of vascular endothelial growth factor and interleukin-8 but inhibited the secretion of endothelin-1 in tumor-associated EC. DMC treatment of glioma xenografts in mice resulted in smaller tumors with a pronounced reduction in microvessel density compared with untreated mice. *In vitro* and *in vivo* analyses confirmed that DMC has antivascular activity. Considering that DMC targets both tumor cells and tumor-associated ECs, this agent is a promising anticancer drug. *Mol Cancer Ther; 9(3); 631–41. ©2010 AACR.*

Introduction

The ability of tumors to progress to more malignant phenotypes is dependent on the tumor microenvironment. As initially proposed by Folkman (1), the vasculature is critical for the growth and progression of solid tumors. Blood vessels provide the tumor with nutrients, oxygen, and a route for metastasis, enabling cancer cells to proliferate and invade the normal parenchyma. Studies show that tumor cells are intimately associated with the vasculature and cannot grow beyond a 1- to 2-mm diameter from the blood vessel (2).

Glioblastoma multiforme, a grade 4 astrocytoma, is the most malignant form of brain tumor and is highly vascular (3). The tumor blood vessels are architecturally and functionally different from those found in the normal brain parenchyma; the tumor vasculature is hemorrhagic, is dilated, and forms disorganized networks (4). We have previously shown that tumor-associated brain endothelial cells (TuBEC) from gliomas are chemoresistant, have increased migration capacity, and secrete high levels of angiogenic factors (5–7). The studies presented here used human EC derived from glioma tissues and control, nontumor brain tissues. These control BEC populations are either subconfluent, actively dividing cells or confluent, quiescent cells, similar to those typically found in the systemic vasculature (8). These EC populations represent the different blood vessels usually associated with tumors: ECs situated within the tumor, actively proliferating EC at the tumor edge, and quiescent ECs in the normal brain parenchyma, which are at a distance from the tumor. Our results indicate that these populations respond differently to drugs.

Many laboratories have reported the antiproliferative, proapoptotic, and antiangiogenic effects of celecoxib, a cyclooxygenase-2 (COX-2) inhibitor (9–12). The mechanism of these antitumor properties remained unclear because celecoxib analogues, which are not COX-2 inhibitors, seemed to have similar properties (13). Recently, our studies had shown that the antitumor effects of celecoxib can be mimicked by the non–COX-2 inhibitor analogue of celecoxib, 2,5-dimethyl-celecoxib (DMC; ref. 14). These reports found that DMC was cytotoxic to tumor cells *in vitro* and reduced glioma tumor growth *in vivo* (15). Studies showed that DMC, which lacks COX-2–inhibitory activity, aggravated the already endoplasmic reticulum (ER)–stressed tumor cells, causing their cell death (16). To more clearly identify the mechanism

Authors' Affiliations: Departments of [1]Pathology, [2]Neurosurgery, and [3]Molecular Microbiology and Immunology, Keck School of Medicine; [4]Department of Clinical Pharmacy and Pharmaceutical Economics and Policy, School of Pharmacy; and [5]Department of Chemistry, College of Letters and Arts, University of Southern California, Los Angeles, California

Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

This work was supported by the cooperative efforts of the Glioma Research Group.

Corresponding Author: Florence M. Hofman, Department of Pathology, University of Southern California, 2011 Zonal Avenue, Hoffman Medical Research Building 315, Los Angeles, CA 90033. Phone: 323-442-1153; Fax: 322-442-3049. E-mail: hofman@usc.edu

doi: 10.1158/1535-7163.MCT-09-0652

Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

of cell death and to better distinguish the effects of ER stress compared with COX-2 inhibition, unmethylated celecoxib (UMC), a derivative of celecoxib with potent COX-2 inhibition and no known association to ER stress, was used (9).

The studies presented here focus on the capacity of DMC to function as an antivascular agent. Our results showed that DMC had significant antiangiogenic properties. This drug reduced EC migration, inhibited EC proliferation, blocked endothelin-1 (ET-1) secretion, and induced cytotoxicity in tumor-associated EC. Furthermore, this cytotoxicity in tumor-associated EC was mediated through the ER stress–initiated apoptotic pathway. *In vivo* data confirmed that DMC had a direct effect on the tumor vasculature.

Materials and Methods

EC Isolation and Culture

Isolation of EC from human glioma and nontumor brain tissues was previously described (5). Tissues were obtained and handled in agreement with the Keck School of Medicine, University of Southern California Institutional Review Board guidelines. ECs were cultured in RPMI 1640 (Life Technologies) supplemented with 100 ng/mL EC growth supplement (Upstate Biotechnology), 2 mmol/L L-glutamine (Life Technologies), 10 mmol/L HEPES (Life Technologies), 24 mmol/L sodium bicarbonate (Life Technologies), 300 units heparin USP (Sigma-Aldrich), 1% penicillin/streptomycin, and 10% FCS (Omega Scientific). ECs were used up to passage 6. In doing the experiments, EC growth supplement was removed from the medium. Digital images were taken using a Canon PowerShot SD880 IS Digital Elph camera and Nikon TMS microscope.

Several experiments were done with confluent cultures of nontumor BEC. These cells were grown to 100% confluence and remained in culture for an additional 48 to 72 h before experiments were done, ensuring that the cells were contact inhibited and quiescent. Unless otherwise stated, experiments were done on 60% to 70% subconfluent BEC.

Reagents

DMC is a close structural analogue of celecoxib; the 5-aryl moiety was modified by replacing 4-methylphenyl with 2,5-dimethylphenyl. DMC and UMC were synthesized in our laboratory according to previously published procedures (9). Celecoxib was obtained from the pharmacy or synthesized, as previously reported (17). Drugs were dissolved in DMSO at a 100 mmol/L stock solution.

MTT Cell Viability Assay

The cell viability assay was done as previously described (5). Percent cell viability was calculated relative to untreated controls.

Cell Death ELISA

The Cell Death Detection ELISA^{Plus} kit (Roche Diagnostics) was used according to the manufacturer's protocol. Percent cell death was calculated based on a 100% positive cell death control.

Apoptosis Assay

Subconfluent or confluent ECs were treated with DMC. After 48 h, cytospin preparations of the cells were made, fixed in 4% paraformaldehyde, and labeled with the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction mixture according to the manufacturer's protocol (Roche).

Western Blot

Western blots were done as previously described (18). The following primary antibodies were used: caspase-7 and caspase-4 (BD Pharmingen) and CCAAT/enhancer binding protein homologous protein (CHOP), glyceraldehyde-3-phosphate dehydrogenase, glucose-regulated protein 78 (GRP78), and survivin (Santa Cruz Biotechnology).

Small Interfering RNA Transfections

Cells were transfected with siGFP and siCHOP, at a concentration of 40 nmol/L, using the Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol. The small interfering RNAs (siRNA) were obtained from Ambion, Inc. At 24 h after transfection, cells were treated with DMC for further analysis.

Trypan Blue Exclusion Assay

Cells (1.5×10^5) were seeded in six-well plates and treated with drugs. At the end of each treatment period, cells were stained with trypan blue dye. Viability was determined by counting the number of unstained cells.

Bromodeoxyuridine Cell Proliferation Assay

Cells (3×10^3) were plated in 96-well plates and treated with drugs for 72 h. The bromodeoxyuridine (BrdUrd) cell proliferation assay was then done according to the manufacturer's instructions (Calbiochem). The absorbance readings of untreated cells were considered to be 100% absorbance.

Cell Migration Assay

The Transwell migration assay was done as previously described (6). The conditioned medium was added 30 min before drug treatments and prepared by collecting 3 mL of medium from 70% confluent U87MG or U251 glioma cells. The collected medium was centrifuged, filtered through 0.22-μm micropore filters, and diluted 1:1 with fresh culture medium.

Growth Factor ELISA

ELISAs were done as previously described (7). After attachment, cells were treated with drugs or tumor necrosis factor-α (TNF-α) for 48 h, and supernatant was

Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

collected and analyzed using the Human VEGF Immunoassay kit (Invitrogen), Human IL-8/NAP-1 Immunoassay kit (Invitrogen), and Human Endothelin-1 ELISA Immunoassay kit (R&D Systems) according to the manufacturers' protocols. The numbers of viable cells were counted, allowing us to express the levels of growth factors per 10^6 cells.

Cytochrome *c* Release Assay

ECs were plated in gelatin-coated chamber slides and cultured in 10% FCS in complete medium for 24 h. The ECs were then treated with vehicle, celecoxib (100 μmol/L), or DMC (60 μmol/L). Celecoxib was used as the positive control based on the literature (19). The chambers were incubated for another 6 h. Subsequently, the cells were fixed in acetone, treated with 0.1% Triton X-100, and incubated with mouse anti–cytochrome *c* antibody (BD Pharmingen) overnight. Cells were then incubated in fluorescein-labeled horse anti-mouse antibody and mounted using 4′,6-diamidino-2-phenylindole mounting medium.

In vivo Implantation and Tumor Sizing

Animal protocols have been approved by the Institutional Animal Care and Use Committee of the University of Southern California. Applicable policies were strictly practiced during the course of this study. Four- to 6-wk-old male athymic *nu/nu* mice (Harlan) were kept in a pathogen-free environment. U87 glioma cells (5×10^5) were injected s.c. into the right flank. Once tumors were palpable, mice were randomly divided into the untreated group or the DMC-treated group, with four mice per group. DMC-treated mice received 1,000 ppm in the animal chow daily. Calipers were used to measure tumor size every 2 to 4 d. Tumor size was calculated by the formula volume (mm^3) = length × width × height × 0.5 and expressed as the percentage of growth compared with the original tumor size at the start of treatment. The experiment was repeated twice.

Immunostaining

Frozen sections from mouse tumor tissues were fixed in acetone and stained with anti-mouse CD31, anti–vascular endothelial growth factor (VEGF), anti–interleukin-8 (IL-8), and anti–ET-1 (BD Pharmingen). Immunostaining was done as previously described (5).

Statistical Analysis

The Student's *t* test was used for statistical analysis. Values are represented as the mean ± SE. A *P* value of <0.05 was considered significant.

Results

DMC Is Cytotoxic to Tumor-Associated ECs

DMC has been shown to induce apoptosis in a variety of tumor cell lines (9, 15, 20). However, the effects of the drug on tumor-associated EC have not been fully determined. To address this issue, human TuBECs derived from glioma tissue and subconfluent control, nontumor BEC were treated for 72 hours with either DMC, celecoxib, or UMC. Celecoxib and UMC exhibit COX-2–inhibitory activity, whereas DMC does not (9, 12, 14). The results (Fig. 1A) showed that DMC caused tumor-associated EC cytotoxicity at an IC_{50} of 50 μmol/L. TuBECs were relatively resistant to celecoxib and UMC, exhibiting no attainable IC_{50} at these doses. The subconfluent BECs (Fig. 1A) had similar sensitivity to DMC (IC_{50}, 50 μmol/L) and were also sensitive to celecoxib (IC_{50}, 70 μmol/L). UMC did not affect the subconfluent control EC at the doses tested. Cell death was also quantified by analyzing the cytoplasmic histone-associatedDNA fragments. The data showed that treatment of TuBEC with DMC was more toxic than celecoxib and UMC ($P < 0.02$; Fig. 1B). Similarly, subconfluent control EC treated with 80 μmol/L DMC and celecoxib caused 72% and 60% cell death, respectively, whereas UMC caused only 22% apoptosis.

The subconfluent ECs, at 60% to 70% confluence, were highly active, proliferating, and analogous to the activated ECs detected at the tumor/brain tissue edge. This is in marked contrast to the continuous layer of quiescent EC normally found in the nontumor vasculature of the body. To test this quiescent EC population, we use confluent EC cultures. To determine the effects of DMC on confluent, quiescent cells, normal ECs were cultured to 100% confluency and allowed to remain confluent for 2 days. Subsequently, the confluent ECs were tested with DMC as described above. Confluent cells treated with DMC (60 μmol/L) for 3 days exhibited minimal cell death compared with subconfluent ECs (Fig. 1C). We confirmed these results using the TUNEL assay. Confluent EC did not display any TUNEL-positive cells, whereas subconfluent cells exhibited notable TUNEL-positive cells (Fig. 1D) with DMC treatment. We did the MTT assay to quantify cell viability. At 60 μmol/L, confluent EC underwent significantly less cell death than subconfluent EC ($P < 0.02$; Fig. 1E). These results showed that whereas DMC caused cytotoxicity in TuBEC and subconfluent, activated BECs, the quiescent ECs were resistant to the drug.

DMC Mediates EC Death through the ER Stress Mechanism

To identify the mechanism of death in TuBEC and subconfluent, activated ECs, cells were treated with 60 μmol/L DMC, celecoxib, and UMC for 72 hours and analyzed for GRP78 and full-length and cleaved caspase-4 and caspase-7. CHOP was analyzed after 24 hours of drug treatment. CHOP is a specific and critical mediator of the ER stress–induced apoptotic pathway (21). GRP78 expression was highly elevated in tumor-associated EC and subconfluent EC after DMC treatment, signifying the induction of ER stress (Fig. 2A; ref. 22). Furthermore, DMC increased CHOP levels in both tumor-associated

Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

and subconfluent ECs. DMC-treated cells also exhibited cleaved caspase-7 and caspase-4, characteristic of ER stress–induced apoptosis. Thus, the cytotoxicity observed in the DMC treatment of tumor-associated and subconfluent EC involved the ER stress mechanism. Further investigation of the mechanism of cell death showed that whereas ER stress is responsible for triggering DMC-mediated cytotoxicity, the mitochondrial and death receptor pathways are involved in the effector phase of cytotoxicity. Normal BEC and TuBEC, incubated with DMC (60 μmol/L) or celecoxib (100 μmol/L), showed release of cytochrome *c* (Supplementary Fig. S1) and the upregulation of death receptor 5 (DR5) expression (Supplementary Fig. S2). These data are consistent with the established notion that cell death initiated by excessive ER stress involves both the intrinsic and extrinsic apoptotic pathways and that DMC may use both processes.

Confluent ECs were also analyzed for the activation of the ER stress–induced apoptotic pathway on drug treatment. DMC treatment (60 μmol/L) did not result in CHOP induction nor the activation of caspase-4 (Fig. 2A). GRP78 exhibited a slight elevation in DMC-treated confluent EC, but this increase was remarkably less than that observed in subconfluent cultures at the same conditions (Fig. 2A). These data suggest that DMC does not trigger ER stress–induced apoptosis in normal, quiescent ECs.

We did a transient knockdown of CHOP expression to confirm the involvement of ER stress–induced apoptosis in the DMC cytotoxicity of EC. Western blot analysis verified the reduced levels of CHOP in tumor-associated and subconfluent, activated ECs (Fig. 2B). The siGFP was used as control. The downregulation of CHOP in tumor-associated EC caused significantly less cell death with 60 μmol/L DMC treatment compared with control (31% versus 63%, respectively; $P = 0.004$; Fig. 2C). DMC was also less potent after the knockdown of CHOP in activated EC at the same concentration (Fig. 2C). This protection was consistently observed in the lower concentration (40 μmol/L) as well; transfected controls exhibited 78% viability, whereas siCHOP in subconfluent EC displayed 92% viability ($P = 0.003$). Thus, the downregulation of CHOP protected both the tumor-associated EC and the subconfluent EC from the cytotoxic effects of DMC, confirming the mechanism of ER stress–induced apoptosis.

DMC Decreases Survivin Expression in Tumor-Associated ECs

Our previous studies emphasized the role of survivin in protecting ECs from drugs (5). Survivin is a member of the inhibitor of apoptosis gene family and is overexpressed in TuBEC (5, 23). Here, we determined the effects of DMC on survivin expression in tumor-associated EC and subconfluent EC (Fig. 2D). We used a low concentration of DMC (20 μmol/L) so that cells remain viable and to ensure that changes in survivin expression are not a result of cell death. DMC reduced survivin in tumor-associated EC after 48 hours. Downregulation of survivin was also observed with celecoxib and UMC. In subconfluent EC, DMC had minimal effects after 24 hours, whereas celecoxib and UMC increased survivin at 24 hours (Fig. 1D). After 48 hours, all three treatments reduced survivin expression. Thus, the regulation of survivin protein expression seemed to be mediated by COX-2–independent and COX-2–dependent mechanisms and is a relatively late response.

DMC Suppresses the Proliferation of Tumor-Associated ECs

We next investigated whether DMC affected EC proliferation. Equal numbers of tumor-associated EC and subconfluent EC were treated with noncytotoxic concentrations (20 μmol/L) of DMC, celecoxib, and UMC. Cells were harvested on different days, and the number of viable cells was counted. The results showed that DMC decreased the proliferation in tumor-associated EC and subconfluent EC (Fig. 3A). The number of tumor-associated EC nearly doubled after 7 days, but the number of DMC-treated cells only increased by 31%. Subconfluent EC, shown to proliferate faster than tumor-associated EC (18), increased in number by 3-fold after 7 days, whereas DMC-treated cells remained close to the number of cells plated on day 0 (20.5×10^4 versus 15×10^4). Moreover, DMC was more potent in inhibiting proliferation, compared with celecoxib and UMC, in both tumor-associated EC and subconfluent EC. These data show that at low noncytotoxic concentrations, DMC was effective in reducing EC proliferation, a critical process in angiogenesis.

These observations were confirmed with the BrdUrd incorporation assay. The results showed that DMC (20 μmol/L) inhibited EC proliferation. This growth-inhibitory effect was more pronounced for DMC than the other drugs tested ($P < 0.03$; Fig. 3B). DMC treatment of tumor-associated EC resulted in a 40% decrease in proliferation, whereas DMC caused a 60% decrease for activated EC.

We also investigated whether DMC altered the proliferation of normal, quiescent ECs. Confluent ECs were treated with 20 μmol/L DMC, celecoxib, and UMC for 72 hours, and proliferation was analyzed through the BrdUrd incorporation assay. DMC treatment did not inhibit the proliferation of confluent EC (7% decrease), although it potently suppressed the growth of subconfluent cultures (50% decrease), compared with untreated cells (Fig. 3C). Therefore, DMC inhibits the proliferation of subconfluent, activated EC and not the confluent, quiescent EC.

DMC Inhibits the Migration of Tumor-Associated ECs

EC migration is an integral component of angiogenesis. Growth factors present in the tumor microenvironment provide the stimulus for this migration process.

Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

To test the effectiveness of DMC on EC migration, the cells were stimulated with conditioned medium derived from the U87MG or U251 glioma cell lines (U87cm and U251cm). As expected, conditioned medium from tumor cells increased EC migration (Fig. 4). Treatment of EC with DMC (20 μmol/L) significantly decreased the migration of both TuBEC ($P < 0.01$; Fig. 4A) and BEC ($P < 0.01$; Fig. 4B). Cells treated with celecoxib and UMC (both at 20 μmol/L) had similar effects.

DMC Blocks ET-1 Secretion but Does Not Affect the Secretion of VEGF and IL-8

The levels of proangiogenic growth factors in the tumor microenvironment regulate angiogenesis. To further study the influence of DMC on angiogenesis, we treated TuBEC and U87MG glioma cells with DMC for 2 days and measured the secretion of proangiogenic cytokines. VEGF is a critical growth factor in the angiogenic process (24). The results showed that DMC, as well as celecoxib and UMC, had no apparent effect on VEGF secretion by the TuBEC or U87 glioma cells at a low-dose nontoxic concentration (20 μmol/L; Fig. 5A). The production of IL-8, a cytokine responsible for EC migration and activation, was also tested (Fig. 5B; ref. 6). The results showed that DMC (20 μmol/L) had no effect on IL-8 secretion by TuBEC. However, celecoxib and UMC decreased IL-8 secretion in glioma cells, with no discernible effects on EC. ET-1 is responsible for vasoconstriction as well as angiogenesis (25). ET-1 secretion was blocked by 20 μmol/L

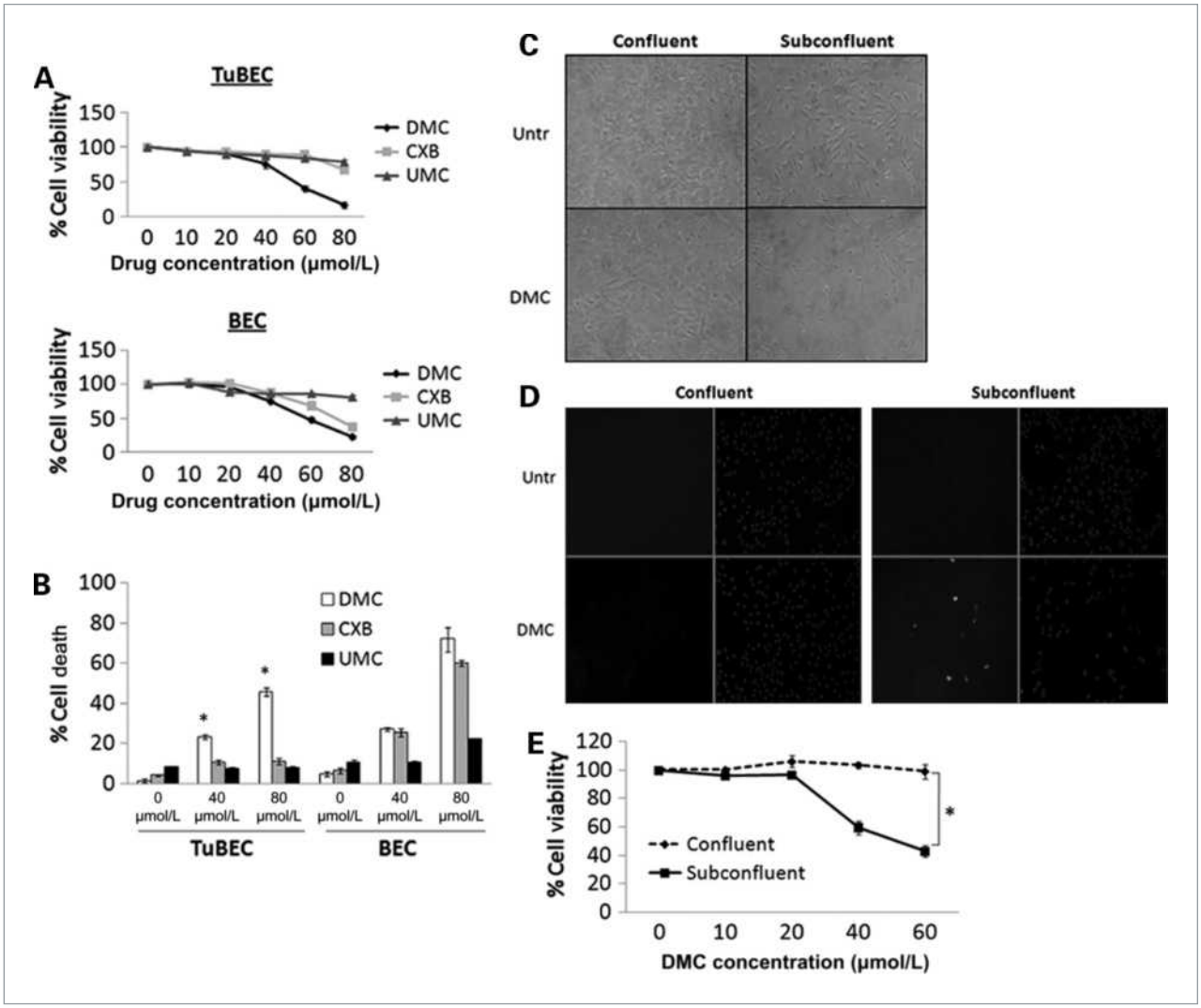

Figure 1. Cytotoxic effects of DMC on TuBECs. TuBECs and subconfluent BECs were treated with DMC, celecoxib (CXB), and UMC for 72 h. Cell viability was assessed using the MTT assay (A) and the cell death ELISA (B). Statistical comparisons were made from the DMC treatments to the celecoxib and UMC treatments. C, photographs of subconfluent and confluent BECs were taken after treatment with 60 μmol/L DMC for 72 h. D, TUNEL assay was done on untreated (Untr) and DMC-treated (60 μmol/L, 48 h) cells. The staining denotes TUNEL-positive cells. E, confluent and subconfluent BECs were treated with DMC for 72 h and analyzed with the MTT assay. Untreated cells were incubated with vehicle. *, $P < 0.02$.

Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

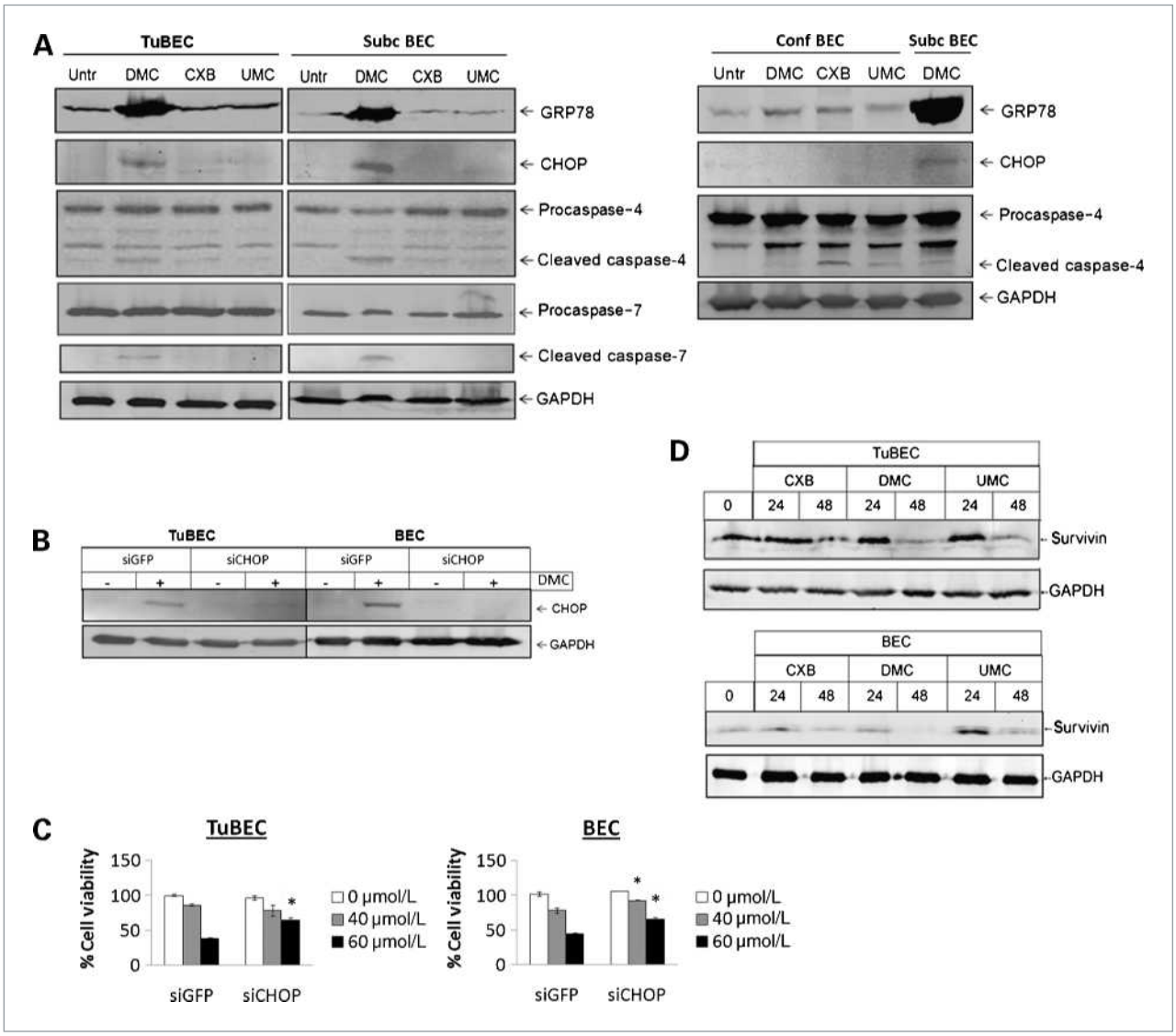

Figure 2. ER stress–induced apoptosis and survivin expression in tumor-associated ECs with DMC treatment. A, TuBECs, subconfluent BECs (Subc BEC), and confluent BECs (Conf BEC) were treated with 60 μmol/L DMC, celecoxib, and UMC and analyzed for GRP78, CHOP, caspase-4, and caspase-7. Western blots for CHOP were done after 24 h of treatment, whereas the other markers were tested after 72 h of treatment. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, TuBEC and BEC were transiently transfected with siGFP (40 nmol/L) or siCHOP (40 nmol/L). After 24 h, cells were treated with 60 μmol/L DMC for an additional 24 h. Western blot analysis was used to detect the relative knockdown of CHOP expression. C, TuBEC and BEC were transfected with siGFP or siCHOP for 24 h and then treated with DMC (40 and 60 μmol/L) for 72 h. MTT assay was used to quantify cell viability. D, TuBEC and BEC were treated with celecoxib, DMC, or UMC (20 μmol/L) for 24 or 48 h. Survivin expression was detected using Western blot. Untreated cells were exposed to vehicle. *, $P < 0.01$.

DMC treatment in TuBEC ($P < 0.05$; Fig. 5C). As expected, stimulation of TuBEC with TNF-α (10 ng/mL) led to an increase of ET-1 secretion. Interestingly, DMC and celecoxib (both at 20 μmol/L) also suppressed ET-1 secretion in the TNF-α–stimulated cells ($P < 0.05$). In sharp contrast to DMC and celecoxib, UMC (20 μmol/L) strikingly enhanced the secretion of ET-1 in both the unstimulated ($P = 0.05$) and the TNF-α–stimulated ($P = 0.02$) TuBEC. DMC, celecoxib, and UMC did not affect the secretion of ET-1 in U87MG glioma cells (data not shown).

DMC Inhibits Angiogenesis in an *In vivo* Glioma Mouse Model

We evaluated the activity of DMC *in vivo*. U87MG glioma cells were implanted s.c. into nude mice. Mice treated with DMC had notably smaller tumors than untreated mice (Fig. 6A). On average, tumors from untreated mice were significantly bigger in size, ~2.3-fold larger, than tumors from DMC-treated mice at the termination of the experiment ($P = 0.03$). To determine whether the tumor vasculature was affected by DMC treatment, tumor tissues were analyzed for blood vessels

Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

by staining for CD31. Immunostaining revealed numerous CD31-positive blood vessels throughout the tumor of untreated mice, whereas fewer CD31-positive vessels were found in DMC-treated tumors (Fig. 6B). Furthermore, the vessels in untreated mice were elongated and large, whereas the vessels in DMC-treated mice appeared thinner and stunted. Microvessel density analysis was done on tumor tissue from each mouse to quantify the relative levels of CD31 positivity and found that DMC treatment had indeed reduced the number of CD31-positive vessels in all tumors (Fig. 6C). The experiment was repeated twice, and similar results were observed; microvessel density was on the average of 35% to 46% less in the DMC-treated tumors. To determine whether DMC had an effect on the production of VEGF, IL-8, and ET-1 *in vivo*, tumor tissues were immunostained with antibodies to these growth factors. The results showed that DMC did not affect VEGF or ET-1 expression; however, DMC treatment seemed to decrease ET-1 staining in the tumor tissue (Supplementary Fig. S3).

Discussion

In this study, we report that the novel compound DMC exerted potent antiangiogenic effects on the tumor vasculature derived from human gliomas. DMC is a close structural analogue of celecoxib but lacks COX-2–inhibitory activities (14). Our previous studies showed that DMC is an antitumor agent (9, 15). DMC potently reduced tumor growth in gliomas, multiple myeloma, lymphoma, and pancreatic and breast cancers (9, 10, 13, 20, 26, 27). We had shown that the mechanism of DMC-induced cytotoxicity in tumors was activation of the ER stress response, which led to the perturbation of intracellular calcium levels, accumulation of polyubiquitinated proteins, and ultimately apoptosis (16). Because it has been well established that tumor growth and survival require the contribution of the tumor vasculature (28), we analyzed DMC for antivascular activity.

Here, we systematically explored the effects of DMC on different angiogenic functions. DMC induced cytotoxicity in both subconfluent, activated BEC and EC isolated from glioma tissues. The vasculature within the tumor has different morphologic and functional characteristics compared with normal, systemic blood vessels (4). The tumor vasculature contains abnormal structures, arteriovenous shunting, a low proliferation rate, and enhanced vascular permeability (4). In contrast to the vasculature within the center of the tumor, blood vessels at the border of the tumor are highly activated, proliferating rapidly, and forming new vessels similar to those in the normal angiogenic process (29). In contrast, the normal blood vessels throughout the body are composed of

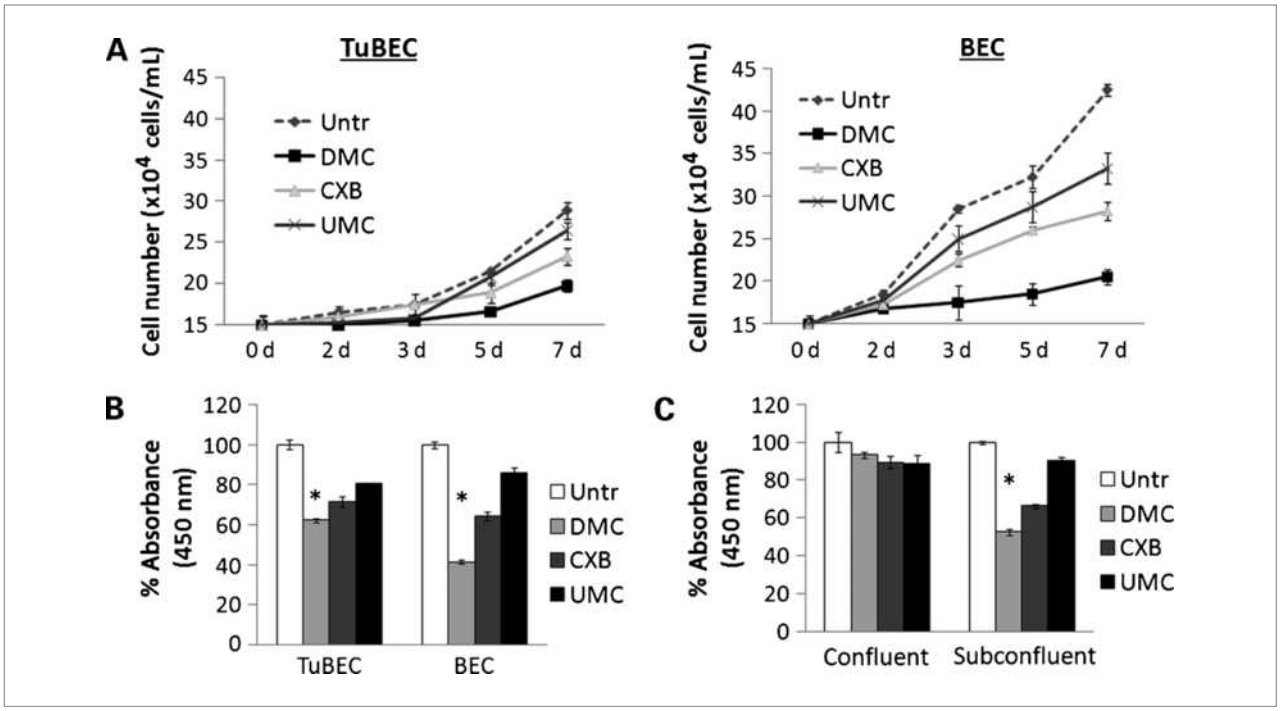

Figure 3. Effects of DMC on the proliferation of tumor-associated ECs. A, TuBECs and subconfluent BECs were treated with vehicle (Untr), DMC, celecoxib, or UMC (20 μmol/L). After 2, 3, 5, and 7 d, cells were stained with trypan blue dye; the unstained, viable cells were counted. B, TuBEC and BEC were treated with DMC, celecoxib, or UMC (20 μmol/L) for 72 h and analyzed with the BrdUrd assay. C, confluent and subconfluent BECs were treated with 20 μmol/L DMC, celecoxib, and UMC for 72 h and assessed through the BrdUrd assay. Statistical comparisons were made from the DMC treatments to the celecoxib and UMC treatments. *, $P < 0.03$.

Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

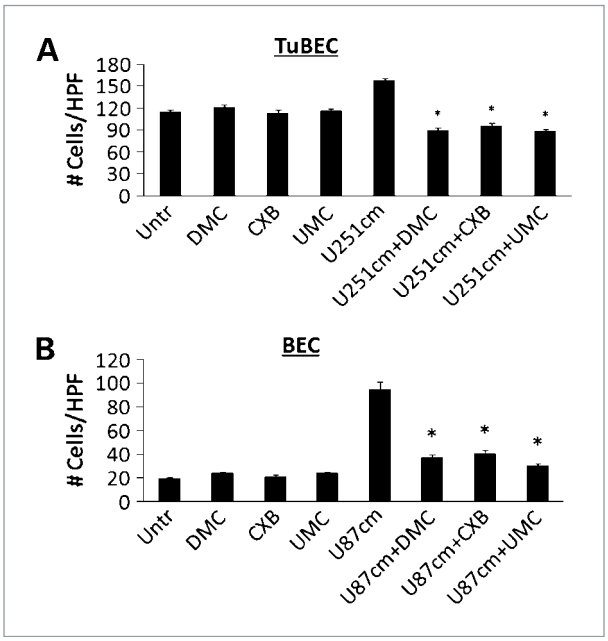

Figure 4. Effects of DMC on EC migration. TuBECs (A) and subconfluent BECs (B) were treated with 20 μmol/L DMC, celecoxib, UMC, and conditioned medium (cm) collected from U87MG or U251 glioma cells; untreated cells were incubated with DMSO. After 16 h, the number of migrated cells was determined. The data are expressed as the number of cells in 10 high-powered fields (HPF) under ×200 magnification. *, $P < 0.05$, drug treatments versus stimulation with glioma cell–conditioned medium.

quiescent ECs that replicate once every 3.1 years (8). The studies presented here show that DMC caused cytotoxicity and inhibited proliferation in tumor-associated EC and subconfluent, activated EC and not in the quiescent EC. These results indicate that DMC exerts its effects on the tumor vasculature and EC undergoing active angiogenesis, but not on normal quiescent EC. *In vivo* studies showed that the administration of DMC produced no apparent toxicity on the vasculature of normal organs (data not shown) but showed reduced microvessel density in tumors. Thus, DMC targeted tumor-associated and activated EC without affecting the normal vasculature throughout the body.

TuBECs are resistant to various chemotherapies (5, 18, 30). We had reported that the mechanism of this drug resistance involved survivin overexpression (5). Here, we found that DMC decreased survivin expression in tumor-associated EC. Therefore, DMC may act not only as an antivascular agent but also as a chemosensitizing agent by targeting survivin expression. For example, DMC with other agents (i.e., nelfinavir) acted synergistically in killing tumor cells (27). We are currently investigating DMC in drug combinations on the tumor endothelium.

Our data showed that the mechanism of EC death is ER stress–induced apoptosis. DMC induced cleavage of caspase-4, an enzyme particularly involved in ER stress–induced apoptosis (31). The elevated GRP78 levels caused by DMC treatment are indicative of ER stress (22). Moreover, CHOP is a specific mediator of ER stress apoptosis, and we observed notable CHOP induction with DMC treatment (21). These studies showed that DMC induced apoptosis using the ER stress pathway. Furthermore, this DMC-induced cytotoxicity may be mediated by several different death pathways, including cytochrome *c* release and the induction of DR5. Other survival pathways, such as the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, may also be involved (32). Essentially, we have shown here that DMC induces CHOP and that the inhibition of CHOP with siRNA blocks the apoptotic outcome of DMC treatment, clearly establishing a cause-and-effect relationship of DMC-induced ER stress and apoptosis. Thus, altogether, these data show that treatment of ECs with DMC triggers ER stress, which

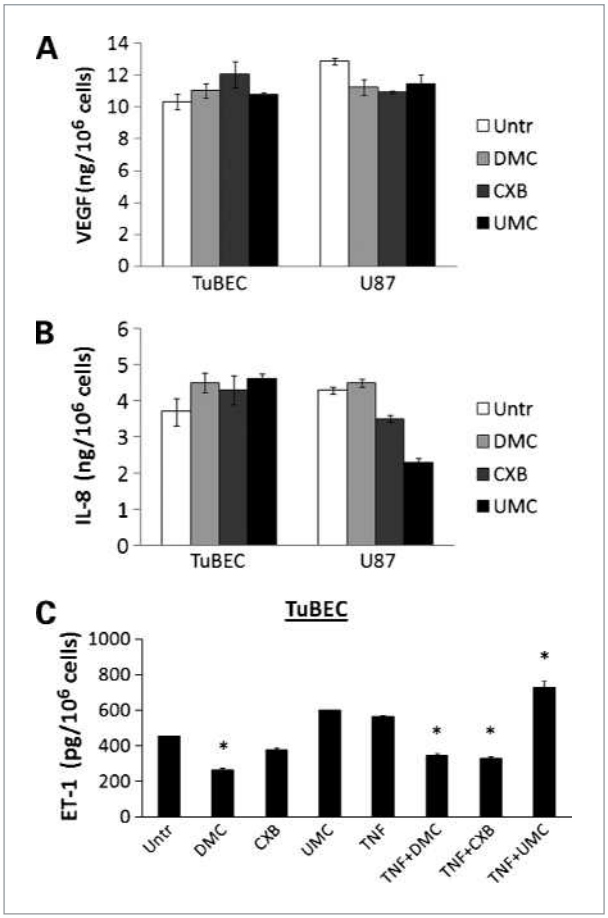

Figure 5. Effects of DMC on the secretion of angiogenic factors. TuBECs and U87MG glioma cells were treated with 20 μmol/L DMC, celecoxib, or UMC. After 48 h, the supernatants were collected and analyzed for VEGF (A) or IL-8 (B) levels. C, TuBECs were treated for 48 h with DMC, celecoxib, UMC (20 μmol/L), or TNF-α (10 ng/mL). The supernatants were analyzed for ET-1 levels. Untreated cells were incubated with DMSO. *, $P < 0.05$, drug treatments versus untreated controls or TNF-α–stimulated controls.

Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

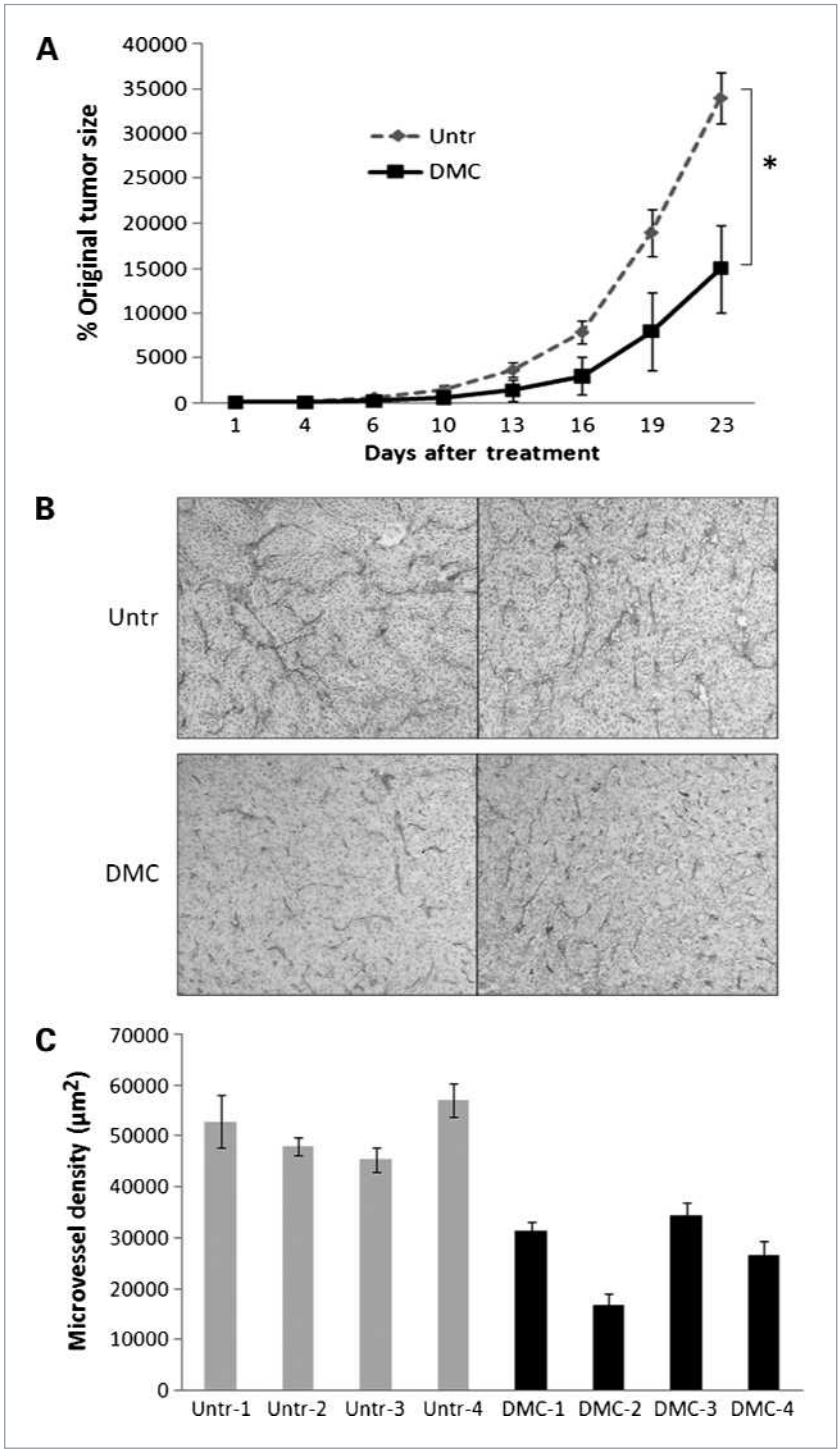

Figure 6. *In vivo* analysis of angiogenesis in a glioma xenograft mouse model. U87MG glioma cells were implanted s.c. into the flank of nude mice. Once the tumors became palpable, the mice were untreated or treated with DMC. A, tumor size was measured every 2 to 4 d. Tumor size is expressed as the percentage of growth compared with the original tumor size. *, $P < 0.05$. B, frozen sections of tumors from DMC-treated and untreated mice were stained for CD31. The dark gray denotes positive staining. Two representative images are shown for the untreated and DMC-treated mice. Magnification, ×200. C, quantitative analysis was done on the microvessel density based on four random fields (200×) per tissue. Four untreated and four DMC-treated tumor tissues were analyzed. The microvessel density for each individual mouse is presented.

results in cell death involving components of the intrinsic and extrinsic apoptotic machinery.

In addition to cytotoxic properties, DMC reduced proliferation and migration of both tumor-associated and subconfluent ECs. DMC inhibited the proliferation more effectively than either celecoxib or UMC. Furthermore, DMC inhibited the proliferation of both the faster-growing and the slower-growing ECs, making this agent useful for the vasculature within the tumor and along the tumor edge. EC migration, a critical aspect of angiogenesis, is potently inhibited by DMC. To replicate the tumor microenvironment, ECs were cultured in conditioned medium from glioma cells. DMC inhibited the migration of these ECs when exposed to tumor-conditioned medium. Similar

Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

findings were observed with celecoxib and UMC, suggesting that EC migration can be regulated through COX-2 and non–COX-2 mechanisms.

The use of DMC as an antitumor compound was validated in this and previous studies using a glioma animal model (15). DMC treatment resulted in smaller tumors, which were attributed to the effects of DMC on tumor cells (16). Our data showed that the blood vessels in DMC-treated tumors were also altered; these vessels were shorter, thinner, and reduced in number. This reduction may be the result of decreased proliferation, suppressed migration, blocked ET-1 secretion, and increased EC death and is most likely the result of the sum of these altered functions. Thus, this novel compound targets both tumor and ECs, making DMC an attractive candidate for chemotherapy.

DMC is a close structural analogue of celecoxib with no significant COX-2–inhibitory functions. This is a great advantage for therapeutic purposes because COX-2 inhibitors have been shown to increase risk of heart attacks and stroke (33). Our data showed that UMC, a COX-2 inhibitor, markedly increased ET-1 secretion in ECs. ET-1 is a vasoconstrictor and a stroke risk factor (25, 34). In contrast, DMC did not exhibit this activity, and even decreased ET-1 secretion in TuBEC, and *in vivo*, in the tumor tissue of DMC-treated animals. These data suggest that DMC can be used as a safe antivascular agent without fear of increased heart disease or stroke.

Our data are consistent with other reports of DMC activity in human umbilical vein ECs (HUVEC). Lin et al. (32) showed that DMC arrested HUVEC at G_1 of the cell cycle, which was mediated through the PI3K/Akt signaling. These studies, however, use HUVECs, which do not realistically depict the tumor endothelium or the endothelium of normal organs. In our studies, we use low-passage primary cultures of ECs isolated from human normal brain and brain tumor specimens. Here, we also extend the findings of Lin et al. and show that DMC affects other aspects of the angiogenic process, including EC viability, migration, and production of ET-1. We also showed that DMC induced cytotoxicity to EC through the mechanism of ER stress–induced apoptosis and inhibited angiogenesis in a glioma xenograft model.

Antiangiogenic drugs are currently being explored as treatments for a wide range of cancers, including glioblastoma multiforme, prostate, and pancreatic carcinomas (35). Bevacizumab, a humanized monoclonal antibody against VEGF-A, was recently approved for the treatment of metastatic breast, non–small cell lung, and colorectal cancers (36). Despite its promising activity in preclinical studies, bevacizumab had only modest effects in patients, and overall patient survival has not considerably improved (37). Therefore, there still remains a compelling need for more effective antiangiogenic drugs. Drugs that dually target tumor cells and the tumor vasculature are definitely welcome in the clinic. Thus, DMC, which has both antitumor and antiangiogenic activity, is a promising anticancer therapeutic agent.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Ligaya Pen and Susan Su for EC isolation.

Grant Support

L.K. Whittier Foundation through the University of Southern California/Norris Comprehensive Cancer Center (T.C. Chen).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 07/21/2009; revised 12/28/2009; accepted 01/04/2010; published OnlineFirst 03/02/2010.

References

1. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182–86.
2. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003;3:401–10.
3. Louis DN, Pomeroy SL, Cairncross JG. Focus on central nervous system neoplasia. Cancer Cell 2002;1:125–8.
4. Nagy JA, Chang SH, Dvorak AM, et al. Why are tumor blood vessels abnormal and why is it important to know? Br J Cancer 2009;100:865–9.
5. Virrey JJ, Guan S, Li W, et al. Increased survivin expression confers chemoresistance to tumor-associated endothelial cells. Am J Pathol 2008;173:575–85.
6. Charalambous C, Pen LB, Su YS, et al. Interleukin-8 differentially regulates migration of tumor-associated and normal human brain endothelial cells. Cancer Res 2005;65:10347–54.
7. Charalambous C, Hofman FH, Chen TC. Functional and phenotypic differences between glioblastoma multiforme-derived and normal human brain endothelial cells. J Neurosurg 2005;102:699–705.
8. Kruger O, Plum A, Kim JS, et al. Defective vascular development in connexin 45-deficient mice. Development 2000;127:4179–93.
9. Chuang HC, Kardosh A, Gaffney KJ, et al. COX-2 inhibition is neither necessary nor sufficient for celecoxib to suppress tumor cell proliferation and focus formation *in vitro*. Mol Cancer 2008;7:38.
10. Pyrko P, Soriano N, Kardosh A, et al. Downregulation of survivin expression and concomitant induction of apoptosis by celecoxib and its non-cyclooxygenase-2-inhibitory analog, dimethyl-celecoxib (DMC), in tumor cells *in vitro* and *in vivo*. Mol Cancer 2006;5:19.
11. Klenke FM, Gebhard MM, Ewerbeck V, et al. The selective Cox-2 inhibitor celecoxib suppresses angiogenesis and growth of secondary bone tumors: an intravital microscopy study in mice. BMC Cancer 2006;6:9.
12. Zhu J, Huang JW, Tseng PH, et al. From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors. Cancer Res 2004;64:4309–18.
13. Kardosh A, Wang W, Uddin NA, et al. Dimethyl-celecoxib (DMC), a derivative of celecoxib that lacks cyclooxygenase-2-inhibitory function, potently mimics the anti-tumor effects of celecoxib on Burkitt's lymphoma *in vitro* and *in vivo*. Cancer Biol Ther 2005;4:571–82.
14. Schönthal AH. Antitumor properties of dimethyl-celecoxib, a derivative of celecoxib that does not inhibit cyclooxygenase-2: implications for glioblastoma therapy. Neurosurg Focus 2006:201–10.

Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

15. Pyrko P, Kardosh A, Liu YT, et al. Calcium-activated ER stress as a major component of tumor cell death induced by 2,5-dimethyl-celecoxib (DMC), a non-coxib analog of celecoxib. Mol Cancer Ther 2007;6:1262–75.
16. Kardosh A, Golden EB, Pyrko P, et al. Aggravated endoplasmic reticulum (ER) stress as a basis for enhanced glioblastoma cell killing by bortezomib in combination with celecoxib or its noncoxib analog, 2,5-dimethyl-celecoxib. Cancer Res 2008;68:843–51.
17. Penning TD, Talley JJ, Bertenshaw SR, et al. Synthesis and biological evaluation of the 1,5-diarylpyrazole class of cyclooxygenase-2 inhibitors: identification of 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benze nesulfonamide (SC-58635, celecoxib). J Med Chem 1997;40:1347–65.
18. Charalambous C, Virrey JJ, Kardosh A, et al. Glioma-associated endothelial cells show evidence of replicative senescence. Exp Cell Res 2007;313:1192–202.
19. Fantappie O, Solazzo M, Lasagna N, Platini F, Tessitore L, and Mazzanti R. P-glycoprotein mediates celecoxib-induced apoptosis in multiple drug-resistant cell lines. Cancer Res 2007;67:4915–23.
20. Kardosh A, Soriano N, Liu YT, et al. Multi-target inhibition of drug-resistant multiple myeloma cell lines by dimethylcelecoxib (DMC), a non-COX-2-inhibitory analog of celecoxib. Blood 2005;106:4330–38.
21. Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ 2004;11:381–9.
22. Ma Y, Hendershot LM. The role of the unfolded protein response in tumour development: friend or foe? Nat Rev Cancer 2004;4:966–77.
23. Altieri DC. The case for survivin as a regulator of microtubule dynamics and cell-death decisions. Curr Opin Cell Biol 2006;18:609–15.
24. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004;25:581–611.
25. Knowles J, Loizidou M, Taylor I. Endothelin-1 and angiogenesis in cancer. Curr Vasc Pharmacol 2005;4:309–14.
26. Schönthal AH, Chen TC, Hofman FM, et al. Celecoxib analogs that lack COX-2 inhibitory function: preclinical development of novel anticancer drugs. Expert Opin Investig Drugs 2008;17:197–208.
27. Cho HY, Thomas S, Golden EB, et al. Enhanced killing of chemo-resistant breast cancer cells via controlled aggravation of ER stress. Cancer Lett 2009;282:87–97.
28. Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 2007;6:273–86.
29. Li J, Zhang YP, Kirsneron RS. Angiogenesis in wound repair: angiogenic growth factors and the extracellular matrix. Microsc Res Tech 2003:60107–14.
30. Virrey JJ, Dong D, Stiles C, et al. Stress chaperone GRP78/BiP confers chemoresistance to tumor-associated endothelial cells. Mol Cancer Res 2008;6:1268–75.
31. Hitomi J, Katayama T, Eguchi Y, et al. Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Aβ-induced cell death. J Cell Biol 2004;165:347–56.
32. Lin HP, Kulp SK, Tseng PH, et al. Growth inhibitory effects of celecoxib in human umbilical vein endothelial cells are mediated through G_1 arrest via multiple signaling mechanisms. Mol Cancer Ther 2004;3:1671–80.
33. Fitzgerald GA. Coxibs and cardiovascular disease. N Engl J Med 2004;351:1709–11.
34. Schiffrin EL. Role of endothelin-1 in hypertension and vascular disease. Am J Hypertens 2001;14:83–89S.
35. Ma J, Waxman DJ. Combination of antiangiogenesis with chemotherapy for more effective cancer treatment. Mol Cancer Ther 2008;7:3670–84.
36. Ferrara N, Hillan KJ, Novotny W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. Biochem Biophys Res Commun 2005;333:328–35.
37. Jain RK, Duda DG, Clark J, et al. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol 2006;3:24–40.

Published OnlineFirst March 9, 2010; DOI: 10.1158/1535-7163.MCT-09-0652

Molecular Cancer Therapeutics

Antiangiogenic Activities of 2,5-Dimethyl-Celecoxib on the Tumor Vasculature

Jenilyn J. Virrey, Zhi Liu, Hee-Yeon Cho, et al.

Mol Cancer Ther 2010;9:631-641. Published OnlineFirst March 9, 2010.