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Tyrosine Kinase Inhibition of Multiple Angiogenic Growth Factor Receptors Improves Survival in Mice Bearing Colon Cancer Liver Metastases by Inhibition of Endothelial Cell Survival Mechanisms[1]

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ABSTRACT

Redundant mechanisms mediate colon cancer angiogenesis. Targeting multiple angiogenic factors simultaneously may improve survival of mice with colon cancer metastases. BALB/c mice underwent splenic injection with CT-26 colon cancer cells to generate liver metastases and received administration of either vehicle alone or a tyrosine kinase inhibitor for vascular endothelial growth factor, basic fibroblast growth factor, and platelet-derived growth factor receptors (SU6668). Mice were sacrificed when they became moribund as determined by a blinded observer. In a parallel experiment, groups of mice were sacrificed at earlier time points to better define the kinetics of the effect of SU6668 on angiogenic parameters over time. SU6668 increased median survival by 58% ($P < 0.001$) and led to a progressive increase in tumor cell and endothelial cell apoptosis that increased over time. In addition, pericyte vessel coverage and tumor vascularity were significantly decreased in mice treated with SU6668. Based on current knowledge of endothelial cell survival, these data suggest that SU6668 may prevent tumor endothelial cell survival directly (vascular endothelial growth factor) and indirectly (pericyte coverage) by affecting endothelial cell survival mechanisms.

INTRODUCTION

The process of angiogenesis involves the formation of new blood vessels from established vasculature and is essential for progressive tumor growth and metastasis (1). In colon cancer, the expression of VEGF,[3] bFGF, and PDGF has also been correlated with the growth and vascularity of colon cancer (2–4). Additionally, TK receptors for several of the above-mentioned factors are expressed by tumor and endothelial cells as well as by pericytes (2–5). We have recently shown that TK inhibitors that target the receptor for VEGF alone (SU5416) or the receptors for VEGF, bFGF, and PDGF (SU6668) inhibit tumor angiogenesis and growth and induce tumor cell and endothelial cell apoptosis in a murine model of colon cancer liver metastasis (6). In that study, the selective inhibition of VEGF activity significantly increased endothelial cell apoptosis, suggesting that VEGF is an *in vivo* tumor endothelial cell survival factor. SU6668 increased endothelial cell apoptosis to a greater degree than selective VEGF receptor inhibition by SU5416. It is well established that VEGF is a survival factor for endothelial cells in both developing tissues and tumor vessels (7–14). However, the exact manner in which multiple cytokines work in coordination to effect endothelial cell apoptosis is less well defined.

Simultaneously targeting TK receptors that are present on endothelial cells (VEGF, bFGF, and PDGF), tumor cells (bFGF and PDGF), and pericytes (bFGF and PDGF) represents a novel approach for antiangiogenic therapy. The purpose of this study was to determine whether the simultaneous inhibition of multiple angiogenic/growth factor receptors by SU6668 would improve survival in a relevant murine model of colon cancer liver metastasis. A secondary goal of this study was to determine the effect of SU6668 treatment on alteration of tumor angiogenesis and endothelial cell and tumor cell apoptosis over time. Finally, we investigated the effect of SU6668 therapy on the extent of pericyte vessel coverage.

MATERIALS AND METHODS

Reagents and Antibodies. Reagents were obtained as follows: MEM, fetal bovine serum, penicillin/streptomycin, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids, trypsin-EDTA, HBSS, and trypan blue were obtained from Life Technologies, Inc. (Grand Island, NY); ketamine, xylazine, and metallic abdominal wall Autoclips were obtained from Clay Adams (Parsippany, NJ); SU6668 was obtained from SUGEN, Inc. (South San Francisco, CA); PEG-300, Tween 80, 27- and 30-gauge needles, 1-ml syringes, and Gill 3 hematoxylin were obtained from Sigma Chemical Co. (St. Louis, MO); sodium monophosphate and diphosphate salts were obtained from EM Science (Gibbstown, NJ); OCT compound was obtained from Miles Inc. (Elkhart, IN); diaminobenzidine substrate and Universal Mount were obtained from Research Genetics (Huntsville, AL); Superfrost slides were obtained from Fisher Scientific Co. (Houston, TX); the TUNEL kit was obtained from Promega (Madison, WI); and DAPI mount from Vector Laboratories, Inc. (Burlingame, CA).

Antibodies for immunohistochemical analysis were obtained as follows: rat antimouse CD31/PEAM-1 antibody was obtained from PharMingen (San Diego, CA); mouse anti-PCNA clone PC 10 DAKO A/S was obtained from Dako Corp. (Carpinteria, CA); mouse antihuman SMA antibody, peroxidase-conjugated goat antirat IgG (H+L), and Texas Red- and fluorescein-conjugated goat antirat IgG were obtained from Jackson Research Laboratories (West Grove, PA); and peroxidase-conjugated rat antimouse IgG2a was obtained from Serotec Harlan Bioproducts for Science, Inc. (Indianapolis, IN).

Cell Culture. CT-26 murine colon carcinoma cells, which are syngeneic to BALB/c mice, were obtained from The National Cancer Institute Tumor Bank (Bethesda, MD), cultured and maintained in MEM supplemented with 5% fetal bovine serum, and harvested from subconfluent cultures as described previously (6).

Animals and Tumor Cell Inoculation. Eight-week-old male BALB/c mice (The National Cancer Institute's Animal Production Area, Frederick, MD) were acclimated for 1 week and caged in groups of five. Each mouse was anesthetized by i.p. injection of 100 μl of ketamine (80 mg/kg) and xylazine (16 mg/kg) and underwent a laparotomy and splenic injection as described previously (6). Mice were randomized into control ($n = 17$) and SU6668 treatment ($n = 18$) groups. All animal studies were conducted according to

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; TK, tyrosine kinase; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; PCNA, proliferating cell nuclear antigen; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; DAPI, 4′,6-diamidino-2-phenylindole; SMA, smooth muscle actin.

institutional guidelines approved by the Animal Care and Use Committee of The University of Texas M. D. Anderson Cancer Center and the Research Animal Facility at Stanford University.

Antiangiogenic Therapy: i.p. Administration of SU6668. Beginning on postoperative day 4, therapy was initiated with daily 200-μl i.p. injections of either control vehicle (30% PEG-300 (w/v) in 0.1 M sodium phosphate buffer (pH 8.2) or SU6668 (60 mg/kg) by using a 27-gauge needle attached to a 1-ml syringe. Mice in the survival study were sacrificed when they were moribund (as determined by a blinded observer). In a parallel study, five control and five treated mice were sacrificed after 14 and 20 days of treatment, and their livers were harvested. The mice were weighed weekly to evaluate for drug treatment-associated weight loss.

Necropsy and Tissue Preparation. The mice were sacrificed by carbon dioxide asphyxiation, their livers were excised and weighed, and the number of surface liver metastases was counted. Tumor sections were either embedded in OCT compound and frozen at −70°C or formalin fixed.

Immunohistochemical Analysis of Paraffin-embedded and Frozen Tissues. Liver tissue sections were pretreated by standard deparaffinization (for formalin-fixed/paraffin-embedded tissues) and acetone and chloroform fixation (for tissues frozen in OCT), and immunohistochemical analysis was performed as detailed previously using 6-μm tissue sections (6). Briefly, endogenous peroxidases were blocked with 3% H_2O_2 in methanol, and slides were washed in PBS, incubated for 20 min with protein-blocking solution (PBS supplemented with 1% normal goat serum and 5% normal horse serum), incubated overnight at 4°C with primary antibodies directed against CD31 or PCNA, washed, incubated with protein-blocking solution, incubated for 1 h at room temperature with peroxidase-conjugated secondary antibodies, washed, incubated with diaminobenzidine substrate, washed, counterstained with hematoxylin, washed, mounted with Universal Mount, and dried on a 56°C hot plate.

Immunofluorescence Staining for CD31 (Endothelial Cell), SMA (Pericytes), and TUNEL Assay (Apoptotic Cells). Frozen sections stained for CD31 and SMA by immunofluorescence followed the same protocol as detailed above with the following modifications. After sections were incubated overnight at 4°C with the primary antibody, washed, and incubated with protein-blocking solution, they were incubated for 1 h at room temperature with a secondary antibody that was conjugated to either Texas Red (red fluorescence) or fluorescein (green fluorescence) and then washed.

TUNEL staining was performed according to the manufacturer's protocol. Briefly, the sections were fixed with 4% methanol-free paraformaldehyde; washed; permeabilized with 0.2% Triton X-100; washed; incubated with the kit's equilibration buffer; incubated with a reaction mixture containing equilibration buffer, nucleotide mixture, and the terminal deoxynucleotidyltransferase enzyme at 37°C for 1 h; incubated for 15 min at room temperature with 2× SSC to stop the terminal deoxynucleotidyltransferase reaction; washed; stained with DAPI mount (nuclei); and applied glass coverslips.

Quantification of CD31-, PCNA-, and TUNEL-positive Cells. Tumor vessels and PCNA-positive cells were evaluated by light microcopy and counted in five random 0.159-mm^2 fields at ×100 magnification, imaged digitally, and processed with Optimas Image Analysis software (Bioscan, Edmond, WA). We quantified apoptosis using immunofluorescence by imaging sections digitally and processing them with Adobe Photoshop software (Adobe Systems, Mountain View, CA). CD31-positive endothelial cells were detected by localized red fluorescence using a rhodamine filter. Tumor and endothelial cell apoptosis was determined by localized green (tumor cell) or green with red (endothelial cell) fluorescence using a fluorescein filter. Apoptotic pericytes were detected by localized red (pericytes) and green (apoptosis) fluorescence. Microvessels covered by pericytes were detected by localized red (CD31) and green (SMA) fluorescence. Nuclei were detected by blue fluorescence of the DAPI with its respective filter. Cell counts were obtained in five random 0.011-mm^2 fields/slide at ×400 magnification. The percentages of apoptotic endothelial cells, tumor cells, and pericytes per field were then determined by the following equation: % apoptosis = [(number of apoptotic cells/total number of cells × 100]. The percentage of pericyte coverage was determined by the method of Eberhard *et al.* (15): % pericyte coverage = [(number of tumor microvessels that demonstrate pericyte colocalization/total number of tumor microvessels × 100]. Large tumor vessels were excluded from pericyte coverage analysis because any colocalized staining for SMA in this setting could be due to expression by smooth muscle cells and not necessarily by pericytes (15).

Statistical Analysis. Survival data were evaluated for statistical significance by log-rank analysis (Statview Software; SAS Institute, Inc., Cary, NC). Because evaluation of tumor angiogenesis by determining the number of endothelial cells per high power field with immunofluorescence has not been widely used, endothelial cell and vessel counts were compared by performing linear regression analysis using the Pearson's correlation coefficient [r (InStat Statistical Software, San Diego, CA)]. All other analyses for statistical significance were determined by the Mann-Whitney *U* test (InStat Statistical Software).

RESULTS

Toxicity, Tumorigenicity, and Quantification of Gross Tumor Burden. No significant differences in body weight were found between treatment and control groups, and no toxic reactions occurred. Necropsy confirmed that 100% of the control mice had formed liver metastases. No significant differences were observed between the liver weights of the control and SU6668 groups after 14 ($P = 1.000$) and 20 ($P = 0.857$) days of therapy or in the number of surface liver metastases after 14 ($P = 0.873$) and 20 ($P = 0.112$) days of therapy.

Effect of SU6668 Administration on Survival. Compared with controls, daily administration of SU6668 (60 mg/kg) significantly increased median survival in mice with colon cancer liver metastases by 58% (15.8 *versus* 24.9 days, respectively; $P < 0.001$; Fig. 1).

Effect of SU6668 Treatment on Tumor Angiogenesis and Tumor Cell Proliferation. We assessed tumor angiogenesis by immunofluorescence staining for CD31 to detect endothelial cells in liver metastases (Fig. 2*A*). Although the difference between the control and treatment groups after 14 days of treatment was not statistically significant ($P = 0.247$), the number of tumor endothelial cells decreased 46.2% in the SU6668-treated group after 20 days of treatment ($P = 0.015$).

We also assessed tumor angiogenesis by immunohistochemical staining for CD31 to detect vessels in liver metastases (Fig. 2*B*). Similar to endothelial cell counts, the difference in vessel counts between the control and treatment groups after 14 days of treatment was not statistically significant ($P = 0.123$). However, after 20 days of SU6668 treatment, the number of tumor vessels decreased 39.6% compared with controls ($P = 0.012$).

Tumor endothelial cell (detected by immunofluorescence staining) and vessel counts (detected by immunohistochemical staining) correlated strongly by linear regression analysis ($r^2 = 0.870$; $P < 0.001$), validating the former method for evaluating tumor angiogenesis.

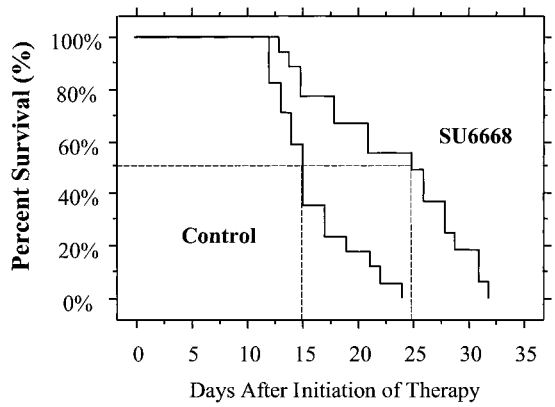

Fig. 1. Effect of SU6668 on survival of mice with liver metastases. Mice with experimentally induced colon cancer liver metastases were treated with either control vehicle or SU6668 (60 mg/kg/day i.p.). As compared with controls, SU6668 significantly improved median survival duration by 58% ($P < 0.001$). *Dashed lines*, median survival.

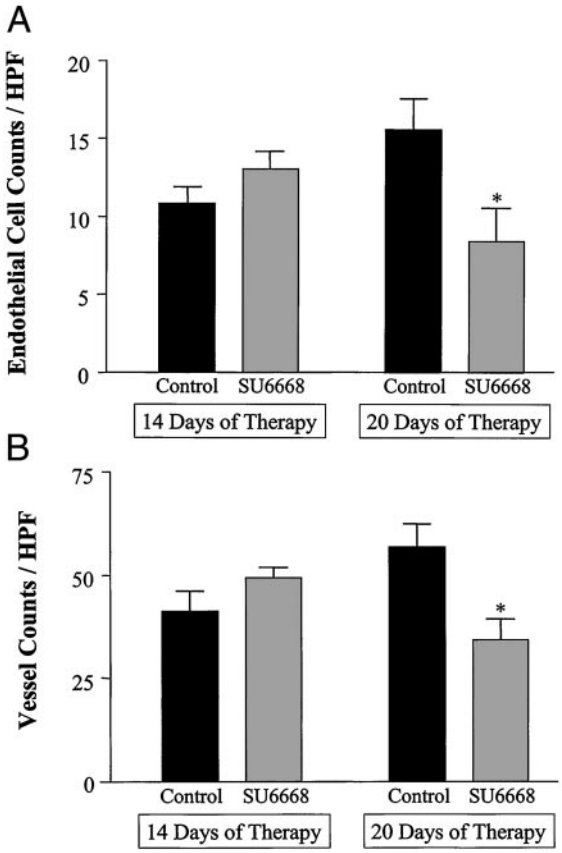

Fig. 2. Effect of SU6668 on tumor angiogenesis in liver metastases. *A*, immunofluorescence staining for CD31 in tumor-bearing liver sections was used to compare the number of tumor endothelial cells between the two groups after 14 and 20 days of therapy. SU6668 therapy led to a 46.2% decrease in endothelial cell number after 20 days (*, $P = 0.015$) but did not have a significant effect at 14 days ($P = 0.247$) compared with control mice (*bars*, SEM). *B*, immunohistochemical staining for CD31 was used to quantify tumor vessel counts. SU6668 therapy led to a 39.6% decrease in vessel counts after 20 days of therapy compared with controls (*, $P = 0.012$).

These findings suggest that tumor angiogenesis can be assessed by counting either tumor endothelial cells or vessels by standard technique (16).

Immunofluorescence staining for PCNA in liver metastases revealed no significant differences in the SU6668-treated group compared with the control group after 14 ($P = 0.411$) and 20 ($P = 0.280$) days of therapy.

Effect of SU6668 on Tumor Cell and Endothelial Cell Apoptosis and Pericyte Vessel Coverage. Immunofluorescent TUNEL staining with and without concurrent staining for CD31 was performed in liver metastases to quantify endothelial cell and tumor cell apoptosis, respectively, as described previously (Ref. 6; Fig. 3*A*). Tumor cell and endothelial cell apoptosis in tumors in the control group was minimal. At 14 days, SU6668 treatment resulted in significantly greater levels of tumor cell apoptosis ($P < 0.001$) and endothelial cell ($P = 0.009$) apoptosis compared with controls. At 20 days, SU6668 led to an even greater increase in tumor cell ($P < 0.003$) and endothelial cell ($P < 0.005$) apoptosis compared with tumors evaluated at 14 days of therapy, as well as a significant increase compared with controls ($P < 0.001$ for both tumor cell and endothelial cell apoptosis). No significant induction of pericyte apoptosis was observed in the treatment group relative to the control group at 14 and 20 days of therapy.

Immunofluorescent TUNEL staining with concurrent staining for SMA was performed in liver metastases to quantify pericyte apoptosis. There were relatively few pericytes undergoing apoptosis in either the control group or the SU6668-treated group. Concurrent immunofluorescence staining for CD31 and SMA was also performed to quantify the percentage of tumor vessels covered by pericytes (Fig. 3*B*; Ref. 15) because pericytes are recognized as endothelial cell survival factors that proliferate in response to PDGF (17, 18). Double staining for pericytes and endothelial cells revealed a significant decrease in pericyte vessel coverage in the SU6668-treated group compared with the control group after both 14 (21.7%; $P = 0.041$) and 20 (53.3%; $P = 0.002$) days of therapy. Representative immunofluorescence images of tumors from the control and SU6668-treated groups demonstrate the observed tumor cell and endothelial cell apoptosis and pericyte vessel coverage (Fig. 4).

DISCUSSION

The current study was undertaken to determine whether SU6668 treatment would improve the survival of mice with colon cancer liver metastases and to determine the biological mechanisms involved. To determine the effect of therapy over time on tumor angiogenesis and tumor and endothelial cell apoptosis, we harvested tumors at early and late time points before mice became moribund. In these temporal studies, significant differences were observed in tumor vascularity

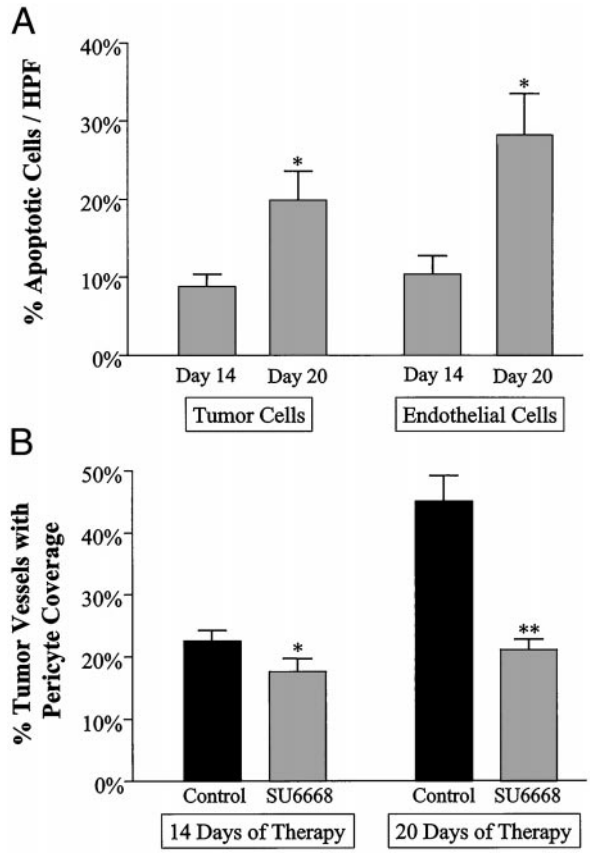

Fig. 3. Effect of SU6668 therapy on tumor cell and endothelial cell apoptosis and pericyte vessel coverage in liver metastases. *A*, immunofluorescence staining for TUNEL (tumor cell apoptosis) and sequential staining for CD31 and TUNEL (endothelial cell apoptosis) were performed in liver metastases after 14 and 20 days of SU6668 therapy. SU6668 therapy significantly increased the percentage of tumor cells and endothelial cells undergoing apoptosis at both time points compared with controls. Tumor cell apoptosis and endothelial cell apoptosis in the control groups after 14 and 20 days of therapy were nearly undetectable (<1%; data not shown). Tumor cell apoptosis and endothelial cell apoptosis were increased at 20 days of therapy compared with 14 days of therapy (*, $P < 0.003$ and $P < 0.005$, respectively). *Bars*, SEM. *B*, concurrent immunofluorescence staining for CD31 (endothelial cells) and SMA (pericytes) was performed to quantify the percentage of tumor microvessels covered with pericytes. SU6668 led to a significant decrease in pericyte vessel coverage compared with the control group after both 14 and 20 days of therapy. *, $P < 0.041$; **, $P = 0.002$.

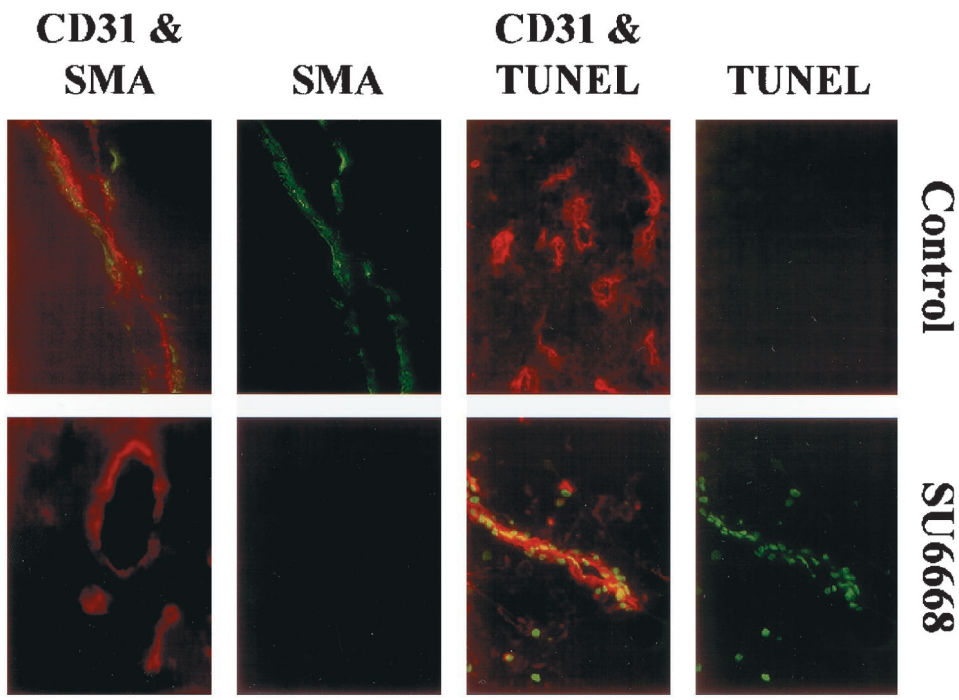

Fig. 4. Immunofluorescence evaluation of colon cancer liver metastases. Immunofluorescence staining for TUNEL (*column 4*, ×200), sequential staining for CD31 (*red*) and TUNEL (*green*; *column 3*, ×200), immunofluorescence staining for SMA (*green*; *column 2*, ×400), and sequential staining for CD31 (*red*) and SMA (*green*) were performed in tumor-bearing liver sections from both the control (*column 1*, *row 1*) and SU6668 (*column 2*, *row 1*) group. Representative images demonstrate that SU6668 therapy led to a significant increase in tumor cell and endothelial cell (*column 4*, *row 2*) and tumor cell (*green*) and endothelial cell (*red* and *green*; *column 3*, *row 2*) apoptosis and a decrease in pericyte coverage (*green*) of tumor blood vessels (*red*; *column 1*, *row 2*) compared with the control group (*column 1*, *row 1*).

(which was decreased) after 20 days of therapy and in tumor cell and endothelial cell apoptosis (which was increased) after 14 days and 20 days of therapy. These biological mechanisms likely contributed to the significantly improved survival observed in this study.

With regard to evaluating the effect of SU6668 on gross tumor burden, we did not observe significant differences in liver weights and the number of surface liver metastases between control and treated mice. This is in contrast to our previous study using a similar model (6). There are, however, several experimental design differences that likely account for the differences observed between these studies. In the current study, we inoculated mice with 25% fewer cells, which altered the growth kinetics of the model. In our previous study, we sacrificed mice when the control group became moribund. The current study was done to assess survival; therefore, liver weights and surface metastases were assessed at a later time point, when it is likely that other angiogenic mechanisms were driving angiogenesis in these tumors. These experimental design differences do not allow for direct comparisons between these studies and likely account for the differences observed at the termination of the studies. We hypothesize that at the time of liver harvest in our current study, selection of cells whose angiogenesis is driven by other angiogenic factors (such as platelet-derived endothelial cell growth factor, angiogenin, interleukin 8, and so forth) had established clonal dominance (19). However, to determine the dynamics of SU6668 on tumor cell and endothelial cell apoptosis, we harvested mice after 14 and 20 days of therapy and analyzed tumors for these parameters. These time points were chosen because we used fewer cells for induction of liver metastases than in our previous study (6); therefore, we expected mice to survive longer than they did in our previous study.

The finding that tumor vascularity was not significantly less than that of controls after 14 days of therapy but was significantly different after 20 days of therapy suggests that the efficacy of SU6668 in inhibiting tumor angiogenesis is dependent on chronic administration. This is consistent with the widely held view that antiangiogenic therapy in humans must be given as chronic therapy (20, 21). Putative antiangiogenic agents must be well tolerated because long-term therapy is likely necessary to inhibit tumor angiogenesis and facilitate disease stabilization. During the course of our study, we found no drug-related toxic reactions or weight loss, suggesting that SU6668 is well tolerated in this model, and we found that the differences between the control and treatment groups were not a result of nonspecific drug-related toxicity.

In addition to being classic angiogenic growth factors, VEGF, bFGF, and PDGF may also be endothelial cell or tumor cell survival factors (7, 22). In two recent *in vivo* studies of the effect of anti-VEGF therapy on colon cancer liver metastases (6, 9), we observed that by inhibiting the action of VEGF alone, we could induce both tumor cell apoptosis and endothelial cell apoptosis. In the study by Bruns *et al.* (9), endothelial cell apoptosis clearly preceded tumor cell apoptosis and was consistent with the hypothesis that VEGF is an endothelial cell survival factor. Therefore, in the study reported here, we sacrificed mice in both the control and SU6668 groups after 14 and 20 days of therapy to determine the effect of therapy over time on tumor cell and endothelial cell apoptosis. We observed that SU6668 therapy led to increased tumor cell and endothelial cell apoptosis at both time points, presumably because SU6668, unlike selective VEGF receptor inhibitors, directly targets receptors located on tumor cells (bFGF and PDGF) and endothelial cells (VEGF, bFGF, and PDGF). This suggests that SU6668 therapy may have contributed to tumor cell apoptosis both directly (by inhibiting TK receptor activity on tumor cell) and indirectly (by decreasing angiogenesis and endothelial cell survival). Although bFGF has been shown to be an endothelial cell survival factor *in vitro* by inducing the expression of the antiapoptotic protein survivin (22), further investigations that use selective inhibitors of bFGF and PDGF activity are necessary to more clearly delineate the roles of bFGF and PDGF in endothelial cell and tumor cell survival *in vivo*.

Several studies have recently suggested that pericytes may play an important role in the regulation of angiogenesis in certain developmental (23–25) and tumor model systems (5, 26, 27). Because colon cancer cells and endothelial cells express PDGF, a known mitogen for pericytes (17), we evaluated liver metastases for alterations in pericyte apoptosis and vessel coverage in tumors. We found that SU6668 therapy *in vivo* led to a significant decrease in the extent of pericyte

vessel coverage. Although this finding has been suggested in certain developmental and other tumor models (23–25, 28), it has not been previously demonstrated that antiangiogenic therapy can lead to a decrease in pericyte coverage of endothelial cells in colon cancer liver metastases. Therefore, we hypothesize that inhibition of PDGF signaling by SU6668 may lead to a decrease in pericyte coverage and its effect on endothelial cell survival (18). However, given the redundancy of the angiogenic process, it is unlikely that inhibition of a single angiogenesis factor will lead to continued inhibition of angiogenesis and/or pericyte recruitment *in vivo*. More importantly, whereas one factor may be responsible for pericyte coverage in one tumor microenvironment, it is possible that a different factor may be responsible for recruitment of pericytes in another tumor microenvironment. Therefore, studies in developmental biology examining "vasculogenesis" may or may not yield similar results to those obtained in studies examining "tumor angiogenesis." Given the inherent redundancy in these processes, we cannot exclude the possibility that inhibition of the VEGF or bFGF receptors by SU6668 is also contributing or is individually responsible for the inhibition of pericyte vessel coverage in our model. In support of this possibility, other recent reports have suggested a role for VEGF and bFGF as putative chemoattractants for pericytes (5, 27).

In conclusion, we have shown that simultaneous inhibition of the VEGF, bFGF, and PDGF receptors by SU6668 significantly improved the survival of mice with colon cancer liver metastases *in vivo*. This improvement was associated with marked decreases in tumor angiogenesis and pericyte vessel coverage and increases in both tumor cell and endothelial cell apoptosis. Our findings support the results of recent studies suggesting that VEGF and bFGF are endothelial cell survival factors (11, 12, 22). The simultaneous inhibition of the activities of several TK receptors located on tumor cells, endothelial cells, and pericytes with no observable toxicity suggests that agents that target the activity of numerous angiogenic factors may be more effective at inhibiting tumor angiogenesis than agents that target a single angiogenic factor (29). However, it is likely that therapy combining antiangiogenic agents with other antineoplastic therapies may further improve response and, ultimately, survival (30, 31).

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