Antiangiogenic Therapy Targeting the Tyrosine Kinase Receptor for Vascular Endothelial Growth Factor Receptor Inhibits the Growth of Colon Cancer Liver Metastasis and Induces Tumor and Endothelial Cell Apoptosis

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Abstract

Increased vascular endothelial growth factor (VEGF) expression is associated with colon cancer metastases. We hypothesized that inhibition of VEGF receptor activity could inhibit colon cancer liver metastases. BALB/c mice underwent splenic injection with CT-26 colon cancer cells to generate metastases. Mice received daily i.p. injections of vehicle, tyrosine kinase inhibitor for Flk-1/KDR (SU5416) or tyrosine kinase inhibitor for VEGF, basic fibroblast growth factor, and platelet-derived growth factor receptors (SU6668), SU5416 and SU6668 respectively inhibited metastases (48.1% and 55.3%), microvessel formation (42.0% and 36.2%), and cell proliferation (24.4% and 27.3%) and increased tumor cell (by 2.6- and 4.3-fold) and endothelial cell (by 18.6- and 81.4-fold) apoptosis (P < 0.001). VEGF receptor inhibitors increased endothelial cell apoptosis, suggesting that VEGF may serve as an endothelial survival factor.

Introduction

Angiogenesis is a dynamic and complex process that involves new blood vessel formations from established vasculature (1). This neo-vascularization is essential for both primary and metastatic tumor growth; therefore, antiangiogenic therapy may provide a novel addition to current antineoplastic approaches (1). The development of effective antiangiogenic therapy must first involve identifying biologically relevant molecular targets that are associated with tumor aggressiveness and metastasis formation. Three such angiogenic factors, VEGF (2), bFGF (3), and PDGF (4), function by binding to specific high-affinity TK receptors (5). Antiangiogenic strategies directed toward inhibiting VEGF activity include neutralizing anti-VEGF antibodies, anti-VEGF receptor antibodies, soluble VEGF receptors, antisense VEGF techniques, and VEGF receptor TK inhibitors (6). VEGF receptor TK inhibitors are small, synthetic, selective molecules that have favorable toxicity profiles, do not induce an immune response, and are not susceptible to enzymatic inactivation (7). Two novel compounds in this class are SU5416 (8), a selective inhibitor of only the VEGF receptor, and SU6668, an inhibitor of the receptors for VEGF, bFGF, and PDGF. The purpose of this study was to investigate the effect of these two inhibitors on the angiogenesis and growth of colon cancer liver metastases. A secondary aim was to investigate the effect of these agents on tumor cell proliferation and the induction of tumor and endothelial cell apoptosis.

Materials and Methods

Cell Culture. CT-26 murine colon carcinoma cells were cultured and maintained in MEM supplemented with 5% fetal bovine serum, 2 units/ml penicillin-streptomycin, vitamins, 1 mM sodium pyruvate, 2 mM L-glutamine, and nonessential amino acids at 37°C in 5% CO2 and 95% air (9). Cells were harvested from subconfluent cultures with trypsin-EDTA for 1 min, washed in suspended in media, centrifuged at 300 × g for 8 min at room temperature, and then resuspended to a final concentration of 1 × 106 viable cells/50 μl HBSS. Trypan blue exclusion was performed to ensure cell viability. All cell culture reagents were obtained from Life Technologies, Inc. (Grand Island, NY).

Animals and Tumor Cell Inoculation. Eight-week-old male BALB/c mice were obtained from the National Cancer Institute’s Animal Production Area (Fredrick, MD), acclimated for 1 week and caged in groups of five, and fed a diet of animal chow and water ad libitum. Mice were anesthetized in a methoxylflurane (Pitman-Moore, Mundelein, IL) chamber, followed by left upper quadrant laparotomy and splenic exteriorization. Using a 30-gauge needle and a 1-ml syringe, 50 μl of the tumor cell suspension were injected beneath the splenic capsule. The skin and peritoneum were closed in a single layer using metallic clips (Autoclip; Clay Adams, Parsippany, NJ), which were removed on POD 7. Mice were randomized to one of three groups (15 mice/group), with no statistically significant difference between the mean weights of the three groups. All animal studies were conducted according to a protocol approved by the Animal Care and Use Committee of The University of Texas M. D. Anderson Cancer Center.

Antiangiogenic Therapy. Beginning on POD 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)], SU5416 [12 mg/kg in 99% PEG-300 (w/v) with 1% Tween 80 (polyethylene sorbitan monooleate detergent)], or SU6668 [60 mg/kg in control vehicle] using a 30-gauge needle attached to a 1-ml syringe. Animals were sacrificed on POD 22 when the control mice became moribund. Mice were weighed weekly to confirm no drug treatment-associated weight loss. SU5416 and SU6668 were provided by SUGEN, Inc. (South San Francisco, CA); PEG-300 and Tween 80 were obtained from Sigma Chemical Co. (St. Louis, MO), and sodium monophosphate and diphosphate salts were obtained from EM Science (Gibbstown, NJ).

Autopsy and Tissue Preparation. Mice were sacrificed by cervical dislocation after adequate sedation with methoxyflurane was confirmed by the toe pinch technique. Livers were excised and weighed, and the number of total surface hepatic metastases was counted using a dissection microscope. For IHC staining, a section of the tumor tissue was fixed in Bouin’s solution for 24 h and then fixed in formalin and embedded in paraffin. Another section was IHC stained, a section of the tumor tissue was fixed in Bouin’s solution for 24 h and then fixed in formalin and embedded in paraffin.

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1The 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 deoxynucleotidyl transferase-mediated nick end labeling; IHC, immunohistochemistry; POD, postoperative day; PEG, polyethylene glycol.

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lowed by 100%, 95%, and 80% ethanol and rehydrated in PBS (pH 7.5). These sections were used for H&E staining and detection of PCNA protein expression. Sections analyzed for PCNA were microwaved for 5 min to increase antigen retrieval. Sections analyzed for tumor cell apoptosis by TUNEL were predigested with pepsin (Biomedica, Foster City, CA) for 15 min at 37°C and washed three times for 3 min each time with PBS (Irvine Scientific, Santa Ana, CA).

Liver tissues frozen in OCT were sectioned (8–10 μm), mounted on positively charged slides, and air-dried for 30 min. Frozen tissues were fixed in cold acetone (5 min), 1:1 acetone/chloroform (5 min), and acetone (5 min) and then washed with PBS three times for 3 min each. After these pretreatment procedures, all samples were incubated with 3% H2O2 in methanol for 12 min at room temperature to block endogenous peroxidase. Sections were washed three times for 3 min each with PBS (pH 7.5) and then incubated for 20 min at room temperature in a protein-blocking solution consisting of PBS supplemented with 1% normal goat serum and 5% normal horse serum. The primary antibodies directed against CD31 and PCNA were diluted 1:200 and 1:50, respectively, in protein-blocking solution, applied to the sections, and incubated overnight at 4°C. Sections were then rinsed three times for 3 min each in PBS and incubated for 10 min in protein-blocking solution before the addition of peroxidase-conjugated secondary antibody. The secondary antibodies used for CD31 and PCNA staining were diluted 1:200 and 1:100, respectively, in protein-blocking solution. After incubating with the secondary antibody for 1 h at room temperature, the samples were washed and incubated with stable diaminobenzidine (Research Genetics, Huntsville, AL) substrate. Staining was monitored under a bright-field microscope, and the reaction was stopped by washing with distilled water. Sections were counterstained with Gill’s No. 3 hematoxylin (Sigma Chemical Co.) and mounted with Universal Mount (Research Genetics) for 15 s. Control specimens were treated with a similar procedure, except that the primary antibody was omitted.

Immunofluorescence Double Staining and Quantification of Apoptotic Endothelial Cells in Tissues. Frozen tissue sections (8 μm) were fixed with cold acetone for 5 min, acetone plus chloroform (1:1) for 5 min, and acetone for 5 min. Samples were washed three times with PBS and incubated with protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS for 20 min at room temperature. Blocking solution was drained, and the samples were incubated with a 1:400 dilution of rat monoclonal anti-CD31 antibody (human cross-reactive antibody; PharMingen, San Diego, CA) for 24 h at 4°C. Samples were rinsed with PBS three times for 3 min each and incubated with protein-blocking solution for 10 min at room temperature. Avoiding exposure to light, the blocking solution was drained, and the samples were incubated with a 1:200 dilution of Texas Red-conjugated goat antirat secondary antibody for 1 h at room temperature. Samples were washed two times with PBS containing 0.1% Brij and washed with PBS for 5 min. TUNEL was performed using a commercial kit (Promega, Madison, WI) with the following modifications. Samples were fixed with 4% paraformaldehyde (methanol free) for 10 min at room temperature. The samples were washed with PBS two times for 5 min and then incubated with 0.2% Triton X-100 for 15 min at room temperature. The samples were washed with PBS two times for 5 min and incubated with equilibration buffer (from the kit) for 10 min at room temperature. The equilibration buffer was drained, and reaction buffer containing equilibration buffer, nucleotide mix, and terminal deoxynucleotidyl transferase enzyme was added to the tissue sections and incubated in a humid atmosphere at 37°C for 1 h, avoiding exposure to light. The reaction was terminated by immersing the samples in 2× SSC for 15 min. Samples were washed three times for 5 min to remove unincorporated fluorescein-dUTP. For quantification of endothelial cells, the samples were incubated with 300 mg/ml Hoechst stain for 10 min at room temperature. The samples were then washed with PBS two times for 5 min at room temperature. Prolong solution (Molecular Probes, Eugene, OR) was used to mount coverslips. Immunofluorescence microscopy was performed using a ×40 objective (Zeiss Plan-Neofluar) on an epifluorescence microscope equipped with narrow bandpass excitation filters mounted in a filter wheel (Ludl Electronic Products, Hawthorne, NY) to individually select for green, red, and blue fluorescence. Images were captured using a cooled charge coupled device camera (Photometrics, Tucson, AZ) and SmartCapture software (Digital Scientific, Cambridge, United Kingdom) on a Macintosh computer. Images were further processed using Adobe Photoshop software (Adobe Systems, Mountain View, CA).

Endothelial cells were identified by red fluorescence, and DNA fragmentation was detected by localized green and yellow fluorescence within the nucleus (visualized by Hoechst stain) of apoptotic cells. Quantification of apoptotic endothelial cells was expressed as the average of TUNEL positive endothelial cells in five random fields at ×40 magnification.

Quantification of Tumor Vessel Counts, PCNA, and TUNEL. To quantify tumor vessel counts, frozen sections were fixed and stained with primary antibodies to CD31. Five random 0.159-mm² fields at ×100 magnification were captured for each tumor by using a Sony three-chip camera (Sony Corporation of America, Montvale, NJ) mounted on a Zeiss universal microscope (Carl Zeiss, Thornwood, NY) and Optimas Image Analysis software (Bioscan, Edmond, WA) installed on a Compaq computer with a Pentium chip, a frame grabber, an optical disc storage system, and a Sony Movigraph UP-D7000 Digital color printer (Tokyo, Japan). To quantify PCNA expression, the number of positive cells (yellow-stained cells) and tumor cells (green-stained cells) in frozen tissue sections under the Olympus microscope, the numbers of apoptotic events were counted in five random 0.159-mm² fields at ×100 per field. More than 95% of cells in these tumor specimens are tumor epithelial cells. Therefore, quantitation of tumor cell apoptosis was made under the assumption that the majority of green-stained cells were tumor cells. This was confirmed by observing the relative amount of apoptotic events in non-CD31 TUNEL-positive cells versus CD31 TUNEL-positive cells when double staining was done in the subsequent study.

Antibodies. Antibodies for IHC were obtained from the following sources: (a) rat antimonoclonal CD31 antibody, PharMingen; (b) mouse anti-PCNA clone PC 10, DAKO A/S; (c) peroxidase-conjugated goat antirat IgG (H+L) and Texas Red-conjugated goat antirat IgG, Jackson Research Laboratories (West Grove, PA); and (d) peroxidase-conjugated rabbit antimouse IgG2a, Serotec, Inc. (Raleigh, NC).

Statistical Analysis. Liver weights; quantification of CD31, PCNA, and TUNEL; and quantification of apoptotic endothelial cells (by sequential staining for CD31 and TUNEL) were compared by using unpaired Student’s t-tests (SAS for Macintosh; GraphPad Software, San Diego, CA).

Results

Tolerance of Therapy and Tumorogenicity. No significant differences were found in body weight among the three groups at the end of the experiment. Until mice became moribund from tumor burden, no toxic reactions were noted. Autopsy confirmed that 100% of the control mice had surface colon cancer liver metastases.

Effect of SU5416 and SU6668 on Liver Metastases. Harvested livers were weighed as a gross measure of tumor burden. Relative to control mice, liver weights were decreased in the SU5416 (31.9%; \( P = 0.002 \)) and SU6668 (35.7%; \( P < 0.001 \)) groups (Fig. 1A). Fewer surface liver metastases were present in the SU5416 (48.1%; \( P < 0.001 \)) and SU6668 (55.3%; \( P < 0.001 \)) groups than in the control group (Fig. 1B).

Effect of SU5416 and SU6668 on Tumor Vessel Counts. Immunohistochemical staining for CD31 to detect vessels in hepatic metastases revealed a significant decrease in tumor vessel counts in the SU5416 (42.0%; \( P < 0.001 \)) and SU6668 (36.2%; \( P < 0.001 \)) groups compared with those in the control group (Fig. 2). Additionally, no significant differences were observed between tumor vessel counts in the SU5416 group and in the SU6668 group.

Effect of SU5416 and SU6668 on PCNA Expression and on Endothelial Cell and Tumor Cell Apoptosis. Immunohistochemical staining for PCNA and immunofluorescent TUNEL staining, with and without concurrent staining for CD31, were performed in tumor-bearing liver sections to evaluate tumor cell proliferation, endothelial cell apoptosis, and tumor cell apoptosis, respectively. SU5416 and SU6668 treatment resulted in a significantly reduced level of tumor cell proliferation (24.4% and 27.3% less than the control group value, respectively; \( P < 0.001 \); Figs. 3 and 4) and a significantly greater level of apoptosis in endothelial cells (2.6- and 4.3-fold increases,
respectively, over that of the control group, \( P < 0.001 \) and tumor cells (18.6- and 81.4-fold increases, respectively, over that of the control group, \( P < 0.001 \)). No differences were found in PCNA expression between the SU5416 and SU6668 groups. However, SU6668 treatment produced 4.4-fold higher endothelial cell apoptosis (\( P < 0.001 \)) and 1.7-fold higher tumor cell apoptosis (\( P < 0.048 \)) than SU5416 treatment.

**Discussion**

Tumor growth and metastasis are angiogenesis-dependent processes (10). Angiogenesis is typically stimulated in response to tumor-secreted angiogenic factors such as VEGF, which bind to high-affinity TK receptors and promote endothelial cell proliferation, invasion, and the formation of new capillaries (11). We have previously shown that the expression of VEGF and its receptor is associated with tumor vascularity, metastasis, and proliferation of human colon cancer (2). Based on these fundamental observations regarding the biology of colon cancer, we postulated that inhibition of VEGF action by inhibiting signaling through the VEGF receptor could represent an important antiangiogenic therapeutic modality for inhibiting the growth of colon cancer liver metastases (12, 13).

Several small molecule inhibitors that target these growth factor receptors are currently being evaluated in clinical trials (7). Protein TK inhibitors are promising agents within this class that demonstrate selectivity with minimal toxicity to the host (7, 8). Our present results show that treatment of mice with the VEGF receptor TK inhibitors SU5416 and SU668 resulted in marked inhibition of the growth, vascularity, and proliferation of colon cancer liver metastases. We did not observe toxic effects at the doses administered, as evidenced by body weight and grooming habits, which remained similar to those of control mice during the treatment. Our results confirm a recent study that reported decreases in tumor vascularity, growth, and proliferation in multiple tumor types after SU5416 administration (8). To better understand the mechanism involved in growth inhibition, we also evaluated tumor cell apoptosis. We found that antiangiogenic therapy by TK inhibitors limited tumor growth in association with an increase in tumor cell apoptosis and a decrease in tumor cell proliferation. This finding contrasts with that of a previous study using a Lewis lung carcinoma model in which the inhibition of tumor growth in the presence of angiogenesis suppression was mediated by an induction of apoptosis, without inhibition of tumor cell proliferation (14). This difference among studies may reflect differences in the model, the agent used, or the duration of the observations.

To determine why antiangiogenic therapy, which specifically targets endothelium, produced an increase in tumor cell apoptosis, we evaluated tumors for endothelial cell apoptosis by combining an immunohistochemical stain for CD31 (vessels) and TUNEL (apoptosis) staining. With this approach, we found a significant induction of...
endothelial cell apoptosis in the SU5416- and SU6668-treated groups as compared to the control groups. We also observed a more marked increase in the extent of endothelial cell apoptosis relative to tumor cell apoptosis. Because, with rare exceptions, VEGF receptors are expressed exclusively on endothelial cells, it is unlikely that SU5416 directly induces tumor cell apoptosis. Therefore, it is possible that inhibiting the action of VEGF may lead to tumor endothelial apoptosis, which could then lead to a subsequent increase in tumor cell apoptosis. These findings suggest that VEGF may act as a direct survival factor for tumor endothelium and an indirect survival factor for colon carcinoma cells. These findings are supported by other recent reports that have purported VEGF to be crucial to the survival of tumor endothelium (15). Additional investigations are necessary to confirm whether these causal and temporal relationships exist between VEGF receptor inhibition and endothelial and tumor cell apoptosis.

In conclusion, we have shown that antiangiogenic therapy targeting the TK receptor for the VEGF receptor inhibits the vascularity, proliferation, and growth of colon cancer liver metastasis and significantly increases endothelial and tumor cell apoptosis. These findings suggest an important role for VEGF as a survival factor for tumor endothelium. Despite the growth inhibition of tumors with no observable toxicity, liver metastases were not eradicated, at least during the brief time that we treated these tumors. Therefore, it remains to be seen whether this growth inhibition could lead to a survival advantage for the treatment groups. Survival studies are currently underway that seek to answer this question. At present, there is a lack of effective systemic therapies that significantly improve survival in patients with metastatic colon cancer. Therefore, our findings suggest that SU5416 and SU6668 are promising antiangiogenic agents that may have clinical utility in the management of colon cancer liver metastases.

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