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Antiangiogenic and Radiation Therapy:

Early Effects on In Vivo Computed Tomography Perfusion Parameters in Human Colon Cancer Xenografts in Mice

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Abstract

Objectives—To assess early treatment effects on computed tomography (CT) perfusion parameters after antiangiogenic and radiation therapy in subcutaneously implanted, human colon cancer xenografts in mice and to correlate in vivo CT perfusion parameters with ex vivo assays of tumor vascularity and hypoxia.

Materials and Methods—Dynamic contrast-enhanced CT (perfusion CT, 129 mAs, 80 kV, 12 slices $\times$ 2.4 mm; 150 μ L iodinated contrast agent injected at a rate of 1 mL/min intravenously) was performed in 100 subcutaneous human colon cancer xenografts on baseline day 0. Mice in group 1 (n = 32) received a single dose of the antiangiogenic agent bevacizumab (10 mg/kg body weight), mice in group 2 (n = 32) underwent a single radiation treatment (12 Gy), and mice in group 3 (n = 32) remained untreated. On days 1, 3, 5, and 7 after treatment, 8 mice from each group underwent a second CT perfusion scan, respectively, after which tumors were excised for ex vivo analysis. Four mice were killed after baseline scanning on day 0 for ex vivo analysis. Blood flow (BF), blood volume (BV), and flow extraction product were calculated using the left ventricle as an arterial input function. Correlation of in vivo CT perfusion parameters with ex vivo microvessel density and extent of tumor hypoxia were assessed by immunofluorescence. Reproducibility of CT perfusion parameter measurements was calculated in an additional 8 tumor-bearing mice scanned twice within 5 hours with the same CT perfusion imaging protocol.

Results—The intraclass correlation coefficients for BF, BV, and flow extraction product from repeated CT perfusion scans were 0.93 (95% confidence interval: 0.78, 0.97), 0.88 (0.66, 0.95),

and 0.88 (0.56, 0.95), respectively. Changes in perfusion parameters and tumor volumes over time were different between treatments. After bevacizumab treatment, all 3 perfusion parameters significantly decreased from day 1 ($P = 0.006$) and remained significantly decreased until day 7 ($P = 0.008$); tumor volume increased significantly only on day 7 ($P = 0.04$). After radiation treatment, all 3 perfusion parameters decreased significantly on day 1 ($P < 0.001$); BF and flow extraction product increased again on day 3 and 5, although without reaching statistically significant difference; and tumor volumes did not change significantly at all time points ($P = 0.3$). In the control group, all 3 perfusion parameters did not change significantly, whereas tumor volume increased significantly at all the time points, compared with baseline ($P = 0.04$). Ex vivo immunofluorescent staining showed good correlation between all 3 perfusion parameters and microvessel density ($\rho = 0.71, 0.66$, and 0.69 for BF, BV, and flow extraction product, respectively; $P < 0.001$). There was a trend toward negative correlation between extent of hypoxia and all 3 perfusion parameters ($\rho = -0.53, -0.47$, and -0.40 for BF, BV, and flow extraction product, respectively; $P = 0.05$).

Conclusions—CT perfusion allows a reproducible, noninvasive assessment of tumor vascularity in human colon cancer xenografts in mice. After antiangiogenic and radiation therapy, BF, BV, and flow extraction product significantly decrease and change faster than the tumor volume.

Keywords

CT perfusion; colon cancer; antiangiogenic therapy; radiation therapy; bevacizumab

With the increasing number of molecularly targeted cancer drugs, imaging techniques for monitoring treatment effects and predicting tumor response are becoming more important because many of these novel drugs have only a small or delayed effect on tumor size.^{1–4} For example, antiangiogenic drugs such as the first US Food and Drug Administration–approved agent, bevacizumab, inhibit the formation of new blood vessels, thereby causing tumor starvation with little effect on tumor size.^{5,6} In addition to decreasing tumor blood vessels and causing long-term tumor starvation, several studies have suggested the concept of short-term tumor vessel normalization early after initiation of antiangiogenic treatment, resulting in temporarily improved tumor perfusion and decreasing tumor hypoxia.^{7–9} However, results from several studies exploring the effects of a wide variety of antiangiogenic agents show controversial results. Radiosensitization because of transiently improved oxygenation of a murine FSaII fibrosarcoma model was observed within 2 days after intraperitoneal administration of thalidomide, an antiangiogenic drug that acts on vascular endothelial growth factor and basic fibroblast growth factor signaling.¹⁰ Similarly, at day 2 and 3 after thalidomide administration, tumor oxygenation increased in syngeneic transplantable mouse liver tumors, potentiating the effect of the chemotherapeutic cyclophosphamide when administered during this short 2-day therapeutic window. In contrast, SU5416, an antagonist of the vascular endothelial growth factor receptor, potentiated tumor response to radiotherapy but not to chemotherapy in both the murine FSaII fibrosarcoma model and syngeneic transplantable mouse liver tumor model. Furthermore, intraperitoneal administration of yet another antiangiogenic agent, DC101, a rat monoclonal antibody specific for mouse vascular endothelial growth factor receptor type 2 caused increased extent of hypoxia in an orthotopic human breast cancer xenograft model in mice during the

entire treatment course of 21 days.¹¹ Given the increasing number of different antiangiogenic agents being tested in current clinical trials in patients with different tumor types, these contradicting results underline the need for novel functional or molecular imaging strategies to optimize combined antiangiogenic, chemotherapeutic, and radiation therapies in cancer patients.

Although several imaging techniques are currently being explored to noninvasively assess tumor vascularity,^{1,12–15} dynamic contrast-enhanced computed tomography (perfusion CT) imaging is advantageous because contrast-enhanced anatomic-morphologic CT is already the most commonly used first-line imaging modality for routine staging and monitoring of cancer in clinical practice and can be easily extended to measure perfusion parameters in the same imaging session.^{16,17} However, there is substantial controversy in the literature as to whether CT perfusion parameters correlate with histologic markers of tumor vascularity.^{18–22} Furthermore, there is lack of experience on the temporal course of CT perfusion parameters within the early time window after antiangiogenic treatment and radiation therapy. This information, however, is of paramount importance to not only predict early treatment response to antiangiogenic therapy but also to potentially combine antiangiogenic drugs with other therapeutic approaches, including chemotherapeutics or radiation therapy, within a presumed early vascular normalization window.^{8,9}

The purpose of our study was to assess early treatment effects on CT perfusion parameters after antiangiogenic and radiation therapy in subcutaneous human colon cancer xenografts in mice and to correlate in vivo CT perfusion parameters with ex vivo assays of tumor vascularity and hypoxia.

MATERIALS AND METHODS

Subcutaneous Human Colon Cancer Xenografts in Mice

All procedures performed with the use of laboratory animals were approved by the Institutional Administrative Panel on Laboratory Animal Care. A total of 123 human colon cancer xenografts were established by subcutaneous injection of 5×10^6 human colon carcinoma LS174T cells (ATCC, Manassas, VA) dissolved in 50 μ L of basement membrane matrix (Matrigel; BD Biosciences, San Jose, CA) on the back and 1 cm laterally to the spine of nude female mice (6–8 weeks old; Charles River, Wilmington, MA). Tumors were allowed to grow for up to 8 days until an average tumor volume of 193 mm³ (range, 100–1209 mm³) was attained, as measured on CT images (mentioned later in the text) and by using the formula for a prolate ellipsoid, $\pi/6 \times (L \times W \times H)$, where L is length, W is width, and H is height.

CT Perfusion Imaging Technique

Before imaging, all animals were anesthetized by intraperitoneal injection of a mixture of ketamine (Ketaject; Phoenix Pharmaceutical, Inc., St. Joseph, MO; 100 mg/kg of body weight) and xylazine (Anased; LLOYD laboratories, Shenandoah, IA; 20 mg/kg of body weight). CT scanning was performed with a clinical 64-detector row CT scanner (Sensation 64; Siemens; Erlangen, Germany). To decrease imaging misregistration of the tumors

caused by respiratory movement, mice were placed and secured with a tape on the CT scanner table in a supine position, with the tumors on the back resting directly on the scanner table. The CT imaging protocol included a digital radiograph (topogram), a nonenhanced CT scan to localize the tumor, and a dynamic contrast-enhanced CT acquisition after intravenous contrast material administration. The following parameters were used for the nonenhanced CT scan: 35 mA tube current, 80 kV tube voltage, 0.5-second rotation time, 1-mm slice thickness, and $60 \times 60\text{-mm}^2$ field of view. Based on the nonenhanced CT data sets, a scan range of 28.8 mm with a field of view of $60 \times 60\text{ mm}^2$ was prescribed for the subsequent CT perfusion study in all animals, covering both the subcutaneous tumor xenografts and the heart of the animals (which allowed using the left ventricle for arterial input function measurements).²³ In all animals, 150 μL of iodinated contrast media (Omnipaque, 350 mgI/mL; GE Healthcare Inc., Princeton, NJ) was injected through a 27-gauge intravenous catheter (Vevo Micromarker tail vein access; Visualsonics, Toronto, ON, Canada) placed in 1 of the 2 tail veins, at a constant injection rate of 1 mL/min (injection time, 9 seconds), controlled by a dedicated injection pump (Genie Plus; Kent scientific corporation, Torrington, CT). Dynamic contrast-enhanced CT (129 mA, 80 kV; 0.5-second rotation time) was initiated 3 seconds before the start of contrast medium injection and acquired every 0.5 seconds for a total duration of 100 seconds. The 28.8-mm scan volume was reconstructed into 12 contiguous slices with a section thickness of 2.4 mm and an image matrix of 512×512 , using a B50s kernel (resulting in a total of 2400 reconstructed CT images per tumor). The resulting voxel size was $0.1 \times 0.1 \times 2.4\text{ mm}^3$.

CT Perfusion Imaging Data Analysis

All reconstructed dynamic contrast-enhanced imaging data sets were transferred to a Multimodality Workplace workstation (Siemens Healthcare, Forchheim, Germany) for volumetric perfusion CT analysis using commercially available software (vPCT; Siemens Healthcare, Forchheim, Germany), which includes both maximum slope- as well as deconvolution-based methods. All perfusion parameters in this study were calculated using the implemented deconvolution algorithm, which uses a parametric impulse residue function.^{24,25} To exclude osseous structures, a processing threshold was set at CT values between -200 and 500 Hounsfield units (HU). The arterial input was obtained from a 2- to 3-mm^2 region of interest (ROI) placed in the left ventricular cavity of the heart (Fig. 1). The following perfusion parameters were calculated: blood flow (BF) (in milliliters per 100 g of wet tissue per minute), blood volume (BV) (in milliliters per 100 g of wet tissue), and flow extraction product (in milliliters per 100 g of wet tissue per minute), and displayed as functional maps. One blinded reader (blinded to the type and timing of treatment) manually drew ROIs over the whole subcutaneous tumor xenografts on each of the 12 image slices in random order. CT perfusion parameters measured from the ROIs of the 12 slices were averaged for each tumor. Tumor lengths, widths, and heights were measured on transverse and coronal nonenhanced CT data sets using an electronic caliper available on the workstation.

Study Design

Reproducibility of CT Perfusion Imaging Technique—To evaluate reproducibility of CT perfusion measurements, CT perfusion imaging was repeated 5 hours after the first

scan in 8 animals, with identical CT imaging and reconstruction parameters (Fig. 1). No treatment was administered in these animals.

Treatment Plan and CT Perfusion Imaging in Mice

CT Perfusion Assessment in Individual Human Colon Cancer Xenografts: Following a baseline (day 0) CT perfusion scan in 100 subcutaneous human colon cancer xenografts, 4 mice were randomly selected and killed to correlate in vivo imaging results with ex vivo assays at baseline day 0. The remaining mice were randomly assigned to 1 of 3 treatment groups (Fig. 2). In group 1 (n = 32), a single intravenous injection of the anti-VEGF monoclonal antibody bevacizumab (Avastin; Genentech, South San Francisco, CA; 10 mg/kg b.w) was administered through the tail vein. Animals of group 2 (n = 32) underwent focal tumor treatment by a single 12-Gy irradiation dose (administered over 20 minutes to the tumor by using a Philips RT 250 x-ray irradiator; Philips Medical System Inc. Shelton, CT). Animals of group 3 (n = 32) served as an untreated control group. On days 1, 3, 5, and 7 after treatment, 8 mice from each of the 3 groups, respectively, were randomly selected and rescanned with the same CT perfusion protocol as described earlier in the text. After the second CT perfusion imaging, mice were killed and tumors were excised for correlating in vivo CT perfusion imaging results with ex vivo analysis (Fig. 2).

Longitudinal Intraindividual CT Perfusion Assessment: To confirm results obtained from individual tumor xenografts at different time points, an additional 15 tumor-bearing mice were randomly assigned to the 3 treatment groups (n = 5 each for antiangiogenic, radiation, or no treatment), and CT perfusion imaging was performed longitudinally in all animals at baseline (day 0) and at days 1, 3, 5, and 7 (Fig. 2). At each time point, CT perfusion imaging parameters were kept identical according to the protocol described earlier in the text.

Ex Vivo Analysis of Tumor Tissues—Mice received an intraperitoneal injection of pimonidazole (60 mg/kg; HPI, Inc., Burlington, MA) 90 minutes before being sacrificed. Tumors were then excised and embedded in optimum cutting temperature (O.C.T. Compound Tissue-Tek; Sakura Finetek USA, Inc., Torrance, CA) and cryosectioned into 10- μ m slices.

Assessment of Microvessel Density: For microvessel density (MVD) analysis, slices were stained for the vascular endothelial marker CD31. Briefly, the sections were first fixed with cold acetone for 3 minutes and allowed to air dry at room temperature for an hour, followed by a rinse in phosphate-buffered saline. Incubation in 5% normal goat serum in phosphate-buffered saline for 5 hours was performed to block nonspecific proteins. Expression of CD31 was assessed by incubating all slices with rat antimouse CD31 (BD Pharmingen; at 1:100 dilution) overnight at 4°C, followed by incubation of corresponding secondary antibodies of goat antirat fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch labs; at 1:300 dilution) for 30 minutes at room temperature. Slices were mounted in aqueous mounting media (BiogeneX, San Ramon, CA), and fluorescent images were directly acquired from microscope slides to a computer equipped with Metamorph software (Universal Imaging Corp., West Chester, PA) using a Zeiss Axioscope microscope

(Axiophot; Carl Weiss AG, Thornwood, NY) attached to a digital camera (AxioCam MRc, Bernried, Germany), at a magnification of $\times 100$. MVD analysis was performed using a standardized protocol.²⁶ The total number of vessels was summed for at least 10 fields of view (single field-of-view area = 0.57 mm^2) covering the whole tumor section for each tumor slice, and MVD was calculated as the average number of vessels per total fields of view area.

Assessment of Tumor Hypoxia: The presence of tumor hypoxia was evaluated by immunofluorescent visualization of pimonidazole, which is a bioreductive chemical probe with activation occurring at pO_2 levels $< 10 \text{ mm Hg}$ and, therefore, can be used to assess tumor hypoxia as described elsewhere.^{11,27} Fixing and blocking steps used for pimonidazole staining were the same as used for CD31 staining. Pimonidazole was then detected by incubating tumor sections with mouse antipimonidazole monoclonal antibody (Hypoxyprobe-1 Mab1, 1:50 dilution; HPI, Inc., Burlington, MA) overnight at 4°C and followed by incubation with FITC-conjugated goat antimouse antibody (Alexa Fluor 488, Invetrogen, Carlsbad, CA; at 1:200 dilution) for 1 hour at room temperature. Fluorescent images were acquired with the same microscope as that for MVD analysis, with a magnification of $\times 100$. Quantification of tumor hypoxia extent was performed by calculating the fraction of the tumor area stained for pimonidazole as described elsewhere¹¹ using ImageJ software (available at: <http://rsb.info.nih.gov/ij/>). Briefly, a contour line was drawn around all areas stained with pimonidazole, and the hypoxic fraction was calculated as the tissue surface stained for the hypoxic marker relative to the total tissue surface of tumor in each field of view (single field-of-view area = 0.57 mm^2). In total, 10 to 15 fields of view were analyzed for each slide (depending on the size of tumor), and hypoxia fractions were calculated as the average from all analyzed fields of views per tumor.

Statistical Analysis

Data were reported as mean $\pm$ standard deviation (SD). The change of each of the 3 perfusion parameters (BF, BV, and flow extraction product) and of the tumor volume at the 4 different time points after treatment (days 1, 3, 5, and 7; either after antiangiogenic, radiation, or no therapy) were compared with the baseline scan on day 0 and expressed as percent change using the following formula: $\text{change (\%)} = (\text{value}_{\text{day N}} - \text{value}_{\text{day 0}}) / \text{value}_{\text{day 0}} \times 100\%$. For assessment of reproducibility of different CT perfusion parameters obtained 5 hours apart, intraclass correlation coefficients were calculated including 95% confidence intervals (CI) based on the bootstrap method. Using the Bland-Altman method, the mean difference between 2 consecutive CT perfusion measurements, SD of the differences, and 95% limits of agreement were calculated for the 3 CT perfusion parameters. Also, within-subject standard deviation and within-subject coefficient of variation (which is the ratio of within-subject SD divided by the average of the replicated CT perfusion measurements) for the 3 perfusion parameters were calculated. Furthermore, the repeatability coefficient was calculated, which is the threshold value below which the absolute difference between 2 measurements on the same subject is expected to lie for 95% of the pairs. The 1-sample Wilcoxon rank test was used to compare CT density measurements and tumor volumes on 2 consecutive CT scans as well as to assess differences of the 3 perfusion parameters and of tumor volumes after antiangiogenic, radiation, or no therapy.

compared with baseline CT scans. The 3 in vivo perfusion parameters were correlated with ex vivo MVD and extent of hypoxia by calculating correlation coefficients including 95% CI (obtained using the Fisher transformation). All statistical analyses were performed with R 2.7.2 software (available at: <http://www.r-project.org/>). The results with a *P* value of less than 0.05 were considered statistically significant.

RESULTS

Reproducibility of CT Perfusion Imaging

Tumor volumes measured at the first CT scans ($521 \pm 393 \text{ mm}^3$) were not significantly different ($P = 0.55$) compared with the consecutive CT scans ($533 \pm 380 \text{ mm}^3$) obtained 5 hours later for the reproducibility study. In addition, CT densities of the tumor xenografts on nonenhanced CT images were not significantly different ($P = 0.55$) at the first ($60 \pm 13.5 \text{ HU}$) and second ($60.9 \pm 13.3 \text{ HU}$) CT scan, indicating clearance of intravenous contrast agent from earlier injection.

The intraclass correlation coefficients for BF, BV, and flow extraction product from the 2 consecutive CT perfusion scans were 0.93 (95% CI: 0.78, 0.97), 0.88 (0.66, 0.95), and 0.88 (0.56, 0.95), respectively. Mean difference, standard deviation of the differences, and 95% limits of agreement for the 3 perfusion parameters are shown in Table 1. Table 2 summarizes within-subject SD, within-subject coefficient of variation, and repeatability coefficient for the 3 perfusion parameters.

CT Perfusion Assessment in Individual Tumor Xenografts

Table 3 summarizes BF, BV, flow extraction product, and tumor volumes of tumors from group 1 (antiangiogenic treatment), group 2 (radiation treatment), and group 3 (no treatment), measured at baseline (day 0) and at days 1, 3, 5, and 7 after treatment initiation.

Group 1—One day after single administration of bevacizumab, BF ($P = 0.002$), BV ($P = 0.006$), and flow extraction product ($P = 0.002$) significantly decreased, compared with baseline scanning (Figs. 3, 4). All 3 parameters remained significantly decreased ($P = 0.008$) at subsequent time points until day 7. In contrast, there was no significant difference ($P = 0.08$) in tumor volumes from baseline up to day 5 after treatment initiation. At day 7, tumor volume was significantly ($P = 0.04$) increased, compared with baseline scanning.

Group 2—After single radiation therapy for tumors, BF, BV, and flow extraction product significantly dropped ($P < 0.001$ for each), compared with baseline scanning (Fig. 4). Although BF and flow extraction product transiently increased at day 3 and 5, BV remained significantly below baseline values at day 3 ($P = 0.03$) and 5 ($P = 0.01$). At day 7, all 3 perfusion parameters again dropped significantly ($P = 0.03$) below baseline values. In contrast, tumor volumes did not significantly change at any time compared with baseline values ($P = 0.3$).

Group 3—Without treatment, the 3 perfusion parameters did not significantly ($P = 0.05$) change, compared with baseline values, except for BF that significantly increased ($P = 0.03$) at day 3 and flow extraction product that significantly decreased ($P = 0.04$) at day 7 (Fig. 4).

Tumor volumes, however, significantly increased ($P = 0.04$) at all time points without treatment.

Longitudinal Intraindividual CT Perfusion Assessment

Intraindividual longitudinal CT perfusion imaging in the subgroup of 15 tumor-bearing animals confirmed the trends of CT perfusion parameters measured in individual tumors at single time points in the 3 treatment groups (Fig. 5).

Correlation of In Vivo CT Perfusion Imaging With Ex Vivo Analysis

There was a significant positive correlation between ex vivo MVD and in vivo BF ($\rho = 0.71$; 95% CI: 0.48, 0.85; $P < 0.001$), BV (0.66; 0.40, 0.83; $P < 0.001$), and flow extraction product (0.69; 0.43, 0.84; $P < 0.001$) measurements (Fig 6). In addition, there was a trend toward negative correlation between extent of hypoxia as assessed by ex vivo pimonidazole staining and in vivo BF ($\rho = -0.53$; 95% CI: $-0.83, 0.001$; $P = 0.05$), BV (-0.47 ; $-0.80, 0.07$; $P = 0.09$), and flow extraction product (-0.40 ; $-0.77, 0.17$; $P = 0.16$) (Fig. 7).

DISCUSSION

Our study showed that CT perfusion parameters including BF, BV, and flow extraction product substantially decreased in human colon cancer xenografts as early as 1 day after antiangiogenic or radiation therapy. Furthermore, all 3 in vivo CT perfusion parameters strongly correlated with ex vivo MVD in treated and control tumors, whereas there was a trend toward negative correlation between CT perfusion parameters and extent of tumor hypoxia. To our knowledge, this study is the first to longitudinally assess early effects of antiangiogenic and radiation therapy on CT perfusion parameters in a large number of tumor xenografts in mice, to assess reliability of CT perfusion imaging in mice, and to correlate in vivo CT perfusion imaging parameters with ex vivo analysis of tumor vascularity and hypoxia.

There are only a few reports on CT perfusion imaging of tumors in mouse models using clinical CT scanners,^{23,28} likely because spatial resolution is not high enough to identify central or peripheral arteries to reliably draw ROIs in the vessel lumen to obtain arterial input functions.^{29,30} An alternative to arteries for obtaining arterial input functions is the left ventricular cavity that can be well visualized in mice using clinical CT scanners. As mice are advantageous compared with other animal cancer models because multiple human disease models exist in mice (eg, transgenic mouse models of cancer) that closely reflect cancer development and progression compared with patients,^{31,32} we explored the potential of CT perfusion imaging in a murine cancer model. Our study confirms feasibility of CT perfusion imaging in mice using a clinical CT scanner.^{23,28} This is further underscored by the good reproducibility of measuring BF, BV, and flow extraction product at 2 CT perfusion sessions 5 hours apart in our study (with intraclass correlation coefficients ranging between 0.88 and 0.92). A waiting time of 5 hours between the 2 consecutive CT perfusion scans was chosen in our study to allow clearance of the intravenous contrast agent between CT scans and to ensure similar tumor sizes and states of tumor progression at both CT scans, considering the rapid growth of the human colon cancer xenografts used in our study. Future

studies using latest-generation CT scanners^{33,34} that allow extended-field-of-view perfusion CT scanning compared with the 64-detector row CT scanner used in our study are warranted to also assess feasibility and reliability of perfusion CT imaging of orthotopic tumors in more remote body parts from the left ventricle such as tumors in the abdomen and pelvis.

There is controversy in the literature as to whether CT perfusion parameters correlate with MVD.^{18–21} For example, a study in an orthotopic F13762 murine mammary cancer in 20 rats showed no correlation between CT perfusion parameters (BF, BV, mean transit time, and flow extraction product surface area) and MVD.²² In contrast, a recent study in 10 rats with a subcutaneous FN13762 murine breast cancer showed substantial correlation between CT parameters and MVD in corresponding tumor regions, but interestingly not with MVD of the entire tumor area.²⁰ Other recent studies on VX2 tumors in rabbits³⁵ and several studies performed in patients with pulmonary nodules,³⁶ colon cancer,¹² renal cell cancer,³⁷ and esophageal squamous cell carcinoma³⁸ showed positive correlation between various CT perfusion parameters and MVD. Our study confirmed a substantial positive correlation between in vivo BF, BV, and flow extraction product and ex vivo MVD in a large number of 100 human colon cancer xenografts in mice.

Furthermore, our study showed that as early as 1 day after initiation of antiangiogenic or radiation therapy, MVD was substantially reduced compared with pretreatment baseline values and that the extent of hypoxia as measured by pimonidazole staining increased after treatment. Our results confirm observations in an orthotopic human breast carcinoma (MDA-MB-231) model treated with the antiangiogenic agent DC101.¹¹ In that study,¹¹ MVD, blood flow (measured by a speckle-variance flow-processing algorithm applied to B-mode images of high-resolution ultrasound imaging), and tumor perfusion (assessed by fluorescence microscopy after injection of the fluorescent dye Hoechst 33342) were also substantially reduced after initiation of DC101 therapy. Similarly to our study, the hypoxic extent, measured with both pimonidazole stains and assessment of hypoxia-inducible factor-1A expression levels, was also increased after antiangiogenic DC101 treatment in that study.¹¹ Therefore, at least in our human colon cancer xenograft model, and using the described CT perfusion technique to measure BF, BV, and flow extraction product, we could not observe a therapeutic window based on changes in CT perfusion parameters after antiangiogenic therapy. Thus, based on our results, we were not able to predict optimized temporal combinations of bevacizumab with radiation therapy or chemotherapy to increase therapeutic effect. However, the vessel normalization hypothesis^{8,9,39} may depend on the type of antiangiogenic agent, the tumor type, and tumor stage; therefore, our results may not be extrapolated to other tumors and treatment approaches. Future studies in different tumor models are therefore needed to further assess whether CT perfusion parameters or other functional or molecular imaging approaches can be used to optimize combined therapies based on noninvasive imaging biomarkers.

The following limitations of our study need to be addressed. First, subcutaneous tumor xenografts may behave differently compared with orthotopic or transgenic mouse models, which better reflect the microenvironment of human cancers. Also, because of the rapid growth of the human colon cancer xenograft model used in the control group, the post-

treatment observation interval was limited to 7 days. Second, because of the limited spatial resolution of the CT scanner, subtle changes in tumor perfusion and tumor heterogeneity (eg, caused by tumor necrosis) could not be measured by the technique used in our study. Third, we measured BF, BV, and flow extraction product using commercially available perfusion software that is based on Abels model and optimized for relatively short acquisition and injection times.²⁴ This may have introduced errors in the estimates of the flow extraction product and BF, and resulted in a strong correlation of BF, BV, and the flow extraction product. We also cannot exclude that changes in the flow extraction product over time and after treatment may have introduced additional errors in BF. Although such errors may affect the accuracy and blur the separate quantitative effects, we believe that the observations with respect to antiangiogenic and radiation therapeutic effects of our model are valid. Other authors have used different software packages and mathematical models and calculated additional parameters such as mean transit time or time to peak from time-intensity curves after intravenous contrast administration.^{40,41} In fact, a recent study showed substantial differences in CT perfusion measurements between software packages of different vendors in stroke patients.⁴² Future studies are needed that fully exploit different CT perfusion parameters using analysis tools from different vendors to assess which perfusion parameter or which combination of different parameters best reflect treatment effects of antiangiogenic and radiation therapy. Finally, the commercial software package that we used in our study has been designed for the calculation of perfusion parameters in humans, and it has not been validated for small animals. Although this may introduce a systematic under- or overestimation of calculated values, we have shown that the data are reproducible and, therefore, believe that the observed longitudinal data are robust.

In conclusion, our study suggests that CT perfusion can quantify antiangiogenic and radiation therapy effects early after treatment initiation. CT perfusion parameters such as BF, BV, and flow extraction product substantially decreased 1 day after single bevacizumab and single tumor radiation treatment. In vivo CT perfusion parameters positively correlated with ex vivo MVD, and there was a trend toward negative correlation between CT perfusion parameters and extent of tumor hypoxia in the human colon cancer xenograft used in our study. With the CT perfusion parameters obtained in our study, we did not observe a vessel normalization window with temporarily increased tissue perfusion or decreased hypoxia in the human colon cancer xenograft model. Future studies are needed to further explore different CT perfusion parameters or combinations of parameters to better individualize antiangiogenic therapy, radiotherapy, and chemotherapy in cancer.

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FIGURE 1.

Time density curves obtained from left ventricular cavity (above) and subcutaneous human colon cancer xenograft (below) in representative tumor bearing mouse. Region of interest (small circle in first insert) was drawn in the left ventricular cavity to obtain arterial input function. Second insert shows region of interest drawn over subcutaneous xenograft in same mouse to obtain time intensity curve in xenograft.

FIGURE 2.

Flow diagram summarizing experimental design of in vivo CT perfusion experiments.

FIGURE 3.

Representative transverse perfusion images of blood flow, blood volume, and flow extraction product in subcutaneous human colon cancer xenograft obtained at baseline (day 0) and 1 day after single administration of bevacizumab (scale bar, 10 mm). Note that all 3 perfusion parameters decreased after antiangiogenic treatment.

FIGURE 4.

Line graphs summarizing mean percentage change of blood flow (A), blood volume (B), and flow extraction product (C) at days 1, 3, 5, and 7 for group 1 (antiangiogenic treatment), group 2 (radiation treatment), and group 3 (no treatment) that was correlated with ex vivo assays. Error bars = standard deviations. *P* values are provided in Table 3.

FIGURE 5.

Line graphs summarizing mean percentage change of blood flow (A), blood volume (B), and flow extraction product (C) at days 1, 3, 5, and 7 for group 1 (antiangiogenic treatment), group 2 (radiation treatment), and group 3 (no treatment) from longitudinal CT perfusion scans. Error bars = standard deviations.

FIGURE 6.

Representative ex vivo CD31-immunostained micrographs ($\times 100$, scale bar = $100\ \mu\text{m}$) of subcutaneous human colon cancer xenografts obtained at baseline (day 0) and 1 day after initiation of antiangiogenic treatment with bev-acizumab. Note that microvessel density substantially decreased 1 day after treatment.

FIGURE 7.

Representative ex vivo pimonidazole-immunostained micrographs ($\times 100$, scale bar = 100 μm) of subcutaneous human colon cancer xenografts obtained at baseline (day 0) and 1 day after initiation of antiangiogenic treatment with bevacizumab. Note that extent of tumor hypoxia substantially increased 1 day after treatment.

TABLE 1

Agreements Between Replicate CT Perfusion Measurements for Blood Flow, Blood Volume, and Flow Extraction Product in 8 Mice With Subcutaneous Human Colon Cancer Xenografts

Perfusion Parameters	Mean (SD)	Mean Difference	SD of the Difference	95% Limits of Agreement
Blood flow (mL/100 mL/min)	7.97 (6.29)	1.08	2.40	(−3.72, 5.88)
Blood volume (mL/100 mL)	3.26 (1.63)	0.26	0.82	(−1.38, 1.90)
Flow extraction product (mL/100 mL/min)	5.19 (1.82)	−0.43	0.87	(−2.17, 1.30)

CT indicates computed tomography; SD indicates standard deviation.

TABLE 2

Measurement Error and Repeatability of Replicate CT Perfusion Scans for Blood Flow, Blood Volume, and Flow Extraction Product in 8 Mice With Subcutaneous Human Colon Cancer Xenografts

Perfusion Parameters	Within-Pair SD	Within-Pair CV (%)	Repeatability Coefficient
Blood flow (mL/100 mL/min)	1.437	24.6%	4.705
Blood volume (mL/100 mL)	0.486	19.2%	1.608
Flow extraction product (mL/100 mL/min)	0.549	10.5%	1.701

CT indicates computed tomography; SD, standard deviation; CV, coefficient of variation.

TABLE 3

Summary of Blood Flow, Blood Volume, Flow Extraction Product, and Tumor Volumes Assessed by CT Perfusion Imaging at Baseline (Day 0) and at Days 1, 3, 5, and 7 After Antiangiogenic Treatment (Group 1), Radiation Treatment (Group 2), or No Treatment (Group 3)

	Blood Flow (mL/100 mL/min) Mean $\pm$ SD (<i>P</i>)	Blood Volume (mL/100 mL) Mean $\pm$ SD (<i>P</i>)	Flow Extraction Product (mL/100 mL/min) Mean $\pm$ SD (<i>P</i>)	Tumor Volume (mm³) Mean $\pm$ SD (<i>P</i>)
Group 1				
Day 0	14.11 $\pm$ 2.87	6.67 $\pm$ 1.22	7.19 $\pm$ 1.01	174.24 $\pm$ 69.11
Day 1	6.25 $\pm$ 1.83 (<i>P</i> = 0.002)	2.24 $\pm$ 0.54 (<i>P</i> = 0.006)	2.6 $\pm$ 0.7 (<i>P</i> = 0.002)	189.9 $\pm$ 72.9 (<i>P</i> = 0.922)
Day 3	6.99 $\pm$ 1.84 (<i>P</i> = 0.008)	2.16 $\pm$ 1.13 (<i>P</i> = 0.008)	3.05 $\pm$ 0.94 (<i>P</i> = 0.008)	196.56 $\pm$ 54.44 (<i>P</i> = 0.109)
Day 5	5.3 $\pm$ 1.67 (<i>P</i> = 0.002)	2.1 $\pm$ 0.74 (<i>P</i> = 0.002)	2.4 $\pm$ 0.78 (<i>P</i> = 0.002)	228.37 $\pm$ 55.61 (<i>P</i> = 0.084)
Day 7	4.88 $\pm$ 2.04 (<i>P</i> = 0.008)	1.77 $\pm$ 0.72 (<i>P</i> = 0.008)	2.14 $\pm$ 0.79 (<i>P</i> = 0.008)	245.76 $\pm$ 84.28 (<i>P</i> = 0.039)
Group 2				
Day 0	14.09 $\pm$ 2.4	7.33 $\pm$ 2.57	7.17 $\pm$ 1.47	208.91 $\pm$ 75.25
Day 1	9.9 $\pm$ 3.75 (<i>P</i> < 0.001)	3.86 $\pm$ 2.23 (<i>P</i> < 0.001)	4.77 $\pm$ 2.06 (<i>P</i> < 0.001)	252.98 $\pm$ 111.09 (<i>P</i> = 0.47)
Day 3	12.21 $\pm$ 3.98 (<i>P</i> = 0.25)	4.90 $\pm$ 1.83 (<i>P</i> = 0.03)	6.24 $\pm$ 2.42 (<i>P</i> = 0.43)	191.99 $\pm$ 43.07 (<i>P</i> = 0.5)
Day 5	11.93 $\pm$ 5.42 (<i>P</i> = 0.32)	4.86 $\pm$ 1.71 (<i>P</i> = 0.01)	5.48 $\pm$ 2.47 (<i>P</i> = 0.16)	206.72 $\pm$ 114.14 (<i>P</i> = 1)
Day 7	6.55 $\pm$ 3.41 (<i>P</i> = 0.02)	2.67 $\pm$ 1.57 (<i>P</i> = 0.03)	3.15 $\pm$ 1.92 (<i>P</i> = 0.02)	182.05 $\pm$ 61.26 (<i>P</i> = 0.3)
Group 3				
Day 0	14.05 $\pm$ 2.67	6.76 $\pm$ 2.13	7.21 $\pm$ 1.46	125.39 $\pm$ 63.1
Day 1	17.79 $\pm$ 4.01 (<i>P</i> = 0.1)	7.12 $\pm$ 1.9 (<i>P</i> = 0.32)	7.64 $\pm$ 1.63 (<i>P</i> = 0.57)	184.61 $\pm$ 62.73 (<i>P</i> = 0.04)
Day 3	18.21 $\pm$ 5.76 (<i>P</i> = 0.03)	7.09 $\pm$ 2.88 (<i>P</i> = 0.97)	8.16 $\pm$ 2.88 (<i>P</i> = 0.18)	199.38 $\pm$ 75.62 (<i>P</i> < 0.001)
Day 5	15.78 $\pm$ 6.64 (<i>P</i> = 0.46)	5.44 $\pm$ 1.66 (<i>P</i> = 1)	6.85 $\pm$ 2.14 (<i>P</i> = 0.74)	238.9 $\pm$ 59.99 (<i>P</i> = 0.008)
Day 7	9.26 $\pm$ 3.85 (<i>P</i> = 0.08)	3.54 $\pm$ 1.64 (<i>P</i> = 0.05)	3.8 $\pm$ 1.54 (<i>P</i> = 0.04)	293.96 $\pm$ 76.57 (<i>P</i> = 0.008)

P values were calculated compared with baseline CT perfusion scans.

CT indicates computed tomography; SD, standard deviation.