Effects of Overexpression of Dimethylarginine Dimethylaminohydrolase on Tumor Angiogenesis Assessed by Susceptibility Magnetic Resonance Imaging

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ABSTRACT

Intracellular factors that regulate nitric oxide (NO) synthesis represent important targets in tumor progression. Overexpression of dimethylarginine dimethylaminohydrolase (DDAH), which metabolizes the endogenous inhibitors of NO synthesis asymmetric dimethylarginine and N-monomethyl-L-arginine, results in C6 gliomas with enhanced growth rate compared with wild type. To investigate the effects of DDAH on tumor vascular morphogenesis in vivo, we have measured the transverse relaxation rates $R_2^*$ and $R_2$ in clone D27 gliomas overexpressing DDAH and C6 wild-type gliomas using intrinsic susceptibility magnetic resonance imaging (MRI), sensitive to changes in endogenous [deoxyhemoglobin], and susceptibility contrast-enhanced MRI using the intravascular blood pool contrast agent NC100150, and we compared the results with fluorescence microscopy of the tumor uptake of the perfusion marker Hoechst 33342. The baseline $R_2^*$ was significantly faster in the D27 tumors, consistent with a greater vascular development ($P < 0.02$, ANOVA). There was no significant difference between the response of the two tumor types to hypercapnia (5% CO$_2$/95% air), used as a probe for vascular maturation, or hypoxia (5% CO$_2$/95% O$_2$), used as a probe for vascular function. NC100150 increased the $R_2^*$ and $R_2$ rates of both tumor types and demonstrated a significantly larger blood volume in the D27 tumors ($P < 0.02$, ANOVA). This correlated with a significantly greater uptake of Hoechst 33342 in the D27 tumors compared with C6 wild-type tumors ($P < 0.02$, ANOVA). Despite the increased tumor blood volume, the $\Delta R_2^*/\Delta R_2^*$ ratio, an index of microvesSEL size, showed that the capillaries in the two tumor types were of a similar caliber. The data highlight the potential of susceptibility MRI-derived quantitative end points to noninvasively assess tumor angiogenesis, and in this regard, the use of intravascular blood pool contrast agents such as NC100150 appears very promising. Overexpression of DDAH results in increased neovascularization of C6 gliomas in vivo. The lack of significant difference in hypercapnic/hypoxic response between the C6 and D27 tumors and the similar vessel caliber are also consistent with a role for DDAH in the initial stages of vasculogenesis.

INTRODUCTION

Angiogenesis is the development of new blood vessels to provide a nutritive blood supply and is a prerequisite for tumor growth and survival (1). Tumor blood vessels can grow by sprouting, intussusception, or the incorporation of endothelial cell precursors. Angiogenesis is stimulated by the release of specific growth factors from tumor cells, endothelial cells, or associated macrophages, in particular VEGF (2). Production of these factors is up-regulated by physiologic conditions associated with tumors, e.g., hypoxia, low glucose, and pH, all of which are consistent with a compromised blood supply.

Numerous angiogenic stimuli also induce the production of NO, and strong positive correlations between nitric oxide synthase expression and human tumor grade have been demonstrated (3–7). Intracellular factors that regulate NO synthesis may therefore represent important targets in the control of tumor growth. We recently showed that C6 glioma cells genetically engineered to constitutively overexpress the enzyme DDAH resulted in tumors that grew twice as fast as the wild type (8). DDAH metabolizes two competitive inhibitors of NO synthesis, asymmetric dimethylarginine and N-monomethyl-L-arginine, indirectly leading to an increase in NO production. Inhibition of DDAH activity leads to decreased NO production both in vitro and in vivo (9). Upon excision, tumors derived from C6 cells overexpressing DDAH bled more profusely compared with wild type. Subsequent analysis showed increased expression and secretion of VEGF in tumors overexpressing DDAH (8).

1H MRI, with its high temporal and spatial resolution, presents itself as an ideal technique as a noninvasive assay of tumor angiogenesis in vivo (10, 11). In particular, two complementary magnetic susceptibility-based MRI approaches have been used in this regard. Firstly, tumor vasculature has been detected using the intrinsic $R_2^*$ contrast produced by paramagnetic deoxyhemoglobin within tumor blood vessels, to which gradient-recalled echo MRI sequences are sensitive (12). This approach, which is truly noninvasive because it relies on endogenous deoxyhemoglobin for contrast, offers an extremely sensitive method of monitoring the dynamics of vascular modeling, function, and regression in vivo (13–16). Secondly, tumor blood volume has been assessed using susceptibility contrast MRI measurements, in which the tumor uptake of USPIO particles is measured. These novel blood pool contrast agents act in a similar way to deoxyhemoglobin but are more powerful in creating susceptibility effects that strongly and locally alter the transverse relaxation rates $R_2$ and $R_2^*$ (17, 18). Contrary to the conventional contrast agent gadoxetate dimeglumine, these high molecular weight USPIO blood pool contrast agents do not leak as readily from the blood vessels. The resulting long intravascular half-life thus simplifies measurements of $R_2$ and $R_2^*$ during steady-state concentrations of the USPIO particles. We have recently shown that estimates of tumor blood volume, derived from measurements of the absolute changes in the relaxation rates $R_2$ and $R_2^*$ after administration of the USPIO blood pool contrast agent NC100150 (Clariscan; Amersham Health), correlate with the tumor blood volume determined by fluorescence microscopy (19). Furthermore, the ratio of relaxivities, $\Delta R_2^*/\Delta R_2$, can be used to derive maps of capillary diameter (19–22) and has shown good agreement with histologically determined vessel size (20).

To validate MRI-derived end points, it is essential that appropriate histological analyses of the tumor vasculature are performed (10). A number of immunohistochemical techniques are available to assess tumor vasculature at a microscopic level, detailed information that is not obtainable by noninvasive, volume-averaging MRI techniques (23–24). One such approach is following the tumor uptake of the perfusion marker Hoechst 33342, from which quantitative histologically derived morphometric parameters of tumors have been deter-
mined and correlated with noninvasive MRI studies of physiological processes (19, 25).

To assess the effects of overexpression of DDAH on tumor angiogenesis, we used both intrinsic susceptibility and susceptibility contrast MRI to assess the tumor vascular architecture and morphology in vivo of tumors derived from C6 glioma cells that constitutively overexpress DDAH and tumors derived from wild-type C6 cells. In addition, fluorescence microscopy of tumor uptake of Hoechst 33342 was quantified to assess the perfused fraction of the tumor.

MATERIALS AND METHODS

Animals and Tumors. Wild-type C6 cells and clone 27 (D27) C6 glioma cells, transfected with the full coding region of the rat DDAH I gene, were used (8). Cells (2 × 10^6) were injected into the flanks of female nude mice under halothane anesthesia. Because tumors derived from mock-transfected cells grew similarly to wild type in vivo (8), MRI was performed only on C6 wild-type and D27 tumors. Two separate cohorts of each tumor type were used for intrinsic susceptibility and susceptibility contrast MRI. All experiments were performed in accordance with the United Kingdom Home Office Animals Scientific Procedures Act 1986.

MRI. Size-matched C6 wild-type (mean volume, 1.05 ± 0.2 cm³) and D27 (mean volume, 1.06 ± 0.2 cm³) tumors were imaged at 23 and 18 days postinjection, respectively. Anesthesia was induced with a 10 ml/kg i.p. injection of fentanyl citrate (0.315 mg/ml) plus sufentanil [10 mg/ml (Hynpoprom; Janssen Pharmaceutical Ltd.)], midazolam [5 mg/ml (Hynnovel; Roche)], and fentanyl citrate (0.315 mg/ml) plus fluanisone [10 mg/ml (Hypnorm; Janssen Research)].

Before imaging, the anesthetized animals were positioned so the tumor hung vertically into the radiofrequency coil and covered with a warm water blanket to maintain the core temperature at 37°C. The anesthetized animals were positioned so the tumor hung vertically into the radiofrequency coil and covered with a warm water blanket to maintain the core temperature at 37°C. Field homogeneity was optimized by shimmin on the water signal for each tumor to a linewidth of 40 Hz.

Tumor vascular development, maturation, and function were assessed by MGRE MRI (26, 27). Images with increasing T_{2*} weighting were acquired using a gradient echo sequence with TE = 5 to 40 ms, echo spacing of 5 ms, 8 echoes, repetition time = 100 ms, and flip angle α = 45°. Images were acquired from three transverse 1-mm slices through the tumor, using 8 acquisitions of 256 phase encode steps over a 4 × 3-cm field of view for each TE. The total imaging time was ~9 min. Air, 5% CO2 in air, or carbogen (5% CO2/95% O2) was administered via a nose piece equipped with a scavenger to prevent the leakage of paramagnetic oxygen into the magnet bore, which could potentially change the magnetic susceptibility around the coil and produce image artifacts. MGRE images were first acquired during air breathing, subsequently during 5% CO2 in air breathing, and finally during carbogen breathing.

Paramagnetic deoxyhemoglobin creates susceptibility perturbations around blood vessels, increasing the magnetic resonance transverse relaxation rate R_{2*} (R_{2*} = 1/T_{2*}, the apparent spin-spin relaxation time). Sampling the MGRE MRI SI at several TEs allows the slope of ln(SI) versus TE to be calculated, which determines R_{2*} and is directly proportional to the tissue content of deoxyhemoglobin and hence a sensitive index of tissue vascularity. A fast R_{2*} is consistent with a high [deoxyhemoglobin] and hence indicative of high blood vessel density. In addition, the intercept of the fitted data, i.e., the SI at TE = 0 (A_0), is exclusively sensitive to changes in perfusion. A decrease in the slope (R_{2*}) implies an increase in oxygenhemoglobin. Blood vessel maturation and function were also assessed by measuring the changes in tumor R_{2*} during hypercapnia (5% CO2/95% air) as a probe for vascular smooth muscle dilation or during hyperoxia (5% CO2/95% O2) to indicate functional blood vessel density, respectively (13, 14, 16). Apparent R_{2*} and A_0 maps were calculated on a pixel-by-pixel basis from the MGRE image data sets (26, 27). The average apparent R_{2*} relaxation rates were calculated for each tumor for a global ROI encompassing the whole tumor image but excluding the surrounding skin and muscle. A relatively fast R_{2*} is consistent with a high [deoxyhemoglobin] and hence vascularity, but cellular paramagnetic debris from necrosis such as hemosiderin can also increase R_{2*}. Therefore, the intercept A_0 maps, which are exclusively sensitive to perfusion, were used to identify local ROIs that showed relatively high SI and hence were vascular, and changes in R_{2*} within them were subsequently assessed (Fig. 1).

RESULTS

Synthesized A_0 and R_{2*} maps obtained from one C6 wild-type and one D27 tumor while the host breathed air, 5% CO2/95% air, and,
finally, 5% CO₂/95% O₂ are shown in Fig. 2. Overall, both D27 and wild-type tumors exhibited heterogeneity in the R₂* maps, with regions of both high and low SI identified within the tumor. Tumors derived from D27-transfected cells were more vascularized compared with tumors derived from wild-type cells, as indicated by the significantly faster average baseline R₂* shown in Fig. 3. No significant change in tumor R₂* over the whole tumor could be detected in either of the tumor lines in response to 5% CO₂/95% air or 5% CO₂/95% O₂.

In an approach analogous to histological vascular hot spot analysis, local ROIs showing relatively high SI in the perfusion-sensitive A₀ intercept maps were identified and were predominantly found within the rim of the tumor (Figs. 1 and 2). The baseline R₂* measured from these local ROIs was also significantly faster in the D27 tumors compared with C6 wild type. Small decreases in R₂* were measured from these vascular hot spots in both tumor lines during hypercapnia and hyperoxia (Fig. 3).

Synthesized R₂ and R₂* maps from one C6 wild-type tumor and one D27 tumor before administration of 2.5 mgFe/kg NC100150 and the R₂ and R₂* maps acquired in the presence of the USPIO particles are shown in Fig. 4. Again, both C6 wild-type and D27 tumors exhibited heterogeneity in the R₂* maps both before and after administration of the blood pool agent, whereas the R₂ maps were more homogeneous. The baseline R₂* and R₂ and the changes induced by NC100150 are shown in Fig. 5. There was no significant difference between the average R₂* of the two cohorts of each tumor type used in this study and those in the MGRE MRI experiments. As before, the average baseline R₂* measured over the whole tumor was significantly faster in the D27 tumors than in the C6 wild-type tumors. NC100150 induced significant increases in both R₂* and R₂ of both C6 wild-type and D27 tumors, and this response was significantly greater in the D27 tumors. This response was sustained in the second set of images acquired after administration of NC100150, suggesting negligible leakage of NC100150 out of the tumor vasculature over the 30-min time period (data not shown).

Tumor vascular morphology was also assessed by deriving maps of the ΔR₂*/ΔR₂ ratio, a ratio that increases with increasing microvessel size. Fig. 6 shows calculated ΔR₂*/ΔR₂ maps of the same tumors in Fig. 4. The intensity of the synthesized ΔR₂*/ΔR₂ maps of both C6 and D27 tumors is similar. The average ΔR₂*/ΔR₂ ratio for both C6 and D27 tumors is also summarized in Fig. 6c, and there was no significant difference between the two tumor types.
Analysis of the tumor perfusion was performed on sections obtained from mice treated with Hoechst 33342. Fig. 7 shows composite fluorescence microscopy images of the perfused vascular architecture obtained from one C6 wild-type tumor and one D27 glioma. A greater abundance of Hoechst 33342 staining was associated with the D27 tumors, consistent with the D27 tumors having more perfused vessels. The mean perfused areas obtained for the two tumor types are summarized in Fig. 7c, which shows that the perfusion of the D27 tumors was significantly greater than that of the C6 wild-type tumors.

**DISCUSSION**

Investigation of tumor angiogenesis in vivo within genetically manipulated tumors expressing a well-defined phenotypic change gives an opportunity to investigate the role of factors implicated in tumor vascular morphogenesis in vivo and the chance to identify, validate, and calibrate MRI-derived prognostic and diagnostic indices that can be translated into the clinic (13, 14, 16, 30). We have used this powerful approach to evaluate the effects of DDAH on tumor angiogenesis in vivo.

MGRE MRI permits efficient, quantitative assessment of the role of factors implicated in tumor blood vessel development. Image contrast arises only from intravascular RBCs, and contrast changes are not sensitive to vascular permeability. The significantly faster baseline $R_2^*$ of the D27 tumors is consistent with these more rapidly growing D27 gliomas having a greater vascular development (i.e., angiogenesis) compared with wild-type C6 gliomas (8).

The maturation state of tumor vasculature has recently been suggested as a marker of tumor progression, with the recruitment of pericytes resulting in tumor blood vessels refractory to withdrawal of vascular growth factors such as VEGF (13, 14). Clinical measures of capillary maturation may thus assist the identification of patients who would benefit from antiangiogenic therapies or vascular targeting treatments (31–33). In this study, the maturation and functional state of the tumor blood vessels were probed by measuring the $\Delta R_2^*$ in response to hypercapnia and hyperoxia, respectively. Small decreases in $R_2^*$ were identified within the periphery of tumors derived from both wild-type C6 and D27 cells, presumably indicating areas of active angiogenesis. These results are consistent with the blood vessels of both tumor types containing (a) some smooth muscle in the vessel walls and (b) deoxygenated blood. There was no significant difference in either response between the C6 and D27 gliomas.

Administration of 2.5 mg Fe/kg NC100150 was a sufficient dose at 4.7 Tesla to induce significant changes in the transverse relaxation rates $R_2^*$ and $R_2$, of both wild-type C6 and D27 gliomas, and this response was heterogeneous over the whole tumor. There was no

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**Fig. 7c** Average baseline ($a$) $R_2^*$ and ($c$) $R_2$ (mean ± 1 SE) measured over the whole tumor and changes in ($b$) $R_2^*$ and ($d$) $R_2$ after administration of 2.5 mg Fe/kg NC100150 measured in wild-type C6 tumors ($n = 6$) and clone D27 gliomas ($n = 5$). There was no significant difference between the average $R_2^*$ of the two cohorts of each tumor type used in this study and those in the intrinsic susceptibility MRI experiments (Fig. 3). As before, the average baseline $R_2^*$ measured over the whole tumor was significantly faster in the D27 tumors than in the C6 wild-type tumors (**, $P < 0.02$, ANOVA). NC100150 induced significant increases in both $R_2^*$ and $R_2$ of both D27 and wild-type tumors (###, $P < 0.01$, Student’s $t$ test), and this response was significantly greater in the D27 tumors (###, $P < 0.01$, ANOVA).

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**Fig. 7d** Average baseline ($a$) $R_2^*$ and ($c$) $R_2$ (mean ± 1 SE) measured over the whole tumor and changes in ($b$) $R_2^*$ and ($d$) $R_2$ after administration of 2.5 mg Fe/kg NC100150 measured in wild-type C6 tumors ($n = 6$) and clone D27 gliomas ($n = 5$). There was no significant difference between the average $R_2^*$ of the two cohorts of each tumor type used in this study and those in the intrinsic susceptibility MRI experiments (Fig. 3). As before, the average baseline $R_2^*$ measured over the whole tumor was significantly faster in the D27 tumors than in the C6 wild-type tumors (**, $P < 0.02$, ANOVA). NC100150 induced significant increases in both $R_2^*$ and $R_2$ of both D27 and wild-type tumors (###, $P < 0.01$, Student’s $t$ test), and this response was significantly greater in the D27 tumors (###, $P < 0.01$, ANOVA).
apparent recovery to baseline values of either $R_2^*$ and $R_2$ within the 30 min after the administration of NC100150, implying negligible permeability of NC100150 from the tumor circulation. The significantly larger changes in $R_2^*$ and $R_2$ measured in the D27 tumors are consistent with a larger blood volume compared with C6 wild type. This was supported by tumor uptake of Hoechst 33342, which showed that the perfused area in the D27 tumors was significantly greater than that in the C6 wild-type tumors (Fig. 7) and confirmed our earlier observations (8). These data highlight the advantages of using histological techniques such as Hoechst 33342 that assay for perfused functional vasculature. These methods appear to correlate with angiogenic potential, whereas with blood vessel density measurements using pan-endothelial markers, the correlation with angiogenesis can be unclear (34). The increased blood volume of the D27 tumors is consistent with clinical observations, in which high-grade human gliomas had a substantially higher relative blood volume compared with low-grade tumors (35).

Evaluation of the $\Delta R_2^*/\Delta R_2$ ratio, an index of the average microvessel size, showed no significant differences between the D27 and wild-type C6 gliomas (Fig. 6). This implies that, despite the clearly increased tumor blood volume in the D27 tumors, the caliber of the vasculature in the two tumor types is similar. Using the methodology of Tropres et al. (22), we have recently shown that estimates of both the tumor fractional blood volume and microvessel size could be calculated using the NC100150-induced changes in $R_2^*$ and the $\Delta R_2^*/\Delta R_2$ ratio, respectively (19). Assuming that the susceptibility change induced by NC100150 was the same for both tumor types, and using a previously reported diffusion coefficient for C6 gliomas of $1.1 \times 10^{-9} \text{m}^2 \text{s}^{-1}$ (36), the fractional blood volume of the D27 and C6 gliomas was estimated to be 3% and 1%, respectively, whereas the average microvessel size for both tumor types was 6 $\mu$m. This is not dissimilar from previous histological measurements (mean $\pm$ 1 SE) of the microvessel diameter of C6 gliomas of $7.8 \pm 0.5 \mu$m (37) and $12.5 \pm 3.9 \mu$m (20).

Taken together, the results are consistent with enhanced tumor
angiogenesis in D27 tumors and suggest that DDAH activity might play a central role in tumor growth by regulating the concentration of tumor-derived NO. The spatial and temporal production of NO is tightly regulated and could be an important factor in determining tumor progression. We have previously shown enhanced VEGF production by D27 tumors compared with C6 wild-type tumors, suggesting that DDAH may enhance tumor growth and angiogenesis via VEGF expression (8). The lack of significant difference in (a) the hypercapnic/hyperoxic response and (b) vessel caliber between the C6 and D27 tumors suggests that DDAH is primarily involved in the initial formation of tumor blood vessels, either through angiogenic sprouting or intussusception, rather than the subsequent stages of vascular remodeling (1). Support for this has come from in vitro invasion assays reported previously (8).

Intrinsic susceptibility-based MRI measurements were made on a tumor system in which DDAH was constitutively overexpressed, and the results were compared with wild type. A similar approach has been used previously to investigate the role of hypoxia-inducible factor 1α in tumor angiogenesis (38). The localized tumor ΔR₂* responses to hypercapnia and hyperoxia measured by MGRE MRI herein were inherently small, a consequence of the reduced contrast: noise ratio of intrinsic contrast-enhanced MRI. Murine erythrocytes in whole blood are typically 6 μm in diameter (19). Thus, the ability of murine erythrocytes, the primary source of changes in R₂* intrinsic contrast, to traverse similarly sized, tortuous capillaries in vivo would be limited, abrogating the hypercapnic/hyperoxic response (19). This would explain why, contrary to the hyperoxic ΔR₂* response, a higher proportion of functional vasculature was measured in the D27 tumors after Hoechst 33342 uptake. Susceptibility contrast MRI has been used previously to show an increased blood volume fraction in murine MCF-7 mammary carcinomas constitutively overexpressing VEGF compared with nontransfected controls (30). In this study, we have clearly demonstrated the utility of NC100150 to show that overexpression of DDAH in C6 gliomas resulted in a greater tumor blood volume compared with wild type, yet the sizes of the vessels in the two tumor types were similar. An alternative approach to investigate both the effects and dynamics of DDAH on vascular remodeling assessed by intrinsic susceptibility MRI and the contribution of DDAH to vascular morphology assessed by the microvessel size index ratio ΔR₂*/ΔR₁ after susceptibility contrast MRI would be to use a tumor system in which DDAH is under inducible control, rather than being constitutively overexpressed, and where each tumor acts as its own control. A C6 cell line in which production of DDAH is under the control of a tetracycline-inducible promoter is currently being engineered.

The development and validation of quantitative, clinically applicable MRI end points of functional/physiological measures of tumor angiogenesis is critical for (a) enhancing our understanding of the incipient tumor vasculature and (b) determining the efficacy of antiangiogenic and antivascular therapies, many of which do not induce a significant growth delay in human tumor xenografts (39). The utility of both MRI methods used herein in the clinic has been demonstrated. Recently, MGRE MRI, which can be implemented on most clinical MRI scanners, was used to measure human tumor R₂* during air and carbogen breathing to assess the prognostic value of ΔR₂* for radiotherapeutic outcome (40). The blood pool agent NC100150 was used recently to measure vessel permeability and blood volume in human breast cancers (41). The methods also present themselves as simpler than dynamic T₁-weighted contrast-enhanced MRI using gado-pentetate dimeglumine approaches to employ in the clinic because there is no need for rapid data acquisition, and the data analysis and interpretation are more facile. In particular, noninvasive suscep-

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