Expression of the Hypoxia Marker Carbonic Anhydrase 9 Is Associated with Anaplastic Phenotypes in Meningiomas

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Abstract

Purpose: Hypoxia in the tumor microenvironment triggers a variety of genetic and adaptive responses that regulate tumor growth. Tumor hypoxia is often associated with more malignant phenotypes, resistance to therapy, and poor survival. The purpose of this study was to evaluate the prevalence of hypoxia in meningiomas using the endogenous hypoxia marker carbonic anhydrase 9 (CA9) and to relate the expression of CA9 to tumor vascularity, histopathologic grade, and clinical variables, such as recurrent tumor status.

Experimental Design: Expression of CA9 and CD34, an endothelial cell marker, was examined in serial paraffin-embedded sections by immunohistochemistry in 25 grade 1, 17 grade 2, and 20 grade 3 meningiomas. Areas of immunoreactivity were semiquantitatively scored and correlated to clinical variables using Statistical Analysis System statistical software.

Results: Approximately 50% (29 of 62) of all meningiomas contained regions of hypoxia as judged by expression of CA9, and this expression was significantly associated with higher-grade histology ($P = 0.001$). In contrast, vascularity, as assessed by the percentage of vascular hot-spots, was inversely associated with tumor grade ($P = 0.023$) and was not associated with CA9 expression. Among lower-grade meningiomas, CA9 expression tended to be more common in recurrent tumors.

Conclusions: Tumor hypoxia is an endogenous feature of meningiomas, and therapeutic regimens should include strategies to target the subpopulation of hypoxic as well as the normoxic cells within the tumor. Hypoxia in meningiomas is associated with an aggressive phenotype. Further studies to define the contribution of hypoxia to meningioma pathophysiology are warranted.

Meningiomas account for ~30% of all primary brain and central nervous system tumors and are associated with extremely variable clinical outcomes (1). Meningiomas are classified into three WHO grades, and ~80% of these tumors are WHO grade 1 (benign; refs. 2, 3). A significant proportion of benign meningioma patients can be cured by surgical resection alone. However, some of these tumors recur despite complete resection and/or occur in locations that are not amenable to complete resection (4). WHO grade 2 (atypical) and WHO grade 3 (malignant) meningioma patients are less common but have worse clinical outcomes (5). Current treatment options for these patients, limited to surgical

resection and/or radiation therapy, are frequently inadequate (5). Local recurrence rates are high and the median overall survival for grade 3 meningioma patients is <2 years (6). A better understanding of the molecular mechanisms and microenvironmental factors driving meningioma tumor growth and survival is essential for the development of more effective therapies for meningioma patients.

In a previous study, we observed increased expression of glycolytic enzymes in high-grade meningiomas (7). One major factor that contributes to enhanced glycolysis is an adaptation to hypoxia in the tumor microenvironment (8). Hypoxia in the tumor microenvironment triggers a molecular response that promotes an aggressive cancer phenotype. Tumor hypoxia has been associated with a poor clinical outcome in many cancers, including astrocytomas, cervical carcinomas, and head and neck carcinomas (9–11). Hypoxic cells in tumors also contribute to resistance to radiation and chemotherapy (12, 13). Hypoxia regulates several important tumor growth-promoting cellular mechanisms, including angiogenesis, cell proliferation, genomic instability, and tissue invasion (14). Although hypoxia plays this critical role during tumor growth and development, its prevalence in meningiomas and its contribution to meningioma tumor progression have not been investigated. We therefore sought to assess the extent of hypoxia in meningiomas and to investigate its relationship to tumor aggressiveness.

Carbonic anhydrase 9 (CA9) is induced by hypoxia in a wide range of tumors, including glioblastoma and cervical, breast,

and head and neck carcinomas, and it is usually suppressed in normoxic conditions (15–18). CA9 expression has been associated with a poor prognosis in lung, breast, and cervical cancer patients (19–21) and to resistance to chemotherapy in head and neck cancers (22). CA9 expression shows colocalization with pimonidazole, a chemical marker of hypoxia, and its expression is usually restricted to perinecrotic areas (15, 23). These features make CA9 an attractive endogenous marker of hypoxia in tumors.

Hypoxic areas in tumors are a consequence of inadequate blood supply due to a disorganized, chaotic, and insufficient vascular network or due to tumor-associated coagulopathies leading to thrombosis of microvessels (24). Chronically hypoxic cells, in turn, secrete angiogenic factors that stimulate new blood vessels. Thus, tumor vasculature and tumor hypoxia are interdependent variables, and we therefore investigated their interrelationship in meningiomas. The mainstay for assessing tumor vasculature has been estimating the intratumoral microvessel density (IMD) in regions of neoangiogenesis or vascular hotspots (VHS; ref. 25). In many, but not all, cancers, IMD is a significant prognostic indicator of overall and recurrence-free survival (26). Meningiomas display variable vascularity, ranging from sparse to the highly angiogenic angiomatous subtype (27). The prognostic and clinical significance of vascular variables in meningiomas are largely unknown.

The purpose of this study was to use CA9 to evaluate the prevalence of hypoxia in meningiomas and to associate the presence of hypoxia with tumor vascularity, histopathologic grade, recurrent or primary tumor. We show that half of all meningiomas contain CA9-positive cells, presumably the subpopulation of cells that are hypoxic. CA9 expression is not associated with tumor vascularity but is significantly associated with grade 3 (malignant) meningiomas. Although the numbers were small, CA9 expression tended to be more common in recurrent tumor in lower-grade meningiomas. Thus, tumor hypoxia is associated with more aggressive meningioma phenotypes and therapeutic regimens for these tumors should include strategies to target both normoxic and hypoxic cells.

Materials and Methods

Tumor samples. All human tissues were collected by the Neurological Surgery Tissue Bank using protocols approved by the University of California at San Francisco Committee on Human Research. A neuropathologist (S.R.V.) graded each case using the revised 2000 WHO grading system. Meningioma cell lines (SF4068, SF4433, and IOMM-Lee) were grown using standard cell culture techniques in DMEM supplemented with 10% fetal bovine serum either in equilibrium with atmospheric oxygen or in a Modular Incubator Chamber (Billups-Rothenberg, Inc., Del Mar, CA) with 0.3% oxygen for the indicated times. SF4068 and SF4433 were derived from benign meningiomas and have been immortalized with the human papillomavirus *E6/E7* genes and hTERT as described earlier (28).

Quantitative PCR. Quantitative PCR was done on cDNA templates with the iCycler machine (Bio-Rad, Hercules, CA) and SYBR Green I (Molecular Probes, Eugene, OR) using PCR conditions and data analysis as described earlier (7). Primers for vascular endothelial growth factor (*VEGF*) were 5′-ACAAGAAAATCCCTGTGGGC and 5′-CCTGGAGAGAGATCTGGTTC, and primers for *CA9* were 5′-AGTGCC-TATGAGCAGTTGCT and 5′-TGCTTAGCACTCAGCATCAC.

Western blot analysis. Western blots on total tissue lysates were done as described earlier (7). Briefly, protein (75 μg) containing polyvinylidene fluoride membranes was incubated with either the anti-CA9 monoclonal antibody (M75; 1:150; gift from Egbert Oosterwijk, University of Nijmegen, Nijmegen, the Netherlands) or the anti-β-tubulin antibody (Invitrogen, Carlsbad, CA) for 30 min followed by incubation with horseradish peroxidase–conjugated goat anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, PA). Bound antibody was visualized by chemiluminescence using the SuperSignal West Pico substrate (Pierce Chemical Co., Rockford, IL).

Immunohistochemistry. Immunohistochemical staining was done on 5-μm formalin-fixed, paraffin-embedded serial tissue sections; the M75 mouse monoclonal antibody (1:250) and the NB 100-417 rabbit polyclonal antibody (1:1,000; Novus Biologicals, Littleton, CO) were used to detect CA9, and the QBEnd/10 mouse monoclonal antibody (1:50, Lot # L121221; Vision BioSystems, Norwell, MA) was used to detect CD34. The slides were deparaffinized and rehydrated, and antigen retrieval was done using solution AR10 as recommended by the manufacturer (BioGenex, San Ramon, CA). Sections were sequentially incubated with horse serum, primary antibody, biotinylated secondary antibody, and streptavidin-conjugated horseradish peroxidase (Supersensitive Detection system, BioGenex). Bound antibody was detected using 3, 3′-diaminobenzidine and permanently mounted. The presence of necrosis was evaluated in the preceding serial section by doing a H&E stain.

Estimation of tumor vascularity. Regions of VHSs were identified by scanning tumor sections at ×50 total magnification. The percentage of the tumor section that had VHS was estimated as low (0-10%), medium (10-50%), or high (50-100%). Three VHSs from different areas were chosen, and individual microvessels in four regions within each VHS were counted at ×200 magnification. All stained endothelial cell or cell clusters that were distinct from adjacent microvessels, tumor cells, and other connective tissue elements were regarded as distinct countable microvessels (26). Microvessel counts from the four ×200 fields were summed and defined as the IMD. The IMD for each meningioma case was defined as the average IMD derived from the three VHSs. Two investigators (H.Y. and S.R.V.) scored the tumor vascularity, and another investigator (S.R.V.) confirmed the overall rating of the VHS percentage and the IMD scores.

Scoring of CA9 immunostaining. Meningioma cases were classified as either CA9 positive or CA9 negative. Tumor sections with strong membranous CA9 staining were considered CA9 positive. CA9-negative cases were those in which CA9 expression was absent or was very weak cytoplasmic with no membranous reactivity. Meningioma tissue sections stained with the M75 mouse monoclonal antibody were graded independently for CA9 expression by two investigators (H.Y. and S.R.V.). There was 100% concordance in the CA9 scores between the two investigators. The reproducibility of the CA9 staining was addressed by staining the same set of meningioma cases with the NB 100-417 rabbit polyclonal antibody. Two investigators (S.V. and A.W.B.) graded CA9 expression in this set, and once again, 100% concordance in the CA9 scores was observed. However, discordance in CA9 immunohistochemical staining was observed between the two antibodies in one (1.6%) case. The tabulated data presented in this article are the results of the CA9 staining done with the M75 antibody.

Statistical analysis. All analyses were done with the statistical package Statistical Analysis System. For all tests, *P* < 0.05 two tailed was considered statistically significant. Association of CA9 positivity with tumor and patient characteristics used the Cochran-Mantel-Haenszel test with stratification for grade as indicated in the results. When the patient or tumor characteristic was ordered or continuous (e.g., age, IMD, and VHS), the analysis was based on the ranks for that characteristic.

Results

We assessed whether CA9 is induced by hypoxia in meningiomas. One of the most well studied responses to
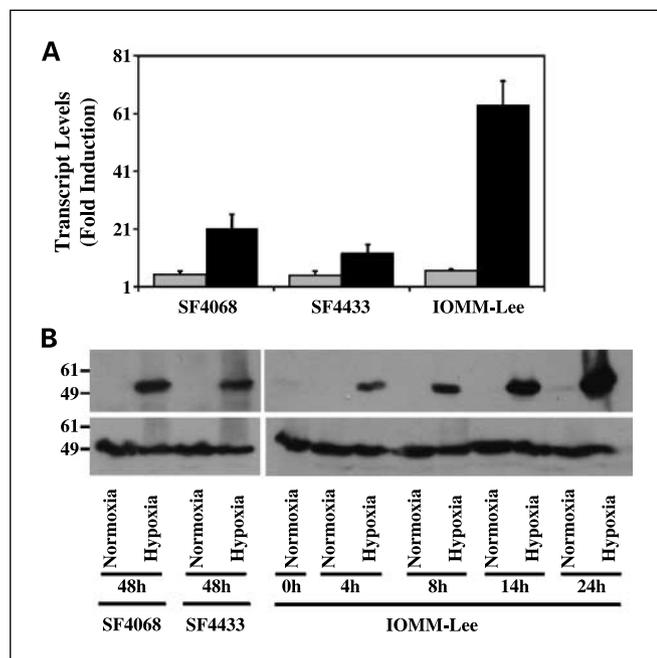
Fig. 1. CA9 is induced by hypoxia in meningioma cell lines. *A,* meningioma cell lines were grown in either atmospheric oxygen or 0.3% oxygen for 24 h and the transcript levels of *VEGF* (*gray columns*) and *CA9* (*black columns*) were determined by quantitative PCR. Fold induction under hypoxia was the ratio of the relative transcript levels at 0.3% oxygen to those at atmospheric oxygen. Columns, mean of three independent hypoxia exposures; bars, SD. *B,* expression of CA9 protein (*top*) was measured by Western blot analysis in SF4068, SF4433, or IOMM-Lee lysates exposed to either atmospheric oxygen or 0.3% oxygen for the indicated times. α-Tubulin (*bottom*) was included as a loading control. Left, molecular weight markers.

hypoxia is the induction of the proangiogenic growth factor VEGF (29). Induction of *CA9* and *VEGF* transcript levels by hypoxia was evaluated and compared using quantitative PCR analysis in two benign and one malignant meningioma cell line exposed to either atmospheric oxygen or 0.3% oxygen for 24 h. Although transcript levels of both *CA9* and *VEGF* were induced by hypoxia in all three cell lines, induction of *CA9* exceeded that of *VEGF* by severalfold (Fig. 1A). Whereas hypoxia caused a 3- to 5-fold induction in the transcript levels of VEGF levels, transcript levels of CA9 were induced by 10- to 60-fold. Using Western blot analysis, we confirmed that CA9 protein was also induced under hypoxic conditions in all three meningioma cell lines (Fig. 1B). A time course of induction in IOMM-Lee revealed that protein levels of CA9 were induced as early as 4 h following exposure to hypoxia, and these levels continued to increase up to 24 h. Importantly, protein levels of CA9 were undetectable under normal oxygen conditions (Fig. 1B).

Next, we evaluated the expression of CA9 in 62 meningiomas by staining paraffin-embedded sections with the M75 mono-clonal antibody. The vasculature was estimated by staining for CD34, an endothelial cell–specific antigen (26). Clinicopath-ologic characteristics of the 62 cases are summarized in Table 1. This series included 25 grade 1, 17 grade 2, and 20 grade 3 meningiomas. Patient age ranged from 23 to 88 years. Of these patients, 42 were female and 20 were male, and as anticipated, the female bias was most prevalent in grade 1 meningiomas. Most meningiomas were located in the convexity (including parasagittal meningiomas) or skull base region, whereas the

remaining meningiomas occurred in falcine, tentorium, intra-ventricular, or spinal regions.

Strong membranous CA9 expression was observed in 29 of 62 (46.8%) cases (Fig. 2). In sections of the tumors that were CA9 positive, the cumulative area of CA9 immunoreactive cells varied from <5% to ∼25%. In all cases, the pattern of CA9 immunoreactivity was heterogeneous and zonal (Fig. 2B). These zones were sometimes in viable cells adjacent to areas of frank necrosis (Fig. 2D). However, often CA9 immunoreactive cells were found in viable tumor tissue that was not directly associated with or adjacent to any necrotic areas (Fig. 2B and F; compare Fig. 2H with Fig. 2G). Although zones of CA9 immunoreactive cells could be found adjacent to sporadic blood vessels, they did not occur within VHS (Fig. 2; compare Fig. 2E with Fig. 2F). Thus, for the most part, regions of prominent tumor vascularity and tumor hypoxia were mutually exclusive.

Two main types of microvascular patterns, with intermediate examples, were observed within VHS in meningiomas. The first pattern included primarily complex hyperplastic vessels with variable endothelial cell hyperplasia (Fig. 3A). The other type of microvascular pattern did not have prominent endothelial cell hyperplasia but rather contained frequent vessels with dilated, prominent lumens and a less complex overall configuration (Fig. 3B). Considerable variability was observed in the VHS of the meningioma cases studied, ranging from <10% to >70%. Whereas the majority of cases (58%) had a medium VHS score, 18% had low values and 24% were highly vascular. The median IMD for the 62 meningiomas was 78 per four ×200 fields with a range varying from 11 to 774. Six meningioma cases had an IMD of >200 per four ×200 fields. Of these, four cases were

Table 1. Clinical variables of meningioma cases and assessment of meningioma vasculature stratified by grade

Variable	Benign (grade 1)	Atypical (grade 2)	Malignant (grade 3)
Total no. cases	25	17	20
Tumor status			
Primary	24	12	10
Recurrent	1	5	10
Age			
Median (range)	50 (30-76)	60 (30-85)	54 (23-88)
Gender			
Male	3	7	10
Female	22	10	10
Presurgical embolization			
Yes	4	2	9
No	21	15	11
Location			
Convexity	12	7	11
Skull base	7	5	6
Falcine	1	2	1
Tentorium	2	0	2
Intraventricular	3	2	0
Spine	0	1	0
VHS			
Low (0-10%)	2	5	4
Medium (10-50%)	14	7	15
High (50-100%)	9	5	1
IMD			
Median (range)	101 (19-774)	62 (11-197)	58 (26-237)

Fig. 2. *In vivo* expression of CA9 and CD34 in meningiomas. Immunohistochemistry using antibodies against CA9 (*B, D, F,* and *H*) and CD34 (*A, C,* and *E*) was used to evaluate and compare regions of hypoxia and tumor vascularity in serial sections (*A* with *B*; *C* with *D*; and *E* with *F*) of meningiomas. CA9 expression was zonal (*B*) and sometimes surrounded regions of necrosis (*D*). n, necrosis. CA9 immunoreactive cells were viable and sometimes not associated with any necrosis (compare *H* with corresponding H&E stain in *G*).
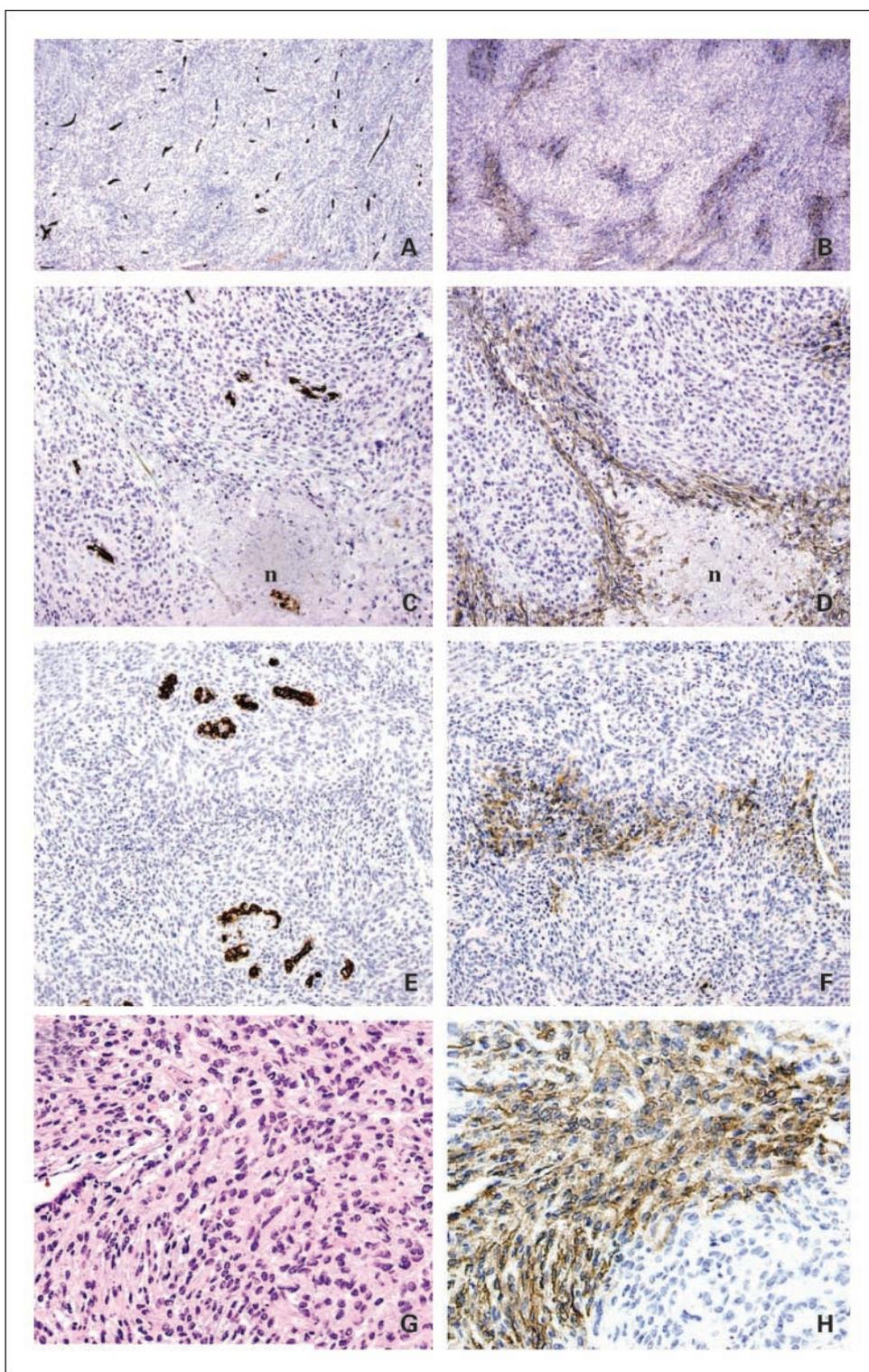
grade 1 meningiomas and two belonged to the angiomatous subtype (Fig. 3C). The remaining two cases were grade 3 meningiomas. Interestingly, both of these highly vascular grade 3 tumors were CA9 positive. These tumors were very heterogeneous, containing regions of high vessel density (Fig. 3D) interspersed with regions devoid of vessels that contained CA9 reactivity.

A summary of CA9 expression as it relates to various clinical and experimental variables is summarized in Table 2. Preoperative embolization of meningiomas is often done to minimize intraoperative bleeding and facilitate surgical excision (30). Because preoperative devascularization induces necrosis and ischemia in tumors, we assessed whether CA9 expression was simply a consequence of embolization. As in
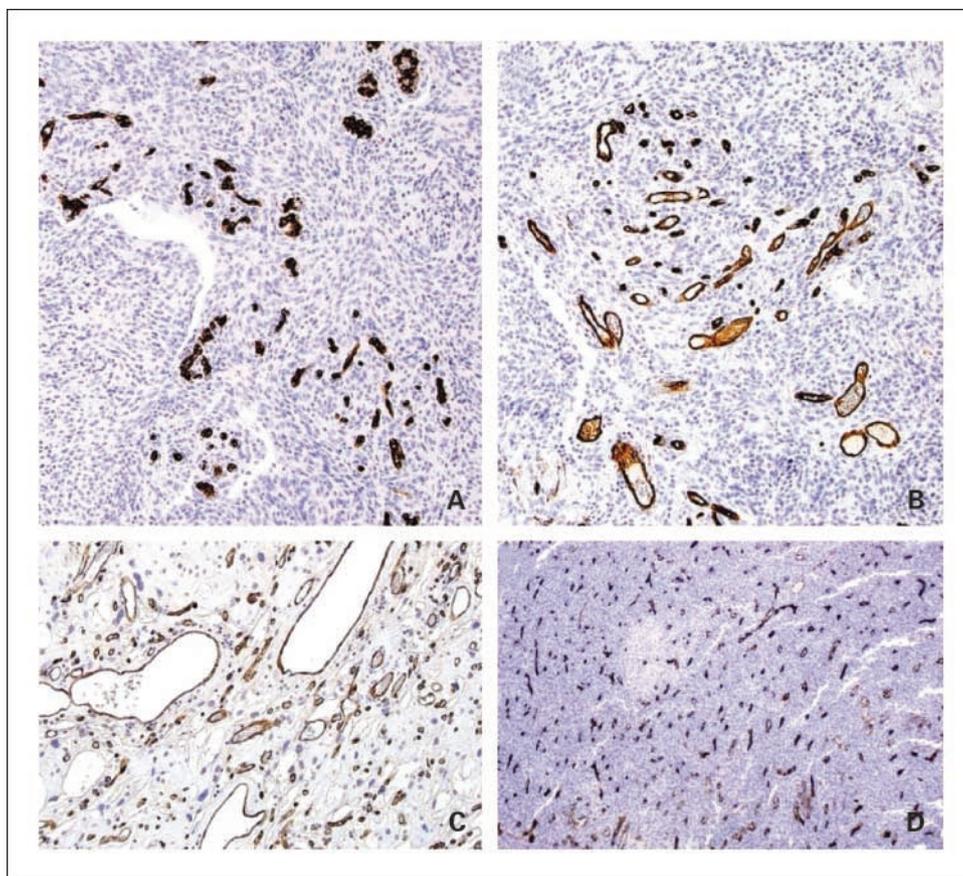
Fig. 3. Patterns of microvasculature in meningiomas. Immunohistochemical stains using antibodies against CD34 were used to assess the vasculature. VHSs contained vessels that were complex, hyperplastic with endothelial cell hyperplasia (*A*), or overall less complex with dilated prominent lumens (*B*). Meningiomas with extremely high intratumoral microvessel densities were either angiomatous (*C*) or sometimes nonangiomatous with regions of dense vascularity (a grade 3 meningioma; *D*).

other series, embolization was done more frequently in high-grade tumors (31). Whereas 50% of grade 3 meningiomas had undergone embolization, only 16% of grade 1 and 11.8% of grade 2 meningiomas were embolized. Adjusted for grade, CA9 expression was not a consequence of presurgical embolization ($P = 0.23$). Approximately half of the grade 3 meningiomas that were CA9 positive had not been embolized. Thus, it seems that expression of CA9 is an endogenous feature of meningioma pathophysiology rather than a consequence of preoperative embolization.

Next, we assessed whether CA9 expression correlated with histopathologic grade. Whereas 32% of grade 1 meningiomas and 23.5% of grade 2 meningiomas had regions of CA9 immunoreactivity, 85% of grade 3 meningiomas had regions of CA9 immunoreactivity ($P = 0.001$; Table 2). There was no difference in the incidence of CA9 expression between grade 1 and grade 2 meningiomas ($P = 0.56$). Hence, grade 1 and 2 meningiomas were grouped together and designated as grade <3 meningiomas; all remaining comparisons of CA9 were adjusted for grade 3 versus grade <3. CA9 expression had no association with age of the patient ($P = 0.23$). Although there was a tendency for CA9 expression to be more prevalent in males, this did not reach statistical significance ($P = 0.08$). In the grade <3 category, 25% of meningiomas from women and 40% of those from men were CA9 positive, whereas, among the grade 3 patients, 70% of female-derived meningiomas and 100% of male-derived meningiomas were CA9 positive. Expression of CA9 had a significant correlation with location of the tumor ($P = 0.027$), prompting us to look at each category

separately. The relationship between CA9 expression in grade 3 tumors and location was not significant ($P = 0.51$), although this relationship was significant in the grade <3 tumor category ($P = 0.032$). In the latter analysis, the significance was primarily due to tumors in the intraventricular region, which were frequently positive for CA9. A loss of statistical significance was observed ($P = 0.25$) when the intraventricular tumors were removed from the analysis.

Among the vascular variables assessed, VHS scores were inversely related to histopathologic grade ($P = 0.023$; Table 1). This significance was mainly due to a reduction in the 50% to 100% VHS group in the grade 3 category. The IMD tended toward an inverse relation with histopathologic grade but did not reach significance ($P = 0.09$). Grade I meningiomas had a higher median IMD when compared with grade 2 and 3 meningiomas, which had very similar IMDs. Interestingly, there was no significant association between CA9 expression and VHS ($P = 0.48$) or IMD ($P = 0.45$). This analysis was adjusted for grade 3 versus grade <3 meningiomas.

The recurrence rates for meningiomas increase with the histopathologic grade of the tumor (6). In this study, a greater proportion of grade 3 tumor samples were recurrent when compared with grade 1 or 2 cases (Table 1). Overall, no correlation was observed between CA9 expression and the recurrence status of the tumors analyzed ($P = 0.55$). However, among the lower-grade (grade 1 and 2) meningiomas, there was a tendency for the CA9-positive tumors to be recurrent ($P = 0.11$). In this subgroup, 50% of the recurrent tumors were CA9 positive, whereas only 25% of the primary tumors were CA9 positive.

Discussion

The effect of microenvironmental hypoxia on meningioma tumor growth is unknown. We show that approximately half of all meningiomas contain subpopulations of hypoxic cells. Tumor hypoxia, as assessed by expression of CA9, was significantly associated with histopathologic grade in meningiomas. CA9 expression was considerably higher among grade 3 meningiomas and may be contributing to the aggressiveness of these meningiomas. Tumor hypoxia was independent of global vascular variables, although regions of prominent tumor vascularity and hypoxia were mutually exclusive. Tumor vascularity was inversely associated with histopathologic grade. Thus, tumor hypoxia, and not high tumor vascularity, is indicative of an aggressive meningioma phenotype.

Previous studies have found a significant correlation between CA9 expression and tumor hypoxia measured either by direct needle electrode measurements (21) or by using bioreductive drug markers, such as pimonidazole (16, 23, 32, 33), supporting the role of CA9 as an intrinsic marker of hypoxia. Although these correlations were significant, they were not absolute. For example, in cervical cancers, the number of cells that expressed CA9 was, on average, twice the number as those that bound pimonidazole (16). In contrast, another study found no correlation between oxygen electrode measurements and CA9 expression in cervical cancers (34). These differences

probably reflect differences in the techniques and the nature of hypoxia being measured. Direct oxygen measurement with polarographic electrodes measures both acute and chronic hypoxia and does not distinguish oxygen levels in areas of necrosis from viable tumor tissue (35, 36). On the other hand, bioreductive drug markers tend to measure the presence of chronic or more long-term hypoxia in metabolically active tumor cells. In addition, the snapshot in time of hypoxia is influenced by the tumor distribution, half-life, and other pharmacokinetic variables of these drugs (36). In fact, some studies have shown that bioreductive drug measurements do not correlate with direct oxygen measurements (37). CA9 expression is a molecular response to hypoxia mediated by the hypoxia-inducible factor-1 transcription factor and is a measure of chronic hypoxia (15).

In the current study, our estimations of hypoxia in meningiomas are dependent on CA9 being an accurate marker of hypoxia. Our data show that CA9 is specifically expressed under hypoxic conditions in all meningioma cell lines tested, suggesting that CA9 is indeed an intrinsic hypoxia marker in meningiomas. However, CA9 expression was induced to varying levels under similar hypoxic environments, suggesting that the tumor phenotype has considerable influence on the nature of the hypoxic response. It is known that malignant cells are more adapted to survive in hypoxic environments (14). Thus, although CA9 expression may not precisely mimic oxygen concentrations as measured by microelectrodes or bioreductive drugs, it is an independent marker of hypoxia.

The presence of necrosis is a criterion for grading malignant meningiomas (6), and perinecrotic regions within tumors are typically hypoxic. Consistent with this, in the present study, CA9 expression is often found in a classic perinecrotic pattern in meningiomas. Additionally, however, CA9 expression is often found in histologically viable regions of tumors not associated with any visible necrosis. CA9 expression was not associated with presurgical embolization, and it is likely that the degree and duration of hypoxia generated by embolization is insufficient to induce CA9 expression.

CA9 expression has been associated with a worse relapse-free survival and overall survival in invasive breast carcinomas (20). In small cell lung cancer, CA9-positive tumors had a significantly shorter overall survival rate (19). Thus, CA9 expression clearly has prognostic significance in a variety of human tumors. Our studies show that CA9 expression is significantly associated with the more aggressive grade 3 meningiomas that have a poor prognosis. Interestingly, there is no difference in CA9 expression between grade 1 and grade 2 meningiomas. Among these grade <3 meningiomas, preliminary indications suggest that CA9 may be a potential marker of aggressiveness because CA9 expression tended to be associated with recurrent tumor status. However, prospective studies with larger numbers are needed to confirm these findings. CA9 expression is strongly associated with grade. Consequently, the number of CA9-negative tumors in the grade 3 category and the number of CA9-positive tumors in the grade <3 category are small. Cumulatively, these results suggest that microenvironmental hypoxia is associated with and probably contributes to the aggressive nature of meningiomas. Further investigations into the role of hypoxia in malignant meningioma biology are warranted.

Table 2. Summary of CA9 expression in meningioma cases stratified by grade

Variable	Benign (grade 1)*	Atypical (grade 2)*	Malignant (grade 3)*
CA9 positive cases	8 (32)	4 (24)	17 (85)
Tumor status			
Primary	7 (29)	2 (17)	9 (90)
Recurrent	1 (100)	2 (40)	8 (80)
Gender			
Male	1 (33)	3 (43)	10 (100)
Female	7 (32)	1 (10)	7 (70)
Presurgical embolization			
Yes	2 (50)	1 (50)	8 (89)
No	6 (29)	3 (20)	9 (82)
Location			
Convexity	4 (33)	1 (14)	10 (91)
Skull base	0 (0)	1 (20)	4 (67)
Falcine	1 (100)	1 (50)	1 (100)
Tentorium	0 (0)	0 (100)	2 (100)
Intraventricular	3 (100)	1 (50)	0 (100)
Spine	0 (100)	0 (0)	0 (100)
VHS			
Low (0-10%)	0 (0)	1 (20)	4 (100)
Medium (10-50%)	7 (50)	2 (29)	12 (80)
High (50-100%)	1 (11)	1 (20)	1 (100)
Age†			
CA9 positive	56 (42-76)	56 (39-74)	54 (23-88)
CA9 negative	48 (30-74)	60 (30-85)	64 (43-73)
IMD†			
CA9 positive	75 (19-119)	66 (46-101)	55 (26-237)
CA9 negative	111 (24-774)	62 (11-197)	91 (86-121)

*Number of CA9-positive cases followed by the percentage of CA9-positive cases in a particular category in parenthesis.
†Median (range) for the CA9-positive and CA9-negative cases for each grade.

Radiation is the only adjuvant therapy available to patients with grade 3 meningiomas (5). Often, these tumors recur even after complete resection and radiation therapy. Hypoxic cells are generally resistant to radiation, and it is possible that one reason for the high recurrence rates of these tumors is the resistance of this subpopulation to current treatment protocols.

Induction of tumor vasculature, also called "the angiogenic switch," is essential to the survival and propagation of tumors (38). Although the angiogenic activity of a tumor, most commonly measured as the IMD, is a prognostic indicator for certain cancers, this is not true for other cancers. Benign meningiomas had significantly more VHS when compared with malignant meningiomas. This held true even after removing the two angiomatous histologic subtypes from the analysis. Thus, the angiogenic switch occurs early in meningioma tumor development and the vascular nature of these tumors is probably a reflection of an easier access to the host vascular system. Meningiomas derive their blood supply predominantly from the meningeal vessels originating from the external carotid circulation (39). It is possible that benign meningiomas are tumors that grow so slowly that they never overgrow their vascular supply, whereas the lower VHS and IMD in faster-growing malignant meningiomas are simply by-products of the higher degrees of cellularity. We have observed increased microvasculature in a region of the brain adjacent to and being compressed by a malignant meningioma (data not shown), suggesting that meningiomas and/or brain parenchyma cells in the affected region are actively secreting angiogenic factors. Previous studies have found a correlation between meningioma grade and VEGF content, which was 10-fold higher in malignant meningiomas when compared with benign meningiomas (27). In contrast to our findings, this study did not find a correlation between IMD and tumor grade. This discrepancy is probably a reflection of the different endothelial cell–specific antibodies used to assess IMD and the different method of scoring IMD. The prior study used antibodies to factor VIII–related antigen, and this antigen has previously shown considerable variability in staining of large vessels and capillaries, identifying only a proportion of capillaries (25, 26). In the current study, we used anti-CD34, an antibody that has been reported to stain small and large vessels with equal intensity (25, 26). Interobserver variation in scoring vascular patterns has been reported to limit the prognostic use of VHS and IMD scores (40).

The observation that VEGF content increases with tumor grade, whereas angiogenic activity decreases with grade, suggests that other angiogenic factors are involved in the development of meningioma vasculature. Other factors, such as placental growth factor, hepatocyte growth factor/scatter factor, and basic fibroblast growth factor, have all been detected in meningiomas but with no differences among tumor grade (27). It is possible that it is the balance of proangiogenic and antiangiogenic factors that drives vessel growth in meningiomas. Elucidating the interplay between these factors has important implications for any potential antiangiogenic therapy development in meningiomas.

In conclusion, we have shown that hypoxia in the tumor microenvironment is a component of grade 3 meningiomas and that tumor hypoxia may be an indicator of poor prognosis for these patients. New therapies for these tumors should include strategies to target both normoxic and hypoxic cells.

Acknowledgments

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