Neutrophils Promote the Malignant Glioma Phenotype through S100A4

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Abstract

Purpose: Antiangiogenic therapy is effective in blocking vascular permeability, inhibiting vascular proliferation, and slowing tumor growth, but studies in multiple cancer types have shown that tumors eventually acquire resistance to blockade of blood vessel growth. Currently, the mechanisms by which this resistance occurs are not well understood.

Experimental Design: In this study, we evaluated the effects of neutrophils on glioma biology both in vitro and in vivo and determined target genes by which neutrophils promote the malignant glioma phenotype during anti-VEGF therapy.

Results: We found that an increase in neutrophil infiltration into tumors is significantly correlated with glioma grade and in glioblastoma with acquired resistance to anti-VEGF therapy. Our data demonstrate that neutrophils and their conditioned media increased the proliferation rate of glioblastoma-initiating cells (GIC). In addition, neutrophils significantly increased GICs Transwell migration compared with controls. Consistent with this behavior, coculture with neutrophils promoted GICs to adopt morphologic and gene expression changes consistent with a mesenchymal signature. Neutrophil-promoting tumor progression could be blocked by S100A4 downregulation in vitro and in vivo. Furthermore, S100A4 depletion increased the effectiveness of anti-VEGF therapy in glioma.

Conclusions: Collectively, these data suggest that increased recruitment of neutrophils during anti-VEGF therapy promotes glioma progression and may promote treatment resistance. Tumor progression with mesenchymal characteristics is partly mediated by S100A4, the expression of which is increased by neutrophil infiltration. Targeting granulocytes and S100A4 may be effective approaches to inhibit the glioma malignant phenotype and diminish antiangiogenic therapy resistance.

Introduction

Gliomas are the most common type of primary brain tumors in adults and classified into four grades by the World Health Organization (WHO) according to their degree of malignancy and histologic features (1). For patients with high-grade gliomas (HGG), such as glioblastoma, the prognosis remains poor with a short survival time (1, 2). Patients with low-grade tumors have a comparative longer term survival, but nearly all low-grade tumors progress to high-grade malignancy (3). Identifying the key contributors and molecular pathways that result in tumor progression is the focus of intense investigation because inhibition of malignant progression of glioma has the potential to significantly extend patient survival.

Over the past two decades, advances made in molecular technologies such as microarray technologies and genome sequencing have made it possible to evaluate the molecular and genetic changes in malignant brain tumors. Recent gene expression profiling studies have revealed three to four molecular subclasses of HGGs based on differential gene expression (4, 5). One tumor subtype is characterized by expression of neural progenitor markers (proneural), which is enriched in low-grade gliomas (LGG; ref. 6), is associated with longer survival, whereas malignant glioma with a mesenchymal signature are aggressive, commonly coincide with disease recurrence, resistant to chemotherapy, and is associated with a shorter survival time. In addition, resistance to chemotherapy is also seen in gliomas that were originally defined as proneural and later have increased mesenchymal genes expression, suggesting a shift to the mesenchymal subtype over time (4, 5). This shift from proneural to mesenchymal transition (PMT) in glioblastoma may drive its aggressive behavior and eventually cause chemotherapy failure (7). Currently, it is unknown what drives this PMT in glioblastoma.

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Recent evidence provided by our group and others point to tumor microenvironment components as potential participants in the generation of tumor malignancy and chemoresistance (7–11). The tumor microenvironment contains large populations of cancer-related inflammatory cells, including tumor-associated neutrophils (TAN). In gliomas, increased infiltration of neutrophils has been observed in high-grade gliomas compared with low-grade gliomas (12). Resistance to chemotherapy is also associated with increased infiltration of neutrophils. Our previous work shows that glioblastoma resistance to anti-VEGF therapy is associated with myeloid cell infiltration and mesenchymal transition, indicating a positive correlation between them (7). Similar findings have been reported for other tumors (13, 14), suggesting that targeting TAN-promoting tumor progression can be a universal method to overcome drug resistance and tumor recurrence. Shojaei and colleagues reported that inherent anti-VEGF refractoriness is associated with infiltration of the tumor tissue by CD11b+/Gr1+ myeloid cells. Combining anti-VEGF treatment with antimyeloid cell therapy inhibits growth of refractory tumors more effectively than anti-VEGF alone (15, 16). Recent work by Acharya and colleagues showed that inhibition of neutrophil recruitment augments the efficacy of chemotherapy against breast tumors and particularly against lung metastasis (8). It has been reported that blocking TAN infiltration into tumor tissues can influence several key aspects of tumor progression, including tumor growth (17–19), angiogenesis (16, 20, 21), and metastasis (8, 11). Identifying the mechanisms by which TANs promote tumor progression and the genetic characteristics associated with these phenotypic changes is essential to identify the key regulators of glioblastoma progression and thus guide the development of new therapies to overcome resistance.

In this study, we make use of an in vitro coculture model to investigate the interaction between glioma cells and neutrophil progenitor cells, especially how neutrophil influence glioma phenotypes and signaling pathways. We found that coculture of neutrophil and glioma stem cells increases the expression of S100A4 in glioma cells, which was also upregulated in anti-VEGF-resistant tumors. Downregulating neutrophil-promoting expression of S100A4 can mitigate the neutrophil-mediated malignant phenotype in vitro and in vivo. Furthermore, S100A4 depletion prolongs the survival of anti-VEGF treated animals and partially reduces glioblastoma resistance to anti-VEGF therapy.

**Materials and Methods**

**Brain tumor tissue microarray**

A human glioma tissue microarray (TMA) was constructed using formalin-fixed, paraffin-embedded archival tissue blocks as described previously (22). The TMA included samples from 232 primary brain tumors of varying grades taken from sites of the most phenotypically representative tumor regions. The array contained 96 glioblastoma tumors, 13 gliosarcoma tumors, 12 anaplastic mixed oligoastrocytoma tumors, 32 anaplastic astrocytoma tumors, 24 anaplastic oligodendroglioma tumors, 29 oligodendroglioma tumors, 11 mixed oligoastrocytoma tumors, and four low-grade astrocytomas. Normal brain tissue samples were included in the array as a negative control. Expression levels of myeloperoxidase-positive (MPO) were evaluated by a standard indirect immunoperoxidase procedure as previously described (7). Mayer’s hematoxylin nuclear staining was used as a counterstain. An intensity score was assigned to each sample that represented the average numbers of the positive staining on an arbitrary scale of 0 to 5. Statistical analysis was done using a Kruskal–Wallis analysis of ranks.

**Cell culture**

Glioma stem cell line GIC23, GIC11, GIC2, and GIC20 were obtained from Dr. Howard Colman (Department of Neuro-Oncology, MD Anderson Cancer Center, Houston, TX). Glioblastoma-initiating cells (GIC) were maintained in suspension in Dulbecco’s Modified Eagle Medium (DMEM) containing EGF, basic fibroblast growth factor (bFGF), and B27 (Invitrogen) at 37°C in 5% CO_2_ atmosphere. Neutrophil progenitor cell line CRL-11422 (AACR) was cultured in Iscove’s modified Dulbecco’s medium with 4 mmol/L L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate containing 10 ng/mL murine granulocyte macrophage colony-stimulating factor (GM-CSF), 80% and heat-inactivated horse serum, 20%. Before collecting conditioned media, cells were changed to serum-free and GM-CSF–free media. Conditioned media were collected after 24 hours incubation.

**Cell-cycle analysis**

For cell-cycle analysis, asynchronous GIC cells were fixed with ethanol and stained with 50 μg/mL propidium iodide (PI) containing 0.1 mg/mL RNaseA (both from...
Immunofluorescence analysis was done as previously described with minor modifications (23). Briefly, formaldehyde-fixed cells were permeabilized with Triton X-100 and blocked with 5% serum diluted in PBS-gel (0.2% gelatin in PBS) for 30 minutes. The primary antibodies were incubated in blocking solution overnight at 4°C. Immunostaining was performed using the primary antibody against Ykl-40 (1:50; Santa Cruz Biotechnology), CD31 (1:50; Abcam) and ly6B.2 (1:50; AbD Serotec). Coverslips were mounted using ProLong antifade reagent (Invitrogen). The images were acquired with an inverted deconvolution microscope. Images were taken with a Zeiss Axioskop 40 microscope equipped with AxioVision Rel.4.2 software.

Animal xenografts

For in vivo experiments, GIC cells (3 x 10⁵) with or without CRL11422 (9 x 10⁵) were implanted intracranially into 4- to 6-week-old female nude mice (12 mice per group). The mice were euthanized at 3, 6, 9, 11 weeks, and their brains were removed and processed for analysis. All experiments were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center. Tumor volume analysis was done using an unpaired two-tailed Student test and groups were compared using the log-rank test. P < 0.05 was determined to be significant.

Immunohistochemistry

Paraffin sections from xenografts were used for immunohistochemical analysis. The slides were deparaffinized and subjected to graded rehydration. After blocking in 5% serum and an antigen retrieval step (citrate buffer, pH 6.0), the slides were incubated with the primary antibodies overnight at 4°C. After washing in PBS with Tween 20, primary antibody reactions were detected using the VECTASTAIN ABC Kit (Vector Laboratories) with the respective secondary antibody.

Transfection

Cells were plated at a density of 3 x 10⁵ per six-well plate 3 hours before transfection. Transfection was carried out using Hyfect reagents according to the vendor’s instructions. Transfected cultures were selected with puromycin (5 µg/mL) for 10 to 14 days. At that time, antibiotic-resistant colonies were picked, pooled, and expanded for further analysis under selective conditions. The pGIPZ control was generated with control oligonucleotide GCITCTAACCCGGAGGTCTT. pGIPZ S100a4 shRNA was generated with TGCTCAGCATCAAGCACGT and TGAGCITGAACCTGTCACC.

The Cancer Genome Atlas analysis

S100A4 gene expression values were determined among glioblastoma datasets in The Cancer Genome Atlas (TCGA), accessed through the cBio Cancer Genomics Portal (http://cbioportal.org) supplied by Memorial Sloan Kettering Cancer Center (website accessed on April 25, 2013). The levels of expression of S100A4 were evaluated in the different TCGA glioblastoma subtypes (5). For each glioma subtype, patients were divided into two groups on the basis of on gene expression (Z score < or ≥ 2). Kaplan–Meier curves
were generated using these data. The difference in survival between patients with high and low expression of S100A4 was calculated for each subtype.

Results

Neutrophil infiltration is correlated with glioma grade and tumor progression

A human glioma TMA was used to evaluate for the presence of neutrophil infiltration using immunohistochemical staining for MPO. The numbers of MPO-positive cells for each tumor sample were calculated from grade 2, 3, and 4 glioma. Eighty-six percent of all glioma samples in our TMA (n = 232) had some evidence of neutrophil infiltration (Fig. 1A). Grade 4 glioblastoma specimens had the highest numbers of infiltration neutrophil [number of neutrophil > 10/200 HPF (high-powered field; Fig. 1A) and Supplementary Table S1]. The level of neutrophil infiltration was significantly positively correlated with glioma grade. To confirm the level of neutrophil infiltration in murine models of glioma, we evaluated the number of neutrophils infiltrated into tumor xenografts injected into the brain of nude mice. The number of murine neutrophils characterized by the marker Ly-6B.2 (24, 25) increased during tumor growth and progression (Fig. 1B).
Neutrophils promote the proliferation of glioma stem cells

To identify the effects of neutrophils on tumor cells, we performed in vitro coculture of glioma stem cells (GIC) with neutrophil progenitor cells (MPRO cells; CRL11422; ATCC). After 2 days of coculture, the number of GFP-positive GIC11 cells was significantly increased compared with controls (Fig. 2A). Conditioned media collected from MPRO cells (24-hour serum-free culture medium) also increased the numbers of GICs (Fig. 2B), suggesting that secreted factors from neutrophils may be partially responsible for the tumor proliferating effects.

Further evaluation by PI staining identified that the increase in cell number resulted from both a decrease in GIC apoptosis (Fig. 2C) and an increase in cell proliferation (Fig. 2D). Western blot analysis data demonstrated that conditioned media upregulated the expression of proliferation-related proteins cyclin D1, cyclin D2, and c-myc (Fig. 2E). In addition, conditioned media increased the phosphorylation of Akt and Erk (Supplementary Fig. S1A). Blocking Akt and Erk pathway activation by their inhibitors decreased the expression of cyclin D1 and c-myc induced by conditioned media (Supplementary Fig. S1B). These data indicate that, at least in our in vitro model, factors secreted by neutrophils promote GIC proliferation.

Neutrophils promote a mesenchymal phenotype

We next investigated the effect of neutrophils on the ability of GIC invasion. Matrigel Transwell assays showed an increase in Transwell migration/invasion for GICs when cocultured with MPRO cells compared with controls (Fig. 3A). GIC11 cells in culture demonstrated a spindle-shaped

Figure 2. Neutrophils promote the proliferation of GICs. A, morphology of GFP-positive GICs with and without coculture with MPRO cells (at 1:1 ratio) for 3 days (×100). Bar graph demonstrating the fold change of GFP-expressing cell numbers analyzed by flow cytometry. B, fold changes of cell numbers after 3 days incubation of GIC11 or GIC23 with serum-free conditioned media (CM) collected from MPRO cells. Cell-cycle analysis using PI staining showed that CM decreased the percentages of cells in sub-G1 stages (apoptotic cells, C) and increased that in G2-M+S stages (growing cells, D). E, expression of cyclin D1, cyclin D2, C-myc, and tubulin were detected by Western blot analyses in GICs incubated with or without CM. **, P < 0.05; ****, P < 0.01; Student t test.
morphology, which is a typical morphology for mesenchymal cells (26), when cocultured with MPRO cells (Fig. 3B). Consistent with a more mesenchymal phenotype, Western blot analyses showed increased expression of mesenchymal markers YKL-40 and MMP2, and less expression of nestin, a proneural marker following coculture (Fig. 3C). To confirm the effects of neutrophils on GICs and to investigate the underlying mechanisms by which neutrophils promote a mesenchymal glioma phenotype, we performed gene expression analysis of GIC23 treated with or without MPRO cells. IPA analysis (Supplementary Fig. S2) showed a significant shift toward a mesenchymal gene signature and increased cell mobility in the coculture group compared with the control group. These data indicate that neutrophils promote a mesenchymal shift in GICs. In addition, gene expression analysis demonstrated that neutrophils also promote an increase in genes related to cell division processes, consistent with the results presented in Fig. 2.

Neutrophils promote a malignant glioma phenotype through S100A4

In our previous work, we found that glioblastoma resistance to anti-VEGF therapy was associated with neutrophil infiltration and a mesenchymal phenotype (7). Gene expression profiling data from these experiments also identified (Fig. 4A) multiple genes that were upregulated during the development of resistance. Several of these genes may be involved in the regulation of neutrophil-mediated mesenchymal transformation. One of the candidate genes S100A4 was found to be highly expressed in GIC with a mesenchymal phenotype (Fig. 4B, GIC2 and GIC20), whereas no expression was observed in GICs of the proneural subclass (Fig. 4B, GIC11 and GIC23). To identify the role of S100A4, we developed a stable GIC23 cell line with a specific shRNA to S100A4 (Fig. 4C).

Consistent with the gene expression analysis, GICs’ coculture with neutrophils increased S100A4 expression, which was not observed in the S100A4 knockdown cell line. Although down regulation of S100A4 did not affect neutrophil-mediated proliferation (Fig. 4E), neutrophil promotion of tumor cell migration (Fig. 4F) and YKL-40 expression (Fig. 4G) were inhibited by S100A4 knock down. To evaluate the impact of S100A4 on tumor growth in vivo, we then coinjected neutrophils with control shRNA or S100A4 shRNA GIC23 into nude mice. Consistent with the in vitro data, neutrophils’ coinjection increased human S100A4 expression in tumor tissue, whereas almost no human S100A4 staining was observed in the S100A4 knockdown group (Supplementary Fig. S3A). Our data showed that coinjection of neutrophils with control shRNA or S100A4 shRNA GIC23 into nude mice. Consistent with the in vitro data, neutrophils’ coinjection increased human S100A4 expression in tumor tissue, whereas almost no human S100A4 staining was observed in the S100A4 knockdown group (Supplementary Fig. S3A). Our data showed that coinjection of neutrophils with control shRNA or S100A4 shRNA GIC23 into nude mice.
S100A4 depletion increases the efficacy of anti-VEGF therapy in gliomas

Because S100A4 depletion may block neutrophil-mediated mesenchymal transformation, which is a prominent feature of anti-VEGF–resistant tumors, we next investigated whether S100A4 depletion would improve the efficacy of anti-VEGF therapy. Our survival study in animals demonstrated that mice treated with bevacizumab had a longer survival time than the no treatment group ($P = 0.0044$, log-rank (Mantel–Cox) test; Fig. 6A), and S100A4 depletion further prolonged survival ($P = 0.0137$, log-rank (Mantel–Cox) test; Fig. 6A). Immunostaining data showed that the specific shRNA dramatically inhibited S100A4 expression in tumor cells (Supplementary Fig. S3B), but this inhibition did not affect the number of infiltrated neutrophils during bevacizumab treatment (Supplementary Fig. S3C). As reported previously (7, 27), we found that the angiogenesis marker CD31 was markedly inhibited in both control cells and S100A4-depleted tumors following 4 weeks of bevacizumab treatment (Fig. 6B, 4 week), indicating that the bevacizumab-treated tumor had not escaped from therapy at that time point. However, at 6 weeks, tumors were bevacizumab-resistant and demonstrated an increase in CD31 staining, which was not observed in the S100A4 depletion group (Fig. 6B, 6 week). Interestingly, YKL-40 staining was increased in bevacizumab-treated tumors as early as 4 weeks, which was also inhibited by S100A4 downregulation at 4 weeks and 6 weeks (Fig. 6C). These
data suggest that the mesenchymal transformation of gliomas was regulated by S100A4 and may be responsible for the increase in angiogenesis during tumor progression on anti-VEGF therapy.

Discussion

Glioblastoma is recognized as the most common and lethal form of brain cancer. Current treatments for glioblastoma include temozolomide paired with radiotherapy, and to more recently the use of antiangiogenic agents (28). However, anti-VEGF agents have not significantly extended the life expectancies of patients diagnosed with glioblastoma (29, 30), which has partially been attributed to drug resistance. The major aim of this study is to determine the mechanisms underlying glioblastoma resistance to antiangiogenic therapy. Although there is strong preclinical evidence supporting the efficacy of antiangiogenic therapy, tumors eventually adopt a highly resistant phenotype. In this study, we demonstrate that neutrophils may play a critical role in tumor-escape from antiangiogenic therapy. Consistent with previous studies (7), we observed an increase in neutrophil infiltration into gliomas, which may be partially driven by tumor hypoxia possibly exacerbated by prolonged anti-VEGF therapy (7, 31). The extent of neutrophil infiltration was positively correlated with glioma grade. Our studies provide evidence that neutrophils promote the glioma malignant phenotype and increase the expression of mesenchymal markers in vitro and in vivo.

Neutrophils have been reported to play an important role in stimulating tumor angiogenesis (32, 33). In patients with myxofibrosarcomas, increased numbers of neutrophils were associated with increased intratumoral microvessel density (34). In vivo studies using various tumor models...
of angiogenesis demonstrated that neutrophils promote neovascularization (20, 21, 35). In our proneural glioma stem-cell xenograft model, we found increased infiltration of neutrophils and expression of mesenchymal marker following 4 weeks of anti-VEGF treatment, with enhancement of angiogenesis at later stages of tumor escape. Mesenchymal tumors are highly vascularized, consistent with the findings that the mesenchymal gene expression

Figure 6. Inhibition of S100A4 increases the efficacy of anti-VEGF therapy in gliomas. A, Kaplan–Meier survival curves for GIC11 Control shRNA and S100A4 shRNA cell-injected mice treated with or without bevacizumab (Bev, 10 mg/kg). GIC11 S100A4 shRNA cell-injected mice treated with Bev have a longer survival duration compared with treated GIC11 Control shRNA cell-injected mice. [P = 0.0137, long-rank (Mantel–Cox) test]. Bar graph demonstrating the mean staining of CD31 (B) and Ykl-40 (C) at 4 and 6 weeks. Data are shown as averages ± SD; n = 9 fields taken from at least 3 mice per group at each time point. *, P < 0.05; **, P < 0.01; P values were determined by t test. D, model showing how anti-VEGF treatment failure is promoted by neutrophils through S100A4.
signature contains multiple proangiogenic genes (4). Our data suggest that neutrophils may promote angiogenesis by inducing a shift of gliomas from proneural to mesenchymal subtype (Fig. 6D). Although activated myeloid cells such as M2-skewed macrophages can directly secrete proangiogenic factors (36–39), TANs may also regulate tumor angiogenesis in an indirect way, such as through shifting stem cells toward a mesenchymal phenotype.

In the present study, we found that a mesenchymal transformation in glioma stem cells was promoted by neutrophils through upregulation of S100A4. S100A4 belongs to the S100 family of proteins that contain two Ca\(^{2+}\)–binding sites, including a canonical EF-hand motif (40). S100A4 is involved in the regulation of a wide range of biologic effects, including cell motility, survival, differentiation, and contractility (41). Several of the S100A4 effects resemble processes that occur during epithelial–mesenchymal transition (EMT), and a direct link between S100A4 expression and EMT has been suggested in a number of organs (42–44). In human patients with cancer, increased expression of S100A4 is positively associated with an increased incidence of metastasis, invasiveness, aggressiveness, and a worse prognosis (45–50). Our mesenchymal glioblastoma stem cells expressed S100A4, whereas GICs with a proneural signature did not. Likewise, a query of the TCGA identified 5.8% of the glioblastoma samples (206 cases) overexpressed S100A4 and most of these samples (58.6%) were of the mesenchymal subtype and associated with a shorter overall survival (P = 0.003964). Conversely, 14.3% proneural glioblastoma samples have downregulation of S100A4 and longer survival (P = 0.009221). Consistent with our results that neutrophils can increase the expression of S100A4 in vitro, S100A4 and CD64, a human monocyte marker, are significantly coexpressed in glioblastoma patient samples from TCGA. Cumulatively, these data suggest that S100A4 may be a key contributor of mesenchymal transition in glioblastoma progression, and the increased expression of S100A4 can be at least partially promoted by an increase in neutrophil infiltration.

Currently, it is difficult to discern the exact mechanisms underlying S100A4-mediated mesenchymal transition because S100A4 can function both intra- and extracellularly. It is likely that the intracellular and the extracellular effects involve distinct mechanisms. S100A4 has been reported to interact with multiple proteins that are known to play a role in tumor progression and metastasis, such as MMP-9 (51). Iwamoto and colleagues showed that patients with glioblastoma with longitudinal increases in MMP-9 had a shorter survival (52). Studies in endothelial cells and tumors demonstrate that S100A4 expression can be promoted by TGF-β (43, 53), which is well known to induce EMT. Consistently, we have observed an increase in the TGF-β pathway in anti-VEGF–resistant tumors (7). Further studies are needed to identify the molecular mechanism by which S100A4 regulate the mesenchymal phenotype.

Although TANs play a critical role in tumor progression, therapeutic targeting of this cell type in cancer will be challenging. Neutrophils are critical mediators of host defense against infection, and depletion of these cells could result in dangerous levels of immunosuppression. However, we found that downregulation of S100A4 inhibited neutrophil-promoting tumor progression independent of the infiltration of neutrophils. Our findings provide a possible alternative strategy to target the specific neutrophil-activated regulator on tumor cells, S100A4. Therefore, the combination of S100A4 inhibitor with standard antiangiogenesis therapy may inhibit glioma progression and anti-VEGF therapy resistance.

Disclosure of Potential Conflicts of Interest

J.F. de Groot received commercial research grant from EMD Serono, Sanofi Aventis, AstraZeneca, Eli Lilly, and Document Pharmaceuticals and is a consultant/advisory board member of Genentech and Novartis. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Liang, Y. Piao, G.N. Fuller, J.F. de Groot.
Writing, review, and/or revision of the manuscript: J. Liang, G.N. Fuller, J.F. de Groot.
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): N. Tiao, J.F. de Groot.

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