Tumorigenesis and Neoplastic Progression

Osteopontin Expression in Intratumoral Astrocytes Marks Tumor Progression in Gliomas Induced by Prenatal Exposure to N-Ethyl-N-Nitrosourea

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To better study early events in glioma genesis, markers that reliably denote landmarks in glioma development are needed. In the present study, we used microarray analysis to compare the gene expression patterns of magnetic resonance imaging (MRI)-localized N-ethyl-N-nitrosourea (ENU)-induced tumors in rat brains with those of uninvolved contralateral side and normal brains. Our analysis identified osteopontin (OPN) as the most up-regulated gene in glioma. Using immunohistochemistry we then confirmed OPN expression in every tumor examined ($n = 17$), including those with diameters as small as 300 μm . By contrast, no OPN immunostaining was seen in normal brain or in brains removed from ENU-exposed rats before the development of glioma. Further studies confirmed that OPN was co-localized exclusively in intratumoral glial fibrillary acidic protein-expressing cells and was notably absent from nestin-expressing ones. In conjunction with this, we confirmed that both normal neurosphere cells and ENU-immortalized subventricular zone/striatal cells produced negligible amounts of OPN compared to the established rat glioma cell line C6. Furthermore, inducing OPN expression in an immortalized cell line increased cell proliferation. Based on these findings, we conclude that OPN overexpression in ENU-induced gliomas occurs within a specific subset of in-

tratumoral glial fibrillary acidic protein-positive cells and becomes evident at the stage of tumor progression. (Am J Pathol 2006, 168:1676–1685; DOI: 10.2353/ajpath.2006.050400)

For many decades, gliomas [defined broadly as primary central nervous system (CNS) neoplasms that display morphological characteristics of macroglia] were considered to arise from dedifferentiation of glial cells.^{1,2} Recently, however, much interest has been directed toward the possibility that these tumors arise from multipotent neural stem cells, including cells that persist in the brain's subventricular zone (SVZ).^{3–6} A less-appreciated aspect of this potential stem cell origin is that it allows one to better address glioma development within the framework of the multistage cancer model. This model, which has been used to describe epithelial cancer formation,^{7,8} proposes that cancer develops in three stages: 1) an initiation stage that presumably occurs through irreversible or stable damage to DNA, 2) a promotion stage that is an epigenetic process bringing about a clonal expansion of initiated cells, and 3) a progression stage that results when genetic instability leads to further mutagenic and epigenetic changes.^{9–11} In this paradigm, the emergence of tumors follows the initiation of multipotent stem cells that are initially suppressed by surrounding normal cells through inhibitory influences that are gradually overcome during the promotion stage, which is the rate limiting step in the carcinogenic process.^{10,11}

To date, little attention has been paid to the promotion stage by those interested in glioma development in part because gliomas are virtually impossible to diagnose before they are fully developed (ie, already entered the progression stage). To begin addressing this question, therefore, we have been using a model of neurocarcino-

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genesis in which gliomas invariably develop several months after a single prenatal exposure to *N*-ethyl-*N*-nitrosourea (ENU).^{12,13} This model is uniquely suited to study this question because ENU itself is rapidly cleared (within minutes), after which tumors are not observed before 90 days of age, a large temporal window in which one can safely assume that pathological changes are because of developing tumor and not from continued exposure to mutagens.

In a previous study, we described distinctive clusters of nestin⁺ cells residing outside the SVZ that could be detected at a very early age (P30) only in rats that were exposed to ENU¹⁴ and hypothesized that these nests represent a very early stage of glioma, analogous to lesions that are frequently seen in epithelial cancers (ie, such as polyps). Consistent with this contention, we were able to isolate nestin⁺ cells from neonatal SVZ/striata of ENU-exposed (but not control) rats that were immortal, aneuploid, and INK4a/ARF-deficient but lacked other tumorigenic properties.¹⁵

To better study whether promotion is an important aspect of glioma development, markers are needed that can reliably identify specific stages of tumor development. We reasoned that additional potential markers could be identified if gene expression arrays were performed on tumors as soon as they became apparent on serial MRI imaging. Even though we could not identify tumors before they were more than 2 mm in size, our analysis nevertheless revealed an overexpression of a number of interesting genes involved in growth and motility, the most overexpressed of which was osteopontin (OPN), a phosphoprotein that has been implicated in tumor progression of a variety of systemic cancers.^{16–18} We then confirmed using immunohistochemistry that OPN was expressed in all ENU-induced gliomas, including those of very small size, whereas it was absent in both normal CNS as well as the earlier described nests. Furthermore, we noted that OPN expression within tumors was confined to a distinct large glial fibrillary acidic protein (GFAP)-expressing intratumoral astrocyte. Based on these data, we conclude that the appearance of this OPN-expressing cell marks a transition from promotion to the progression stage in this glioma model.

Materials and Methods

All procedures involving live rodents were approved by the University of Massachusetts Medical School and Stanford University Medical School's Animal Regulatory Committees.

Transplacental Administration of ENU

Timed pregnant Sprague-Dawley rats (Taconic Farms, Germantown, NY) were placed in a restrainer and injected intravenously via tail vein with 50 mg/kg of ENU (Sigma, St. Louis, MO) at gestational days 17 to 19 through a 26-gauge needle throughout a several minute period. Pups were housed two per cage (same sex) after weaning and observed weekly for signs of illness. At specified times after birth or when illness appeared, rats

were deeply anesthetized with ketamine HCl (Fort Dodge, Overland Park, KS) and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). Selected rats received three intraperitoneal injections of 50 mg/kg of bromodeoxyuridine (BrdU) (Sigma) before sacrifice to label cells in S phase.

Punch Biopsies for Gene Array

Two rats exposed *in utero* to ENU as well as one exposed to saline were imaged every 30 to 45 days after P90 on a 4.7 T 40-cm bore MR scanner (Bruker, Billerica, MA) using heavily weighted T2 images, which had been determined in an earlier study as the most rapid and sensitive screen to identify tumors. When tumors were identified by imaging, rats were immediately sacrificed and the brain removed. Using the MR image to prepare a coronal brain slice that encompassed the tumors, the tumor area (which appeared as a gray discoloration) was then punch-biopsied with an 11-gauge bone marrow biopsy needle (Medical Device Technologies, Gainesville, FL). The punch was immediately frozen in liquid nitrogen. In addition, the contralateral normal appearing side of both rat brains as well as an equivalent area from the control rat were punch-biopsied using similar technique. The remaining brain tissue was then fixed and stained with hematoxylin and eosin (H&E) to confirm that the area punched encompassed a tumor-bearing area.

Stab Wound Injury

Stab wound injury was performed essentially as described by Jang and colleagues.¹⁴ Briefly, adult male Sprague-Dawley rats (250 to 280 g) were anesthetized with a mixture of ketamine HCl and xylazine via intraperitoneal injection. The stab wounds were created by inserting a 20-gauge needle at 0.3 mm posterior to bregma, 3 mm lateral to the sagittal suture, and 5 mm deep to dura. Rats were allowed to survive for 4 and 14 days after the lesion.

Gene Expression Profiling

We used methodologies recommended by the Expression Analysis Technical Manual published by Affymetrix (Santa Clara, CA). Frozen tissue samples were homogenized in extraction buffer (QuickPrep Micro mRNA purification kit; Amersham Biosciences, Piscataway, NJ). Poly(A)⁺ RNA was isolated from the samples using the QuickPrep mRNA purification kit. cDNA synthesis was performed using Superscript Choice cDNA synthesis kits (Invitrogen, Carlsbad, CA) using T7 promoter-containing oligonucleotides. cDNA was then used for *in vitro* transcription reactions with T7 RNA polymerase and biotinylated UTP to create biotin-labeled RNA transcripts. Twenty μ g of each sample was hybridized onto Affymetrix rat genome U34A microarray cartridges at 45°C for 16 hours. The cartridges were then placed into a GeneChip Fluidics Station 400 (Affymetrix) and subjected to an automated washing procedure that included a

staining subprotocol using streptavidin-phycoerythrin for the detection and quantitation of hybridized cRNA bound to the microarray. The cartridge was then read on a GeneArray scanner (Affymetrix), which uses an argon laser to read and quantitate the fluorescence levels of the hybridized, streptavidin-phycoerythrin-stained microarrays. Hybridization data were acquired and processed on a GeneChip Analysis Suite.

Data Analysis

Gene expression values for all experiments were calculated from raw CEL data using the method of Li and Wong.¹⁹ Raw data from the RGU34A chips were normalized and processed using dChip. Low and negative values were truncated upward to a uniform value of 150 and genes that had at least one *P* designation were used for further analysis. For a given gene, the mean gene expression value x_t (log units) for tumor tissue punches was compared with the mean gene expression x_c (log units) of the mean of the contralateral controls and control brain using a cumulative distribution function, where s is the SD (log units) of the tumor samples.

$$\text{cdf} = \int \frac{1}{2\pi s} \exp\left(-\frac{(x_t - x_c)^2}{2s^2}\right) dx$$

In the case where x_t is less than x_c , the *cdf* is the integral taken from minus ∞ to x_t and represents the area of the tail of the distribution to x_t . Conversely when x_t is greater than x_c , the area of the tail is $(1 - \text{cdf})$ and represents the area of the tail from x outward. Genes in which the tail area is less than 0.001 for a given x_t were considered different in expression from those in the control tissues.

Immunohistochemical Procedures

Rat brains were postfixed in 4% paraformaldehyde overnight. Fifty- μm sections were cut from the anterior commissure through the posterior hippocampus using a vibratome. The following antibodies and dilutions were used: murine anti-OPN (MPIIB10₁, 1:2000; Developmental Hybridoma, Cambridge, UK), rabbit anti-OPN (1:500; Abcam, Iowa City, IA), mouse anti-nestin (0.1 $\mu\text{g}/\text{ml}$; Pharmingen, La Jolla, CA), rabbit anti-GFAP (1:2000; Chemicon, Temecula, CA; or 0.0041 $\mu\text{g}/\text{ml}$; DAKO, Carpinteria, CA) and mouse anti-BrdU (0.1 $\mu\text{g}/\text{ml}$, Chemicon). For single antibody staining, brain sections were washed with PBS and pretreated with H_2O_2 before overnight incubation with primary antibody. After another wash, sections were incubated with species-specific biotinylated secondary antibodies and ABC reagent (Vector Laboratories, Burlingame, CA). Diaminobenzidine (Vector Laboratories) was used to visualize immunoreactivity. For double immunofluorescence, after a PBS wash, brain sections were incubated overnight in a mixture of two primary antibodies raised in different species (ie, mouse anti-OPN and rabbit anti-GFAP, mouse anti-nestin and rabbit anti-

OPN). Species-specific fluorescein isothiocyanate- and tetramethyl-rhodamine isothiocyanate-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were used to visualize double-fluorescent immunostaining with a Leica DMIRB fluorescent microscope. Confocal microscopy was performed with a LSM 510 Meta (Carl Zeiss, Thornwood, NY) and images were obtained at 2.2- μm optical sections. Specificity of immunostaining was confirmed by omitting the primary antibodies.

Quantitative Analysis of OPN Expression by Real-Time Polymerase Chain Reaction (PCR)

Poly A⁺ RNA was obtained using the QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech). cDNA was synthesized from 0.1 μg poly(A)⁺ RNA using the SuperScript first-strand synthesis system (Invitrogen). For quantitative real-time PCR, reaction mixtures (50 μl) contained cDNA derived from 0.005 μg of poly(A)⁺ RNA, 0.4 $\mu\text{mol}/\text{L}$ each of forward and reverse primers, and 1 $\times$ SYBR Green PCR Master Mix (Qiagen, Valencia, CA). Primers for OPN were 5'-TGA TGA CGA CGA CGA TGA CGA TGG-3' (forward) and 5'-ACG CTG GGC AAC TGG GAT GAC CTT-3' (reverse). Values were normalized to the expression of the housekeeping gene, GAPDH (forward primer: 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3', reverse primer: 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3'). Samples were preincubated for 15 minutes at 94°C in an MJ Research DNA engine continuous fluorescence monitor (Waltham) followed by 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute. Identity of the experimental PCR products were confirmed by comparing the melting points (T_m ; determined throughout the range of 55 to 90°C) to those of the standards. For standard curves, the respective templates were obtained by PCR amplification of cDNA from C6 rat glioma cell cultures followed by gel purification (QIAquick gel extraction kit, Qiagen) and spectrophotometric quantitation.

Enzyme-Linked Immunosorbent Assay (ELISA) of OPN Levels

Cells (1×10^6) were washed in phosphate-buffered saline and placed in six-well plates containing 1 ml of N2 medium supplemented with 20 mg/ml epidermal growth factor and incubated at 37°C in a 95%/5% CO₂ humidified atmosphere for 5 days. Medium was collected, centrifuged at 900 $\times g$ to pellet any cell material, and the supernatant frozen at -20°C. Cells were harvested by trypsin-ethylenediaminetetraacetic acid treatment, washed in PBS, and counted in a hemocytometer by light microscopy. The supernatant fluids were analyzed for rat OPN using a commercially-available ELISA kit (Assay Designs).

Transfection of ENU3.2 Cells with OPN-Expressing Plasmid

Full length OPN was purchased from Open Biosystems (clone no. 14978-b-3). The sequence was generated from a rat liver library and cloned into the pExpress 1 vector with a CMV promoter to drive expression. The plasmid was linearized by *ScaI* digestion before electroporation. Fifty μg of OPN and 10 μg of neomycin-resistance plasmid were added to a suspension of 1×10^6 ENU 3.2 cells suspended in 800 μl of cold PBS. Ten minutes later, cells were electroporated once at 800 V and 3 μF , after which cells were plated in a 10-cm dish in N2 media supplemented with 5% serum without drug selection. One week later, G418 was added to media. Half of the media was changed every 2 days and entire media completely replenished every 4 days.

In Vitro BrdU Incorporation

Cells (5×10^3) in 250 μl of media (N2 supplemented with 5% fetal bovine serum) were added to an eight-well culture slide that was coated with 0.1% gelatin for 15 minutes before the experiment. After a 1-hour incubation at 37°C, 250 μl of media containing BrdU (5 $\mu\text{g}/\text{ml}$) was added for 6 hours. BrdU was then removed and cells incubated in media without BrdU for an additional 30 minutes after which media was removed and cells fixed in 500 μl of ice-cold 4% paraformaldehyde for 15 minutes. After three 10-minute washes in PBS, slides were exposed to 2 N HCl for 15 minutes at 37°C and washed three times in PBS. After preblocking with normal donkey serum (Jackson ImmunoResearch) for 1 hour, slides were incubated in mouse anti-BrdU primary (Jackson ImmunoResearch) 1:500 overnight in the cold room. Cells were then washed three times and exposed to Cy3 (Jackson ImmunoResearch) donkey anti-mouse secondary 1:250 for 1 hour. After three PBS washes, slides were exposed to 4,6-diamidino-2-phenylindole (DAPI)-containing mounting media (Vector Laboratories). For each slide, photomicrographs of five randomly chosen fields were taken at $\times 20$. A blinded observer then counted both BrdU- and DAPI-labeled cells within the fields. Proliferation index was expressed as number of cells BrdU⁺/number of cells DAPI⁺.

Results

We examined serially cut coronal sections spanning an area encompassing the anterior corpus callosum to the posterior hippocampus in 38 rats, aged 15 to 163 days, exposed as embryos to ENU. No tumors were ever noted in rats less than 90 days of age ($n = 19$). Small tumors began being noted from P90 onwards. The size of the largest tumors observed increased as a function of age so that by P150 all rats examined had tumors greater than 1 mm (Figure 1, A–D).

To attempt acquisition of the smallest tumors possible, we began imaging two ENU-treated rats at 30-day intervals after P150 with MRI using heavily weighted T2 se-

Figure 1. A–C: H&E sections demonstrating early neoplastic proliferation (ENP) (A), microtumor (B), and macrotumor (C) that were noted in 90-, 115-, and 150-day-old rats, respectively, after ENU exposure *in utero*. Similar lesions are never observed in non-ENU-exposed rats. Tumor (T) margins are demarcated with dotted lines. D: Graphic depiction of tumor size in serially sectioned brains from ENU-exposed rats that were stained with H&E. Each data point represents an individual brain ($n = 38$) (there are less than 38 data points visualized because of overlap at 0 and >1 mm sizes). Line and r value represent results of a regression analysis.

quencing. We could not visualize a tumor mass until days 180 and 210, a time when their diameters exceeded 2 mm, suggesting that it is not possible to visualize tumors below this size using these particular sequence parameters. Both tumors (which were composed primarily of astrocytic appearing cells) were successfully punch-biopsied using the methodology outlined above (Figure 2).

Samples of frozen tissue were processed into cRNA, which was hybridized onto Affymetrix rat genome U34A microarray cartridges and read on a GeneArray scanner. We first performed a cluster analysis that revealed that the sample results split into two groups: the two sides contralateral from the localized tumors formed one cluster with the normal brain area while the two tumor samples formed the other. We then compared the average results of the two clusters after assigning a minimum target intensity of 150 for each result to dampen small differences.

Table 1 lists the 10 genes that were most over- and underexpressed in tumor relative to the normal brain cluster. Among the underexpressed genes were genes

Figure 2. Heavy T2-weighted image of an asymptomatic P210 rat exposed to ENU *in utero*. Tumor that was sampled is located in left cerebral cortex (arrow). Imaging 30 days before this study did not reveal tumor. Right: H&E of tumor sample. Pathology verified astrocytoma.

Table 1. Genes Most Differentially Expressed between Tumors and Normal Brain

GenBank no.	Gene name	Tumor/normal (fold)
Up-regulated in glioma compared to normal brain		
M14656	Osteopontin	21.3
X75207	Cyclin D1	15.7
M11596	Calcitonin-related polypeptide β	14.1
X62952	Vimentin	13.2
AA998683	Heat shock 27-kd protein	12.8
AI012030	Matrix Gla protein	12.3
AI169327	Tissue inhibitor of metalloproteinase (TIMP) 1	9.7
AI232379	Platelet-derived growth factor- α	7.5
X13016	CD48 antigen	7.5
X76489	CD9 mRNA	7.5
Down-regulated in glioma compared to normal brain		
S81478	PTPase = oxidative stress-inducible protein	0.25
U19866	Activity regulated cytoskeletal-associated protein	0.25
D10392	Syntaxin 1a	0.27
Y17048	Calcium binding protein 1	0.29
M62752	Statin-like protein	0.29
AF058795	G protein-coupled receptor 51	0.30
AA858621	CaM-kinase II inhibitor α	0.30
J03754	ATPase isoform 2, Na ⁺ K ⁺ transporting	0.33
U27767	Regulator of G-protein signaling 4	0.34
X83231	Pre- α -inhibitor, heavy chain 3	0.34

associated with the postmitotic state (statin) as well as genes expressed primarily by neuronal cells (syntaxin). By contrast, a number of genes associated with cell proliferation and increased motility were overexpressed in the tumor cluster. Of these, the most overexpressed gene (over 20-fold) was OPN.

Because our primary goal in this gene array analysis was to identify genes or proteins that could be used as markers of glioma development, we next sought to characterize the evolution of OPN expression in ENU-induced gliomas. Although we initially used two anti-OPN antibodies, our early studies revealed that the murine monoclonal antibody MP1110₁, obtained from Developmental Hybridoma, was superior, so this was then used exclusively for further studies. Using this antibody, we evaluated 17 gliomas in rats aged 115 to 163 days with tumor diameters ranging from 300 to 2200 μ m on adjacent H&E sections and noted OPN expression in all, leading us to conclude that this protein is universally expressed within established gliomas (Figure 3). Staining was identified

predominantly within large cells that were diffusely located within the tumor parenchyma (Figure 3B, inset). As tumor size increased, immunostained cells tended to be confined more toward the tumor edges. By contrast, no OPN staining was seen in CNS areas outside of the tumor bed, consistent with the very low levels of OPN expression noted in the microarray results of normal brain or uninvolved brain regions. Further, OPN staining was not observed in the two neuroinomas that we examined (data not shown), suggesting that increased expression is a characteristic specific to gliomas in this model.

We next sought to characterize OPN expression in normal brains as well as in ENU-exposed rats before the development of tumors (ie, before 90 days of age). No OPN expression was noted in normal brain sections, even in areas in which proliferative and migratory activity is high, such as SVZ (Figure 4). Furthermore, we did not detect OPN staining in ENU-exposed brains before the development of small tumors (ie, before P90, $n = 6$). Importantly, we did not observe any staining even in sections adjacent to ones that contained characteristic nests.¹⁴ Thus, OPN expression appears confined to established gliomas in this model.

Figure 3. Representative examples of OPN staining in ENU-induced gliomas. Tumors in **A–C** correspond to those depicted in Figure 1. **Insets** in **B** represent typical OPN-expressing cells visualized within all gliomas examined. In addition, a fine speckled pattern of staining is noted in extracellular spaces. Below each panel (**D–F**) are the same sections counterstained with the hematoxylin method to demonstrate tumor in more detail.

Figure 4. OPN is not expressed in normal brain, even in areas such as the SVZ, an area containing migratory, proliferating cells. Adjacent coronal sections at the level of the striatum stained with H&E (**left**), BrdU and nestin (**middle**), and OPN (**right**). No OPN staining is noted.

Figure 5. No evidence of co-localization of OPN and nestin in ENU-induced gliomas. **Arrows** delineate nestin⁺ cells expressing tetramethyl-rhodamine isothiocyanate (**A**) and **arrowheads** OPN⁺ cells expressing fluorescein isothiocyanate (**B**). A merged figure (**C**) with **arrows** and **arrowheads** included reveals no evidence of co-localization.

We next sought to determine further the characteristics of the OPN-expressing cells. Using double-staining techniques (described in Materials and Methods), we determined that there was no co-expression of OPN with nestin (Figure 5). However, we did note a close concordance with cells within the tumor that expressed GFAP (Figure 6), a finding further verified using confocal microscopy (Figure 7). These GFAP-expressing cells were much larger than the normal astrocytes noted in the surrounding parenchyma (Figure 6), suggesting that they were not normal astrocytes. OPN expression was exclusively and consistently observed only in GFAP⁺ cells: of 83 GFAP⁺

Figure 6. Co-localization of OPN and intratumoral GFAP-expressing cells. **A:** Brain-glioma interface (glioma is to the **left** of the **dotted line**) illustrating increased size of GFAP⁺ cells within tumor (**arrows**) compared to normal astrocytes (**arrowhead**). **B** and **C:** Higher magnification photomicrograph of area enclosed within the **inset** in **A** illustrating GFAP (green) and OPN (red) co-localization. A similar result was noted when the fluorophores were reversed.

intratumoral cells, 81 (98%) expressed OPN. In contrast, we could not identify OPN expression in any GFAP^{neg} cell.

Because the possibility remains that these GFAP⁺OPN⁺ cells represented reactive astrocytes, we also performed stab wounds in adult rat cortex and examined astrocytes in the area of injury 4 and 14 days later. Although areas of stab wound injury contain abundant GFAP⁺ cells, we could not identify a single instance of co-expression with OPN (which was barely detected under these conditions) (Figure 8). Furthermore, unlike the situation in reactive astrocytosis in which the rate of BrdU positivity within GFAP⁺ cells was negligible, we noted within gliomas a BrdU labeling rate of 23.4% in GFAP⁺OPN⁺ cells. Although not confirmatory, these findings are consistent with the contention that OPN is a marker for tumor progression.

To address when OPN begins to be overexpressed in tumor cells, we also compared *in vitro* OPN mRNA expression and protein secretion in normal SVZ/striatal stem cells as well as those that had been immortalized after ENU exposure *in utero*. A previous study from this laboratory indicated that, although immortal, these latter cells did not possess other typical characteristics of fully transformed cells, such as the ability to form tumors after transplantation.¹⁵ Using real-time PCR analysis, C6 rat glioma cells expressed more than 20-fold more OPN mRNA than either the immortalized cell lines or normal neurosphere cells [210.1 ± 19.8 fg OPN/20 fg GAPDH (SD) compared to values ranging from 0.47 ± 0.01 to 14.25 ± 2.5 , $P < 0.001$, one-way analysis of variance Bonferroni *t*-test]. Similarly, C6 secreted almost 20-fold more OPN into the media when analyzed by ELISA [187.1 ± 35.1 ng/ml (SD) compared to values ranging from 1.6 ± 0.9 to 10.0 ± 2.1 , $P < 0.001$, one-way analysis of variance Bonferroni *t*-test] (Figure 9). Interestingly, no difference was noted between the immortalized cell lines and normal neurospheres on analysis of variance analysis, suggesting that, consistent with our histological analysis, OPN overexpression does not occur until a full neoplastic phenotype is attained.

Finally, to ascertain whether OPN overexpression was correlated with cellular function, we introduced an OPN-expressing plasmid into ENU3.2 cells. ENU3.2^{OPN} cells expressed 10 times more OPN mRNA than native ENU3.2 cells, although these levels were still significantly less than C6 cells (54.2 ± 15.4 , 485.7 ± 60.4 versus 3327 ± 464.6 fg OPN/20 fg GAPDH for ENU3.2, ENU3.2^{OPN}, and C6, respectively; $P < 0.004$, one-way analysis of variance on ranks, $P < 0.05$ for all comparisons, Student-Newman-Keuls method) (Figure 10). In association with OPN overexpression, we noted a more rapid proliferation rate for ENU3.2^{OPN} cells compared to native ENU3.2. Thus, using BrdU incorporation as a measure, we noted a significant increase in the number of ENU3.2^{OPN} cells incorporating BrdU relative to ENU3.2 (Table 2). Therefore, even a relatively modest increase in OPN levels compared to C6 was enough to alter ENU3.2 cell proliferation, supporting the contention that OPN overexpression is functionally significant.

Figure 7. Confocal 2.2- μ m optical sections of GFAP and OPN staining in ENU-induced tumor cells of 150-day-old rat. GFAP (**A**) and OPN (**B**) co-exist in the same cell (**D**). Cell nucleus is stained blue with DAPI in **C**. **Arrows** in panels designate location of nucleus of OPN- and GFAP-labeled cell. Scale bar, 20 μ m. Original magnifications, $\times 800$.

Discussion

To adequately study the epigenetic phenomenon of glioma promotion *in situ*, tumors must invariably develop in an environment that is not continually exposed to muta-

Figure 8. Representative section of reactive astrocytosis produced by a stab wound illustrating no co-expression of GFAP with OPN. Normal rat was sacrificed 14 days after wounding and the section double stained for GFAP (green) and OPN (red). Cell density is higher in the wound area on the **left** of photograph. Reactive GFAP cells are aggregated at the edge of the injured brain in **center** of photograph. Hypertrophic GFAP-expressing cells similar to what was noted within ENU-induced gliomas were not seen. No OPN is visualized in the area; reddish substance within the wound reflects nonspecific staining of blood products. **Arrow** points to fluorescein isothiocyanate-labeled GFAP⁺ cell within the stab wound.

Figure 9. C6 glioma cells express more OPN mRNA as assessed by real-time PCR and secretes more OPN protein as assessed by ELISA than either immortalized cell lines ENU3.2, ENU4.30, and ENU4.1 (described in Savarese et al¹⁵) or normal neurosphere cells (RNS) isolated from P1 rat pups (isolation methods described in Savarese et al¹⁵). * $P < 0.001$, one-way analysis of variance. There is no statistical significance in the levels of OPN mRNA or protein between normal neurosphere cells and immortalized lines.

genic stimuli (so that the investigator can be confident that changes observed are not an effect of newly created mutations). Because ENU is cleared within minutes, after which a several-week latency period occurs before gliomas are noted, this model is optimal for studying this process and is perhaps the only one in which it may be possible to observe the transition from a preneoplastic state to a fully developed tumor. Nevertheless, despite its tractability from a temporal standpoint, the pathological study of this process crucially depends on the identification of suitable informative markers.

In our first study,¹⁴ we identified nestin-expressing cells that often appeared in clusters only in ENU-exposed brains as early as P30. Such cells did not have characteristics of reactive astrocytes and the size of these nests increased throughout time. The fact that ENU-induced gliomas are invariably nestin⁺ led us to propose that these cells and nests represented tumor precursor lesions. However, the ubiquitous presence of nestin in other brain areas, including the SVZ and endothelial cells,^{20–23} made this intermediate filament of limited use as a marker of either tumor development or progression.

Figure 10. ENU3.2^{OPN} expresses intermediate levels of OPN mRNA as assessed by real-time PCR compared to native ENU3.2 and C6 glioma. The differences in expression between each line is significant (* $P < 0.05$, Student-Newman-Keuls method).

Table 2. Proliferation Indices of ENU3.2, ENU3.2^{OPN}, and C6 Glioma after 6 Hours of BrdU Incubation

Line	No. cells counted	No. cells BrdU ⁺ (%)	<i>P</i> value (χ -square) versus ENU3.2
ENU3.2	627	167 (26.7)	
ENU3.2 ^{OPN}	625	293 (46.9)	<0.001
C6 glioma	959	430 (44.8)	<0.001

We reasoned that another way to identify potential markers would be to identify small ENU-induced tumors using MRI so that we could then compare their gene expression patterns with that of uninvolved brain regions. To determine the optimal time to do such imaging, we graphed glioma size as a function of time of sacrifice in 38 rats exposed to 50 mg/kg of ENU at E17/18. Consistent with previous pathological studies,^{24–30} we did not identify tumors before P90, at which time some rats harbored small tumors. After this time, tumors were seen with greater frequency until by 150 days of age, every brain examined histologically had a tumor >1 mm in size.

Using this information, we therefore began imaging rats at 150 days of age so as to be able to detect small lesions. It is interesting therefore that we could not visualize tumors by MRI until much later, when they had attained a diameter of at least 2 mm each. This suggests that it is difficult, if not impossible to view smaller tumors even using sensitive sequences such as heavily weighted T2. This result is consistent with an earlier report that suggested that ENU-induced gliomas below this size cannot be visualized.³¹

Although several genes of interest were noted to be markedly up-regulated in glioma compared to normal brain, we were especially intrigued that the most overexpressed gene was OPN, a protein that has been noted to be markedly up-regulated in several systemic tumors. Although its name reflects its important role in bone remodeling, OPN is a pleiotropic molecule that was originally described as a marker of epithelial cell transformation.³² It plays important roles in diverse systems ranging from the immune system, where it regulates cytokine production and cell trafficking, to the vascular system, where it inhibits ectopic mineralization and macrophage function.^{33,34} Although its expression is generally low within the CNS, up-regulation of OPN expression as assessed by either mRNA or protein levels has been reported in either astrocytes or microglia in experimental autoimmune encephalomyelitis, demyelination, and cortical injury.^{35–40}

OPN has also been proposed to play a significant role in tumorigenesis.^{41–44} Because it neither transforms normal cells nor is mutated in cancer,⁴⁵ OPN is considered more involved with tumor progression than tumor initiation. Several groups have noted elevated OPN expression associated with a metastatic phenotype of breast cancer cells^{16,17,45} and a microarray expression profiling study of a large number of colorectal tumors identified OPN as the lead candidate for a marker of tumor progression based on the observation that it was the most consistently up-regulated gene during sequential stages

of tumor progression.¹⁸ Although it has not been extensively studied to date as a mediator of glioma pathobiology, OPN is known to be overexpressed in human high-grade gliomas⁴⁶ and has been identified as one of the most overexpressed genes in a SAGE analysis comparing C6 rat glioma cells with normal rodent astrocytes.⁴⁷

Using a commercially available monoclonal antibody that was originally raised against a minor rat bone fraction, but which has subsequently been shown to specifically recognize OPN in many studies including those addressing CNS,^{48,49} we noted OPN expression in all tumors examined, including those that qualified as early neoplastic proliferations.³⁰ The cellular staining pattern was characteristic: several large cells were noted dispersed throughout small tumors while expression was confined more to the brain-glioma interface in larger ones. By contrast, no OPN staining was noted in uninvolved brain areas, even those in which there is increased proliferative and migratory activity, such as the SVZ.

Interestingly, especially in light of its putative role as a mediator of tumor aggressiveness,⁴⁵ we could not detect OPN in ENU-exposed brains before the development of early neoplastic proliferations (ie, <300 μ m in largest diameter) and found no evidence that it is expressed in the nests that appear as early as 30 days in this model, and which we hypothesize may represent an early precursor lesion. These findings suggest that the appearance of OPN expression is associated with the acquisition of the full glioma phenotype.

We suspected that OPN expression would be found in many cell types within the tumor, because its production has been reported in both tumor and stromal compartments.^{50–56} We were therefore surprised to find expression confined to a GFAP-expressing cell. We believe these cells are neoplastic based on their large size that was clearly distinguished from surrounding astrocytes (Figure 6) and the absence of OPN immunoreactivity in reactive astrocytes (Figure 8). This suggests that gliomas are developing through an orderly process in which nestin⁺ cells first appear followed by the appearance of a second GFAP⁺OPN⁺nestin^{neg} cell that arises later in the setting of increased cell growth. Consistent with this contention is the observation that increased OPN expression is a feature of a fully tumorigenic nitrosourea-induced cell line (C6) but not in initiated but not yet promoted cells isolated from neonatal ENU-exposed rats.¹⁵

Within the context of the multistage model, we therefore propose that the appearance of the GFAP⁺OPN⁺nestin^{neg} cells marks a transition point in the development of glioma in which tumors enter the stage of progression. This in turn raises several questions for further study. The first is whether the GFAP⁺OPN⁺nestin^{neg} cells are descendants of the nestin⁺ ones, eg, they represent differentiated progeny of nestin⁺ cells. Based on the fact that fully developed tumors are felt most often to be monoclonal,⁵⁷ this would seem likely. However, two alternative scenarios might be proposed. In one, the nestin⁺ cell is not in fact a preneoplastic cell. For example they may mark a tumor cell niche. On the other hand, although the

weight of evidence supports the contention that the GFAP⁺OPN⁺ cell is a neoplastic astrocyte, the alternative possibility that it represents a novel type of reactive astrocyte still needs to be resolved.

Finally, a second more important question, especially from a therapeutic standpoint, is whether OPN expression is necessary and sufficient to induce tumor progression. The observation that inducing increased OPN levels in immortalized cells results in increased proliferation is consistent with a functional role of OPN in this process. Thus, further studies are in progress to determine whether tumor development can be modified by either stimulation or inhibition of OPN expression.

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