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Elevation of osteopontin levels in brain tumor cells reduces burden and promotes survival through the inhibition of cell dispersal

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Abstract

Osteopontin (OPN) is a pleiotrophic molecule that has been associated with multiple disorders of the central nervous system (CNS). Its roles in CNS malignancy are unclear but suggest that higher levels of OPN expression correlate with increased tumor grade and increased migratory capacity of tumor cells. In this study OPN cDNA was cloned into a retroviral vector and used to infect F98 Fischer rat-derived glioma cells and U87 human-derived glioblastoma multiforme (GBM) cells in vitro. Cells expressing high levels of OPN migrated less distance than control cells in vitro. This

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effect was not RGD mediated, but was reversed in the presence of c-Jun N-terminal kinase (JNK) inhibitor suggesting that JNK1 is an essential component of a negative feedback loop affecting OPN activated signaling cascades. Implantation of tumor cells expressing high levels of OPN into adult Fischer rats and nude rats resulted in morphologically distinct tumors and prolonged host survival relative to controls. We propose that local produced, high level OPN expression limits the malignant character of glioma cells and that the downstream mechanisms involved represent pathways that may have therapeutic value in the treatment of human CNS malignancy.

Keywords

F98 cells; Glioma; Glioblastoma multiforme; Osteopontin; Tumor cell dispersal; U87 cells

Introduction

Osteopontin was initially identified as a protein secreted by malignant epithelial cells [14,25,32,33]. It is constitutively secreted in diverse tissues and inducible in T-lymphocytes, epidermal cells, bone, macrophages and tumor cells of various origins [11,16,23,36]. Multiple cellular functions are influenced by OPN including apoptosis, cellular proliferation, angiogenesis and cell migration within a pro-survival paradigm leading to the hypothesis that OPN expression represents a generalized cellular response to stress [13,21]. The pleotropic character of OPN may reflect its complex post-translational modification, extracellular protease cleavage and multiple cell-surface receptor interactions. Specifically, three isoforms are produced with subsequent translation producing a protein from 41 to 75 kDA. The various isoforms are associated with distinct tissue expression patterns and may mediate different cellular functions. Isoforms are further subjected to variable levels of phosphorylation, glycosylation and trans-glutamination [26]. After secretion, full length OPN is cleaved by thrombin resulting in an amino terminal fragment which contains a highly conserved RGD tripeptide sequence that facilitates binding to $\alpha v \beta 3$, $\alpha v \beta 1$ and $\alpha v \beta 5$ integrins [15] as well as a SVVYGLR sequence which mediates binding to $\alpha 9 \beta 1$ and $\alpha 4 \beta 1$ integrins [2,37]. The absence of the RGD binding-domain does not prevent OPN fragments from binding cell surface receptors [18]. Additionally OPN has multiple matrix metalloprotease (MMP) cleavage sites and MMP3 and MMP7 produced OPN fragments each have unique receptor binding profiles [1]. Given the number of possible integrin heterodimeric associations and CD44v combinations it is not surprising that diverse functions have been attributed to OPN.

Variability in cell surface receptor binding capacity and activation of signal transduction cascades may underlie differences in cellular responses to OPN [19,38]. Binding of the COOH-fragment to CD44 promotes cell survival and motility by activating PLC γ /PKC/PI 3-kinase pathways that are downregulated by PTEN [20]. Binding of OPN to $\alpha v \beta 3$ integrin stimulates a complex downstream signaling cascade mediated by PI 3-kinase/AKT dependent NF κ B activation and urokinase plasminogen activation (uPA). This pathway can also induce NIK-dependent NF κ B activation through IKK/ERK mediated pathways stimulating uPA-dependent MMP9 activation [9]. A further signaling cascade involves the stimulation of c-Src dependent epidermal growth factor receptor (EGFR) transactivation and activation of FAK, paxillin and vinculin which regulate cell attachment and migration [10,34].

The wide range of molecular interactions of OPN has hindered characterization of its role(s) in disease processes. Expression of OPN has been consistently associated with human malignancy, it is one of the most abundantly expressed genes in animal models of melanoma and brain tumors and experimentally has been demonstrated to play a crucial role in tumor

models of progression, invasion and metastasis [4]. Tumor grade has been correlated with expression level in humans, as has prognosis in patients with breast and lung cancers [7,30,31]. These observations, combined with low expression levels in benign tumors, suggest that OPN dysregulation is important in cell transformation or that elevated OPN expression contributes to malignant and metastatic character. In the brain OPN is intimately associated with the extracellular matrix (ECM) and its expression is developmentally regulated, suggesting that it may facilitate neuronal or glial cell migration [12,29]. Consistent with its role in the stress response, OPN has been implicated as a possible mediator of CNS inflammatory diseases, as a neuroprotective agent in acute stroke and is up-regulated in models of CNS degenerative disease and epilepsy [6,22,35].

This study was designed to determine if OPN over-expression in CNS-derived tumor cell lines would affect the malignant nature of the brain tumor. F98, Fischer rat-derived, high-grade glioma cells and U87, human-derived, GBM cells were transfected with a retrovirus expressing the OPN cDNA and subsequently implanted into adult Fischer rats or nude rats respectively. Rats receiving implants of cells expressing OPN at high levels survived longer than rats receiving wild type implants or cells expressing lower levels of OPN. We demonstrate that cells expressing high levels of OPN have decreased migratory capability in vitro. Furthermore, we show that JNK contributes to this effect as JNK inhibitor reverts the migratory capacity of high expressing OPN tumor cells to that of control cells. Taken together the data suggests that elevated levels of OPN actually limit the malignant character of CNS tumor cells through a JNK1 mediated negative feedback pathway.

Materials and methods

Cloning of retroviral vectors

The retroviral expression vector pLXCGN was constructed by ligation of the enhanced green fluorescent protein (EGFP) cDNA (Clontech, Palo Alto, CA) into the prototype mouse Moloney Leukemia Virus retroviral vector pLXCN upstream and in frame with the neomycin phosphotransferase gene (supplemental data). The OPN cDNA was obtained via RT-PCR amplification using RNA isolated from a human primitive neuroectodermal tumor cell line. Primers were as follows: OPN forward: (5'GACCAAGGAAAACCTACTA3') and OPN reverse: (5'CTCCTT TTAATTGACCTCA3'). RT-PCR amplicon was blunt end ligated upstream of the CMV promoter sequence in the parent vector pLXCGN resulting in the new vector pL(OPN)CGN.

Harvesting of amphotrophic retrovirus

PA317 cells were transfected with pLOPN/CGN and selected in 800 µg/ml G418. Individual subclones were identified that produced high titer viral supernatant ($\geq 1 \times 10^6$ colony forming units/ml) from which virus was harvested.

Osteopontin ELISA

A 96-well plate was coated with 2-µg/ml anti-human OPN antibody (Santa Cruz, Santa Cruz, CA; SC21742). After a 16-h incubation at room temperature (RT), the plate was washed with buffer (PBS + 0.05% Tween 20) and blocked (PBS + 0.05% Tween20, 0.5% bovine serum albumin) for 1 h. Media from various cell cultures was added to wells and incubated for 2 h. Wells were washed and OPN antibody (Santa Cruz; SC21742; 1:5,000) added to each well for 2 h. After washing three times biotinylated secondary (Santa Cruz; SC21742; 1:50,000) was added and incubated for 2 h. Finally, the plate was incubated in streptavidin–HRP complex, incubated in tetramethylbenzidine: H₂O₂ (1:1) for 30 min at RT and absorbance determined at 450 nm on a microplate reader. Samples and standards were

run in triplicate and results expressed as mean concentration of OPN per milliliter of cell culture supernatant per 24 h per million cells. The entire experiment was replicated twice.

In vitro apoptosis assay

The ability to induce apoptosis was determined using the Cell Death Detection Elisa Plus (Roche Inc). Cells were plated into a 96-well plate and cultured overnight in topotecan (Sigma) at concentrations ranging from 1 to 4 μ M. Cells were then washed and incubated in lysis buffer for 30 min. Lysate was harvested and centrifuged at 200 g for 10 min. Supernatant was transferred to the supplied streptavidin coated wells and 80 μ l of the supplied immunoreagent added to each well followed by a 2-h incubation period with gentle shaking. Wells were subsequently washed with incubation buffer and incubated with ABTS substrate for 10 min. Photometric analysis was performed at 405 nm with a reference wavelength of 490 nm using an ELISA plate reader. Final absorbance was calculated by subtracting absorbance generated from wells containing only culture media, but no cells, and expressed in mU. From these measures an enrichment factor (mU of sample divided by mU of untreated cells) could be calculated. The enrichment factor expressed the degree of increase in apoptotic activity following the addition of topotecan in that particular cell culture.

All sample lysates generated for ELISA analysis were produced and analyzed in duplicate. The entire experiment was repeated to confirm results.

In vitro cell viability assay

Cell viability was quantified using a MTT [(3-[4,5-dimethylthiad-2-yl]-2,5-diphenyl tetrazolium bromide)] cell viability kit (Roche). Cells were plated in a 96 well plate prior to the administration of 10 μ l of labeling reagent. Labeling reagent was incubated in culture for 4 h followed by the addition of 100 μ l of solubilization buffer and overnight incubation at 37°C in a humidified, 5% CO₂ incubator. Cell culture metabolic activity was quantified using an ELISA reader at 550–600 nm. All cells were plated and evaluated in duplicate. The entire MTT assay was replicated once to confirm results.

In vitro cell proliferation assay

Briefly, 10³ cells were plated on cover slips and cultured overnight. BrdU solution was added to a final concentration of 10 μ M and cells grown an additional 16 h. Cells were permeabilized with acid methanol and hydrochloric acid and incubated with anti-BrdU antibody (Roche Inc.) in 10% NGS/0.5% Tween20/0.1 M PBS for 30 min. Cells were then incubated with secondary IgG-HRP in 10% NGS/0.5% Tween 20/0.1 M PBS for 30 min. After a final wash in PBS, slides were incubated for 5 min in DAB (Sigma) and viewed with light microscopy. The proportion of cells incorporating BrdU was determined by manual counting. The analysis was repeated three times per cell population and a mean proliferative index determined.

In vitro migration assay

The migratory capacity of cells was analyzed in vitro using an agarose gel drop assay and a slice culture model. Cells were trypsinized, counted and resuspended in serum-free DMEM with 0.3% low melting point agarose pre-heated to 37°C at a concentration of 4×10^7 cells/ml. Subsequently, 2 μ l of cells were dropped onto coverslips coated with fibronectin (10 μ g/ml) or laminin (20 ng/ml) in a 24 well plate. The 24 well plate was incubated at 4°C for 20 min to allow the agarose to solidify after which DMEM/5%FBS was added to the 24-well plate and cells placed in a 5%CO₂ room air incubator. The distance from the edge of the

agarose gel was measured at 24 and 48 h. The JNK inhibitor, SP600125 (Sigma), was added to culture medium at 50 μ M concentration.

For slice culture experiment rats were anesthetized and perfused with 100 ml of 0.9% sterile saline at 4°C. Intact brains were harvested and imbedded in 2% low melting agarose/serum free DMEM and then 500 μ m sections prepared using a vibrating microtome. Sections were transferred to membrane inserts (Millipore) and placed in 6-well plates containing injection media: NB + B27 + PSF. Cells were then injected (3,000 cells in 1 μ l media) into the lateral aspect of the corpus callosum. Upon completion of injections, media was changed to 10%FBS/DMEM and cultures maintained in a 5%CO₂ room air incubator.

Isolation of adult primary astrocytes

Astrocytes were obtained by surgical implantation of gelatin sponge (Gelfoam) into the brain white matter of adult Fischer rats. A scalp incision was made in the anesthetized rat followed by the formation of a burr hole. A cannula was advanced with stylet in place to established coordinates (3.2 mm anterior and -3.0 mm lateral to bregma at a depth of 2.0 mm). Eventually, the stylet was removed and deeper cannula penetration with suction allowed for the formation of a biopsy cavity which was filled with Gelfoam.

Three days later Gelfoam was removed and manually minced, incubated in trypsin and DNase, passed through a 20 μ m mesh and suspended in DMEM/20% FBS.

Cell implantation

Implantation of cells was conducted according to approved protocols in accordance with NIH guidelines. Rats weighing 260–300 g were anesthetized with ketamine (80 mg/kg) and xylazine (4 mg/ml) and placed in a stereotactic frame. A scalp incision was made and a right-sided burr hole through the skull, 1.0 mm anterior and -2.0 mm lateral to bregma at a depth of 5.5 mm was made. 1×10^5 tumor cells were injected in 5 μ l of serum free media using a 10 μ l Hamilton syringe/No. 26S-gauge needle. Co-implantation experiments were conducted by implanting 2.5×10^4 F98/WT cells and 2.5×10^4 F98/OPN cells in 5 μ l serum free media or 2.5×10^4 F98/WT cells and 2.5×10^4 adult, primary astrocytes engineered to express osteopontin (AA/OPN) in 5 μ l serum free media.

Analyses of rat brains post implantation

Animals were euthanized, brains removed, snap frozen in liquid nitrogen and stored at -70°C until processing. Tissue sections were prepared using a cryostat.

BrdU labeling index

Four adult Fischer rats were implanted with F98/OPN 12 $\times$ cells and four with F98/GFP cells. On day 13 post-implantation animals received an intraperitoneal injection of 100 mg/kg BrdU labeling mix (Sigma). Twenty-four hours later animals were sacrificed and immunohistochemistry performed with anti-BrdU antibody (Roche Inc.). The number of positive cells was counted within the borders of the tumor mass identified by GFP signal. Three sections from each animal were analyzed and positive cells counted within three visual fields at 100 $\times$ magnification resulting in an apoptotic BrdU+ cell/visual field figure that was used in statistical calculations.

Calculation of tumor volume

Four adult Fischer rats were implanted with F98/OPN12 $\times$ cells and four with F98/GFP cells and electively sacrificed on day 14 post-implantation. Rat brains were removed in whole and then serial sectioned at 10 μ m until tumor was no longer visualized on sections under

fluorescent microscopy. Every 100 μm a thin section with tumor cells was photographed, the GFP positive region outlined and the tumor area per section calculated. The tumor volume was calculated and re-constructed using the FDA approved 3D-Doctor software (Able Software Corp., Lexington, MA).

TUNEL assay for apoptosis in vivo

Tissue was analyzed for apoptosis using the Insitu Cell Death Detection Kit (Roche Inc.). Tissue sections were fixed in 4% paraformaldehyde for 20 min, washed and then permeabilized with 0.1% TritonX-100, 0.1% sodium citrate on ice TUNEL reaction mixture was added and incubated for 1 h at 37°C. Slides were washed and then visualized using the fluorescent microscope. Three sections from each animal were analyzed and positive cells counted and averaged producing an apoptotic cell/visual field figure that was used in statistical calculations.

Immunohistochemical staining

Immunohistochemical staining was conducted using antibodies to lymphocyte common antigen (Santa Cruz, Santa Cruz, CA; SC1178), ED-1 (Chemicon MAB435), CD-31 (Chemicon MAB1393), BrdU (Roche Inc. 1170376) and Osteopontin (Santa Cruz; SC21742).

Statistical analysis

Based on the number of days the rat survived after tumor implantation a Kaplan–Meier plot was produced, hazards ratio was determined and Chi Square was computed to determine the significance between groups for survival time based on the curves. The end point for these statistics was death prior to 90 days. Differences between cell populations for the MTT assay and apoptosis studies were compared using a standard two-tailed *t*-test.

Results

Expression of osteopontin in cell lines in vitro

To assess the effects of expression of different levels of osteopontin on the behavior of brain tumor cells, cell cultures expressing significantly different levels of OPN were generated from two parent cell lines. The amplification of OPN cDNA was confirmed by sequencing and comparing the results to GenBank OPN sequence data. The cloned OPN amplicon contained exons: 2, 3, 4, 5, 6 and 7 (compared to GenBank-D14813). Cells were exposed to viral supernatant and determined to be 100% GFP positive after three rounds of infection with further rounds increasing OPN expression levels. Supernatants derived from overnight cell cultures were then used to quantify the level of OPN produced by transfected cells via ELISA (Fig. 1a). F98 cells infected 12 times (F98/OPN12 $\times$) with pL(OPN)CGN virus produced an average of 9.03 ng of OPN per milliliter/million cells cultured. Cells infected three times (F98/OPN3 $\times$) and six times (F98/OPN 6 $\times$) produced 3.34 ng of OPN per milliliter/million cells cultured and 5.21 ng of OPN per milliliter/million cells cultured respectively while those infected with pLXCGN (F98 WT) produced an average of 3.15 ng of OPN per milliliter/million cells cultured (Fig. 1a; $P < 0.001$). Similarly, U87 cells infected with pL(OPN)CGN virus nine times produced an average of 6.42ng of OPN per milliliter/million cells cultured (U87/OPN) while those transfected with pLXCGN produced an average of 2.87 ng of OPN per milliliter/million cells cultured (U87/WT) ($P < 0.01$). Finally, adult astrocytes (AA) infected with pL(OPN)CGN virus (AA/OPN) twelve times produced an average of 4.03 ng of OPN per milliliter/million cells cultured compared to controls transfected with pLXCGN that produced an average of 0.93 ng of OPN per milliliter/million cells cultured (Fig. 1a; $P < 0.01$).

Expression levels of OPN did not affect cell viability

To determine whether expression levels of OPN altered cell viability an MTT assay was used. Cell viability was not significantly different between F98/OPN12 $\times$ expressing cells and F98 cells expressing only GFP (Fig. 1c). A comparison of cell proliferation by BrdU incorporation assay did not reveal any significant difference between F98 WT ($43 \pm 5\%$) and F98/OPN12 $\times$ ($38 \pm 4\%$) cell populations at 16 h (Fig. 1b). U87/OPN cells had significantly less viability than U87/GFP cells using the MTT assay (data not shown) while BrdU incorporation failed to detect significant differences in proliferation rates between the U87/OPN and U87/GFP cells (Fig. 1b).

Baseline and topotecan induced enhanced apoptosis were compared. A trend towards higher levels of baseline apoptosis in F98/OPN12 $\times$ and U87/OPN cells was seen (Fig. 1d); however induction of apoptosis after topotecan addition was significantly higher ($P < 0.05$) in control populations compared to OPN expressing cells (data not shown). These data suggest that overexpression of OPN in F98 or U87 cells does not markedly alter the intrinsic metabolism, proliferation or cell death levels in vitro.

OPN levels dynamically regulate tumor cell migration

The ability of tumor cells to migrate was tested using both an agarose drop method and a slice culture model (Fig. 2a, a'). Migrational analyses were restricted to F98 cells since they were inherently more motile than U87. In both models, cells expressing high levels of OPN did not migrate as far as F98/WT ($P < 0.01$). F98 WT cells migrated an average of 649 μm ($n = 6$) compared to 315 μm for OPN WT cells ($N = 6$) over 48 h in an agarose drop model. The response to OPN did not appear to be linear. Cells expressing intermediate levels of OPN (F98/OPN6 $\times$) migrated more readily (average migration 1,272 μm ; $n = 6$; $P < 0.5$) than wild type cells.

To assess the contribution of OPN cleavage and subsequent integrin binding to decreased migration in high expressing cells, RGD peptide was added to culture media and the agarose drop assay repeated ($n = 6$). The addition of RGD peptide did not affect the migratory capacity of F98/GFP cells or high expressing OPN/F98 cells (Fig. 2b, b') This suggests that the inhibitory affect of high level OPN expression is not mediated through OPN binding to integrin receptors at the cell surface.

To determine the mode of action of OPN, conditioned media was obtained from a culture of F98/OPN cells and applied to cells in the agarose drop assay system. The extent of migration was not different for either cell population in conditioned media compared to control media suggesting that high levels of OPN inhibit migration either through an autocrine mechanism or via an intracellular mechanism (Fig. 2c, c').

JNK1 has been shown to inhibit the downstream cascade initiated by OPN binding to cell surface receptors. To assess the role of JNK1 in mediating decreased migration, the agarose drop assay was performed in the presence of the JNK1/JNK2 inhibitor, SP600125. In the presence of SP600125 the extent of migration of high expressing OPN cells, F98/OPN12 $\times$, increased such that they migrated 671 μm ($n = 3$) comparable to F98 WT cells (average migration 703 μm ; $n = 3$). The ability of JNK inhibitor to revert the migratory capacity of F98/OPN12 $\times$ to control levels suggests that JNK mediates the inhibition of cell migration observed in high expressing OPN cells (Fig. 2d, d').

To determine whether OPN regulated tumor cell dispersal in adult brain parenchyma cells expressing different levels of OPN were injected directly into slice preparations (Fig. 3). Cells expressing high levels of OPN (F98/OPN12 $\times$) dispersed through the corpus callosum 72-h post-implantation but failed to disperse throughout the rest of the brain parenchyma

even after 10 days. By contrast F98 WT cells migrated throughout the ipsilateral corpus callosum and by day 10 post-implantation cells were identified at midline while the more migratory intermediate OPN expressing cells (OPN6 $\times$) were widely disseminated and located in the contralateral corpus callosum 10 days after implantation. These data suggest that the level of expression of OPN regulates the migratory behavior of brain tumor cells both in vitro and ex vivo.

Elevation of OPN expression levels in tumor cells results in extended host survival following intracranial implantation

The days to death (significant tumor burden as defined by Institutional Animal Care and Use Committee at Case Western Reserve University) was recorded for animals receiving intracranial implants of equivalent numbers of either F98/GFP ($n = 9$) cells or F98/OPN12X ($n = 9$). All animals that received an intracranial implant of F98/GFP cells developed neurological impairment and were sacrificed within 30 days of implantation. By contrast only 2 of 9 rats receiving intracranial implants of F98/OPN cells were sacrificed secondary to tumor burden. The remaining animals were electively sacrificed 90 days post-implantation. A Kaplan–Meier survival curve was generated (Fig. 4a) and Chi square analysis revealed the two survival curves were statistically different ($P < 0.0001$) with a hazards ratio = 0.0903 (95% CI, 0.0091–0.1687).

Parallel studies were conducted on nude rats that received intracranial implants of equivalent numbers of U87/GFP cells ($n = 11$) or U87/OPN cells ($n = 11$). All animals in the control group developed neurological impairment by 32 days post implantation. By contrast, 7 of 11 rats receiving U87/OPN died prior to the endpoint of 90 days. A Kaplan–Meier survival curve was generated (Fig. 4b) and Chi square analysis revealed the curves were statistically different ($P < 0.0001$) with a hazards ratio = 5.69 (95% CI, 7.0380–101.1102). The differential survival of experimental animals may reflect either tumor cell intrinsic or environmental effects. To distinguish between these possibilities F98/WT cells were co-implanted with AA/OPN. The survival of rats after co-implantation (Fig. 4c) of F98 WT cells with AA/OPN cells ($n = 5$) was not significantly different from control rats ($n = 5$) co-implanted with F98/WT and AA/GFP ($P < 0.7634$ and hazards ratio = 1.1792; CI, 0.2968–5.2369). Finally, the survival of rats co-implanted (Fig. 4d) with F98 WT and F98 OPN/12 $\times$ ($n = 6$) was not significantly different from control rats ($n = 6$) implanted with F98/WT ($P < 0.1031$ and hazards ratio = 0.4532; 95% CI, 0.0749–1.2691). The inability of environmentally elevated levels of OPN to promote survival of animals receiving WT F98 cells suggests that the effects of OPN are largely tumor cell intrinsic.

Elevated expression of OPN alters size, tumor morphology and cellular characteristics after intracranial transplantation

Animals that received F98/GFP cells had a characteristic large volume, compact tumor mass with scattered areas of necrosis (Fig. 5). The average tumor volume 14 days post-implantation for F98/GFP derived tumors was 12.02 mm³. In contrast, F98/OPN12 $\times$ -derived tumors had more extensive central areas of necrosis. In non-necrotic regions OPN12 $\times$ tumors had decreased cellularity with increased extracellular matrix material. In general cells comprising the tumor appeared larger than control tumor cells and overall tumor volume (6.32 mm³) was less than control tumors (Fig. 5). The majority of animals implanted with F98 OPN12 $\times$ cells survived to 90 days post-implantation, although dispersed GFP positive cells were seen near the original site of implantation along with decreased cellularity, no clearly defined tumor mass was detectable (data not shown). U87/OPN-derived tumors were also characterized by decreased cellular density relative to U87/GFP tumors with increased ECM deposition.

To determine the extent and relative levels of OPN expression in the different tumors frozen sections were labeled with anti-OPN antibody. Tumors from F98/OPN12 $\times$ and U87/OPN implanted rats both had high levels of anti-OPN labeling in the tumor mass that did not extend beyond the tumor mass (data not shown). U87/GFP and F98/GFP tumors had reduced expression within tumors which was variable and in some cases extended beyond the tumor mass. Differences in the appearance of OPN tumors may reflect the infiltration of phagocytic cells since OPN has been proposed to be chemoattractive for these cells. Labeling with anti-ED1 antibodies demonstrated extensive infiltration in both control and experimental groups. While in control animals microglial/macrophage infiltrates were largely confined to the border of the tumor and the surrounding tissue (data not shown). Staining with LCA to detect lymphocytic infiltration showed no significant differences.

OPN alters tumor cell proliferation and death

BrdU labeled cells and TUNEL positive cells were quantified (Fig. 6). A significant increase in the number of BrdU positive cells were identified within the F98/OPN12 $\times$ tumor when compared to controls ($P < 0.001$). In contrast, the number of apoptotic cells identified through TUNEL assay were significantly less in F98/OPN12 $\times$ tumors compared to controls ($P < 0.05$). Staining for blood vessels using anti-CD31 antibody revealed a decrease in the number of vessels in OPN12 $\times$ derived tumors compared to controls (Fig. 6). Taken together these data indicate that tumors derived from cells that over express OPN differ in a number of important characteristics from those derived from wild type cells.

Discussion

Osteopontin is a highly conserved molecule with a diverse spectrum of cell surface receptors and biological functions. In many types of human malignancies and multiple animal models of tumor formation, OPN is significantly overexpressed suggesting it contributes to tumor formation or progression [4,7,32,33]. Based on these observations we anticipated that animals receiving intracranial implants of tumor cells expressing high levels of OPN would trend towards decreased survival time relative to controls. In contrast, here we show that tumor cells expressing high levels of OPN failed to generate aggressive tumors in vivo resulting in significantly prolonged survival, and in some animals, no signs of tumor formation.

To provide insights into this phenomenon the potential mechanisms of OPN in CNS tumor formation have been examined both in vitro and in vivo using two distinct cell lines. In vitro analyses of cell proliferation, metabolism and cell viability failed to reveal any significant tumor cell intrinsic effects. The migration of cells expressing high levels of OPN, however, was significantly reduced in agarose drop and brain slice assays. In vivo, high OPN expressing cells demonstrated an increase in proliferative activity and a significant decrease in apoptosis consistent with previous studies demonstrating OPN stimulates cell proliferation and inhibits apoptosis. Unexpectedly, host animals implanted with cells expressing high level of OPN had longer survival intervals, altered tumor morphologies, differential recruitment of macrophages and smaller tumor masses. These alterations were not seen when OPN secreting astrocytes were co-injected with tumor cells suggesting an intracellular or autocrine OPN signaling cascade is mediating these responses. Consistent with this, the diminished dispersion observed in migration assays was not replicated in control cells cultured in conditioned media containing high-levels of OPN further supporting an autocrine or intracellular mechanism of action.

One of the most striking effects of altering OPN expression levels was on tumor cell migration. Compared to wild type cells, cells expressing high levels of OPN were less migratory both in agarose drop and slice migration assays. The migratory/invasive

properties of a tumor are particularly important clinically as it prevents adequate surgical debulking contributing to the dismal prognosis associated with high-grade CNS malignancies. Previous studies indicate that in other systems OPN enhanced migration is directly dependent upon upregulation of uPA, MMP2 and MMP9 by distinct cell signaling pathways. Pre-treatment of cells with MMP2 and MMP9 antibody or uPA inhibitors effectively reduces OPN stimulated cell migration. Three major pathways have been described and are activated through binding of OPN to either integrin receptor combinations or CD44 receptor variations. These pathways converge resulting in upregulation of ECM degrading enzymes [28]. The mechanisms underlying the differential effect of intermediate and high levels of OPN expression in the current study is unclear. In intermediate OPN expressing cells the enhanced migration was blocked by addition of the RGD peptide, directly implicating the involvement of integrin receptors in this response. By contrast, the reduced migration observed in high expressing cells was not altered by RGD peptide suggesting that the inhibitory mechanism was not mediated by cleaved OPN binding to integrin receptors at the cell surface.

The influences of OPN appear to be negatively regulated by distinct signaling pathways. For example, PTEN has been proposed to downregulate OPN induced Akt activity thereby reducing its anti-apoptotic influence [24]. Similarly a significant negative cross talk has been demonstrated between the NIK/ERK and MEKK1/JNK1 pathways resulting in a down regulation in OPN stimulated ECM degradation [27]. Here we demonstrate that in the presence of the JNK inhibitor the suppression of migration by high levels of OPN is reversed and the cells revert to wild type migratory behavior. JNK1 is a member of the mitogen-activated protein kinase family, which includes isoforms JNK2 and JNK3. Although JNKs have shared substrates, they also maintain isoform selective substrates and binding partners as well as distinct tissue distributions and subsequently support a complex interplay among diverse signaling cascades and downstream effectors that seem to be conflicting [3]. JNK1 had been implicated in cell responsiveness to apoptotic signals, microtubule and cytoskeletal organization, focal adhesion and cell migration. Sustained levels of JNK1 in tumor cells has been associated with increased apoptosis, decreased cell survival [5] and in neural stem cells—altered migratory capacity, neural dendritic processes and synapse formation. Implantation studies indicate increased tumorigenicity of U87 cells engineered to overexpress JNK2 α 2, while the same cell line overexpressing JNK1 isoforms were found to be G1 arrested with decreased ability to form colonies on soft agar, an inability to activate AKT and a decreased tumor volume in vivo [8].

Altering the expression levels of OPN in tumor cells altered the morphology, cellular characteristics and size of the tumors that formed. In the presence of high levels of OPN tumors had increased necrosis and a thin appearing ECM. Evaluation of tumor volume at 2 weeks post-implantation revealed a significant reduction in tumor mass (nearly 50%) relative to tumors derived from WT cells. The volume limitation and increased necrosis suggested a decrease in neo-angiogenesis. This was confirmed with anti-CD31 staining as the number of vessels in high OPN expressing tumors was significantly reduced compared to that in tumors derived from wild type cells. The mechanisms by which OPN modulates angiogenesis are poorly defined but it seems likely this is an indirect effect dependent in part on the release of cytokines and chemokines from infiltrating cells [17]. Given the more uniform distribution of macrophages in the OPN derived tumors and increase in neovascularization might have been predicted, however the precise spectrum of cytokines elicited by the two types of tumor has not been assessed and specific differences are likely to account for reduced angiogenesis. Alternatively, the decreased number of vessels identified in OPN expressing tumors may be a consequence of tumor volume reduction rather than being directly related to OPN expression.

Finally, macrophages were actively recruited into OPN tumors but not WT tumors. This is consistent with the reported macrophage-chemoattractant properties of OPN. In our work the ability of exogenous OPN to recruit macrophages in the absence of prolonged animal survival suggests that macrophage mediated immuno-surveillance did not contribute to the OPN-mediated anti-tumor effect. Recruitment of macrophages and microglial cells in athymic nude male rats (rnu/rnu) was similar to that seen in euthymic counterparts as the nude rats used in our studies have normal macrophage numbers.

Conclusion

In summary, we propose that the effects of OPN on migration are concentration dependent and that once a threshold expression is exceeded OPN negatively impacts the malignant character of brain tumors. The presence of a negative feedback loop is not surprising given that the pro-survival signals, which OPN expression mediates, are pertinent to cell transformation and tumor progression. The negative feedback appears to be mediated through JNK1 activity resulting in diminished migration and neoangiogenesis which dramatically altered survival in two models of GBM. The enhancement of JNK1 negative feedback on OPN stimulated cellular processes may therefore represent an important therapeutic target for human CNS malignancy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Expression of elevated levels of OPN does not significantly alter the proliferation or death of tumor cells in vitro. **(a)** Infection with pL(OPN)CGN resulted in increased expression of OPN in multiple cell types. F98 cells transfected with pL(OPN)CGN expressed significantly higher levels (9.03 ng/ml/ 10^6 cells; $P < 0.001$) of OPN than F98 cells transfected with control virus pLXCGN (3.15 ng/ml/ 10^6 cells). Likewise U87 cells infected with pL(OPN)CGN expressed significantly higher levels (6.42 ng/ml/ 10^6 cells; $P < 0.01$) of OPN than U87 cells infected with pLXCGN (2.87 ng/ml/ 10^6 cells). Finally, adult primary, rat astrocytes (AA) infected with pL(OPN)CGN expressed significantly higher levels (4.03 ng/ml/ 10^6 ; $P < 0.01$) of OPN than AA cells infected with pLXCGN (0.93 ng/ml/ 10^6). **(b)** Elevated expression of OPN did not significantly alter cell proliferation rate. No significant differences were detected in the proportion of BrdU at 16 h between F98 and U87 OPN expressing cells and controls. **(c, d)** Elevated levels of OPN did not alter cell viability and cell death. No significant difference between F98 WT and F98 OPN12 $\times$ cells was detected using an MTT assay. Apoptosis levels were not significantly different in F98 OPN12 $\times$ cells relative to control. Error bars represent ± 1 standard error

Fig. 2.

Diminished cell migration is reverted in the presence of JNK inhibitor but not RGD peptide. Agarose drop migration assay was repeated in the presence of JNK inhibitor, SP600125 ($n = 3$). JNK inhibitor reverted the migratory capacity of high expressing OPN (12 $\times$) cells to that of WT cells suggesting the JNK1 activity mediates the OPN12 $\times$ diminished migratory phenotype. The presence of RGD peptide in culture media ($n = 6$) did not affect the migratory capacity of OPN12 $\times$ cells suggesting that OPN binding to integrin receptors was not responsible for the diminished migratory capacity of these cells. Finally, media obtained from OPN12 $\times$ cell culture applied to WT F98 cells did not inhibit migration suggesting that the diminished migratory capacity of OPN12 $\times$ cells is mediated by an intracellular or autocrine mechanism (arrows represent distance traveled from agarose drop with mean distance noted above)

Fig. 3.

Expression of OPN alters the migration of tumor cells. Cells expressing high levels of OPN (F98 OPN12 $\times$) failed to migrate (a) as far as F98 WT cells (c) at 48 h (lower panels). In contrast intermediate expressing OPN cells (b) migrated more extensively ($n = 6$). This data was confirmed in brain slice cultures which were examined at 3 days (represented as green on brain slice image) and 10 days post-implantation (represented as red on brain slice image). At 3 days post implant F98 OPN12 $\times$ appeared to have dispersed from the site of implantation while F98 OPN 6 $\times$ and F98 WT appeared localized near the site of implantation. However, when examined at 10 days F98 OPN12 $\times$ cells had failed to migrate further while F98 WT and F98 OPN6 $\times$ migrated through the corpus callosum

Fig. 4.

High levels of OPN in tumor cells prolongs survival time after implantation to the CNS. **(a)** The number of days to death (significant tumor burden) was determined and data represented with a Kaplan–Meier plot. A hazards ratio was calculated (0.0903, 95% CI 0.0091–0.1697) and Chi square analysis computed ($P < 0.0001$) for F98 implanted into Fisher rats. Solid line (—) represented animals implanted with OPN12 $\times$ expressing cells and the dashed line (---) represented control animals. **(b)** U87 cells implanted into nude rats produced a Chi square analysis that demonstrated the two survival curves were statistically different ($P < 0.0001$) with a hazards ratio = 5.69 (95% CI, 7.0380–101.1102). Solid line (—) represented animals implanted with OPN12 $\times$ expressing cells and the dashed line (---) represented control animals. **(c)** The survival of rats co-implanted with F98 WT and AA/OPN cells (solid line,—) was not significantly different from control rats (dashed line, ---) co-implanted with both F98 WT and AA/GFP cells ($P < 0.7634$ and hazards ratio = 1.1792; CI, 0.2968–5.2369). **(d)** Similarly the co-implantation of F98 OPN12 $\times$ with F98 WT (solid line,—) did not significantly alter the survival of rats compared to controls (dashed line, ---) implanted with F98 WT cells ($P < 0.1031$ and hazards ratio = 0.4532; 95% CI, 0.0749–1.2691).

Fig. 5.

Tumors derived from cells expressing high levels of OPN have decreased cellular density and increased necrosis. H&E staining was performed using representative sections from animals in the survival study. F98 WT tumors were extensive with central areas of necrosis (**a**). F98 WT cells small with dense extra cellular matrix material (200 $\times$; day 19 post-implantation). F98 OPN12 $\times$ tumors demonstrated increased areas of necrosis, increased extracellular matrix material and larger cell morphology (**b**, 200 $\times$; day 87 post-implantation). Similarly, tumors developing in nude rats after U87/GFP intracranial implantation appeared to have less extracellular matrix material and a smaller cellular morphology (**c**, 200 $\times$; day 22 post-implantation) compared to tumors developing after implantation of U87/OPN cells (**d**, 200 $\times$; day 53 post-implantation)

Fig. 6.

Tumors derived from high expressing OPN cells had less apoptosis, less blood vessels and more BrdU incorporation compared to controls. Immunohistochemical staining of thin sections derived from rats implanted with either F98 OPN12× or F98 WT cells and sacrificed 14 days post-implantation were subjected to staining with anti-CD31 antibody (top row). F98 OPN12× tumors had less blood vessels (mean = 5.3 vessels/visual field) compared to F98 WT tumors (mean = 12.47 vessels/visual field; $P < 0.01$). In vivo BrdU labeling index was obtained for F98 OPN12× tumors and F98 WT tumors at 14 days post-implantation (middle panel) F98 OPN12× tumors contained more BrdU positive cells compared to F98 WT tumors ($P < 0.001$). Finally, there was a significant decrease in the number of cells undergoing apoptosis (bottom panel) in F98 OPN12× tumors compared to F98 WT tumors ($P < 0.01$). Error bars represent standard error