Bone-Related Genes Expressed In Advanced Malignancies Induce Invasion And Metastasis In A Genetically Defined Human Cancer Model*

Jeremy N. Rich‡§, Qing Shi¶, Mark Hjelmeland§, Thomas J. Cummings‖, Chien-Tsun Kuan‖, Darell D. Bigner‖, Christopher M. Counter**‡‡ and Xiao-Fan Wang**§
Departments of ‡Medicine, ¶Surgery, ‖Pathology, **Pharmacology and Cancer Biology, and ‡‡Radiation Biology, Duke University Medical Center, Durham, NC 27710.

§To whom correspondence should be addressed, at Duke University Medical Center, Box 2900, Durham, NC 27710. Phone: (919) 681-1693; Fax: (919) 681-7152; Emails: rich0001@mc.duke.edu and wang0011@mc.duke.edu.

Abbreviations used are: ECM, extracellular matrix; EGFR, epidermal growth factor receptor; hTERT, human telomerase catalytic subunit; IGF-1R, insulin-like growth factor-1 receptor; PDGFR, platelet-derived growth factor receptor; T-Ag, large T antigen; MMP, matrix metalloproteinase; SPARC, secreted protein acidic and rich in cysteine.

Running Title: Osteonectin and Osteoactivin-Induced Tumor Invasion

Key Words: brain tumor, glioma, osteonectin, SPARC, osteoactivin, GPNMB, stromelysin, gelatinase
ABSTRACT

Differential gene expression analyses of human cancers have generated enormous amounts of data. However, the functional significance of many genes remains to be determined. To address this problem, we employed a genetically defined human cancer model to investigate the contributions of two different candidate genes upregulated in several cancers to phenotypic changes associated with late stages of tumorigenesis. Specifically, tumor cells expressing two structurally unrelated bone-related genes, osteonectin and osteoactivin, acquired a highly invasive phenotype when implanted intracranially in immunocompromised mice. Mimicking a subset of gliomas, tumor cells invaded brain along blood vessels and developed altered vasculature at the brain-tumor interface, suggesting production of those two proteins by tumor cells may create a complex relationship between invading tumor and vasculature co-opted during tumor invasion. Interestingly, the same tumor cells formed massive spontaneous metastases when implanted subcutaneously. This dramatic alteration in tumor phenotype indicates that cellular microenvironment plays an important role in defining the specific effects of those gene products in tumor behavior. In vitro examination of tumor cells expressing either osteonectin or osteoactivin revealed that there was no impact on cellular growth or death but increased invasiveness. Biochemical analysis revealed that expression of osteonectin and osteoactivin was accompanied by increased expression of MMP-9 and MMP-3. Specific pharmacologic inhibitors of MMP-2/9 and MMP-3 blocked the increased in vitro invasion associated with osteoactivin expression but only MMP-3 inhibition altered the invasive in vitro phenotype mediated by osteonectin. Results from this genetically defined model system are supported by similar findings obtained from several established tumor cell lines derived originally from human patients. In sum, these results reveal that the expression of a single bone-related gene can dramatically alter or modify tumor cell behavior and may confer differential growth characteristics in different microenvironments. Genetically defined human cancer models offer useful tools in functional genomics to define the roles of specific genes in late stages of carcinogenesis.
INTRODUCTION

Gene expression analyses of human cancers have yielded tremendous quantities of data. Unfortunately, the phenotypic consequences of many changes in gene expression pattern between cancers and their corresponding normal tissues are largely unclear. To address this problem, we took a functional genomics approach by using a genetically defined glioma model system to investigate genes involved in the acquisition of malignant phenotype associated with late stages of tumorigenesis. Particularly, we were interested in genes whose expression is not associated with normal brain tissues or astrocyte cultures but which are nevertheless overexpressed in gliomas. Among those candidate genes, several bone-related genes were noticeably overexpressed in a high proportion of gliomas. Of note, two structurally unrelated genes, osteonectin and osteoactivin, have been previously found to be overexpressed in several other types of cancers, but their precise contribution to the development of specific cancer phenotype has yet to be elucidated.

Osteonectin, also known as secreted protein, acidic and rich in cysteine (SPARC) or BM-40, is a 43-kDa extracellular matrix (ECM) protein. Osteonectin was originally discovered as one of the most abundant non-collagenous components of bone but it is also expressed in a number of other cell types that are involved in active remodeling of tissues (1). Thus, the primary physiological role of osteonectin has been postulated to be an important modulator of cell-ECM interactions during the processes of tissue remodeling (2, 3). Osteonectin is also abnormally expressed in many cancers, including gliomas (4, 5), medulloblastomas (6), meningiomas (7) and cancers of the gastrointestinal tract, breast, lung, kidney, adrenal cortex, prostate and bladder (3, 8-11). In gliomas, osteonectin is expressed in all tumor grades, usually at the tumor-brain margin and sites of neoangiogenesis (4), suggesting that osteonectin expression may be involved in tumor cell invasion (14). In contrast, increased osteonectin expression in other tumor types is associated with a conversion to invasive and metastatic tumors and a correlation in some instances with elevated expression of matrix metalloproteinases (MMPs) that is linked to increased tumor malignancy (11-13). Importantly, reduced expression of osteonectin by an antisense approach was found to correlate with a reduction in tumor formation by melanoma cells (15). Thus, while the precise role of osteonectin in the pathological process of carcinogenesis remains to be elucidated, osteonectin overexpression is intimately correlated with the progression of tumorigenesis of multiple types of human cancers.

Osteoactivin, also known as GPNMB or dendritic cell-associated, heparan sulfate proteoglycan-integrin ligand (DC-HIL), is a type I transmembrane glycoprotein that is localized to the cell surface and lysosomal membranes (16), as well as in a secreted form (17). Highly expressed in bone, the physiological function of osteoactivin is postulated to be involved in the regulation of osteoblast maturation (16). Interestingly, osteoactivin is found to be overexpressed in both melanomas (18), gliomas (19), and cancers of the breast, stomach, and pancreas. While the precise role of osteoactivin in cancer development remains unknown, it is tempting to suggest that the significant elevation of this bone-related protein in gliomas in brain tumor progression.
To determine the specific contributions of osteonectin and osteoactivin expression to human cancer development, we employed a genetically defined model system in which the genetic effects of increased gene expression could be directly linked to phenotypic changes of tumorigenesis. In this system, transformed human astrocytes by the sequential introduction of the simian virus-40 large T antigen, the human telomerase catalytic subunit hTERT, and oncogenic Ha-Ras display a phenotype mimicking that of low-grade gliomas in the formation of non-invasive tumor mass in immune-compromised animals (20). The characteristics of this human tumor model system allowed us to test specifically the phenotypic changes associated with tumor progression derived from the expression of genes under investigation. Consequently, we found that expression of osteonectin or osteoactivin was associated with angiocentric intracranial invasion and increased production of MMPs by the tumor cells. Furthermore, osteonectin and osteoactivin expression caused the development of spontaneous metastases systemically when the tumor cells were implanted subcutaneously. Our results suggest that the expression of bone-related genes in advanced human cancers may represent a novel mechanism by which tumor cells acquire capabilities that are associated with the phenotypic changes in late stages of tumorigenesis dependent on tumor microenvironment. Moreover, the data strongly support the notion that the genetically defined model of human cancers (20-23) offers a useful tool for functional genomics in defining the pathological contributions of specific genes to late stages of tumorigenesis.
EXPERIMENTAL PROCEDURES

Generation of cell lines. A genetically defined human glioma cell line was generated as previously described (20). A 1.5-kb cDNA fragment (a generous gift from Sandra Rempel, Henry Ford Hospital) and a full-length cDNA fragment of GPNMB (a generous gift from H.P. Bloemers, University of Nijmegen, The Netherlands) were each cloned into a retroviral backbone with a bleo selection marker. Cells underwent a positive selection with Zeocin (400 µg/ml, Invitrogen, Carlsbad, CA). U87MG, U251MG (American Type Culture Collection, Manassas, VA) and D54MG (Duke) were infected with a retrovirus expressing either a puromycin resistance gene or osteonectin and puromycin resistance. Selection was undertaken with puromycin (1 µg/ml). Early passage polyclonal cultures were used for all experiments.

Western Analysis. Cells were analyzed for the expression of osteonectin, growth factor receptors, and matrix metalloproteinases (MMPs) by Western analysis. A 10-cm plate was lysed and 50 µg of total cellular protein was used for each sample. Samples were subjected to SDS-PAGE analysis and transferred to a PDVF membrane. The membrane was blocked in tris-buffered saline with 0.05% Tween 20 and 5% albumin. Primary antibodies for anti-osteonectin (Haematologic Technologies, Essex Junction, VT), anti-GPNMB (gift of Carol Wikstrand, Duke University), anti-actin (Santa Cruz Biotechnology), anti-tubulin (Sigma Chemicals, St. Louis), anti-MMP-9 (Calbiochem, La Jolla, CA), anti-MMP-3 (Calbiochem), anti-MMP-2 (Calbiochem), anti-epidermal growth factor receptor (EGFR) (gift of Carol Wikstrand, Duke University), anti-platelet-derived growth factor receptor β (PDGFRβ) (Santa Cruz Biotechnologies, Santa Cruz, CA), anti-PDGFRα (Santa Cruz), or anti-insulin-like growth factor-1 receptor (IGF-1R) (Calbiochem) antibodies were used. Secondary antibodies were either goat anti-rabbit (BioRad, Hercules, CA) or sheep anti-rabbit antibodies (Amersham, Piscataway, NJ).

Growth Inhibition Assays. Cells were plated into 12-well plates at a density of 2x10^4 cells per well and labeled for the last 6 hours with 4 µCi of [3H]thymidine, fixed in 10% trichloroacetic acid, and lysed in 0.2 N NaOH. [3H]thymidine incorporation into the DNA was measured with a scintillation counter. Each measurement was performed in triplicate.

FACS Analysis. Cells were plated into 10-cm plates at a density of 5x10^5 cells per well, serum starved overnight after attachment, and then fed with media containing serum for 24 hours. Cells were then trypsinized, fixed in 70% ethanol, washed once in phosphate buffered saline, and resuspended in RNaseA (100µg/ml) and propidium iodide (50 µg/ml). Samples were analyzed on a FACScan (Becton Dickinson) flow cytometer. Each experiment was performed in triplicate.

Soft agar assays. For these assays, 35-mm plates were prepared with a base layer of Dulbecco’s minimal essential media (DMEM) with 10% calf serum (Life Technologies, Rockville, MD) and 0.6% bacto agar (Becton, Dickinson and Co., Franklin Lakes, NJ). Cells were plated at a density of 5x10^4 cells per plate in a mix of DMEM with 10% calf serum and 0.4% bacto agar. Plated cells were fed once a week with 0.5 ml of DMEM plus 10% calf serum and 0.4% bacto agar. After 14 days the plates were stained with 0.5 ml of 0.005% crystal violet. On each plate, colonies with more than 30 cells were counted. Each measurement was performed in triplicate.
**Tumor Formation Assays.** Intracranial tumor formation was tested with SCID-beige mice injected with $1 \times 10^6$ glioma cells in 10 µl Methocel (Dow Chemical Co., Midland, IL). Mice were sacrificed when they developed any neurological abnormalities. Brains were serially sectioned and examined by histopathology. Immunohistochemistry was performed on all tumors with hematoxylin and eosin, factor VIII, collagen IV, Ki-67. Vascular characteristics of tumors were compared through an examination of vessel number and size in selected high-power fields. Fields were selected to represent maximal tumor diameters without significant areas of necrosis. Internal organs were completely removed and evaluated grossly for macroscopic metastases. Selected livers and lungs were examined for microscopic metastases. SCID-beige mice (Taconic, Germantown, NY) were subcutaneously injected in the flank with $10 \times 10^6$ glioma cells per mouse in 100 µl of Matrigel (Becton, Dickinson, and Co., Franklin Lakes, NJ) (20). Mice were regularly checked for tumor formation. Tumor volume was calculated with the formula $0.5 \times (\text{length}) \times (\text{width})^2$. Tumors were removed and examined by immunohistopathology. Mice that were noted to have significant weight loss were sacrificed regardless of primary tumor size. All internal organs including the brain were removed at the time of sacrifice and grossly examined for metastases. Selected lungs, livers and brains were sectioned and examined for microscopic metastases.

**Matrigel Invasion Assays.** Kits were purchased from BD Biosciences and used according to instructions. Briefly, $2.5 \times 10^4$ tumor cells were incubated in selected conditions (serum-free media, 1% DMSO control, 100 µM MMP-2 Inhibitor I [Calbiochem], 100 µM MMP-2/9 Inhibitor I [Calbiochem] or 100 µM MMP-3 Inhibitor II [Calbiochem] and then allowed to attach and invade. Cells were fixed 22 hours after plating. The invasion was calculated as a ratio of that shown by control (uncoated) inserts to that shown by Matrigel-coated inserts. Experiments were performed in triplicate.

**Statistical Analysis.** Wilcoxon Rank Sum Test was used in all analysis.
RESULTS

Osteonectin and Osteoactivin Expression Induce Brain Invasion Associated with Penetrating Vessels.

Differential gene expression analyses of primary brain tumors and normal brain by the SAGE technology have permitted the identification of numerous genes that are increased in expression in malignant gliomas, including osteonectin and osteoactivin (4, 19). To understand the role of these two genes in the pathogenesis of gliomas, we determined the effects of overexpressing osteonectin or osteoactivin by using a genetically defined human glioma cell line (hereafter referred to as the THR glioma line) developed in our laboratory (20). When implanted intracranially in immune-compromised mice, THR cells display a non-invasive phenotype. This phenotype is in contrast to that of gliomas in patients that are universally invasive, suggesting that additional genetic alterations beyond those already present in this cell line are required to develop an invasive phenotype \textit{in vivo}. Thus, the THR cells represent an ideal model system in which to study the contributions of specific genetic changes to late stages of glioma development.

To determine the potential impact of osteonectin or osteoactivin expression on tumor development, we implanted these engineered THR cell lines intracranially in suspensions. In this nature environment, the genetically defined glioma cells formed large extra-axial masses with histologic features consistent with a malignant neural tumor including pseudopallisading necrosis (Fig. 1C), regardless whether the tumors expressed the vector, osteonectin, or osteoactivin. Tumors derived from vector control cells were located predominantly within the subarachnoid space, and rare foci superficially invaded the Virchow-Robin spaces but extended no deeper than the molecular layer of the superficial cerebral cortex. The Virchow-Robin spaces and vasculature remained delicate without expansion by tumor, increased numbers of vessels, or hypertrophy of the endothelium (not shown).

However, in 23 of 33 mice implanted with the osteonectin-expressing glioma cells, tumors were located within the subarachnoid space and displayed a striking phenotype of invasion (Fig. 1D), as well as expansion of the perivascular Virchow-Robin spaces by masses of tumor cells that extended into the deep layers of the cerebral cortex (Fig. 1E) similar to previous reports of glioma invasion in mouse models (24). In sharp contrast to the tumors derived from vector control glioma cells, blood vessels associated with the tumors were increased in size and number (Fig. 1F). These features readily contrasted with adjacent brain tissues not invaded by the tumor. Neoplastic cells in rare nests and single cells were seen adjacent to the Virchow-Robin spaces in the parenchyma, a finding that may represent either true invasion of brain parenchyma or formation of an angiocentric perivascular zone outside the limits of the pia-glial membrane. The prominent tumor associated vasculature was readily detected by immunohistochemical staining of the Factor VIII antigen, which showed enhanced proliferation and endothelial hypertrophy of the osteonectin expressing glioma tumors in comparison to the normal vasculature seen in adjacent brain, as well as the tumors derived from the vector control cells.
Osteoactivin-expressing cells formed intracranial tumors in a fashion similar to those expressing osteonectin but with a lower rate of brain invasion (Fig. 1D, G) and less associated vascular alterations (Fig. 1H). Taken together, our data suggest that the expression of osteonectin and osteoactivin induce glioma invasion in the context of brain environment associated with penetrating blood vessels.

Osteonectin and Osteoactivin Expression Induces an Invasive Phenotype In Vitro

Having documented the dramatic effects of osteonectin and osteoactivin expression on tumor invasion in xenograph experiments, we next investigated the mechanism by which these genes exert tumorigenic effects. While in vitro systems are incomplete models of tumor invasion and metastasis as they lack normal stromal interactions, they allow selected analysis of potential changes at the cellular level, including cell proliferation, apoptosis, and invasion. It was previously reported that osteonectin inhibits cell proliferation (25) and induces apoptosis in some ovarian carcinoma cell lines (26). However, we find that expression of osteonectin or osteoactivin in our THR glioma cells did not cause any significant changes in cell proliferation (Fig. 2A), apoptosis (Fig. 2A), or DNA synthesis (Fig. 2B). Disruption of osteonectin expression has been linked to loss of insulin-like growth factor-1 receptor (IGF-1R) expression (27), but we found no differences in the expression levels of IGF-1R, platelet-derived growth factor receptor (PDGFR-α or -β), or epidermal growth factor receptor (EGFR) between control and osteonectin-expressing cells (Fig. 2C). Osteoactivin expression was associated with an increase in EGFR expression but a decrease in PDGFR-α expression and no change in PDGFR-β or IGF-1R expression (Fig. 2C). In addition, the ectopic expression of osteonectin or osteoactivin in human astrocytes expressing SV40 T antigen and hTERT failed to provide a mitogenic stimulus to transform these cells in the absence of oncogenic Ras as measured by soft agar colony formation assays (Fig. 2D). These results suggest that osteonectin and osteoactivin expression is mainly associated with the acquired abilities by the tumor cells in invasion rather than tumor initiation. To test this hypothesis, we examined the impact of osteonectin and osteoactivin expression on tumor cell invasion in vitro. As shown in Figs. 3A and 3B, osteonectin or osteoactivin expression significantly increased cell invasion in all cell lines tested as measured by the Matrigel invasion assay. Constitutive expression of osteonectin or osteoactivin appeared to increase the ability of tumor cells to degrade components of the ECM and increased motility, a critical aspect of invasive cancers.

Osteonectin and Osteoactivin Expression Is Associated with Increased MMP Expression.

To further explore the mechanisms that mediate the invasive effects of osteonectin and osteoactivin, we examined the expression profiles of proteins intimately associated with tumor invasion, specifically the MMPs. As shown in Fig. 3C, we found that osteonectin and osteoactivin expression significantly increased the production of MMP-9 and MMP-3 by the tumor cells with minimal changes in MMP-2 expression. To test if this upregulation of MMPs is directly linked to the invasive behavior of tumors expressing osteonectin and osteoactivin, we examined the effect of specific MMP inhibitors in the Matrigel invasion assay. Treatment of cells expressing osteonectin with an MMP-2/9 inhibitor had no effect (Fig. 3D), suggesting that the increase in MMP-9 expression was not a major contributing factor to the changes in invasive behavior by the osteonectin-expressing tumor cells. However, MMP-3 inhibitor treatment
Cells Expressing Osteonectin and Osteoactivin Display a Spontaneous Metastatic Phenotype.

Since osteonectin and osteoactivin are expressed in other types of advanced cancers, we investigated the potential effects of these two genes on tumor progression in a different growth microenvironment. To do this, osteonectin-expressing and osteoactivin-expressing THR glioma cells were subcutaneously injected into the flanks of SCID-beige mice. The primary subcutaneous tumors derived from the glioma cells expressing osteonectin have an identical latency period (approximately 29 days) to a vector control cell line, but grow to a larger volume (Fig. 4A). Tumors from the osteoactivin-expressing cell line display a longer latency (approximately 39 days) but subsequently grow to on average a larger size than vector controls (Fig. 4A). Tumors expressing osteonectin and osteoactivin did not exhibit a difference in angiogenic features from the control cells (data not shown). Strikingly, both types of tumor cells developed massive spontaneous intrathoracic and/or intraperitoneal metastases (Fig. 4B, C). Metastases were found in 50% (16 of 32) of mice injected with osteonectin-expressing glioma cells and 14% (3 of 15) of osteoactivin-expressing glioma cells (Fig. 4D). In contrast, the THR vector control cell line exhibited a single small, discrete metastasis in only 1 of 15 mice, and no metastases developed in 25 mice implanted with the THR parental cell line (not shown). Interestingly, the development of metastases was independent of primary tumor size. Three of 16 mice that developed metastases from osteonectin-expressing cells had no grossly evident subcutaneous tumors at the initial site of injection at the time of euthanasia. Therefore, the data suggest that the development of a metastatic phenotype from osteonectin-expressing glioma cells is associated with an early stage of tumor development inside the animals.

Upon close pathological examination, we found that tumors derived from osteonectin-expressing glioma cells display a phenotype of solid and circumscribed neoplasms capable of invading the pancreas, peripancreatic soft tissues, subcutaneous tissues, and liver (Fig. 4E, F). Examination of hematoxylin and eosin stained sections of tumors derived from osteonectin-expressing cells revealed a malignant phenotype of poorly differentiated neoplasm. Within the tumor mass, there were numerous areas of atypical mitoses and necrosis pseudopalisaded by neoplastic cells. Taken together, histological analyses of tumors derived from osteonectin-expressing cells revealed typical pathological features that are consistent with metastatic disease. Interestingly, we found that the metastasized tumors did not possess the features of prominently increased vasculature and hypertrophic endothelium that are often associated with the invasive brain tumors. Consistent with this observation, lymphatic/vascular invasion and perineural extension were not detected in the metastatic sites.
To test if the effects of osteonectin described above were cell line-specific, we investigated the behavior of tumor development by the human glioma cell lines D54MG, U87MG, and U251MG engineered to express higher levels of osteonectin (Fig. 4G). Importantly, we found that ectopic expression of osteonectin by these glioma cell lines also induced spontaneous metastases when they were injected subcutaneously in the flanks of scid-beige mice (Fig. 4D). These results suggest that osteonectin expression can induce a non-metastatic cancer cell line to adopt a metastatic phenotype with the alteration in the expression of only a single gene.
DISCUSSION

Technological improvements now permit comprehensive analysis of gene expression patterns in cancer specimens. The resulting data have created a wealth of information to be mined, but utilization of the data remains difficult due to a lack of functional information for specific genes. Here we demonstrate the use of a genetically defined human cancer model system to investigate the function of specific genes differentially expressed in human cancers. Previously developed model systems of cancer have included cell lines created from human tumors and murine models. Although there has been great benefit of each model system to our understanding of the contribution that specific genetic alterations play in the development of specific phenotypes of cancer, each system has significant drawbacks. Human cell lines are generally derived from advanced cancers that have widespread genetic changes and genomic instability as well as changes from long passages in cell culture. Murine models permit genetic control but are labor intensive and suffer from potential species-specific differences in the process of transformation and gene function (28). Thus, a genetically defined human cancer model system offers a useful tool to determine the roles of specific genes in carcinogenesis, particularly the late stages of this multistep process.

Using this system, we investigated genes that normally appear to be highly expressed in bone without presence in brain tissues, but have significantly increased expression in human gliomas. Although the expression of both osteonectin and osteoactivin has been linked to tumor progression in previous studies, their precise contributions to late stages of tumorigenesis remain unclear. Through an ectopic expression strategy in this model system, we revealed that osteonectin expression induced a highly malignant phenotype with significantly increased brain invasion associated with vascular proliferation and spontaneous systemic metastasis, whereas osteoactivin expression resulted in a more modest increase in malignant phenotype with less frequent metastases and lower degrees of vascular change. Although the highly metastatic characteristics displayed by the tumor cells in our study do not fully replicate the precise behavior of gliomas in humans, the data strongly support the notion that expression of osteonectin and osteoactivin could significantly alter or modify tumor behavior. Considering the fact that these genes are also overexpressed in other types of human cancers that do display a metastatic phenotype, this finding is clearly relevant and significant to our understanding of the mechanism underlying metastasis associated with those types of human cancers.

Furthermore, our results indicate that cellular microenvironment potently modulates the phenotypic behavior of cancer cells that express those two genes, since glioma cells expressing osteonectin and to a lesser extent, osteoactivin, formed large spontaneous metastases with subcutaneous implantation, but remained localized to the brain with intracranial implantation. The vascular and extracellular environment in the brain is radically different from that of other organs, with the presence of the blood-brain barrier, absence of lymphatics, and brain-specific ECM and cell-cell interactions associated with neuronal and glial migration during development. These differences may partially account for the differential behavior of cells expressing osteonectin implanted in the brain verses other parts of the body. This finding strongly suggests that contributions to the development of specific tumorigenic phenotypes by the expression of specific genes are dependent on the specific characteristics of microenvironment associated with
tumor progression, adding another layer of complexity to the molecular mechanisms underlying late stages of tumorigenesis.

Another potentially important finding is that osteonectin and osteoactivin appear to promote a specific invasive phenotype intracranially involving tumor cell invasion along pre-existent blood vessels in the Virchow-Robin spaces and development of altered vasculature at the brain-tumor interface. This phenotype mimics the behavior of a subset of human gliomas and many invasive medulloblastomas. Osteonectin has been reported to have an effect on angiogenesis through regulation of VEGF activity (29). In our studies, neither osteonectin nor osteoactivin induced significant changes in the vasculature as measured by vessel number or diameter within the primary tumors, whether formed subcutaneously or intracranially. In contrast, intracranial tumor cells expressing osteonectin and osteoactivin grow along penetrating blood vessels, and the blood vessels at the invasive front were markedly abnormal with vessel hypertrophy and hyperplasia. These results suggest that the production of osteonectin and osteoactivin by glioma cells may create a complex relationship between invading tumor and normal vasculature that may be co-opted during tumor invasion, consequently allowing expansion of the tumor mass without the induction of angiogenesis at a significant level.

The malignant phenotypes mediated by osteonectin and osteoactivin likely involve multiple mechanisms at the molecular level, with the induction of MMP expression as an important component. The rapid increase in MMP-3 protein levels in response to osteonectin treatment by the THR tumor cells strongly suggests that osteonectin may regulate the production of this specific MMP through a more direct mechanism.

Taken together, our results validate the use of a genetically defined human cancer model system in investigating the contributions of specific genes to late stages of tumorigenesis. This *in vivo* system permits the application of a functional genomics approach in defining the specific activities of genes that have been identified to be abnormally expressed in human cancers, particularly those associated with tumor invasion, metastasis, and angiogenesis.

We thank G. J. Riggins, J. Herndon and R. McLendon for helpful discussions, S. Rempel for osteonectin cDNA, M. Hjelmeland, S. Keir, Y. Yu, and R. Nelson for technical assistance. J. Parsons provided editorial support.
This work was supported by NIH K08 NS02055 (to J.N.R.), R01 CA83770 (to X.F.W.), R01 CA94184 and CA82481 (to C.M.C.) and NS20023 (to D.D.B.); the Pediatric Brain Tumor Foundation (to J.N.R.); DOD Breast Cancer Research Program grant DAMD17-00-1-0230 (A.B.H.). C.M.C. is a Leukemia and Lymphoma Society Scholar.

NCBI SAGE Genie Website.
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Figure Legends

Figure 1. Osteonectin and Osteoactivin Expression Increases Brain Invasion. (A) A genetically defined human glioma cell line was engineered to overexpress osteonectin. Western analysis of conditioned media (30 µl of media/sample) revealed that cells appropriately expressed and secreted osteonectin. Par, parental; Vec, vector control; ON, osteonectin. (B) A genetically defined human glioma cell line was engineered to overexpress osteoactivin. Cellular lysates were resolved by SDS-PAGE and analyzed for osteoactivin expression by Western analysis. (C) Genetically defined glioma cells were injected intracranially in immunocompromised mice. Histology of the tumors expressing osteonectin reveals necrosis (*) with pseudopallisading tumor cells consistent with a grade IV glioma. (D) The rate of brain invasion of vector control tumor cells (VEC), osteonectin expressing cells (ON), and osteoactivin expressing cells (OA) was measured on serial sections. The total number of brains examined per point (N) is indicated. Invasion was graded either as no invasion (NONE), limited invasion into the molecular layer (MIN), or widespread and/or deep brain penetration (SIG). *, p = 0.011; **, p = 0.15. (E-H) In general, tumors grew largely separately, but those tumors expressing osteonectin developed brain invasion (indicated by arrows in E, hematoxylin and eosin) following penetrating blood vessels (arrows in F, Factor VIII staining). Osteoactivin expression was associated with significant tumor invasion into normal brain (indicated by arrows in G, hematoxylin and eosin) but with less significant vascular changes than osteonectin (arrows in H, factor VIII staining).

Figure 2. Phenotype Associated With Expression With Osteonectin And Osteoactivin. Genetically defined glioma cells were engineered to express vector control (VEC), osteonectin (ON), or osteoactivin (OA). (A) Cell cycle analysis by flow cytometry revealed that the expression of osteonectin and osteoactivin was associated with minimal changes in the cell cycle fractions. (B) DNA synthesis measured by thymidine incorporation revealed minimal impact by osteonectin or osteoactivin expression. (C) Equal amounts of protein from genetically defined human glioma cells expressing osteonectin or osteoactivin were subjected to Western analysis. Osteonectin expression was not associated with changes relative to vector control while osteoactivin expression was associated with a moderate increase in EGFR expression and decrease of PDGFR-α expression. (D) Transformation of human astrocytes expressing SV40 T antigen and the human telomerase catalytic subunit with vector control, osteonectin, osteoactivin, or oncogenic Ha-ras (RAS) were tested in a soft agar colony formation assay.

Figure 3. Osteonectin and Osteoactivin Expression Is Associated with Increased Invasion and Expression of MMP-9 and MMP-3. (A) Matrigel invasion assays were performed with the genetically defined THR glioma cell line, D54MG, U87MG, and U251MG, each engineered to express osteonectin (ON). The expression of osteonectin was clearly linked to increased invasion relative to vector control (VEC). *, p = 0.01. (B) Matrigel analysis of glioma cells expressing osteoactivin with specific MMP-2, MMP-2/9, and MMP-3 inhibitors ablated the increase in invasion associated with osteoactivin expression (OA) relative to vector controls (VEC). *, p = 0.01 relative to vector control; **, p = 0.01 relative to untreated osteoactivin-
expressing tumor. (C) Western analysis of a genetically defined glioma cell line with vector control (V), osteonectin (ON), or osteoactivin (OA) expression revealed increased expression of gelatinase B (MMP-9) and stromelysin-1 (MMP-3) but minimal change of gelatinase A (MMP-2). (D) Matrigel analysis of glioma cells expressing either vector control (VEC) or osteonectin (ON) showed no change in invasion with an MMP-2/9 inhibitor, but an MMP-3 inhibitor ablated the increase in invasion associated with osteonectin expression (*, p = 0.01 relative to untreated). (E) Parental genetically defined THR glioma cells were treated with 50 µg/ml of purified human osteonectin (Haematologic Technologies, Essex, VT). Conditioned media were collected simultaneously with either no osteonectin or after treatment with osteonectin for specific times. The media (50 µl) was resolved by SDS-PAGE and immunoblotted for stromelysin-1 (MMP-3).

Figure 4. Osteonectin and Osteoactivin Expression Induces Spontaneous Metastases. (A) A genetically defined human glioma cell line was engineered to overexpress a vector control (VEC), osteonectin (ON), or osteoactivin (OA). 10^7 cells were implanted subcutaneously into the right flank of scid-beige mice. Tumor volumes were measured twice a week and calculated by a formula of 0.5(width)^2(length). Tumor volumes were plotted from mice that were not sacrificed due to development of metastases as the tumors of these mice were frequently small at the time of euthanasia. (B, C) A genetically defined glioma cell line engineered to ectopically express osteonectin (B) or osteoactivin (C) developed large spontaneous metastases (indicated by white arrows) in the thorax and peritoneum when implanted subcutaneously in the flanks of immunocompromised mice. (D) Osteonectin and osteoactivin expression increased the rate of spontaneous metastases of human glioma cell lines. The total number of mice examined per point is indicated. *, p < 0.0001; **, p = 0.2; ***, p = 0.15; ****, p = 0.058. (E, F) Pathology was consistent with a malignant neural tumor (E, with normal pancreas) and with a high Ki-67 index (F). The percentage of Ki-67 positive cells was not significantly different between the vector control and osteonectin or osteoactivin expressing tumors (data not shown). (G) Expression of osteonectin was assayed by Western analysis of cellular lysates of human glioma cell lines engineered to overexpress osteonectin.
Figure 1
Figure 2
Figure 3
Figure 4
Bone-related genes expressed in advanced malignancies induce invasion and metastasis in a genetically defined human cancer model
Jeremy N. Rich, Qing Shi, Mark D. Hjelmeland, Thomas J. Cummings, Chien-Tsun Kuan, Darell D. Bigner, Christopher M. Counter and Xiao-Fan Wang

J. Biol. Chem. published online February 17, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M211498200

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