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TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Role of Collagen Matrix in Tumor Angiogenesis and Glioblastoma Multiforme Progression

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Glioblastoma is a highly vascularized brain tumor, and antiangiogenic therapy improves its progression-free survival. However, current antiangiogenic therapy induces serious adverse effects including neuronal cytotoxicity and tumor invasiveness and resistance to therapy. Although it has been suggested that the physical microenvironment has a key role in tumor angiogenesis and progression, the mechanism by which physical properties of extracellular matrix control tumor angiogenesis and glioblastoma progression is not completely understood. Herein we show that physical compaction (the process in which cells gather and pack together and cause associated changes in cell shape and size) of human glioblastoma cell lines U87MG, U251, and LN229 induces expression of collagen types IV and VI and the collagen crosslinking enzyme lysyl oxidase and up-regulates *in vitro* expression of the angiogenic factor vascular endothelial growth factor. The lysyl oxidase inhibitor β-aminopropionitrile disrupts collagen structure in the tumor and inhibits tumor angiogenesis and glioblastoma multiforme growth in a mouse orthotopic brain tumor model. Similarly, D-penicillamine, which inhibits lysyl oxidase enzymatic activity by depleting intracerebral copper, also exhibits antiangiogenic effects on brain tumor growth in mice. These findings suggest that tumor microenvironment controlled by collagen structure is important in tumor angiogenesis and brain tumor progression. *(Am J Pathol 2013, 183: 1293–1305; http://dx.doi.org/10.1016/j.ajpath.2013.06.026)*

Glioblastoma multiforme (GBM), a highly malignant glioma that exhibits various glial lineages such as a mixed oligodendroglial–astrocytic phenotype,[1] is the most common primary brain tumor. Every year in the United States, GBM is diagnosed in about 8000 persons. Despite a great deal of effort to develop effective therapies targeting GBM, current approaches including surgery, radiotherapy, and chemotherapy have not been successful, with median survival of only 1 to 2 years.[2] Angiogenesis, the formation and development of new blood vessels, is an important step in tumor growth and metastasis.[3,4] Since GBM is a highly vascularized tumor and the presence of neovascularization is a key diagnostic criteria for GBM, antiangiogenic therapy is thought to represent a promising strategy for treatment of GBM.[4] Indeed, the antivascular endothelial growth factor (VEGF) agent bevacizumab improves progression-free survival of GBM.[5] However, antiangiogenic therapy for brain tumors potentially induces neuronal cytotoxicity, invasiveness of the tumor cells,[6,7] and resistance to therapy,[3–5,8,9] which diminish the benefits of current antiangiogenic therapy for GBM. Thus, to establish safe and efficient therapies that have long-term anti–tumor activity and fewer adverse effects, we need to understand the comprehensive mechanisms that govern brain tumor angiogenesis.

Most of the work in tumor angiogenesis has been focused on identifying the genetic and chemical signals that control neovascularization.[3,10] Thus, most US Food and Drug Administration (FDA)–approved angiogenesis inhibitors target soluble angiogenic factors such as VEGF.[5,8,9,11] These chemical signaling cascades are clearly necessary for tumor

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growth and vascular development; however, the insoluble extracellular matrix (ECM) and mechanical forces have equally important roles in tumor angiogenesis and progression.[12–19] Cell shape controls the growth of capillary endothelial cells,[20] and mechanical forces such as matrix stiffness control cell migration, angiogenesis, tumor progression, and metastasis, both *in vitro* and *in vivo*.[15,21–23]

Normal brain ECM is composed of hyaluronan, proteoglycans, and tenascin-C and is devoid of rigid ECM structures formed by fibrillar collagens,[24] whereas ECM in GBM is associated with a large increase in components such as collagens, laminin, and fibronectin,[25,26] primarily in the basement membrane of the blood vessels, which suggests that modifying these tumor-specific ECM components could be a potentially promising strategy for treatment of brain tumors.

Physical compaction is the process in which cells gather and pack together, causing associated changes in cell shape and size. Mesenchymal condensation, in which dispersed mesenchymal cells are tightly packed together, results in differentiation of these cells into specific tissue types that occur during early development of various organs (eg, tooth, cartilage, bone, muscle, tendon, kidney, and lung) in mice.[27,28] During the course of mesenchymal condensation, cell shape is changed, cell size is decreased, and cell density is increased, which results in changes in mechanical and chemical signaling in the cells and dictates various cellular behaviors such as proliferation, migration, and cell fate determination that are critical for organ-specific morphogenesis. Physical compaction of the mesenchyme during early organ development increases collagen expression and governs subsequent organogenesis.[29] In addition, changes in cell shape control cell growth through the Rho signaling pathway in capillary endothelial cells,[20] and mechanical forces elicited by ECM stiffness, which also change cell shape and size, control angiogenesis and vascular function.[22,30] Proliferating cancer cells in a confined space undergo compressive stress,[16,31] and physically compacted cancer cells exhibit aggressive phenotypes.[32,33] For example, rapidly growing mammary tumor cells in confined spaces become smaller and packed (ie, physically compacted),[32] which affects tumor cell behavior and collagen VI expression.[34] Therefore, in addition to soluble factors, mechanical forces such as physical compaction of the cells and subsequent changes in expression and structure of collagen may contribute to tumor angiogenesis and GBM progression. However, the role of physical properties of the tumor microenvironment on brain tumor angiogenesis and progression has not been well elucidated.

The present study was initiated to examine whether tumor cell compaction and associated changes in cell shape and density control collagen and angiogenic factor expression using *in vitro* experimental methods (ie, microcontact printing system and mechanical compressor) (Supplemental Figure S1). We have previously used these methods to examine the effects of physical compaction in normal development,[29] in which we could precisely change cell size, shape, and density to mimic physical compaction *in vitro*. We also altered collagen expression and structure by changing the crosslinking ability of collagens and examined whether it controls brain tumor angiogenesis and tumor growth *in vivo*. Compaction of GBM cells changes collagen expression and structure, resulting in increased VEGF expression *in vitro*. Inhibition of collagen crosslinking attenuated the effects of physical compaction of GBM cells on tumor angiogenesis and progression of GBM in a mouse orthotopic brain tumor model. Because this novel approach targets tumor-specific ECM structures, it may lead to development of more stable and efficient antiangiogenic therapy for GBM and other types of hypervascular brain tumors.

Materials and Methods

Materials

Anti–collagen IV, anti–collagen VI, anti–Ki-67, anti-HIF2α, and anti–lysyl oxidase (LOX) polyclonal antibodies were purchased from Abcam (Cambridge, MA); anti-CD31 monoclonal antibody from BD Biosciences (San Jose, CA); anti-VEGFR2 polyclonal antibody from Cell Signaling Technology, Inc. (Boston, MA); anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH), anti–matrix metalloproteinase (MMP)–2, and anti–MMP-9 antibodies from Millipore Corp. (Billerica, MA); and anti–β-actin antibody, β-aminopropionitrile (BAPN), and D-penicillamine from Sigma (St. Louis, MO). U87MG, U251, and LN229 human glioblastoma cell lines (ATCC, Manassas, VA) were cultured in minimum essential medium containing 10% fetal bovine serum.[35]

Fabrication of Micropatterned Substrates

The microcontact printing method for producing glass substrates containing micrometer-sized ECM islands has been described previously.[29,36] In brief, polydimethylsiloxane (PDMS) stamps were cast, cured, and removed from master templates, which were created using photolithographic methods. Stamps were coated with 50 μg/mL fibronectin for 1 hour and dried using compressed nitrogen. Flat and thin PDMS substrates were prepared on the cover glass and UV oxidized for 5 minutes (Ellsworth Adhesives, Germantown, WI), stamped with fibronectin, blocked with Pluronic F108 (Sigma) for 2 hours, and rinsed three times with PBS before plating various GBM cells. The cells were cultured on the fibronectin island at low (0.2×10^5 cells/cm^2) or high (2.4×10^5 cells/cm^2) plating density to recapitulate the physical compaction of the tumor cells *in vivo* (Supplemental Figure S1). After 16 hours of culture, densities and projected cell areas of the cells attached to the ECM islands were measured under a phase contrast microscope.