Medical Research Archives

ESMED
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OPEN ACCESS

Published: September, 30, 2022

Citation: Crawford SA and Hayward-Piatkovskyi B, 2022. Quantitation of A "Malignancy Index" of the Relationship between Glioblastoma Microtumor Mass and Invasive Potential Using A Chicken Egg Albumen Matrix to Define Biomechanical Density Parameters that Promote Malignant Progression, Medical Research Archives, [online] 10(9). https://doi.org/10.18103/mra.v10i9.3002

ISSN: 2375-1924

RESEARCH ARTICLE

Quantitation of a "Malignancy Index" of the Relationship between Glioblastoma Microtumor Mass and Invasive Potential Using A Chicken Egg Albumen Matrix to Define Biomechanical Density Parameters that Promote Malignant Progression

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ABSTRACT

Although many factors contribute to the low translational success of pre-clinical data into human trials, one major factor is the failure of pre-clinical models to recapitulate essential physiological components of malignant tumors. An important body of clinical research indicates that the surrounding cellular microenvironment involving stromal tissue, matrix and the resident immune system play a critical role in the genesis of brain tumors of many diverse types. The research presented in this paper specifically addresses the role of biomechanical components in the brain extracellular matrix that may play a critical role in the development and spread of central nervous system malignancies, specifically gliomas. The data suggest that unfertilized chicken egg albumen, as a novel three-dimensional culture medium, provides a biologically relevant microenvironment that can support dynamic tumor formation and growth. Chicken egg albumen supplemented culture media produced compaction effects on tumor density that varied inversely with invasion zone expansion parameters. Based on this observed relationship, a ratio was extrapolated from primary data measurements to assess more quantitatively the relationship between microtumor spheroid surface area and invasion zone diameter. The ratio of microtumor invasion zone diameter divided by microtumor surface area was calculated and designated a "Malignancy Index" based on the premise that the relationship between the peripheral invasion zone diameter relative to surface area changes reflecting changing compaction parameters of the tumor mass represents a relevant assessment of tumor invasiveness, a fundamental hallmark of malignancy.

INTRODUCTION

Preclinical brain tumor models that more fully represent the process of malignant transformation and the growth properties of brain tumors in the central nervous system (CNS) are needed to advance our understanding of the biology, treatment and prevention of these therapy resistant malignancies. Current *in vitro* tissue culture models in widespread use generally display poor predictive translational applications in human clinical trials. Among therapeutic areas, oncology clinical trials have the highest failure rate[1]. Only a very small percentage of cancer therapeutics that enter phase I clinical trials ultimately display therapeutic efficacy in patients with cancer[2]. Although many factors contribute to the low translational success of pre-clinical results into human trials, one major factor is the failure of pre-clinical models to recapitulate essential biological components of malignant tumors [1-3].

Importance of the Extracellular Matrix (ECM)

An important body of clinical research indicates that the surrounding cellular microenvironment involving stromal tissue, matrix and the resident immune system play a critical role in the genesis of brain tumors of many diverse types. The research presented in this paper specifically addresses the role of biomechanical components in the brain extracellular matrix that may play a critical role in the development and spread of CNS malignancies, specifically gliomas. The extracellular matrix (ECM) is a network of proteins that is secreted by cells that provide support for surrounding tissue[4]. ECM composition varies among the diverse tissues of the body and may play a dramatic role in regulating cell phenotype. For example, mammary tumors cultured in ECM obtained from normal breast tissue exhibit a normal phenotype, despite having genetic mutations associated with breast tumors[5]. Conversely, an altered cellular microenvironment plays a crucial role in tumor progression[6]. Microenvironmental factors constitute an extracellular environment that can either support dormancy or active proliferation. Pre-malignant lesions are the result of monoclonal expansion of cells with genetic mutations that promote mitosis. As these cells multiply, additional genetic mutations may accumulate due to an increased, dysregulated proliferation, resulting in a genetically heterogenous population of abnormal cells[7]. Increased cell density generates changes in the biomechanical parameters that regulate tissue and organ homeostasis. The transition from a premalignant lesion to an invasive phenotype is associated with an altered biomechanical environment that results from abnormal tissue expansion. This, in turn, may activate ECM remodeling to accommodate the expanding tumor. Thus, a premalignant lesion becomes a tumor when cells acquire the capacity to remodel the ECM to promote cell expansion/invasion into nearby tissues. Gliomas remodel newly degraded ECM to promote migration through normal brain tissue[8]. Given the documented importance of the stromal ECM in tumor invasion, it is essential that tumor models that incorporate essential matrix parameters be utilized to study tumor biology *ex vivo*. Currently, many pre-clinical assessments of novel therapies for glioblastoma (GBM) use the mouse model, which, despite its oft-cited superiority over tissue culture assessment models, lacks many fundamental properties critical to an accurate determination of clinical efficacy. Grafting brain tumors into rodents often results in a loss of invasiveness, the deadliest aspect of GBM, which is considered the cause of the poor translation of preclinical response rates into human trials[9]. A biologically relevant model that can accurately preserve the tissue architecture, mechanical and biochemical cues, cell-to-cell communications, and invasiveness of GBM must be developed in order to predict more accurately the ability of novel therapies to impact patient survival.

Pre-clinical Tissue Culture Tumor Models

2-Dimensional Monolayer Culture

Standard monolayer tissue culture pre-clinical models fail to reprise adequately the *in vivo* environment or phenotypes of human tumors, leading to artificial chemotherapy responses[1,2]. Scaffold based cell culture uses ECM coated culture dishes to produce gene expression profiles more representative of protein expression profiles *in vivo*[10]. Scaffold culture models, however, are also a two-dimensional system. Certain cell types, such as fibroblasts, retain phenotypes not observed *in vivo* when grown in this system[3].

3-Dimensional Multicellular Tumor Spheroids (MTS)

Another widely used approach utilizes scaffold-free three-dimensional models of culturing cells. Liquid overlay is a popular method that has been around since the 1970's that uses substrate manipulation to generate microtumor spheroids with diameters up to 1mm (**See Figure 1**)[6,11]. Coating plastic dished with a hydrophobic polymer, such as a polymerized agarose solution, hinders cell adhesion to a substrate and leads to spheroid

formation. This method produces disc-like spheroids through forced aggregation in the absence of ECM components, which is not representative of the *in vivo* tumor matrix microenvironment. Research on the biological properties of multicellular tumor spheroids (MTS) that often form spontaneously in tissue culture when cells are prevented from binding to a substrate, has identified distinct regions in MTS formed from diverse tissue origins that are similarly identified in patient tumors, indicating that 3-dimensional microtumors are a more representative model of tumor biology than monolayer cultures. **(Figure 1)**[6, 12, 13]. These regions are observed also in human resected tumors. The presence of necrotic loci is associated with higher grade tumors [14, 15, 16]. Research suggests that tumor cells within the necrotic center may regress to form cancer stem cells (CSCs), that may play an important role in drug resistance and tumor recurrence[6]. The increasing use of microtumor spheroids and organoid culture systems has addressed some of the fundamental discrepancies encountered in monolayer culture; however, there is still an overwhelming need to reconstitute critical microenvironmental factors involved in tumor development and progression to facilitate a more rational approach to chemotherapy drug development.

Use of Chick Egg Albumen to Model Brain ECM

Chicken egg albumen has been utilized as a substrate replacement for reconstituted basement membrane and matrigel[17, 18]. Culture dishes are coated with sterile albumen that is heated heated to facilitate binding to the dish plastic to form a gel-like solid for cell-to-substrate attachment. Epithelial breast tumor cell lines cultured on this egg white matrix exhibited phenotypes comparable to matrigel, including acini-like tumor formation. These studies concluded that the unfertilized chicken egg albumen contains ECM-like proteins that act as a substrate for attachment and tumor formation, but have not addressed the 3-dimensional components of solid tumor physiology.

Water and proteins are the major components of the albumen[19]. The most abundant is ovalbumin, which accounts for 54% of the total proteins[17, 19]. Ovalbumin is an inert biomolecule that has antibacterial and immunomodulating activities. Yet another protein, ovomucin, accounts for 3.5% of the total albumen proteins and creates a viscosity barrier that protects the embryo from microbes. Most albumen proteins have antimicrobial, antiviral, or immunomodulating properties to protect a developing embryo, such as ovotransferrin, ovomucoid, lysozyme, avidin, cystatin, ovoinhibitor, and ovomacroglobulin.

The research presented here is a step further in the use of albumen as part of a semisolid medium to promote the growth of solid tumor spheroids in three dimensions in a extracellular matrix with similar biophysical parameters in terms of viscosity density and matrix protein content as the human brain. This novel extracellular matrix is comprised of chick egg albumen and cell culture medium mixed together to form an extracellular environment with viscosity/density parameters similar to the matrix composition of brain tumors in the CNS. The data suggest that unfertilized chicken egg albumen, as a novel three-dimensional culture medium, provides a biologically relevant microenvironment that can support dynamic tumor formation and growth.

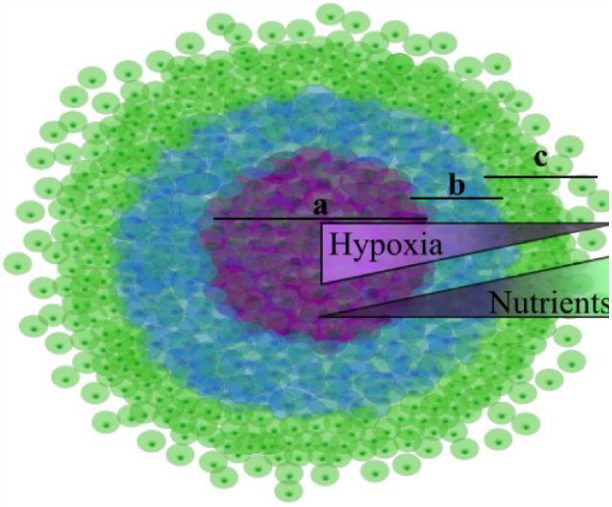


Figure. 1. Microtumor Spheroid Regions. Microtumors generated in liquid overlay cultures display distinctive regions or layers of cells with differing degrees of cellular compaction. These regional density differences generate physiological differences in cells comprising each layer. The outermost layer (green) consists of more loosely attached cells that can proliferate and invade locally to surrounding matrix. The middle layer (blue) is termed the quiescent zone due to its greater degree of compaction, lower proliferation rate and decreased access to oxygen and nutrients from the surrounding vasculature (or culture medium). The innermost region is termed the necrotic core since hypoxia and cell death result from poor oxygenation and nutrient access. Cell division is virtually non-existent due to spatial and physiological constraints, although cancer stem cells have been identified in the core. These regions of differential compaction, the product of tissue overgrowth, generate physiological differences that trigger epigenetic reprogramming of the tumor to a state of inflammation/stress response associated activities linked to tumor progression.

Materials and Methods

Cell Line

The DBTRG-05MG (CRL-2020) cell line used in this study was purchased from the American Type Culture Collection (ATCC©). Cells were cultured in Leibovitz's L-15 medium (Gibco) supplemented with 10% FBS (Gibco) at 37C.

Liquid Overlay Cultures

24 well culture dishes were coated with 1% agarose in phosphate buffered saline (PBS), forming a hydrophobic surface once the solution polymerized. Plates were stored at 4°C prior to use. Monolayer cultures were used to seed spheroids on agarose coated culture plates at a density of $6x10^4$ cells per ml of media. Cultures were grown at 37°C/ 90% humidity. Multicellular tumor spheroid (MTS) formation was routinely observed between 24-48 hours post-plating.

Semi-Solid Culture Conditions

Multicellular tumor spheroids generated using the liquid overlay technique were transferred to one of three experimental conditions (1) liquid overlay in Leibovitz L-15 /10%FBS (LO), (2) L15/10%FBS supplemented with 0.3% agarose "semi-solid" medium (SSM) (prepared in (Hank's Balanced Salt Solution)HBSS supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$); at concentrations of 10-60% to give final concentration of agarose are 0%, 0.06%, 0.12%, 0.18%,%. or (3) L15/10%FBS supplemented with egg albumen (ALSM) obtained from fresh, unfertilized eggs at final concentration of 10-60%. Using a ball and cylinder test to determine density, a 0.3% agarose solution was chosen as an albumen substitute.

Each of these culture preparations (standard media overlay, semi-solid agarose and albumen-supplemened media cultures) was prepared in culture dishes coated with 1% agarose polymer to prevent cell/substrate attachment. Preformed 48 hour spheroids were transferred to agarose supplemented media immediately after plate preparation, in warm agarose prior to polymerization or 20%-60% egg albumen in L-15 medium + 10% FBS.

Image Capture and Analysis

Images were captured using SPOT software. Image settings used in the SPOT software were 0.6 gamma, 0.03 contrast, 0.6 saturation, and 5000 color temperature. These settings were chosen because they provided the clearest photos with optimal contrast across culture medium types. Measurements of the spheroids were extrapolated using Image J software, calibrated from micrometer images captured with various objective lenses. Spheroid area was determined by outlining the spheroid. Invasion zone measurements were calculated as the distance from spheroid tumor mass to the farthest extensions of individual cells loosely attached or separated from the mass. The average of three measurements was used for area calculations.

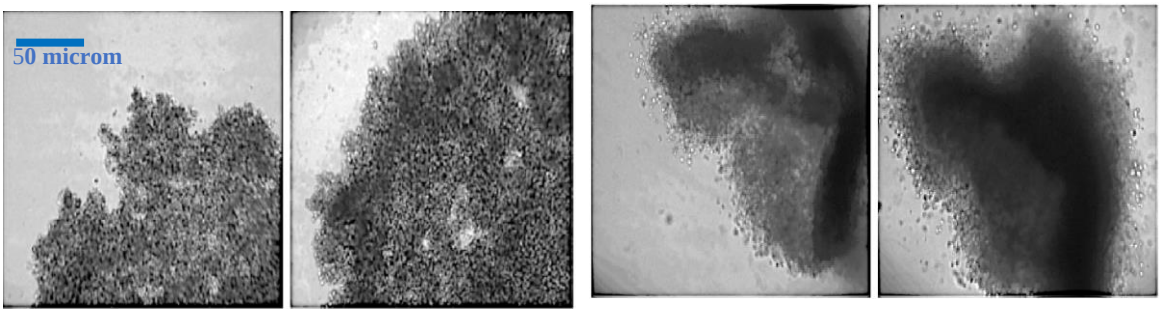
Statistical Analysis

Measurements were exported to Numbers for analysis of spheroid area and invasion zone. Each experiment was carried out at least two times and up to four times. Statistical significance was determined with the two-tail, two equal samples student t-test function in the Numbers software. Data were considered significant at $p < 0.05$.

RESULTS

Effect of gradients of chicken egg albumen-supplemented medium (ALSM) on glioblastoma (GBM) microtumor morphology

Glioblastoma spheroids were seeded from trypsinized cells 48 hours prior to transfer to semi-solid culture conditions. Individual spheroids were injected into culture dishes containing Leibovitz's L-15 medium supplemented with 0-60% albumen (ALSM). Live microtumor images were obtained 7 and 14 days post plating using SPOT imaging software (**See Figure 2a**). Average microtumor surface area measurements were calculated using Image j software (**Figure 2b and 2c**).



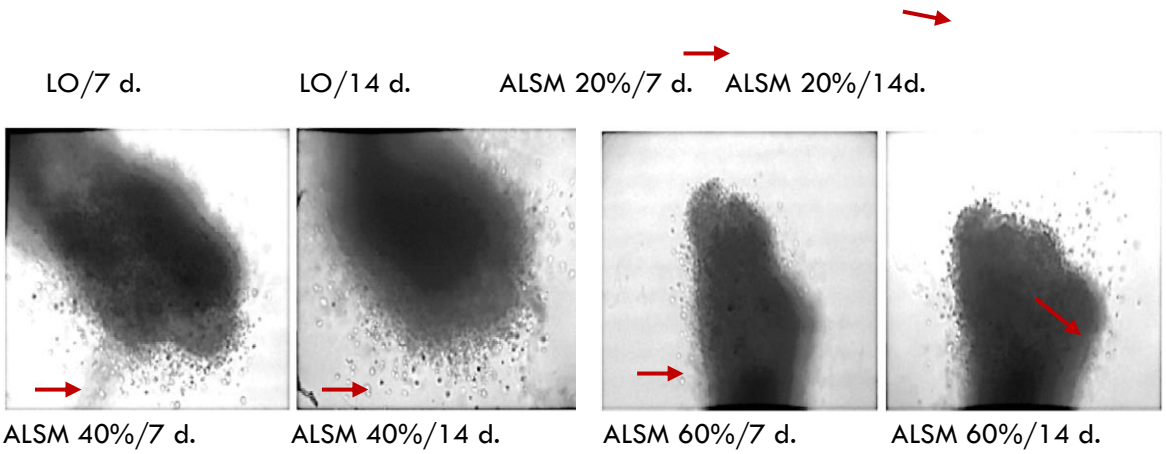


Figure 2a. Liquid overlay (LO) microtumors imaged at 7 and 14 days (d) on agarose-coated plates. Note uniform spacing between cells and disc-like, flattened microtumor morphology. In contrast, microtumors cultured in albumen supplemented media (ASLM) overlay display distinct regions of higher density cell compaction in the interior of the tumor surrounded by a layer of more loosely connected cells. Moreover, the periphery of the tumor displays extensive cell migration from the primary tumor mass. These effects are more pronounced in microtumors at day 14. Microtumors in 40% ASLM overlay showed a greater area of high-density cell compaction and a smaller perimeter of more loosely connected cells. In addition, the absolute migration distance of individual cells shed from the tumor mass is greater for tumors cultured in 40% versus 20% albumen overlay. These effects are more pronounced at day 14. Similar effects were observed were microtumors in 60% overlay. These cultures displayed the largest region of uniform high-density compaction with a smaller periphery of more loosely attached cells. However, migrating tumor cells can be observed; once again, this effect is more pronounced at 14 d. Red arrows point to loosely, connected dispersed cells in invasion zone.

%Albumen Medum Overlay	**Days In Culture**	**Microtumor** Average Surface Area (Mm^2)	Standard Error	**%Albumen Medum Overlay**	**Days In Culture**	Average Surface Area (Mm^2)	Standard Error
0	0	54	4.6	40	0	60.8	1.1
	8	112.9	1.7		8	.111.9	39.8
	15	151.1	2.9		15	68.7	5.2
20	0	54.6	3.4	60	0	56.8	2.9
	8	48.4	3.0		8	78.1	21.8
	15	51.6	1.3		15	43.8	0.5

b.

c.

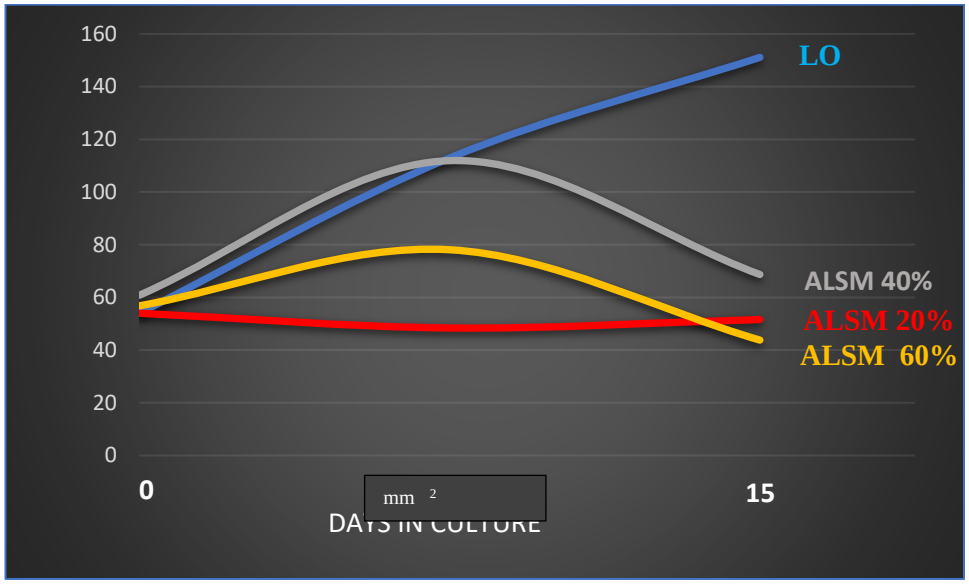


Figure 2b, c. Average surface area of glioblastoma spheroid microtumors in albumen supplemented medium (ALSM). A calibration slide was used to correlate pixels with distance. Each condition was done in duplicate (n=2). Measurements were taken at day1, 8 and 15 for microtumors in liquid medium only (L15 +10%FBS) control=0; liquid medium (L15 + 10%FBS + 20% ALBUMEN=20%), (L15 +10%FBS + 40% albumen)=40%; and (L14+10%FBS + 60% albumen)=60%. Microtumors (MTS) cultured in ALSM expanded in surface area during week one, though not to the same extent as LO MTS. In contrast to LO MTS, whose surface area continued to increase throughout the duration of the experiment, MTS in 40% and 60% ALSM displayed significant surface area contraction associated with increased cell density at 15 days.

The average surface area of spheroid microtumors increased over the two-week incubation under all culture conditions (**Figure 2b, c**). There were, however, distinct morphological and spatial differences in the growth patterns observed in microtumors cultured in liquid overlay (LO) as compared to microtumors cultured in albumen supplemented media (ALSM). LO microtumors displayed a uniform cell morphology from the exterior edges to the inner portions of the mass with similar density of cells throughout, generating a disc-like appearance. In contrast, microtumor spheroids cultured in ALSM displayed distinctive microtumor regions ranging from a densely packed tumor core to a less dense periphery edged with a region of loosely adherent cells that could be seen at the outermost portion to be separated from the main tumor mass. These regions of heterogeneous cell density could be observed clearly after 7 days in culture. Moreover, unlike LO cultures, where the microtumor edges displayed smooth margins, the microtumor edges in ALSM cultures displayed jagged margins comprised of loosely adherent and separated cells, characteristic of an invasive phenotype. These morphological differences were more pronounced over time and with increasing concentrations of albumen. LO microtumors showed a 2.4-fold increase in surface area at day 6 in culture. Microtumors in 20-40% ALSM displayed a less substantial increase in surface area, but higher cell density during the same time period.

Microtumor Invasion Zone Parameters in liquid Overlay (LO) Versus Albumen-Supplemented medium (ALSM)

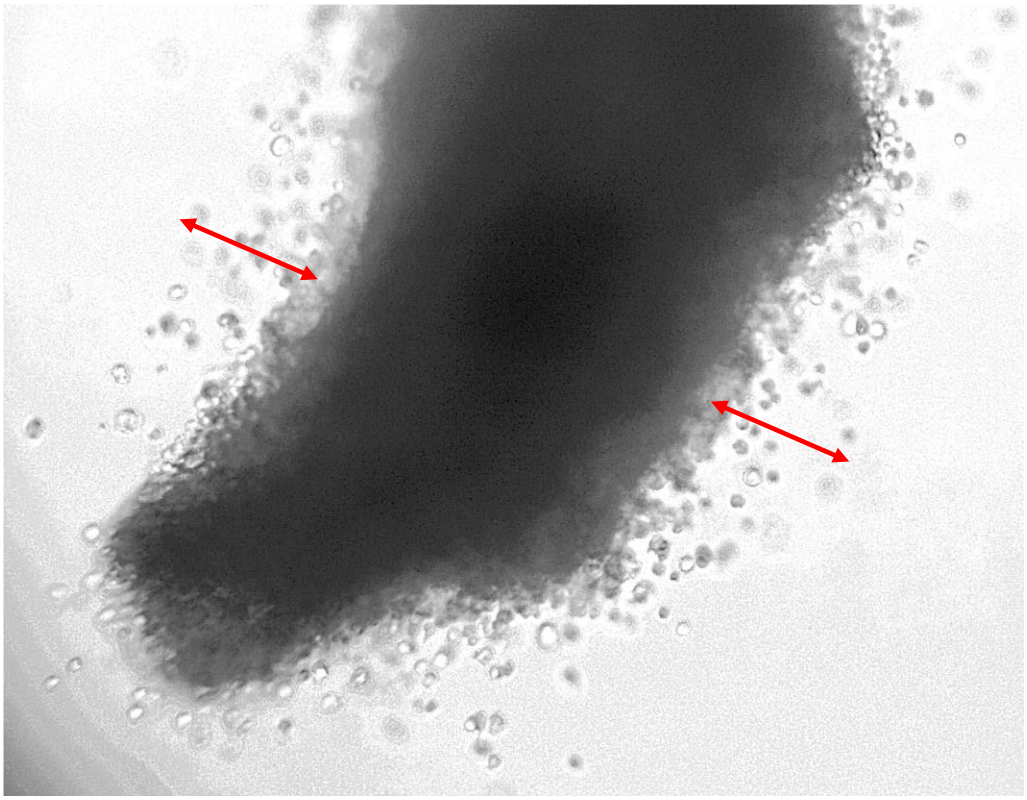
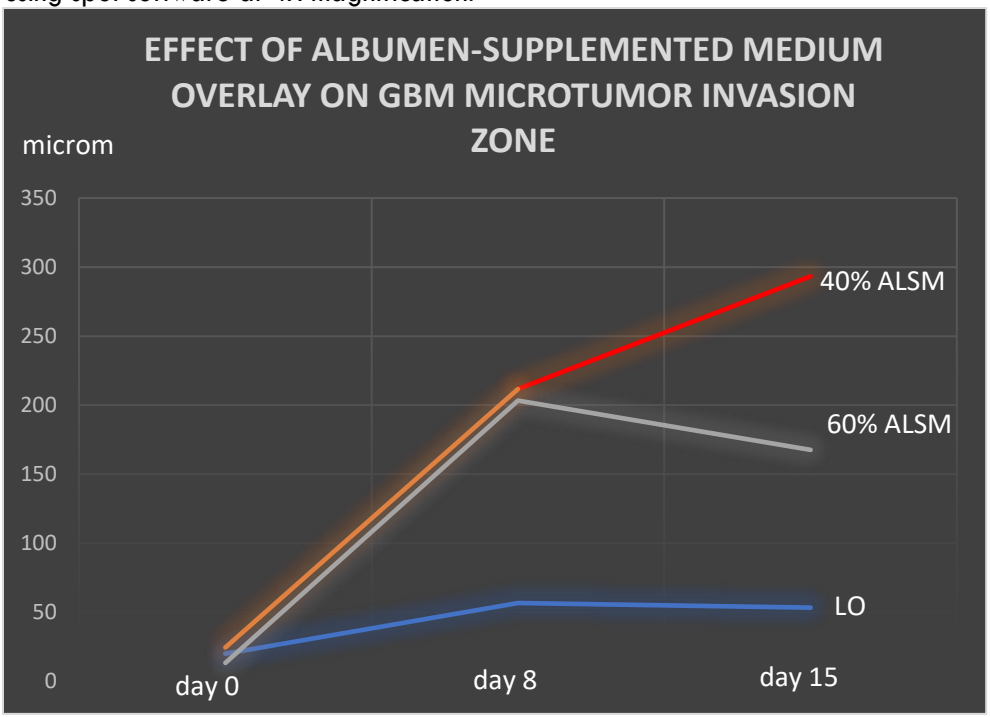


Figure 3a. GBM microtumor cultured in 60% albumin overlay at 11 days. Arrows indicate approximate diameter of loosely connected and dispersed cells at the tumor margins, termed the "invasion zone". Imaged using spot software at 4X magnification.



b.

%Albumen Medum Overlay	Days In Culture	Average Invasion Zone Diameter (µm)	Standard Error (µm)	Condition	Day	Average Invasion Zone (µm)	Standard Error (µm)
0%	0	20	16.1	60% Albumen/ Media	0	13.3	16.3
	8	56.7	43.1		8	203.3	72.5
	15	53.3	35.4		15	167.5	92.9
40%	0	24.5	18.2				
	8	211.7	96.3				
	15	293.3	12.7				

c.

Figure 3 b, c. Glioblastoma (GBM) invasion zone diameter measurements in liquid overlay (LO) or albumen supplemented medium (ALSM) over 15 days post plating. Three measurements were taken per spheroid using Image J. Conditions were done in triplicate with 2-4 spheroids per experiment (n=10). An order of 3 polynomial trendline was fit to the data using Numbers software, each with a R^2 value of 1.

Microtumors cultured in ALSM displayed distinctive regions of higher cell density surrounded by a more loosely attached periphery, in contrast to the more uniform disc-like morphology of microtumors cultured in LO. The loosely attached peripheral border was observed to increase over time, generating an expansive region of semi-attached and individual cells separating from the primary tumor mass. This dynamic region of tumor expansion was designated the "tumor invasion zone" since its diameter increased over time as tumor cells expanded into the albumen-media matrix. Measurements of the diameter of the loosely attached periphery were taken in duplicate over a time interval of approximately two weeks for microtumors cultured in both standard liquid overlay (LO) and medium supplemented with varying concentrations of albumen (ALSM) (**See Figure 3).** These data showed that microtumor invasion zone diameter size was greater (up to 6-fold) in ALSM than in LO cultures.

Comparison of Albumen ALSM Versus Semi-Solid Agarose (SSA) Culture Medium Supplementation on Glioblastoma (GBM) Microtumor Morphology and Invasion Zone Parameters

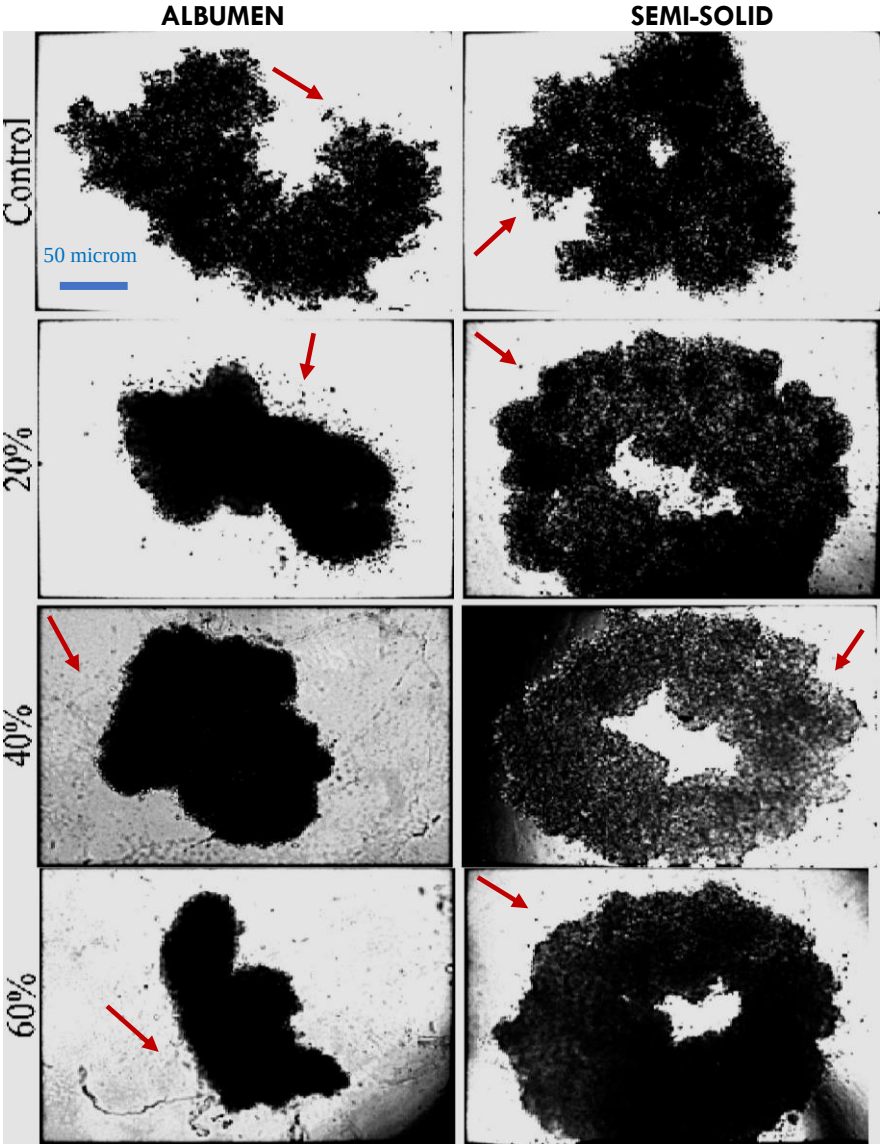


Figure 4a. Comparison of glioblastoma (GBM) microtumors at day 12 in semi-solid agarose (SSA) medium or albumen supplemented culture medium (ALSM) at comparable densities. Note more uniform spacing between cells and disc-like, flattened microtumor morphology In both liquid overlay and semisolid agarose cultures. In contrast, microtumors cultured in albumen supplemented media (ALSM) display distinct regions of higher density cell compaction in the interior of the tumor surrounded by a layer of more loosely connected cells. Moreover, the periphery of the ALSM tumors (especially 20%) display more extensive cell migration from the primary tumor mass. Red arrows point to loosely, connected dispersed cells in invasion zone.

Condition	Day	Average Invasion Zone (µm)	Standard Error (µm)	Condition	Day	Average Invasion Zone (µm)	Standard Error (µm)
0% LIQUID OVERLAY (LO)	0	19	9.2	**0% LO**	0	18	8.7
	4	37.1	17.4		4	27.5	15.3
	7	26	10.4		7	27	13.3
	12	42.5	21.5		12	30.8	19.6
20% ALSM	0	3.7	3.8	**20% SSA**	0	34.3	12.5
	4	87.9	20.7		4	110.4	22.6
	7	143.3	21.4		7	96	17.8
	12	239.2	42.6		12	100	30.8
40% ALSM	0	2	2.3	**40% SSA**	0	33	12.1
	4	67.9	13.9		4	55	15.4
	7	99.3	15.1		7	71.3	14.6
	12	141.7	25.8		12	49.2	28.8
60% ALSM	0	0	0	**60% SSA**	0	28.5	11.9
	4	18	15.7		4	75.7	12.7
	7	39	17.3		7	94.8	19.2
	12	62.2	33.5		12	142.2	33.1

Figure 4b. Average invasion zone of Glioblastoma spheroids in albumen (ALSM) or agarose (SSA) supplemented medium. Three representative measurements were taken for each spheroid using Image J software and averaged. Condition averages represent three experiments with 2-4 spheroids per experiment (n=10).

Control experiments were performed to assess the role of culture medium density per se on microtumor growth parameters using agarose, a biologically inert material, in place of albumen supplementation at comparable solution density (0.3%). In contrast to microtumor spheroids cultured in ALSM, microtumor spheroids cultured in semi-solid agarose (SSA) retained a disc-like appearance (similar to spheroids cultured in standard media) with a relatively uniform density throughout with donut-shaped empty centers (**See Figure 4a**). Although invasion zones were present at the periphery of some microtumors, the defined regional density differences observed in ASM culture were less pronounced in SSA cultures. SSA cultures displayed a smaller compaction factor, between 0.68-0.86 diameter of liquid culture controls, whereas ASM microtumors were between 0.38-0.62 diameter of liquid overlay controls (Figure 5b). In both ASM and SSA supplemented cultures, the degree of compaction increased with increasing culture media density up to 60% agarose or albumen concentration.

Tumor Malignancy Index Quantifies Inverse Relationship between Tumor Surface Area and Invasion Zone as a Function of Microenvironmental Density

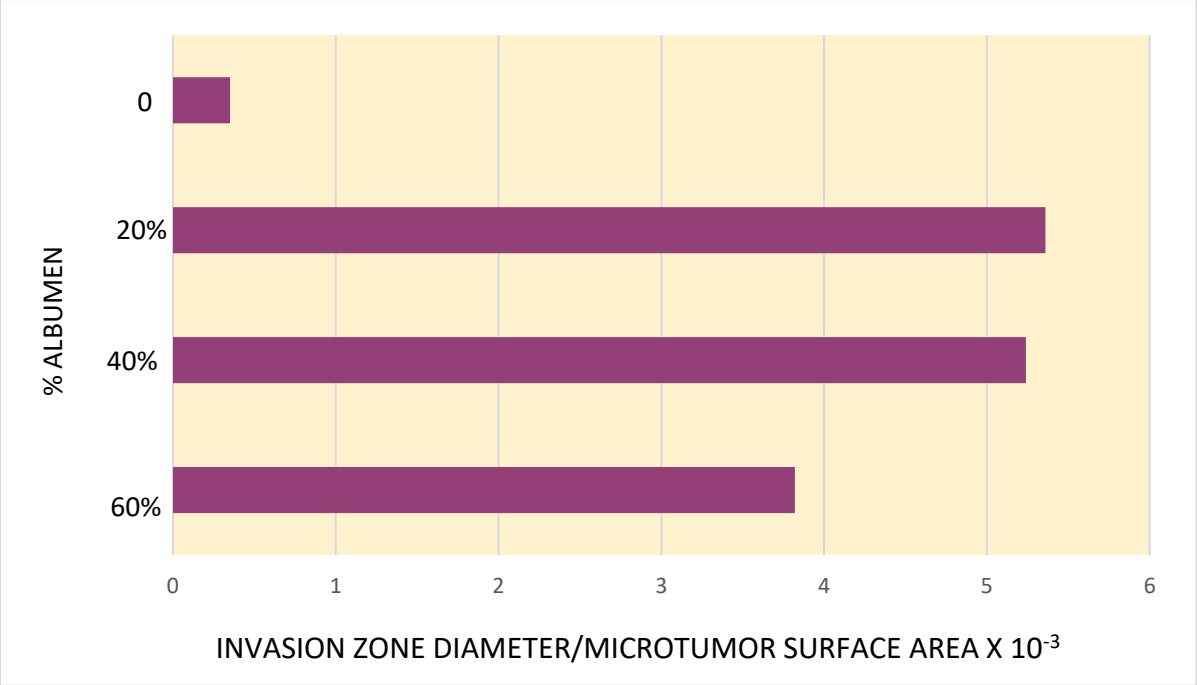


Figure 5a. Malignancy index of Glioblastoma spheroids grown in albumen supplemented medium (ALSM). GBM microtumors cultured in agarose supplemented L15 +10%FBS at 2 weeks. Malignancy Index (MI) for each microtumor was calculated as average invasion zone diameter/microtumor surface area. Each experimental condition was carried out in duplicate (n=2).

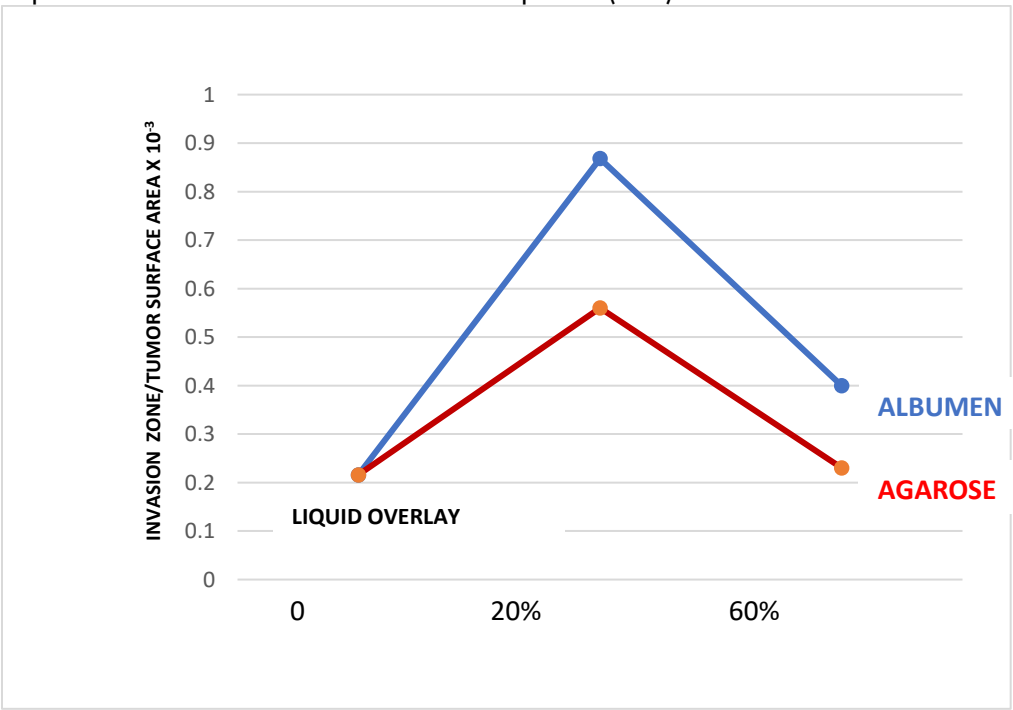


Figure 5b. Calculated Malignancy Index = average invasion zone diameter/microtumor diameter after 12 days in culture in standard culture medium (0%) or medium supplemented with 20%-60% albumen or agarose (see text for details).

media produced compaction effects on tumor density that varied inversely with invasion zone expansion parameters. Based on this observed relationship, a ratio was extrapolated from primary data measurements to assess more quantitatively the relationship between microtumor spheroid surface area and invasion zone diameter. The ratio of microtumor invasion zone diameter divided by microtumor surface area was calculated and designated a "Malignancy Index" (MI) based on the premise that the relationship between the peripheral invasion zone diameter relative to surface area changes reflecting changing compaction parameters of the tumor mass represents a relevant assessment of tumor invasiveness, a fundamental hallmark of malignancy.

This relationship is shown:

Invasion Zone Diameter (IZD)/Microtumor Surface Area (MTSA) =Malignancy Index (MI)

This simple relationship demonstrates that a higher malignancy index defines a larger invasion zone relative to the solid tumor mass to produce a quantifiable assessment of tumor invasive parameters directly linked to physiological determinants of tumor aggressiveness with respect to invasion and spread. This index (invasion zone over spheroid area), i.e., "malignancy index" (MI), whereby the fractional percentage of the tumor that displays invasive behavior serves as a quantitative assessment of the primary physiological characteristic of solid tumor malignancies that distinguishes them from all other types of abnormal growth, including benign tumors. As the tumor total surface area relative to the invasion zone diameter decreases and the invasion zone increases, the malignancy index increases. The greater the index, the greater the invasiveness of the microtumor. Changes in the malignancy index over time or in response to therapeutic intervention may demonstrate important physiological components of this critical parameter. In this study, malignancy index calculations showed that microtumor spheroids maintained in culture media supplemented with 20-60% albumen displayed the highest index by day 15, that was more than ten-fold higher than microtumor spheroids cultured without albumen supplementation **(Figure 5a).** A comparison of malignancy index determinations showed that ASM cultures displayed a 1.5 fold higher index than SSA microtumors at their maximum values (20% ALSM and 60% SSA) **(Figure 5b).** These data suggest that ALSM medium supports a more robust invasive phenotype than SSA.

DISCUSSION

Novel GBM models must replicate defining characteristics of the tumor phenotype *in vivo* before being considered for relevant pre-clinical studies. One of the critical factors that contributes to GBM malignancy is the surrounding tissue architecture. Many preclinical tissue culture models involve the use of microtumors, spheroidal masses or tumor organoids suspended in a liquid culture medium, absent any biomechanical or biophysical interactions that characterize *in vivo* tissue tumor/stromal interactions. The purpose of this research was to establish a simple preclinical microtumor growth environment that would reconstitute some aspects of the matrix properties of the biological system in the brain where glioblastomas develop.

Chicken egg albumen is both viscous and biologically active. Both components may contribute to a more representative *in vitro* microenvironment supportive of critical components of tumor physiology. This notion is supported by data presented in this paper demonstrating that albumen supplementation of standard culture media, at densities comparable to that of the brain tumor microenvironment, promoted microtumor compaction and invasive properties *in vitro*. The fact that media supplementation with agarose produced similar effects, despite the fact that agarose is a chemically inert substance, suggests that the more invasive phenotype displayed by GBM microtumors under these conditions is, at least in part, attributable to tumor cell/stromal interactions driven by biomechanical forces. Agarose and albumen concentrations that were chosen for liquid medium supplementations were selected to provide culture density conditions comparable to that of human brain tissue *in vivo*. Increasing concentrations of albumen and agarose were designed to replicate *in vivo* conditions where tumor spread, due to increases in cell mass, locally produce increases in the density and consequent biomechanical forces of the surrounding tumor microenvironment. These data show that, under conditions reflective of *in vivo* tissue density, increasing microenvironmental density not only produces increased tumor compaction, but facilitates the formation of a more prominent invasive perimeter, a primary requirement for tumor spread. These data, therefore, support the notion that biomechanical forces regulate malignant tumor spread as an independent variable of the tumor microenvironment. Further support is provided by research by Tse *et al.*[20] which demonstrated that mechanical compression of solid tumors produced a

more invasive phenotype. Moreover, Spencer *et al.*[21] showed that gene expression profiles of breast cancer cells were altered to block cell proliferation and enhance vascular adhesion, as well as other effects linked to enhanced malignancy, including therapeutic resistance, all of which occurred in response to mechanical tension induced by increased fluid sheer stress.

The data have been analyzed to calculate a "malignancy index" (MI) reflecting the proportion of the solid tumor mass that constitutes the area of tumor cell expansion and migration. More than tumor size alone, this concept of a malignancy index is critical to defining the relationship between tumor size and aggressive potential. These include an invasive phenotype and compaction within the tumor itself that generates distinctive regions of proliferation, quiescence and necrosis. These phenomena, so characteristic of malignant tumor growth, are interdependent and generate a microcosm of inflammation, oxygen deprivation, altered energy metabolism and necrosis - all of which contribute to the invasion and spread of malignant tumors to unleash their destructive systemic effects. Dysregulation of proliferation, as a consequence of genetic dysregulation, drives incipient tumor formation whose expansion ultimately generates a unique microenvironment with altered bioenergetic, biomechanical, and metabolic properties causing the emergence of the malignant phenotype. In the absence of these critical biological parameters, many of which are epigenetic consequences of the altered growth environment, the ability to reconstitute tumors in biologically relevant *in vitro* models is extremely limited. Both compaction and invasion result directly from the increased density of the culture medium that creates a biomechanical environment similar to that of the brain where tumor formation occurs **(See Figure 7)**.

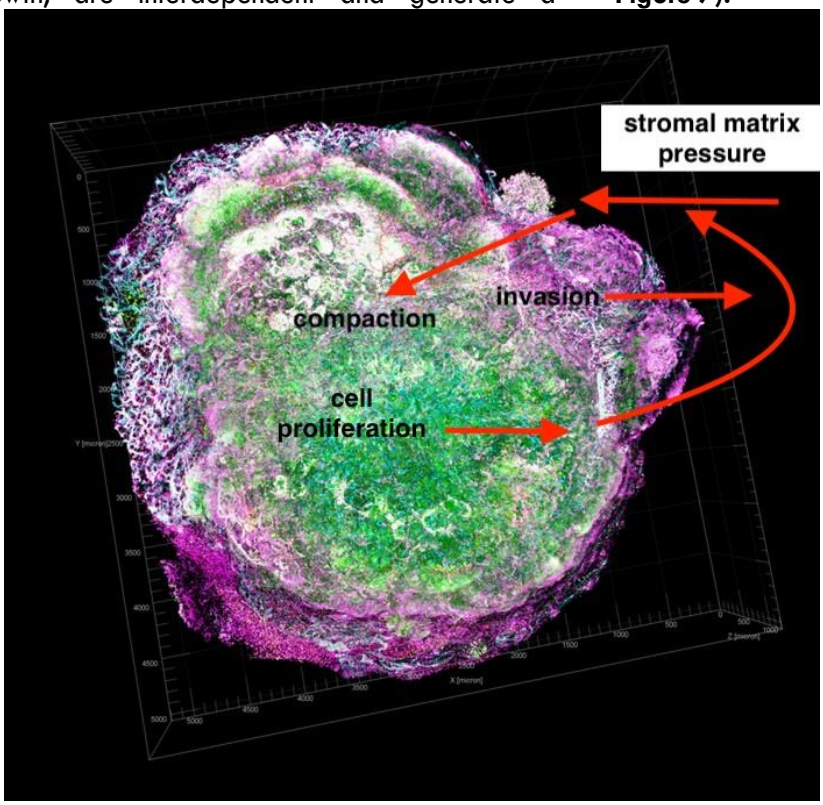


Figure 7. Malignancy Index Model. Diagram depicts the biomechanical forces affecting solid tumor biology. The key driving force is the external pressure generated by the unregulated proliferation of tumor cells from the primary mass. Tumor expansion meets with resistance exerted by the fixed stromal matrix pressure that begins to exert a counterforce causing tumor compaction as the external pressure exerted by the growing tumor mass increases. The biomechanical pressure is relieved only by expansion of the tumor periphery "invasion zone" into the stromal matrix. The malignancy index reflects the composite effects of the inward pressure driving tumor compaction/compression versus the outward pressure driven by cell proliferation towards invasion. The greater the invasion net force relative to the compaction net force, the greater the invasive potential of the tumor. (Breast tumor image courtesy of Steve Seung-Young Lee, National Cancer Institute, University of Chicago Comprehensive Cancer Center, 2015.)

Albumen supplementations produced both greater compaction and invasion zone parameters as compared to agarose at similar concentrations. Undoubtedly, there are multiple explanations for these differences, since both the chemical and physical properties of the two materials are substantively different. It is not surprising that albumen would produce a more significant biological effect on tumor growth parameters since it is a biological substance that plays a very important role in chick development. One may speculate, moreover, that the biomolecular composition of albumen may exert microenvironmental effects on tumors cultured in this medium. Unfertilized chicken egg albumen has a heterogeneous composition of ovomucin, a protein that is responsible for the viscous nature of the albumen, resulting in a heterogenous viscosity and variations in stiffness[19]. Albumen is also rich in avidin, a protein that contains a RYD motif that closely mimics the RGD motif found in vitronectin, the ECM protein attributed to the aggressive malignancy associated with GBM [16, 17]. These biomolecules, either singly or in combination, may produce important microenvironmental interactions with cultured tumor cells. Additionally, the albumen of chicken eggs is transparent, a requirement for image analysis. All these factors, in combination, make the unfertilized chicken egg albumen a useful addition to the design of three-dimensional culture systems that replicate critical components of the tumor stroma.

CONCLUSION

The purpose of this study was to investigate a novel *in vitro* system that utilizes unfertilized chicken egg albumen as a three-dimensional culture system to model stromal biomechanical parameters critical to malignant tumor growth and invasion. This study established that spheroid microtumors could be successfully cultured in albumen supplemented medium to demonstrate a more *in vivo*-like morphology and biological properties that define malignant tumor growth and spread. In addition, a novel physiologically relevant calculation termed the "malignancy index" (MI) is presented as a tool for empirical quantitative assessment of malignant tumor aggression/invasion parameters.

ACKNOWLEDGEMENT

The research was funded by a Connecticut State University Research Grant awarded to S. A. Crawford.

REFERENCES

1. Begley CG, Ellis LM. Raise standards for preclinical cancer research. *Nature* 2012; 483:531-533.
2. Pampaloni F, Reynaud EG, Stetzer EHK. The third dimension bridges the gap between cell culture and live tissue. *Nat Rev*. 2007; 8:839-845.
3. Yamada KM, Cukierman, E. 2007. Modeling tissue morphogenesis and cancer in 3D. *Cell* 2007; 130:601-610.
4. Chiquet M, Gelman L, Lutz R, Maier S. From mechanotransduction to extracellular matrix gene expression in fibroblasts. *Biochim Biophys Acta* 2009; 1793:911-920.
5. Bissell MJ, Jones, PL. Extracellular matrix and integrin signalling: The shape of things to come. *Biochem J*. 1999; 339(3):481-488.
6. Chandrasekaran S, King MR. Gather round: In vitro tumor spheroids as improved models of in vivo tumors. *J Bioeng Biomed Sci*. 2012; 2(4):e109.
7. Yokota J. Tumor progression and metastasis. *Carcinogen*. 2000; 21(3):497-503.
8. Alves TR, Lima FRS, Kahn SA, et al. Glioblastoma cells: A heterogeneous and fatal tumor interacting with the parenchyma. *Life Sci*. 2011; 89:532-539.
9. Holland EC. Glioblastoma multiforme: The terminator. *P Natl Acad Sci*. 2000; 97(12):6242-6244.
10. Bissell MJ, Barcellos-Hoff MH. The influence of extracellular matrix on gene expression: Is structure the message? *J Cell Sci*. 1987; 8:327-343.
11. Weiswald L, Guinebretiere J, Richon S, Bellet D, Saubamea B, Dangles-Marie V. *In situ* protein expression in tumour spheres: Development of an immunostaining protocol for confocal microscopy. *BMC Cancer* 2010;10: 106-117.
12. Kerbel RS. 2003. Human tumor xenografts as predictive preclinical models for anticancer drug activity in humans: Better than commonly perceived—But they can be improved. *Cancer Biol Ther*. 2003; 2(4):S134-S139.
13. Zanoni M, Piccinini F, Arienti C et al. 2016. 3D tumor spheroid models for *in vitro* therapeutic screening: A systematic approach to enhance the biological relevance of data obtained. *Sci Rep*. 2016; 6:19103.
14. Brown MC, Staniszewska I, Lazarovici P, Tuszynski GP, Valle LD, Marcinkiewicz C. Regulatory effect of nerve growth factor in $\alpha_9\beta_1$ integrin-dependent progression of glioblastoma. *Neuro Oncol*. 2008; 10(6):968-980.
15. Burger PC, Green SB. Patient age, histologic features, and length of survival in patients with glioblastoma multiforme. *Cancer* 1987: 59:1617-1625.
16. Grobben B, De Deyn PP, Slegers H. Rat C6 glioma as experimental model system for the study of glioblastoma growth and invasion. *Cell Tissue Res*. 2002; 310:257-270.
17. Kaipparettu BA, Kuiatse I, Chan BT, Kaipparettu MB, Lee AV, Oesterreich S. Novel egg white-based 3-D cell culture system. *BioTechniques* 2008; 45:165-171.
18. Mousseau Y, Mollard S, Qiu H et al. *In vitro* 3D angiogenesis assay in egg white matrix: Comparison to Matrigel, compatibility to various species, and suitability for drug testing. *Lab Invest*. 2014 94:340-349.
19. Kovacs-Nolan J, Phillips M, Mine Y. 2005. Advances in the value of eggs and egg components for human health. *J Agr Food Chem*. 2005; 53:8421-8431.
20. Tse J, Cheng G, Tyrrell J, et al. Mechanical compression drives cancer cells toward invasive phenotype. *PNAS* 2011;109 (3) 911-916.
21. Spencer A, Sligar AD, Chavarria D *et al.* 2021. Biomechanical regulation of breast cancer metastasis and progression. *Sci Rep* 2021; 11, 9838.