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Culture on three-dimensional chitosan-hyaluronic acid scaffolds enhances stem cell marker expression and drug resistance in human glioblastoma cancer stem cells

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Abstract

The lack of *in vitro* models that support the growth of glioblastoma (GBM) cancer stem cells (GSCs) that underlie clinical aggressiveness hinders developing new, effective therapies for GBM. While orthotopic patient-derived xenograft models of GBM best reflect *in vivo* tumor behavior, establishing xenografts is a time consuming, costly and frequently unsuccessful endeavor. To address these limitations, we synthesize a three-dimensional porous scaffold composed of chitosan and hyaluronic acid (CHA), and compared growth and expression of the cancer stem cell (CSC) phenotype of the GSC GBM6 taken directly from fresh xenografts grown on scaffolds or as adherent monolayers. While 2D adherent cultures grow as monolayers of flat epithelial cells, GBM6 cells proliferate within pores of CHA scaffolds as clusters of self-adherent ovoid cells. Growth on scaffolds is accompanied by greater expression of genes that mediate epithelial-mesenchymal transition and maintain a primitive, undifferentiated phenotype, hallmarks of CSCs. Scaffold-grown cells also display higher expression of genes that promote resistance to hypoxia-induced oxidative stress. In accord, scaffold-grown cells show markedly greater resistance to

Supporting Information

Supporting Information is available from the Wiley Online Library or from the authors.

clinically utilized alkylating agents compared to adherent cells. These findings suggest that our CHA scaffolds better mimic *in vivo* biological and clinical behavior and provide insights for developing novel individualized treatments.

Graphical Abstract

A three-dimensional porous scaffold composed of chitosan and hyaluronic acid (CHA) can support the growth of glioblastoma stem cells harvested from tumor tissue. The CHA culture promotes expression of genes that mediate epithelial-mesenchymal transition and represent hallmarks of cancer stem cells. Scaffold-grown cells also have greater resistance to clinically utilized alkylating agents when compared to adherent cells cultured on 2D.

Keywords

chitosan; hyaluronic acid; scaffold; glioblastoma stem cell; drug resistance

1. Introduction

Glioblastoma (GBM) is the most aggressive and deadly human primary brain tumor.^[1] Even with complete surgical resection followed by adjuvant radio-chemotherapy, the prognosis for patients remains dismal with median survival of 12 to 15 months after diagnosis.^[2] The long-standing poor outcome reflects, in large part, extreme genetic and phenotypic intra-tumoral heterogeneity that drives treatment resistance.^[3] GBMs also display a cellular hierarchy with a core of primitive precursor cells, glioblastoma stem cells (GSCs), which give rise to a restricted number of lineages that differ in their biological and clinical behavior, with the mesenchymal lineage being most aggressive.^[4, 5]

The definition of GSCs is primarily functional, reflecting the ability for self-renewal in response to trophic factors elicited by environmental stimuli, and for tumor formation in immune-compromised mice.^[5, 6] While emerging genetic evidence indicates that GSCs frequently express normal neural stem cell developmental pathways,^[7, 8] genetic and

phenotypic features that are unique to GSCs remain poorly defined. Unfortunately, the lack of inexpensive, physiologically relevant methods to culture GSC *in vitro* has greatly limited their characterization. To date, laboratory study of GSCs has employed cell lines grown as adherent cultures on coated plastic surfaces or in suspension as tumorspheres.^[9, 10] These techniques have the advantage of facilitating high throughput analysis for drug screening and novel treatment development. However, establishing long-term cultures is not successful for the majority of tumors, and successful culture, especially in the presence of serum, is accompanied by persistent, non-physiologic genetic and phenotypic changes, including loss of xenograft formation.^[11] The failure to faithfully recapitulate GSC behavior likely reflects the absence of a supporting microenvironment that regulates tumor cell behavior and phenotypic heterogeneity. To circumvent this limitation, considerable effort has been made to develop patient-derived, orthotopic xenograft models of GBM.^[12] While GBM xenografts retain the ability to initiate new tumors by serial passage in nude mice, xenografts are time consuming to establish, labor intensive to maintain, and limited by the expense of housing animals and meeting regulatory compliance. Moreover, many tumors fail to develop as xenografts.

These limitations have stimulated interest in using three-dimensional (3D) biomaterial scaffolds as GBM cell growth substrates that mimic tumor physical and biochemical microenvironment. Naturally derived polysaccharide polymers are attractive materials for constructing scaffolds in view of observations that human tumor cells grown on such substrates better reflect clinical behavior (*e.g.*, greater malignancy and treatment resistance) than cells grown as adherent monolayers.^[13–16] We hypothesize that chitosan and hyaluronic acid (HA) scaffold (CHA) can serve as a cost-efficient and convenient means to culture GSCs that better reflect true tumor behavior for basic science and pre-clinical studies. Chitosan, a biocompatible, biodegradable polycationic linear polysaccharide composed of randomly distributed D-glucosamine and N-acetyl-glucosamine monomers,^[17] is chemically similar to glycosaminoglycans (GAG), major components of tumor extracellular matrix (ECM).^[18] HA is a natural anionic polymer found in synovial fluid, skin, and cartilage, and more importantly is a major GAG component in brain ECM.^[19] We previously demonstrated that CHA scaffolds enabled growth in 3D of a human GBM cell line that had been maintained long term in an adherent 2D culture.^[20] Growth on CHA was also accompanied by changes in gene expression indicative of enhanced malignancy and of a more primitive phenotype characteristic of GSCs. These findings strongly indicated that CHA scaffolds are capable of providing an environment that fosters GBM cell growth that better reflects that observed *in vivo*.

In this study, we compared the growth and gene expression of a documented GSC, GBM6, cultured on CHA scaffolds and poly-L-lysine-coated plastic plates. Both substrates were inoculated with single cell suspensions of GBM6 tumors that were taken directly from fresh xenografts. GBM6 grew as 3D masses of ovoid cells in the pores of CHA suggesting that scaffold interstices provide a niche that favors tumor cell proliferation. Additional evidence that culture on CHA scaffolds better reflects GBM physiology is provided by the higher levels of mRNA expression for genes associated with tumor malignancy and cancer stem cell behavior, including genes that mediate epithelial to mesenchymal transition (EMT). Importantly, expression of EMT genes is reflective of hypoxia, an environmental stimulus

that enhances GSC survival and tumor aggressiveness.^[4, 6] In accord, scaffold cultured cells also showed higher expression of genes known to foster treatment resistance, a finding accompanied by greater survival of alkylating agent-treated scaffold-grown cells compared to their adherent culture counterparts. We propose that CHA scaffolds can serve as a cost-efficient and convenient means to culture GSCs that better reflect true tumor behavior for basic science and pre-clinical studies.

2. Results and Discussion

2.1. Growth and Morphology of GBM6 on CHA Scaffolds

We evaluated the ability of CHA scaffolds to support the *in vitro* growth of GBM6, a line that meets the functional definition of a cancer stem cell (CSC) by readily forming xenografts in nude mice.^[10, 21, 22] Xenograft-derived GBM6 cells suspended in DMEM supplemented with 2.5% FBS were plated on either poly-L-lysine coated 12 well plates or CHA scaffolds. Proliferation was evident on both substrates within 24 to 48 hr after plating (Figure 1). However, cells on CHA scaffolds grew at about half the rate of adherent 2D cultures, a difference we have previously observed for other GBM cell lines grown as 3D cultures.^[20, 23] The difference in growth rate may reflect how cells contact substrate and one another in 2D *versus* 3D. After 24 hr incubation, GBM6 had adhered to the plates as epithelioid-like cells with multiple elongated processes that contacted neighboring cells (Figure 2e–f), a morphology similar to that of many GBM cell lines grown continuously in the presence of serum on coated plastic substrates. In contrast, GBM6 plated on CHA scaffolds grew as clusters of cells (Figure 1 and Figure 2a–d) displaying the characteristic ovoid morphology of undifferentiated cells (Figure 3b) such as seen in cells grown in suspension as “tumor spheres” in serum-free defined medium. The spheroids were well distributed on the scaffolds. Cell-cell interaction was much more extensive on CHA scaffolds as evident by the greater surface area and the number of neighboring cells in contact with one another. Scanning electron microscopy revealed that clusters approximately 50–80 μm in diameter, comparable to the average scaffold pore size,^[20] were located in the interstices of the scaffold (Figure 3a–b). Immunohistochemistry also revealed that clusters were localized in the pores formed by the scaffold matrix, completely filling the volume (Figure 3c–e). Notably, many GBM6 cells appear to have no contact with the scaffold, providing further evidence that cell-cell contact is sufficient to support growth in 3D. Also, some cells in Figure 3b displayed apical processes indicating cellular polarity. Our findings indicate that CHA scaffolds support the growth and maintain the undifferentiated morphology of human GBM CSCs despite the presence of serum. However, spheroids were seen only in the outmost rim of the scaffolds suggesting that the GBM6 cells have limited ability to migrate deep into the scaffolds. In total, these results provide evidence that culture on CHA scaffolds better replicates growth patterns and cell morphology characteristic of cancer stem cells than 2D cultures.

We have previously reported that non-CSC GBM cells usually cultured as adherent monolayers can grow as clusters of ovoid cells on chitosan scaffolds derivatized with either alginate or HA.^[20, 23] However, the same cells cultured on scaffolds made of polycaprolactone or polystyrene failed to grow in 3D. These data strongly suggest that

chitosan plays an important role in supporting GBM6 growth on CHA scaffolds. This conclusion is supported by our earlier work showing that coating a chitosan-based scaffold with polycaprolactone suppressed 3D growth of the GBM line U-87 MG.^[24] Notably, the pattern of growth on CHA scaffolds may reflect a Young's modulus (~ 1.3 kPa^[20]), which is similar to that of brain and is significantly lower than 2D surfaces.^[25] The presence of the brain ECM component HA^[26] may also determine cellular morphology mechanically by binding directly to the cytoplasmic membrane. Alternatively, interaction of scaffold HA with membrane elements may signal changes in the expression of genes that control cell shape and growth patterns (Table 1). In addition to these considerations, the slower growth of GBM6 on CHA scaffolds may also reflect limitations on the diffusion of oxygen and nutrients into and expulsion of metabolic waste from the interior of 3D cell spheroids. Altogether, our findings suggest that CHA scaffolds are capable of recapitulating aspects of the *in vivo* tumor environment that influence cell growth and morphology.

A number of 3D growth matrices have been developed to culture GBM cells in order to facilitate study of tumor cell drug resistance, invasiveness and stem cell markers.^[27–29] Our CHA scaffold differs from these reports in that design was primarily guided by consideration of the biology of GBM and potential clinical utility. As a result, our CHA scaffold affords a number of advantages for experimental investigations and clinical characterization of GBM. Using chitosan-HA polymer better mimics the chemistry of tumor ECM and affords scaffold biocompatibility. The mechanical properties of polymerized CHA permit synthesis across a broad range of diameters appropriate for a variety of multi-well culture plates, facilitating use in high through put screening assays. Importantly, CHA scaffolds are sufficiently robust to be cut into thin sections insuring uniformity of scaffold properties within analyses. The chemistry of the CHA matrix is amenable to modification by other biomolecules that may allow the fabrication of scaffolds to examine specific aspects of GBM biology. For example, inclusion of other known ECM proteins (*e.g.*, periostin) may enhance GBM stem cell invasion and migration on CHA scaffolds as has been observed in other experimental models.^[30]

2.2. Scaffold Growth Promotes EMT in GBM6 Cells

The dramatic change in cell morphology and growth pattern of GBM6 grown on CHA scaffolds is suggestive of the acquisition of a more primitive CSC phenotype, which is maintained by a number of cell signaling pathways.^[3–8] Of particular importance, is the expression of EMT, an epigenetic process that fosters the differentiation of primitive epithelial cells during normal development.^[4] In the context of cancer, EMT is induced by a variety of signals from the tumor environment (*e.g.*, hypoxia, inflammation, trophic signals from tumor and normal tissue) that are believed to play a central role in the genesis and maintenance of the undifferentiated phenotype of GSCs.^[4] The TWIST and SNAIL families of transcription factors mediate EMT by regulating gene expression for a host of stem-like characteristics. TWIST1 and TWIST2 are conserved helix-loop-helix transcription factors that promote dedifferentiation, and enhance cancer stem cell survival, cytoskeletal reorganization, motility and invasiveness. The zinc finger transcription factors SNAIL1 and SNAIL2 also facilitate transition to a mesenchymal phenotype by promoting proliferation,

motility and invasion. We therefore examined whether transition to 3D growth of GBM6 cells on CHA scaffolds was accompanied by altered expression of these mediators of EMT.

Figure 4a compares the relative mRNA abundance determined by quantitative RT-PCR (qRT-PCR) of TWIST and SNAIL family members in GBM6 grown either on CHA scaffolds or as adherent 2D cultures. Content of TWIST and SNAIL mRNA is markedly greater for scaffold-cultured cells, ranging from greater than 40- to 400-fold more than that of adherent cells. These findings strongly indicate that growth on CHA scaffolds promotes expression of a mesenchymal phenotype. Additional evidence is illustrated in Figure 4b that shows that growth on CHA scaffolds is accompanied by loss of expression of E-cadherin, a hallmark of EMT^[4]. Increased expression of FN1 (Figure 4a, far right) coding for fibronectin that plays a critical role in supporting cell adhesion and migration in malignant cells.^[31] These findings indicate that altered morphology of GBM6 on CHA scaffolds is accompanied by EMT and suggest that growth on CHA scaffolds generates stimuli necessary to initiate expression of cell signaling pathways that promote EMT (*e.g.*, Table S1, Supporting Information). Conceivably, these stimuli are generated by scaffold-induced changes in morphology as well as its chemical composition, or by local environmental signals discussed above that are generated as cells form mature clusters. These findings provide additional evidence that scaffold-grown cells better replicate the malignant phenotype of GCSs *in vivo* than 2D adherent cells.

2.3. Growth on CHA Scaffolds Promotes CSC Gene Expression

To further investigate the extent to which growth of GBM6 on CHA scaffolds mimics *in vivo* physiology, we compared the relative mRNA content of a panel of 80 cancer stem cell genes in scaffold and adherent monolayer grown cells. The panel consists primarily of genes that have prominent roles in normal embryonic development, including that of brain, and are frequently co-opted during tumorigenesis.^[3, 6–8, 32] Relative mRNA content differed by less than 2-fold between CHA scaffold and adherent GBM6 cultures for 41 of the CSC genes (Figure S1a, Supporting Information). However, scaffold growth is accompanied by a 1.8- to 26-fold increase in relative expression for 32 genes and by a decrease ranging from 2.3 to ~100-fold lower for 7 genes (Table 1 and Table S3, Supporting Information). The majority of the 39 genes participate in one or more signaling pathways (Table S1, Supporting Information) that promote CSC proliferation, self-renewal, phenotypic preservation, migration and invasion.^[3, 6] Growth on CHA scaffolds alters expression of genes that have roles in EMT (*e.g.*, ZEB2, SNAIL1), stem cell oncogenesis (*e.g.*, LIN28B, MYCN), maintenance of GSC phenotype (*e.g.*, NANOG, ALDH1A1), regulation of motility and invasion (*e.g.*, MRTK, AXL), and cell adhesion (*e.g.*, MUC1, EPCAM). Importantly, the relatively greater mRNA content was accompanied by elevated protein expression as illustrated for the cancer stem cell markers CD44 and SOX2 (Figure S3, Supporting Information), providing evidence that changes in gene expression were mediated by *de novo* protein synthesis.

Table S1 (Supporting Information) also reveals coordinate expression of genes in signal pathways that promote or suppress GSC phenotype. This was evident by the greater expression of genes associated with EMT being accompanied by a 100-fold lower content of

KLF17, a suppressor of EMT. Similarly, mRNA content of WNT which can promote maturation of stem cells^[8] was greatly reduced on scaffolds while abundance of genes that inhibit WNT signaling (*e.g.*, DDR1, DDK1) were increased. We note that examining mRNA using the CSC assay plate revealed mRNA elevation changes in scaffold-grown relative to 2D-grown GBM6 of the EMT genes TWIST1, TWIST2, SNAIL1, and SNAIL2. The results in Table 1 provide confirmatory evidence for the Twist and SNAIL family members shown in Figure 4a. We note that 6 additional genes involved in EMT (Table S1, Supporting Information) were altered when cells grown in scaffolds, suggesting that EMT plays an important role in promoting and maintaining GBM proliferation in scaffolds. Similar coordinated gene expression was also observed for the Hippo pathway, which functions during early development to establish cellular polarity.^[32, 33] Other pathway members showing modest increased expression include Notch2,^[7] STAT3 which promotes acquisition of mesenchymal phenotype,^[34] IKB1B a modulator of the NFKB stress response pathway,^[35] and SAV1, a modulator of the Hippo pathway.^[32] These findings are strong evidence that CHA scaffolds provide an environment that promotes the growth of CSCs that are the progenitors of GBM. Unexpectedly, scaffold-grown GBM6 also had greater content of ENG and IL8, two genes that promote angiogenesis (data not shown), providing additional evidence that CHA substrates provide an environment that better duplicates *in vivo* physiology than 2D adherent cultures.

Whether the acquisition of CSC phenotype reflects chemical and/or physical interaction with the scaffold matrix or response to changes in the local environment as cell clusters form awaits future experiments. Cell growth in scaffolds is characterized by greater expression of genes involved in EMT and response to oxidative stress, a documented environmental stimulus that induces expression of EMT.^[4] Conceivably, growth in CHA scaffolds may also replicate the hypoxic conditions observed in GBM *in vivo* that promote tumor stem cell genesis mediated by EMT.

2.4. Growth on CHA Scaffolds Promotes Treatment Resistance Gene Expression

Accumulating evidence supports the hypothesis that CSCs are uniquely resistant to adjuvant radiation and chemotherapy and are responsible for tumor recurrence.^[3, 6] Impaired treatment response is believed to be the result, at least in part, to CSC responses to hypoxia that promote expression of genes that enhance resistance to apoptosis caused by oxidative free radicals as well as a wide variety of chemotherapeutic agents.^[3, 4, 6] We therefore examined relative mRNA content for a panel of 85 resistance-associated genes in GBM6 grown either on CHA scaffolds or as 2D adherent cultures. As shown in Table 2, Table S3, Figure S1b, and Figure S3 in Supporting Information, greater mRNA content ranging from 1.8- to 20-fold accompanied growth on scaffolds for 29 genes while 4 gene showed 2.1- to 2.9-fold lower content.

As was the case for the CSC gene panel, many of genes in Table 2 have prominent roles in the normal development of brain and other organs that are exploited to enhance the resistance of CSCs to endogenous (*e.g.*, oxidative stress) and exogenous (*e.g.*, tumoricidal agents) insults (Table S2, Supporting Information). Eight of the genes have roles in drug detoxification and drug efflux. Other resistance markers include four cytochrome P450

(CYP) genes, members of a large family that catalyze the hydroxylation of a wide variety of hydrophobic compounds,^[36] and ABCC5, ABCC3 and MVP, components of the multi-drug resistance phenotype responsible for exporting exogenous agents from cells.^[37] Members of both resistance pathways are found in vascular endothelial cells, constituting an element of the blood-brain barrier that protects the central nervous system.^[38] Also showing greater expression are the tyrosine kinase receptors (*e.g.*, ESR1, RARG, ERBB4, MET) which when activated elicit a signaling cascade that results in greater resistance to apoptosis,^[39] as well as tryrosine kinase receptor independent anti-apoptotic genes (*e.g.*, BCL2, B2M). It is notable that a sizeable number of genes promote resistance against oxidative stress, including NFKB family members, PPAR genes and SOD1. Of particular note, is the increased expression of HIF1A (hypoxia-inducible factor-1) that is the master transcriptional regulator of cellular response to hypoxia.^[40] The need for greater protection against oxidative free radicals likely reflects heightened metabolic demands of growth in 3D as well as hypoxia at the core of the clusters of GBM6 cells. Several DNA repair genes that have important roles in preventing or repairing apoptosis-inducing double-strand breaks also display greater relative content. These include MGMT, critical to GBM resistance to temozolomide-based therapies,^[41] XPC, a DNA damage sensor protein, TOP2B and BRCA2 which participate in the non-homologous and homologous recombination repair of double-strand breaks and CDKN13 and CCND1 which arrest DNA replication in response to DNA damage. As illustrated in Figure S3 (Supporting Information), elevated mRNA content was accompanied by increased protein expression for the resistance genes MGMT and HIF1A providing evidence that changes in gene expression were mediated by de novo protein synthesis. Notably, the four genes of scaffold-grown GBM6 showing reduced expression compared to adherent cultures were homologs of genes displaying elevation. These findings strongly suggest that growth on CHA scaffolds mimics physiological conditions that foster expression of cellular defense and anti-apoptotic genes that limit the effectiveness of radio- and chemotherapy.

2.5. Growth on CHA Scaffolds Elevates Alkylating Agent Resistance of GBM6 Cells

Despite their limited efficacy, alkylating agent-based chemotherapies remain the mainstay for post-operative treatment of GBM and other malignant gliomas.^[42] We next examined whether enhanced resistance accompanied greater expression of genes suspected of promoting treatment failure of clinically utilized alkylating agents in CHA scaffold-grown GBM6 cells. As shown in Figure 5a, estimated LD₅₀ for the methylating agent TMZ (temozolomide) of scaffold-grown cells was 3-fold greater than that for adherent cells (Figure 5e). Differences in resistance were even more pronounced for the chloroethylating agents BCNU (carmustine; Figures 5b and 5e) and CCNU (lomustine, Figures 5c and 5e). For the chloroethylating agents, doses that killed more than 90% of 2D adherent monolayers reduced survival of scaffold-cultured cells by no more than 20%. The greater alkylator resistance displayed by scaffold-grown cells likely reflects, at least in part, the elevated expression of MGMT, a DNA repair protein that is the sole mechanism that removes cytotoxic O⁶-alkylguanine adducts from DNA.^[43] Additionally, increased expression of BRCA2, which has a central role in DNA damage response and promotes homologous recombination of lethal double strand breaks, may also promote alkylating agent resistance.^[44, 45]

Additional evidence that differences in drug sensitivity between scaffold and adherent cultures of GBM6 reflect altered gene expression is provided by the identical LD₅₀ for the targeted therapeutic everolimus (Figure 5e). Early clinical trials of everolimus, an inhibitor of mammalian target of rapamycin (mTOR) which activates the AKT pathway,^[46] failed to show activity against GBM which was attributed to p-glycoprotein-mediated multi-drug resistance pathway expression,^[47] drug detoxification, and/or the redundancy of growth pathways that circumvented inhibition of AKT.^[48] Notably, mRNA content for more than half of genes examined that are involved in multi-drug resistance (*e.g.*, ABCB1) and detoxification (*e.g.*, CYP3A4) was not appreciably different between scaffold and 2D cultures of GBM (data not shown), providing a likely explanation for the similar sensitivity between the two growth conditions.

Validation of the biological relevance and utility of our CHA scaffolds is provided by the observation that scaffold-grown GBM6 cells taken directly from flank tumors display cellular morphology, growth as clustered cells, and drug resistance that better reflect cancer stem cell phenotype and clinical malignancy than monolayer-grown cells. Our analyses of mRNA content suggest that the primitive phenotype and enhanced drug resistance is a consequence of altered gene expression. This supposition is supported by the elevated expression of a number of genes that define CSC behavior and phenotype, and promote resistance to endogenous and exogenous cytotoxic insults. Examples include genes that foster cell-matrix and cell-cell adhesion (*e.g.*, MUC1, EPCAM; Table S1, Supporting Information), as well as genes that promote cell proliferation, survival and maintenance of CSC phenotype (*e.g.*, SOX2, TAZ, NANOG; Table S1, Supporting Information). Similarly, the greater alkylating agent resistance displayed by scaffold-grown GBM6 (Figure 5a–e) accompanies greater expression of genes that abrogate the cytotoxic effects of endogenous and exogenous agents (*e.g.*, MGMT, HIF1A, SOD1; Table S2, Supporting Information). Overall, our findings suggest that the biological behavior of scaffold-grown GBM6 cells results from changes in gene expression.

The CHA scaffold also affords a number of advantages for pre-clinical and clinical characterization of GBM. We have previously demonstrated that established GBM cell line cultured on chitosan-alginate scaffolds can be safely implanted in mice.^[23] Because of the biocompatibility of chitosan and HA, we envision that implanting GBM cells attached to CHA scaffolds either intra-cranially or in the flank will also facilitate xenograft formation from human surgical specimens. CHA scaffolds will also allow characterization of signaling pathways that drive GSC malignant behavior, providing pre-clinical evidence for potential new targets for directed therapy as well as facilitate analyses of genomic and phenotypic heterogeneity within and between GBMs. Associating these parameters with patient outcome in future studies will likely identify signatures associated with treatment outcome. This goal will also be served by using scaffold-grown cells to evaluate drug and radiation sensitivity. Such analyses will likely reveal intra-tumoral heterogeneity of response to cytotoxic agents that could guide the development of multi-agent regimens for GBM. Thus, CHA scaffolds have great potential to speed the development of personalized therapies to counter the dismal prognosis of GBM.

Conclusions

We provide strong evidence that primary human GBM6 cell taken directly from flank xenografts when grown 3D on a porous scaffold composed of chitosan derivatized with HA display cellular morphology and patterns of growth that better reflects malignant behavior compared to cells grown as adherent monolayers. Notably, growth on the scaffolds is accompanied by elevated expression of genes that define the undifferentiated, primitive phenotype that characterizes CSCs and promote resistance to endogenous and exogenous cytotoxic insults. The gene expression patterns strongly indicate that scaffold grown cells experience hypoxic stress, an *in vivo* stimuli originating in the tumor microenvironment that fosters CSC development. The ability to replicate critical features of CSC physiology *in vitro* suggests that the scaffolds can provide a facile method to propagate and characterize GSCs in the laboratory. Culturing GSCs directly from surgical specimens will facilitate screening assays to identify the most effective approach to treatment using conventional therapies. More importantly, growth on the scaffolds will facilitate defining the role of various signaling pathways that drive GSC malignant behavior, thus identifying new targets for directed therapy.

3. Experimental Section

Materials

All chemicals and reagents were purchased from Sigma-Aldrich and all tissue culture reagents were purchased from Life Technologies unless otherwise specified. Chitosan (from shrimp shells, practical grade, >75 % deacetylated) and HA (HA sodium salt, from *Streptococcus equi*) were used as received.

Scaffold synthesis

CHA scaffolds were produced as described previously.^[20] In brief, chitosan and HA were dissolved separately into a 1 wt% acetic acid solution. Chitosan was dissolved to obtain a 4 wt% solution and HA was dissolved into a 1 wt% solution. The solutions were mixed using a Thinky mixer (ARM-300) at 2000 rpm for 3 min for a minimum of three times or until completely dissolved. The solutions were then allowed to age overnight at room temperature to ensure complete dissolution. After aging, the solutions were mixed together at 2000 rpm for 3 min four times before being cast in 24-well tissue culture plates. The plates were then refrigerated at 4°C for 12 hr, frozen at -20°C for 24 hr, and lyophilized for 36 hr with a Labconco Freezone 6 Plus freeze drier. The scaffolds were sectioned to obtain 2 mm thick discs, neutralized in 25 v% ammonium hydroxide for 1 hr under vacuum, washed four times with excess DI water, and soaked in Dulbecco's phosphate-buffered saline (DPBS) overnight to ensure the removal of any remaining base. The neutralized scaffolds were then sterilized with 70% ethanol for 1 hr under vacuum before being transferred to sterile DPBS and placed on an orbital shaker overnight to remove any remaining ethanol before use.

Cell culture

GBM6 was obtained from the laboratory of J.N. Sankaria. This line was established GBM6 cells were isolated from tumors and cultured in Dulbecco's Modified Eagle's Medium

(DMEM) supplemented with 2.5% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO₂ as previously described.^[21] The medium was changed every 4–5 days. For CHA scaffold culture, 50,000 cells were seeded on each scaffold in 12-well plates and allowed to grow for 14 days before harvest for quantitative RT-PCR (qRT-PCR) or drug response study. GBM6 cells were seeded at 50,000 cells per well in 12-well plates as the 2D control for *in vitro* assays.

Cell proliferation assay

The Alamar blue assay was used to evaluate cell proliferation on both 2D cultures and CHA scaffolds following the manufacture's protocol (Life Technologies). Briefly, GBM6 cells were cultured in 2D and CHA scaffolds as described previously. Cells were washed with DPBS three times before adding 10% AB solution in the complete growth medium to the wells. The samples were incubated at 37°C for a predetermined time for each culture method (1 hr for 2D and 2 hr for CHA scaffolds). Then the AB solution was transferred to a 96-well plate, and the fluorescence at an excitation wavelength of 556 nm and an emission wavelength of 586 nm was measured on a SpectraMax M5 microplate reader (Molecular Devices). The cell number was calculated according to the previously generated standard curves for 2D and CHA cultures individually.

qRT-PCR

Prior to the evaluation of gene expression, we firstly identified an appropriate reference gene for qRT-PCR since 3D culture could alter expression of the standard reference genes.^[49, 50] The gene expression levels of candidate reference genes were determined using a panel of 14 genes expected to show low variability between experimental conditions (Bio-Rad, Catalog No. 10025898). Expression stability, reflected by the M value calculated as the average pairwise variation between a particular gene and all other reference genes remaining in the gene panel, was determined. The lowest M value represents the most stable transcription.^[51] As shown in Figure S2 (Supporting Information), 11 out of 14 evaluated reference genes had an M value below the limit of 1.0. TFRC and ACTB showed the most stability between 2D and CHA culture of GBM6 cells with the lowest M values of 0.541. Therefore, we chose ACTB as the reference gene for the qRT-PCR studies.

RNA was extracted from GBM6 cells cultured in 2D or CHA scaffolds for 14 days using the Qiagen RNeasy kit. cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's protocol. SYBR Green real-time PCR array were performed using the commercially available predesigned 96-well panel (Bio-Rad, catalog No. 10047231 and 10034111) profiling the expression of 80 and 85 genes encoding CSC and chemotherapy drug resistance markers following the manufacture's manual. All qRT-PCR data were analyzed using the CFX Manager software (Bio-Rad). The EMT primers are indicated in Table 3.

Western blot

GBM6 cells were cultured in 2D or CHA scaffolds for 14 days. Then cells were collected and lysed by incubation for 15 min on ice in 0.1% Triton X-100 in PBS containing 2% β -mercaptoethanol. Samples were boiled in Laemmli sample loading buffer at 100°C for 5

min. 10 µg of extract protein was electrophoresed through SDS-PAGE gels and transferred onto nitrocellulose membranes. Membranes washed three times with TBS were blocked with 3% QuickBlocker (Chemicon) in TBS for 1 hr at room temperature and then incubated overnight at 4°C with primary antibodies against SOX2, CD44, MGMT, and HIF1A (Abcam) in the blocking buffer. Membranes were washed with TTBS before being incubated for 1 hr with alkaline phosphatase-conjugated secondary antibody (Bio-Rad) at room temperature. Membranes were then washed thrice with TTBS and antibody binding visualized by chemiluminescence (Immun-Star detection kit; Bio-Rad) and quantified using the ChemiDoc system running the Quantity One software package (Bio-Rad).

Scanning electron microscopy (SEM)

SEM analysis was conducted on cell cultured scaffolds after the samples were fixed with 10% formalin at 4°C overnight. CHA scaffolds containing GBM6 tumor spheroids were then dehydrated using a series of ethanol washes (0%, 30%, 50%, 70%, 90%, 100%) and allowing the samples to soak for 1 hr. The scaffolds were critical point dried, sectioned and mounted on stubs using carbon tape. The scaffolds were then sputter-coated with Au/Pd for 80 sec before imaging with a FEI Sirion XL30 Field Emission SEM.

Histological analysis

CHA scaffolds containing GBM6 tumor spheroids were fixed with formalin at 4°C overnight, and then dehydrated with sequential incubations in 70%, 85%, 95%, 100% ethanol, and xylene for 1 hr at each step. Scaffolds were embedded into paraffin, cross sectioned into 4µm-thick sections. The sections (4 µm thickness) were stained with hematoxylin and eosin (H&E) and photographed under a Nikon ECLIPSE TE2000-S microscope.

Drug response study

GBM6 cells grown on 2D surfaces and CHA scaffolds for 14 days were treated with TMZ (temozolomide), BCNU (carmustine), CCNU (lomustine), and everolimus with three samples for each trial. DMEM containing drug was replaced with fresh medium at 24 hr after treatment. Cell viability was examined on 7 days after treatment using the Alamar blue assay as described previously. Cell viability was reported as percent of viable cells relative to an untreated control.

Statistical Analysis

All of the data was statistically analyzed to express the mean ± standard deviation (SD) of the mean. Statistical significance was set at $p < 0.05$ and tested with Student's *t*-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Growth kinetics of GBM6 cells in 2D adherent and 3D CHA scaffold-grown cells as determined by the Alamar blue assay. Inset bright-field images indicate tumor spheroids cultured in CHA scaffolds for 4, 7, 10, and 14 days. Scale bar = 50 μm.

Figure 2. Bright-field images of GBM6 cells growing on CHA scaffolds (a–b, low magnification; c–d, high magnification) and as 2D adherent cultures (e–f) for 7 and 14 days. Scale bars represent 50 μm . Arrows and circles indicate tumor spheroids.

Figure 3. SEM images of representative GBM6 cells growing in CHA scaffolds. Scale bars represent 200 μm in (a) and 20 μm in (b). Note the apical appearance of cells on the exterior of the cluster. (c–e) H&E staining of GBM6 tumor spheroids in CHA scaffolds. Scale bars represent 50 μm .

Figure 4. mRNA expression of EMT genes in GBM6 primary cells quantified by qRT-PCR. (a) Expression of EMT genes. (b) E-cadherin expression of scaffold-grown relative to 2D adherent grown cells. Expression was normalized to that of the beta-actin (ACTB) that was chosen as a reference gene as described in Experimental Section.

Figure 5. GBM6 cells grown in 2D cultures and CHA scaffolds in response to drugs (a) TMZ, (b) BCNU, (c) CCNU, (d) Everolimus, and (e) LD₅₀ of the four chemotherapy drugs.

Relative CSC mRNA content (mean $\pm$ SD) in GBM6 from 3D CHA scaffold compared to 2D adherent cultures. Expression was normalized to that of the actin B (ACTB) that was chosen as a reference gene by a process described in Experimental Methods. See Table S1 for gene function and pathway involved.

Table 1

ZEB2	PTPRC	TWIST1	NANOG	MYCN	DDR1	IL8	POU5F1	SNAIL1
26 $\pm$ 0.11	17 $\pm$ 0.21	14 $\pm$ 0.11	14 $\pm$ 0.10	13 $\pm$ 0.13	13 $\pm$ 0.09	11 $\pm$ 0.08	11 $\pm$ 0.12	9.1 $\pm$ 0.21
MUC1	LIN28B	EPCAM	MERTK	FZD7	B2M	NFKB1	ALDH1A1	TWIST2
4.9 $\pm$ 0.31	4.1 $\pm$ 0.13	4.0 $\pm$ 0.15	3.7 $\pm$ 0.24	3.1 $\pm$ 0.23	3.0 $\pm$ 0.17	2.8 $\pm$ 0.08	2.8 $\pm$ 0.06	2.8 $\pm$ 0.19
DKK1	ATXN1	MAMML1	ZEB1	SMO	SOX2	THY1	PLAT	ITGB1
2.7 $\pm$ 0.11	2.7 $\pm$ 0.09	2.6 $\pm$ 0.10	2.6 $\pm$ 0.07	2.5 $\pm$ 0.09	2.5 $\pm$ 0.08	2.4 $\pm$ 0.10	2.3 $\pm$ 0.14	2.3 $\pm$ 0.07
IGGB1	YAP1	ENG	TAZ	NOTCH2	IKB1B	STAT3	SAV1	
2.3 $\pm$ 0.29	2.2 $\pm$ 0.26	2.1 $\pm$ 0.23	2.0 $\pm$ 0.15	2.0 $\pm$ 0.10	2.0 $\pm$ 0.15	2.0 $\pm$ 0.07	2.0 $\pm$ 0.09	
MYC	CD44	WWC1	PLAUR	AXL	ITGA4	WNT1	KLF17	
0.47 $\pm$ 0.002	0.42 $\pm$ 0.008	0.36 $\pm$ 0.006	0.34 $\pm$ 0.003	0.25 $\pm$ 0.004	0.22 $\pm$ 0.007	0.08 $\pm$ 0.01	<< 0.01	

Table 2

Relative resistance gene mRNA content (mean $\pm$ SD) in GBM6 from 3D CHA scaffolds compared to 2D adherent cultures. Expression was normalized to that of the actin B (ACTB) that was chosen as a reference gene by a process described in Experimental Methods. See Table S2 for gene function and pathway involved.

CYP2B6	CYP2C19	BRCA2	ESR1	CYP2D6	NAT2	FOS	RARG	ERBB4
21 $\pm$ 0.10	16 $\pm$ 0.21	13 $\pm$ 0.22	13 $\pm$ 0.12	6.0 $\pm$ 0.08	5.8 $\pm$ 0.31	5.0 $\pm$ 0.23	4.8 $\pm$ 0.08	4.4 $\pm$ 0.13
BCL2	MET	XPC	B2M	NFKB1B	PPARD	CYP3A5	PPARG	CDKN1B
4.1 $\pm$ 0.16	4.0 $\pm$ 0.19	3.6 $\pm$ 0.29	3.5 $\pm$ 0.13	3.4 $\pm$ 0.11	3.2 $\pm$ 0.25	3.1 $\pm$ 0.01	2.8 $\pm$ 0.05	2.7 $\pm$ 0.12
TOP2B	RPLP0	ABCC5	NFKB1	MVP	RELB	SOD1	MGMT*	APC
2.7 $\pm$ 0.09	2.6 $\pm$ 0.08	2.6 $\pm$ 0.10	2.4 $\pm$ 0.11	2.2 $\pm$ 0.12	2.0 $\pm$ 0.15	2.0 $\pm$ 0.13	2.2 $\pm$ 0.20	2.0 $\pm$ 0.01
HIF1A	ARNT				RARB	ESR2	ABCC3	CCND1
1.8 $\pm$ 0.14	1.8 $\pm$ 0.22				0.47 $\pm$ 0.005	0.42 $\pm$ 0.003	0.36 $\pm$ 0.002	0.34 $\pm$ 0.001

* mRNA content determined independently of other genes

Table 3

Primers for EMT-associated genes.

gene	Forward	reverse
<i>ACTB</i>	CGGTTCCGATGCCCTGAGGCTC	CGTCACACTTCATGATGGAATTG
<i>CDH1</i>	AGATGACACCCGGGACAACGT	ACTGGCTCAAGTCAAAGTCCT
<i>SNAIL1</i>	TTTCTGGTTCTGTGTCCTCTG	TTCCCAGTGAGTCTGTCAGCC
<i>SNAIL2</i>	TTTCTGGGCTGGCCAAACATA	ACACAAGGTAATGTGTGGGTC
<i>TWIST1</i>	ACCATCCTCACACCTCTGCAT	TTCCTTTCAGTGGCTGATTGG
<i>TWIST2</i>	ACTGGACCAAGGCTCTCAGAA	TTCCAGGCTTCCTCGAAACAG
<i>FN1</i>	AAACTTGCATCTGGAGGCAAA	AGCTCTGATCAGCATGGACCA