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Review Article

In Vitro Reconstruction of Brain Tumor Microenvironment

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Abstract The cancer cells in brain tumors interact with their microenvironment, which includes stromal cells, the extracellular matrix (ECM), and the physical properties of tissues. The reciprocal interaction between cancer cells and the surrounding microenvironment regulates the biological behavior of cancer cells. To improve our understanding of the progression of brain tumors, it is useful to construct physiologically relevant brain tumor models. Consequently, versatile *in vitro* tumor models ranging from simplistic two-dimensional (2D) cultures to three-dimensional (3D) cultures have been developed to mimic the microenvironments of the brain. This review covers the recent progress in the in vitro reconstruction of brain tumor microenvironments.

Keywords: Brain tumor, Microenvironment, In vitro model

Introduction

A brain tumor is a growth of abnormal cells in the brain tissues that multiply in an uncontrolled manner. Clinically, the World Health Organization (WHO) classifies brain tumors into four grades according to histological and molecular parameters.[1] Grades I and II are benign tumors that grow slowly and are the least aggressive. Malignant, high-grade (grades III and IV) brain tumors grow rapidly and consist of abnormal-appearing cells that infiltrate the surrounding tissues and have a poor prognosis.[2] The standard treatments for brain tumors are surgical resection, radiotherapy, and chemotherapy with alkylating agents.[3] However, these therapies focus on inhibition of the neoplasm or proliferating cells, and not on the cells infiltrating the brain.[4,5] Therefore, these conventional therapies lack efficacy in most high-grade brain tumors and the patients have poor outcomes or develop recurrent tumors.

To enhance treatment effectiveness and predict the prognosis, it is important to understand the characteristics of brain tumors, including their growth and invasion. The brain tumor microenvironment, including blood vessels, immune cells, inflammatory cells, signaling molecules, and the extracellular matrix (ECM), can regulate tumor progression by interacting directly with cancer cells.[6-8] Brain tissues have unique microenvironments that distinguish them from other tissues with low stiffness and loosely connected cellular network,[9] including the composition of the ECM, anatomical structures, and specialized cell types, such as neurons, astrocytes, and microglia. The ECM component of brain tissue contains high amounts of hyaluronic acid (HA), glycosaminoglycans (GAGs), and proteoglycans, but lacks fibrous materials such as collagen, fibronectin, etc.[9,10] The interaction between cancer cells and the unique extracellular microenvironment of the brain can affect the progression of aggressive tumors. Clinical and experimental data have demonstrated that diffusive invasion of cancer cells is regulated by several independent mechanisms including different anatomic and molecular pathways.[11-13] Therefore, an understanding of these complex interactions is essential for developing new therapeutic strategies.

Recently, versatile *in vitro* tumor models have been developed to mimic the brain tumor microenvironment, reflecting the unique features of the brain stroma,

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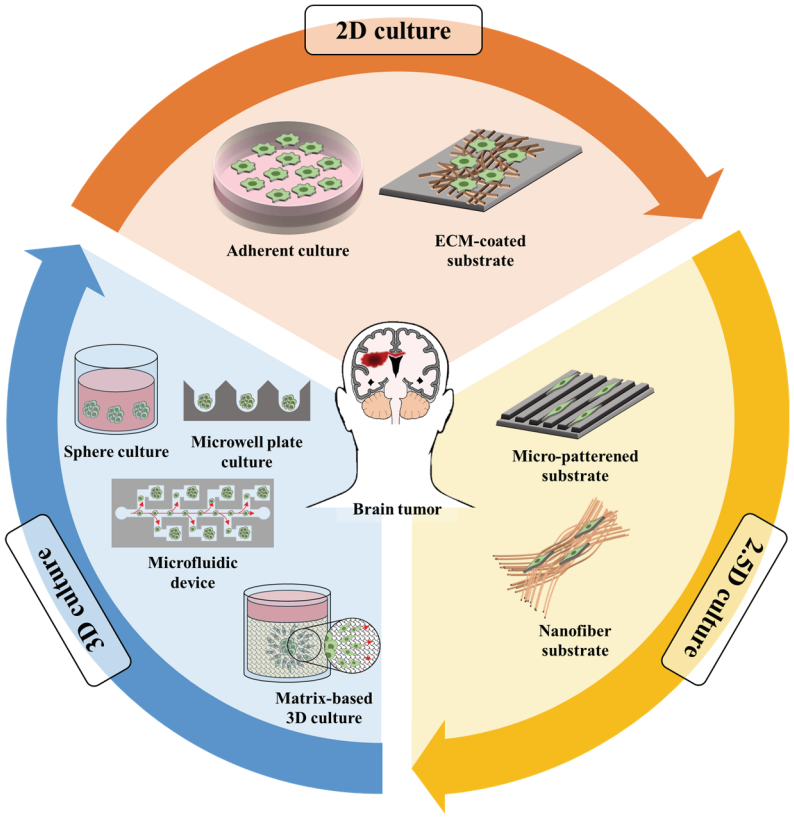

Figure 1. Advancement of *in vitro* brain tumor models. 2D adherent cultures on culture plate and ECM-coated substrate (top). 2.5D cultures on micro-patterned substrate and nanofiber substrate (right). 3D spheroid culture and matrix-based 3D culture (left).

including its structure and the ECM composition. In this review, we introduce the latest *in vitro* brain tumor models (Figure 1) used to reconstruct complex brain microenvironments. Then, future perspectives for recapitulating the brain microenvironments are suggested.

Two-dimensional (2D) Models

Over several decades, various *in vitro* tumor models have been developed to study cancer biology and drug screening. In the simplest approach, 2D tumor models were used for a wide variety of fundamental cancer research. However, 2D culture models are too simple to reflect the complexity of the *in vivo* environment.[14,15]

To replicate the microenvironment features of the brain in the 2D model, researchers have been culturing cancer cells on substrates coated with ECM biomolecules or materials that exhibit the characteristics of native brain tissues (Table 1). This enables cell–ECM interactions within brain tumor microenvironments. In the brain, the ECM contains few fibrous proteins and large amounts of PG, GAG, and glycoproteins. The greatest volume of brain parenchyma is filled with HA, a negatively charged, unbranched GAG, which is the main organizer of the ECM, interacting with proteins and PG. By contrast, fibrous proteins such as collagen, fibronectin, and laminin are expressed only in the brain vasculature.[10,12,16] Therefore, a substrate coated with an HA-based hydrogel[17-19] is preferred to elucidate the effects of the brain-specific ECM microenvironment on cell behaviors. On HA-coated substrate, the Caucasian glioblastoma (GBM)–astrocytoma cell lines U87MG and U373MG showed increased migration speed.[17] Moreover, to elucidate the effects of substrate stiffness on cell behavior, many studies have cultured cells on 2D substrates with different mechanical properties, such as polyacrylamide,[11,20] silicon rubber,[21] and HA.[17-19] A rigid substrate tends to increase the motility, actin formation, adhesion, and proliferation of brain tumor cells.[22]

2.5D Models

In addition to the brain-specific ECM composition, the brain has unique anatomical structures, including the

grey and white matter. The grey matter is composed of neurons and the white matter is formed from bundles of aligned axons; both are fibrous structures with submicron-sized fibers.[23] Specific anatomical structures called Scherer structures may significantly increase the speed of invasion and distance traveled by cancer cells through the brain parenchyma, such as along white matter tracts and capillaries.[24-26] These topological features of the brain can be reproduced using micro-engineered fabrication techniques, including micro-patterned substrates,[20,27] and aligned nanofibers.[28-30] On these substrates, cancer cells display increased polarity and migration speed compared to on a flat substrate. For instance, in micro-tracts smaller than 3 μm, cancer cells exhibited the native characteristics and behavior induced by topographic cues, allowing saltatory migration.[26]

Three-dimensional (3D) Culture Models

Although 2D culture models have been used in basic research to provide various types of information, the absence of the complexity of the *in vivo* microenvironment, such as cell-cell, results in the high failure rate of drug screening studies.[14,15] Recent studies have shown that 3D culture allows a systemically designed microenvironment that includes cell type, dimensionality, ECM, and the enrichment of soluble factors (*i.e.*, growth factors). In addition, 3D culture models exhibit several *in vivo*-like features, including cell-cell interactions,[31] hypoxia,[32] oxygen/medium penetration,[33] and drug resistance.[14,15] Therefore, these culture models have been used increasingly for a wide variety of basic cancer and pre-clinical research.[34-36] Here, we present an overview of the latest 3D *in vitro* brain tumor models (Table 1) used to mimic brain microenvironments.

Multicellular Tumor Spheroid (MCTS)

Since Sutherland and coworkers introduced MCTS, one of the simplest 3D culture methods, in the early 1970s,[37,38] they have provided important insights into tumor biology because of their *in vivo*-like features (cell-cell interactions, proliferation, and nutrient/oxygen gradients). There are versatile culture models for MCTS formation, including the culture of cells on non-adherent plates, spinner flasks, or rotary cell culture systems.[39-41] To enhance production efficiency and size uniformity, various methods have been developed, including hanging drops,[42,43] microwells,[44] and microfluidic devices.[45]

Indeed, cells within tissues or organs interact with the surrounding microenvironment, such as resident cells, the ECM, soluble factors, and nutrient/oxygen gradients. These interactions establish a communication network that regulates tissue function and homeostasis.[46] MCTS re-establish such cell-cell interactions and nutrient/oxygen gradients, which mimic *in vivo*-like features better than 2D cultures. MCTS can be used with matrix-free/matrix-based culture models for basic cancer research and pre-clinical research on brain cancer. Most scaffold-free culture models can be used to characterize MCTS[39,47], or for drug screening.[41,45] For instance, using a microfluidic device, uniformly sized MCTS were produced in mass and could be used for examining multiple-simultaneous drug treatment and testing drug responses.[45] In addition, GBM spheroids were genetically more representative of the parental tumor profile than 2D cultures.[47] Nevertheless, it is hard to replicate cell–ECM interactions or interactions with other cells (immune cells, fibroblast, etc.) within the MCTS. Therefore, matrix-based culture models have been developed to examine cell–ECM interactions and are introduced in the next section.

Matrix-based Culture Models

As mentioned above, the ECM of the brain has a composition distinct from that of peripheral tissues, with few fibrous proteins and large amounts of PG, GAG, and glycoproteins.[12,16] Most of the brain parenchyma is composed of HA, which interacts with CD44 and RHAMM receptors, promoting the proliferation, invasion, and drug resistance of brain tumors.[18,48,49] Moreover, there is accumulating evidence that specific ECM components such as HA, vitronectin, and tenascin-C are dysregulated in brain tumors, which may alter cellular invasiveness.[10,26] Indeed, dysregulation of ECM remodeling is common in cancer and fibrosis.[50] Therefore, it is important to examine the brain ECM features of tumors to understand brain tumor biology, including biophysical and biochemical characteristics.

To study the effects of biophysical cues on tumor cell behavior, it is important to control the mechanophysical properties of the 3D matrix, including stiffness, degradability, and pore size. Alginate,[51] chitosan-alginate hydrogels,[52] collagen-agarose hydrogels,[53] matrix metalloproteinase (MMP)-degradable poly (ethylene glycol) (PEG) gels,[54,55] gelatin methacrylate (GelMA),[56] and HA-based hydrogels[19,57] are used to investigate biophysical impacts on cancer cell behaviors in 3D brain tumor models. Use of these materials showed that biophysical cues play an important

Table 1. Representative in vitro models to mimic brain microenvironments.

2D culture model				
Substrate	Mimicking Environmental Factor	Cell	Finding	Ref
Polyacrylamide	Biophysical cue	U373MG, U87MG	Increasing substrate stiffness and confinement increased cell migration	9, 15
Silicon rubber		SNB-19	Increasing substrate stiffness increased cell adhesion and migration	16
HA-methacrylate	Biophysical cue ECM component	U373MG	Increasing stiffness and fibronectin increased cell migration	17
HA-methacrylate (with RGD peptide)		U373MG	Cell invasion that involves a balance between formation and turnover of cell adhesions	18
2.5D culture model				
Micro-patterned substrate	Anatomical structure	U87MG, U251MG	Topological structure induced saltatory migration	25
Nanofiber		U251MG, X12	Fiber directionality influence directional migration of cells	26
		U87MG, A172	Aligned nanofiber elevate GBM cell migration	27
		OSU-2 (patient derived cell)	Mimic the topological feature of white matter tract	28
3D culture model				
Matrix-free culture model				
Culture method	Mimicking Environmental Factor	Cell	Finding	Ref
Non-adherent culture plate	Dimensional effect Cell-Cell interaction	BGM-1	Spheroid displayed intricate/complex nature of endogenous as well as induced stress resistance that could exist in tumors	37
Agar-coated 96well plate		Patient-derived cells	GBM spheroid displayed genetically more representative of parental tumor profile compared to 2D culture	44
Microfluidic device		U87MG	Chip is capable of high-throughput GBM spheroids formation, multiple-simultaneous drug administration, and a massive parallel testing of drug response	39
Matrix-based culture model				
Matrix	Mimicking Environmental Factor	Cell	Finding	Ref
Alginate (with RGD peptide)	Biophysical cue (dimensionality, stiffness, pore size, matrix degradability)	U87MG, U51	Cells were more susceptible to toxins in softer hydrogels	48
Chitosan-Alginate		C6, U87MG, U118MG	GBM cells display more malignancy in 3D	49
Collagen		C6	Pore size is a key determinant of glioma invasive speed in collagen gels	45
Collagen-agarose		U87MG, U373MG, U251MG	Softer hydrogels promote GBM cell migration	50
Polyethylene glycol (PEG) (with MMP-cleavable prptide)		U87MG	Increased concentration of MMP-degradable site promotes cell migration	52
Gelatin methacylate (Gel-MA)		U87MG	Biophysical factors play in the etiology, growth, and subsequent invasive spreading of gliomas	53
Thiolated-HA	Biophysical cue ECM component	U87R, U118	Increasing stiffness reduced migration distance.	54
HA-methacrylate (with RGD peptide)		U373MG, U87MG	In 3D sphere, glioma cells invaded HA hydrogels with morphological patterns distinct from those observed on flat surfaces or in 3D collagen-based ECMs but highly reminiscent of those seen in brain slices.	19
HA-methacrylate		U87MG	Increased HA content reduces cell proliferation	55
Collagen I-HA (IPN)	ECM component	Patient-derived cells	GBM cell invasion was increased in collagen I-HA hydrogel	56
Decellularized brain matrix		Patient-derived cells	Within the decellularized ECM, GBM cells displayed heterogeneous invasion strategies and upregulated HA-related genes	61

role in regulating brain tumor progression, including proliferation, gene expression, and invasion. For example, the increased MMP-degradable sites of PEG promote the invasion of GBM.[55] U87R and U118 showed reduced migration distance on increasing the stiffness of HA-based hydrogels.[57]

HA-based hydrogels have been widely utilized in *in-vitro* 3D culture models to mimic the ECM components of the brain because HA is the most abundant component of the ECM of the brain. However, HA cannot form cross-links alone and must be mixed with chemically modified HA, such as thiolated[57] or methacrylated[18,19,58] HA, or with other materials, such as interpenetrating polymer networks.[59,60] Within these HA-based hydrogels, brain tumor cells were not only highly invasive and proliferated via CD44-mediated adhesion,[60] but also increased oncogenic markers.[61]

Advanced strategies to mimic the brain ECM have used decellularized matrix obtained from brain tissues to reconstruct *in vitro* models. Decellularization removes the cellular components from tissues or organs, leading to the production of cell- or tissue- derived ECM that preserve the complex mixture of *in vivo* ECM components and structure without antigenicity.[62] In brain research, the decellularized porcine brain is widely used to reconstruct the brain ECM.[63,64] Recently, an *in vitro* model that utilized patient-derived brain tissues and GBM was introduced.[65] Within the decellularized ECM, GBM cells displayed heterogeneous invasion strategies and upregulated brain ECM-specific component-related genes, such as HA.

Conclusion

This review provided an overview of the latest *in vitro* brain tumor models used in biomedical research. The use of biomimetic *in vitro* brain tumor models enables the investigation and evaluation of the characteristics of brain tumors, such as invasion and proliferation, and could be applied to drug screening. For further progress, *in vitro* brain tumor models should incorporate other environmental factors, such as intratumoral heterogeneity, growth factors, and interactions with surrounding cells, to improve our understanding of the characteristics of brain tumors. In the future, we expect that the integration of various environmental components of the brain will enhance our understanding of brain tumor biology and inform the choice of ECM-targeted therapeutic options for patients.

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Conflict of Interests The authors declare no competing financial interests.

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