3D Tumor Spheroid to Model Tumor Microenvironment

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The tumor microenvironment (TME) is a complex and dynamic entity composed of malignant and non-malignant cells, including innate and adaptive immune cells, fibroblasts, adipocytes, vascular and lymphatic endothelial cells, pericytes, and the extracellular matrix (ECM). Both the physical and biochemical features of the tumor microenvironment (TME) play a critical role in promoting the differentiation, proliferation, invasion, and metastasis of cancer cells. It is therefore essential to understand how malignant cells interact and communicate with an assortment of supportive tumor-associated cells including macrophages, fibroblasts, endothelial cells, and other immune cells. To study the complex mechanisms behind cancer progression, 3D spheroid and organoid models are widely in favor because they replicate the stromal environment and multicellular structure present within an in vivo tumor. It provides more precise data about the cell–cell interactions, tumor characteristics, drug discovery, and metabolic profile of cancer cells compared to oversimplified 2D systems and unrepresentative animal models.

Keywords: 3D ; tumor ; TME

1. 3D Modeling of the Tumor Microenvironment

Most research in cancer biology is based on experiments involving 2D cultures; however, 2D cell culture techniques have many limitations. Direct comparisons indicate that the tumor microenvironment (TME) is better simulated through 3D cultures compared to 2D tumor models, as 2D methods fail to mimic cell–cell and cell–extracellular environment interactions [1][2][3]. Additionally, the modes of cell division and adhesion are limited under 2D conditions [1]. As the traditional monolayer cell culture model cannot accurately test drug resistance and may display misleading results, efforts have been directed toward the field of new in vitro tumor models to better represent the TME [1][3][4][5][6]. Modern approaches of multicellular layers, 3D scaffolds, 3D bioprinting, microfluidics, and spheroid models were established to model the TME through relatively inexpensive and convenient methods [1][4][5][6][7][8][9][10][11][12].

In the last century, multicellular layers (MCLs, also referred to as multicellular models or MM models) were developed. This in vitro model mimics the TME and is based on culturing cells at tissue-like densities on collagen-coated microporous Teflon membranes [7]. Since cells cultured via MCLs are physiologically similar to cells observed in in vivo tumors, researchers have used them to study the penetration of chemotherapy anticancer drugs [7][13]. In addition, 3D bioprinting–a novel technology–can be further utilized to construct a 3D scaffold, preserving the tumor network structure and extracellular matrix (ECM) architecture, while manipulating the microenvironment with high reproducibility [8]. Microfluidic cell culture, in contrast, can independently modify all parameters of the synthetic culture system, such as cell types, cell positions, and precise orientation of tissue–tissue interfaces [10]. Additionally, the Tumor-on-a-Chip Device allows the interaction study among breast cancer cells, monocytes, and endothelial cells to examine T-cell infiltration [11].

While engineering-based approaches emphasize model structure and composition, cell-based approaches utilize the inherent capabilities of cells to organize into 3D aggregates without many external cues [12]. Three-dimensional spheroids were initially developed as a more cost-effective and simple approach to 3D modeling compared to prior attempts; this technology has been implemented to facilitate the production of cell spheroids under reproducible conditions in a high-throughput manner [5][12]. The primary features found in solid tumors in vivo and in other 3D models, such as cellular heterogeneity, cell–cell signaling, ECM interactions, growth kinetics, and drug resistance, can all be successfully replicated in 3D spheroids [5]. Further advances using patient-derived tissues have led to the development of organoid models that mimic the structural heterogeneity of the TME with even greater precision [14]. Moreover, comparable in vivo models face certain limitations, including high costs, high labor intensity, and the lack of functional immune systems in xenograft mice models [12][15]. Therefore, 3D spheroids and organoids can complement current therapeutic development strategies, filling in the gap between in vitro and in vivo research.

2. Cell Line-Derived Tumor Spheroids

2.1. Culture Methods

A variety of methods have been implemented to assemble tumor-derived spheroids; both anchorage-independent and -dependent models have been explored to varying degrees of success [16]. An experiment conducted by Al-Hity et al. utilized ultra-low attachment 96-well plates to culture spheroids formed from murine breast cancer cell lines (i.e., either 66CL4 or 4T1) and verified their integrity and fidelity through structural comparison to in vivo samples [17]. Not only was their architecture extremely similar but they also displayed similar vulnerability to immune cell infiltration [17]. Other strategies taking advantage of cancer cells' ability to spontaneously aggregate in nonadherent conditions include the magnetic levitation, hanging drop, and spinner flask methods [6][18]. However, as historically observed, ultra-low attachment substrate highly restricts the number of spheroids formed, provides inconsistent development, and creates variation in spheroid ECM content, constraining testing consistency [19]. Nevertheless, the ability to assemble spheroids provides an avenue for a wide array of testing, including drug sensitivity and penetration, immune cell infiltration, and tumor architecture.

To overcome these limitations, variations on standard spheroid formation have implemented various scaffolding types to control spheroid structure [20]. For example, successful cultures have been accomplished using ultra-porous cellulose for PC3 prostate cancer epithelial cells [21], fibrous scaffolding for MCF-7 breast cancer cell line [22], and alginate encapsulation for the NCI-H157 NSCLC cell line [18]. The adoption of biocompatible scaffolds to support spheroid development has produced greater reproducibility, titration of physical and chemical conditions, and nutrient factor transport that contributed to greater spheroid throughput and control [23]. There are still some limitations overshadowing scaffold use; for instance, porous scaffolds have restrictions on their ability to diffuse nutrients and fibrous scaffolds do not completely represent a 3D ECM due to promoting cell growth along fiber strands [20][23].

Besides scaffolding, another four approaches have been utilized for spheroid formation in a scaffold-free culture [24]. In agitation-based methods, cells are kept in stirring condition to avoid cell adhesion to surfaces for enhanced spheroid aggregation [24]. The hanging drop technique grows spheroids within drops of culture medium, taking advantage of the surface tension of liquid [24][25]. In the liquid overlay technique, non-adhesive surfaces such as agarose are seeded with cells for spheroid formation without cell attachment [26]. In contrast, the microfluidic system has cell culture microchambers and hemispherical microwells with a concentration gradient generator [27]. The complex system provides controlled mixing, chemical concentration gradients, lower reagent consumption, continuous perfusion and precise control of pressure and shear stress on cells [28]. It was reported by Ruppen et al. that spheroids formed with continuous perfusion of drugs would have higher drug resistivity [29]. With some further enhancement, long-term monitoring and high-throughput drug screening platforms could also been achieved through the microfluidic system [27][30].

2.2. Tumor Microenvironment Modeling Capabilities of Spheroids

As metabolic products accumulate (e.g., lactate), acidic environments in tumors have been well observed in the interstitial space and ECM, displaying a heterogeneous acid—base phenotype [31]. Acidic conditions inside the TME, which may subside to as low as a pH of 5.6, contribute to drug resistance for both chemotherapy and radiotherapy [24][31][32]. As shown by Carlsson and Acker in 1988, 3D spheroids similarly display a low pH within their deepest regions, indicating their potential usage as a drug resistant screening platform [33]. In a separate study by Swietach et al., HCT116 cells were cultured as multi-cellular 3D spheroids. A radial pH gradient was established with the lowest level at the core, as the extracellular space restricts the diffusion of metabolically produced acids [34]. With the assistance of spheroids, the efficacy of doxorubicin—a weakly basic drug—was found to be pH-dependent in the TME due to its reduction upon drug entry and drug accumulation [34]. The protonated form of doxorubicin crosses cell membranes slower than the unprotonated form, implying that other basic chemotherapeutic drugs may also be hindered by the acidic environment [34]. Moreover, 3D spheroids were utilized to study the role of Carbonic anhydrase IX, a hypoxia-inducible tumor-associated cell surface enzyme, in the TME acidification procedure [35].

The development of hypoxic conditions from high lactate production and poor tumor vascularization usually occupies an overlapping gradient similar to the acidic microenvironment discussed above [36][37]. Since some anticancer drugs require oxygen to exert their antitumor effects, hypoxia, in combination with acidosis, reduces the formation of reactive oxygen species (ROS) that target cancer cells [38]. Although hypoxic regions can be modeled in 2D cell cultures with a hypoxic chamber, physiological gradients of the TME hypoxia are better simulated by 3D spheroids through the replication of an anoxic core [37]. Therefore, the generation of 3D spheroids produced an effective method to screen for compounds that could target the TME's hypoxic phenotype [37][39][40]. The 3D, high-content screening platform proposed by Wenzel et al. enables an efficient identification procedure of compounds that may induce cell death specifically in inner spheroid

regions with low oxygen availability [39]. This platform provides an advantage over traditional 2D-based screening for the identification of substances that would be otherwise overlooked in the absence of the hypoxic gradient [39]. In another study, nanoimprinting 3D spheroids, together with cancer cells extracted from patient tumors, indicated a similar formation of hypoxic regions in comparison to in vivo tumors, further enhancing the reliability of the 3D screening platform [40].

Due to hypoxic conditions in the TME, tumor cells generate energy through an alternative metabolic pathway than untransformed cells, which standardly undergo mitochondrial oxidative phosphorylation [24][38]. The anaerobic metabolism pathway, instead of the normal tricarboxylic acid (TCA) cycle, was first observed in carcinoma cells by Otto Warburg in 1925 [41]. Accordingly, glucose is utilized to produce lactate as a by-product inside the TME, resulting in lactate accumulation and pH depression [24][38]. In 3D spheroids, Khaitan et al. discovered that glucose consumption and lactate production were much higher in spheroid cells than in monolayer cells. Meanwhile, ROS levels were largely reduced in the spheroids with increasing age and size. These features in 3D spheroids allow researchers to evaluate tumor response to metabolic inhibitors such as 2-deoxy-D-glucose (2-DG), an inhibitor of glucose transport and glycolysis [42]. Moreover, due to their similar metabolism environment as the TME, 3D spheroids were used to study amino acid (i.e., leucine and glutamine) function in melanoma tumor growth, and to map lipid distribution and profile in breast cancer progression [43] [44].

As described above, the TME is characterized by high heterogeneity, consisting of multiple cell types, and displaying various kinds of cell–cell interactions. In addition, 3D spheroid models can culture different cell types together to generate multicellular spheroids, mimicking the cellular heterogeneity of solid tumors and tumor-stromal cell interactions [24]. Tumor cells, fibroblasts, endothelial cells, and immunocompetent cells have all been cocultured in the spheroid generation process [45]. Early attempts co-cultured a single cell line tumor spheroid with polarized TAMs, demonstrating TAM migration and spheroid infiltration [46][47]. Further modeling has shown that TAM migration plays a strong role in tumor pathogenesis by promoting tumor infiltration into the surrounding matrix, having a mechanistic role in tumor progression and metastasis [48]. More sophisticated approximations using multicellular culturing have been accomplished through the generation of 3D spheroids composed of a mixture of tumor cell line, CAFs, and monocytes with appropriate nutrient factors [49]. Interestingly, the 3D TME spheroid captured the de novo polarization of naïve monocytes towards a TAM immunosuppressive phenotype, displaying the fidelity under which multicellular spheroid model operates [49].

Contributing to the success of 3D spheroids, cell–cell interactions can be more clearly observed in 3D spheroids than in 2D cultures, influencing cancer cell signaling, survival, proliferation, and drug sensitivity [24]. For example, Xu et al. demonstrated that E-cadherin, as one of the widely known adhesion receptors, would increase its expression in the multicellular spheroids of ovarian cancer cells [50]. These results indicate that E-cadherin induces spheroid formation, maintenance, and drug resistance in ovarian cancer [50]. Consistently, the administration of an E-cadherin blocking antibody on spheroids led to improved cellular death in liver cells [51]. Another vital feature in solid tumors is the uncontrolled ECM, a noncellular component present in all tissues composed primarily of structural proteins, nonstructural proteins, and other components such as growth factors and Matrix Metalloproteinases (MMPs) [52]. The dysregulation of the ECM's composition, structure, stiffness, and abundance contributes to fibrosis or cancer pathogenesis [53]. Nederman et al. studied the ECM proteins in spheroids of a human glioma cell line (U-118 MG) and a human thyroid cancer cell line (HTh-7) and found that 3D spheroids show increased expression of ECM proteins, including fibronectin, laminin, and collagens [54]. Notably, spheroids may be further engineered to better capitulate the ECM feature of the TME [55][56]. The ECM amount (thickness) between cells was modulated by Tao et al. by replenishing ECM components and polysaccharides into spheroids with the maintenance of cell viability, enabling a more precise fabrication of spheroid models [55].

Cancer stem cells (CSCs) are defined as a fraction of cancer cells capable of generating entire cancer structures due to their self-renewal and differentiation potential [57]. As the formation of spheroids in vitro is regarded as a convenient marker to identify CSCs, tumor-derived spheroids exhibit a close relationship to CSC research [57]. Breast cancer spheroids were widely used to study CSCs and their response to chemotherapies [58][59]. Three in vitro models (a 3D collagen embedded multicellular spheroid tumor model; a 3D collagen model with a single cell-type diffusely embedded; and a 2D monolayer) were compared directly to culture two breast cancer cell lines. As expected, only the 3D spheroid model displayed the enriched CSC content and high chemoresistance observed in in vivo models [58]. It was also reported in a different study that CSCs lose their drug resistance when grown in monolayers. However, CSCs and cells grown in spheroids are highly resistant to chemotherapeutic agents [59]. These comparisons clearly indicate that 3D spheroids provide the best model to study CSC populations.

The internal structure of spheroids contains an external layer of highly proliferative cells, a middle layer of senescent cells, and a core of necrotic cells [5][60]. In combination with the features of hypoxic regions, increased ECM mass, and close

cell–cell interactions, the architecture of spheroids forms a physical barrier that limits the penetration and delivery of drug compounds [24]. This resistance has been well observed and defined above.

Despite their large similarity to the real TME, 3D spheroid models continue to have certain limitations [12][25]. The lack of control on the spheroid architecture may lead to the formation of heterospheroids, which have an opposite hierarchy compared to the solid tumor [12]. In addition, spheroid formation is influenced by cell type, culture technique, medium composition and volume, and cell density, which could introduce variability in production [25]. Further, the culturing and growth time required in spheroid formation is lengthy, which may be improved with the help of novel technologies such as microfluidic chips [25].

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