3D Microenvironment Cell Culture in Snake Venom Research

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Snake venoms are a natural biological source of bioactive compounds, mainly composed of proteins and peptides with specific pathophysiological functions. The diversity of protein families found in snake venoms is reflected by the range of targets and toxicological effects observed, and consequently, a wide variety of potential pharmacological activities. In this context, in vitro biomimetic models such as spheroid and organoid systems, which are three-dimensional (3D) cell culture models, enable extensive screening and identification of substances with pharmacological potential and the determination of the mechanisms underlying their activities.

Keywords: snake toxins ; 3D cell culture ; spheroids ; organoids

1. Introduction

Three-dimensional (3D) in vitro models have wide applications in several fields of biology, including cancer research, stem cell research, drug discovery, pharmacological research, etc. The 3D models provide an in-vivo-like microenvironment that better reflects physiological properties, such as cell–cell and cell–matrix interactions, tissue-specific architecture, cell differentiation, cell metabolism, signal transduction, gene and protein expressions, etc., as opposed to traditional two-dimensional (2D) monolayer cell models [1][2][3][4][5]. The 2D cell models are currently the standard technology applied in life science research as these serve as useful tools for the selection and evaluation of a drug candidate in terms of its efficiency and toxicity toward several targets and pathways [6][7]. However, due to the lack of tridimensionality, 2D cell models do not effectively imitate the cell–cell or cell–matrix interactions and the spatial architecture and microenvironments observed in vivo [8]. Therefore, 3D cell culture is emerging as an attractive tool to recapitulate the in vivo architecture and microenvironment of healthy tissue and organs, as well as those of solid tumors, to evaluate the in vivo-like response to drug candidates and obtain better insights into the molecular mechanisms becoming an interesting preclinical model [9][10][11]. Several 3D cell cultures have become available currently, including scaffold-free models such as spheroids and organoids, as well as scaffold-based technologies such as hydrogels and bioprinting [1][6].

Natural products obtained from animal venoms, especially snake venoms, have demonstrated significant therapeutic potential in this regard. Therefore, due to varied classes of molecules exhibiting a wide range of pharmacological activities, snake venom compounds were used as a design for novel therapeutic agents [11]. A classic example of drug development is an anti-hypertensive agent, Captopril, the first approved drug that was designed based on the structure of a bradykinin-potentiating peptide isolated from *Bothrops jararaca* venom [12][13][14].

The traditional 2D in vitro models have been applied extensively for characterizing the biological functions of the constituents from animal venoms, including their anti-inflammatory, immunomodulation, anti-viral, anti-microbial, and anti-cancer activities. However, in recent studies, 3D models have been gaining preference for understanding the functional role of these venoms in biomimetic microenvironments, thereby emerging as important tools for the development of novel drugs.

2. Spheroid Technology

Spheroids are cellular aggregates that self-assemble in a scaffold-free manner, thereby preserving cell–cell interactions and the tissue-specific phenotype [1][6][15][16]. Spheroids may be obtained using different strategies, such as liquid overlay, microfluidic-based assembly, magnetic levitation, spinner flasks, the hanging drop method, etc. [1][4][8]. The principle underlying these techniques is to induce spontaneous cellular adherence and assembly by minimizing cellular interaction with the substrate via physical forces, such as gravitational or centrifugal forces, thereby allowing the formation of a compact, well-defined structure [17]. The hanging drop method is the most commonly used technique for preparing spheroids owing to its simplicity and low cost [8]. Spheroids recapitulate the physiological characteristics of tissues and

tumors, as this model reproduces the cell–cell and cell–matrix interactions, cellular heterogeneity, the nutrient, metabolite, and oxygen gradients, gene expression, and drug resistance observed in the in vivo conditions [4]. Moreover, the extracellular matrix found in spheroids is synthesized by the cells of the model, without interference from an external hydrogel or scaffold allowing for natural cell–matrix interactions [16][18]. Tumor spheroids resemble the initial avascular aggregates of malignant cells and/or micrometastatic regions in vivo and are, therefore, very useful in cancer research and drug screening [19]. Importantly, when using tumor spheroids, the tumor size is correlated to its function, drug penetration, and transport [15]. While larger spheroids (>400 µm) allow for imitating the oxygen, nutrient, and catabolite gradients and hypoxic regions in the poorly vascularized tumors, smaller spheroids (<200 µm) may be used for drug evaluations [15].

The compound PLA2 derived from different snake venoms has demonstrated the potential for antitumor, antimetastatic, and antiangiogenic effects in vitro in 2D monolayer culture [20][21][22][23]. Further, Azevedo and colleagues demonstrated the antitumor and antimetastatic effects of BthTX-II, an Asp49-PLA2 isolated from the venom of *Bothrops jararacussu*, on MDA-MB-231 human triple-negative breast cancer cells using the 3D culture technique [24]. The authors monitored the development of spheroids in Matrigel for 7 days and reported that the presence of different concentrations of BthTX-II (1, 10, and 50 µg/mL) impaired spheroid formation and tumor growth compared to the non-tumorigenic MCF10A cell line [24]. In their recent study, these authors demonstrated the antiangiogenic effect of the BthTX-II molecule by co-culturing MDA-MB-231 spheroids with an HUVEC vessel network on Matrigel. The authors reported that during the interaction between tumor spheroids and endothelial cells, BthTX-II promoted the disruption of the HUVEC vessel network by inhibiting endothelial cell aggregation, exhibiting a complete inhibition of cell co-culture migration and proliferation compared to the control cells cultured in Matrigel [25].

Another class of snake venom components is disintegrins, a family of small, non-enzymatic substances containing the arginine-glycine-aspartic acid (RGD) peptide sequence. Disintegrins have been detected in the venoms derived from the *Viperidae*, *Crotalidae*, *Atractaspididae*, *Elapidae*, and *Colubridae* snake families. Disintegrins bind specifically to certain integrins expressed by tumor cells and the endothelial cells in the tumor microenvironment, such as αvβ3, αvβ5, α5β1, and αIIbβ3 [26][27][28].

3. Organoid Technology

The term organoid was initially used to refer to a specialized and individual organ-like structure constructed in vitro within 3D gels from small tissue fragments derived from the patients' tissue separated from stroma [1]. Currently, however, the organoid technology involves a variety of tissue culture techniques for preparing self-organizing and self-renewing 3D cultures from embryonic stem cells (ESC) or organ-restricted adult stem cells (ASC) [29]. Both ESC and ASC approaches exploit the seemingly infinite expansion potential of normal stem cells in culture to recapitulate the cell functionality and morphology similar to that exhibited by the native tissue [16]. In order to induce a self-organizing structure, cells are embedded in a hydrogel rich in matrix extracellular proteins, such as Matrigel or Basement Membrane Extract (BME), to simulate the appropriate physiological microenvironment [16][30].

In the context of drug development, organoids serve as a reliable 3D model in a physiologically relevant manner for investigating the pharmacokinetics and toxicity of drug candidates [9]. Since the phenotype, genotype, and metabolic profiles of organoids are highly similar to those of the native tissues, such as cancer tissues, tumor organoids may be utilized as a valuable model for drug candidate screening and understanding the pharmacodynamics of a potential pharmacological drug candidate [1].

Recently, an organoid platform with the potential for in vitro snake venom production has been established. Yorick and collaborators reported developing functional snake venom glands from nine different species belonging to two major families of venomous snakes—the *Elapidae* (*Naja pallida, Naja annulifera, Naja nivea, Naja atra,* and *Aspidelaps lubricus cowlesi*) and the *Viperidae* (*Echis ocellatus, Deinagkistrodon acutus, Crotalus atrox*, and *Bitis arietans*) [31]. The authors dissected both late-stage embryo and adult specimens to obtain the venom gland tissue cells, which were then cultured into mini-organs in long-term culture [31]. First, the venom gland tissue was dissociated to release cells, which were homogenized and then embedded into a 3D environment BME for generating organoids that were allowed to expand [31][32][33]. The technology produced organoids that were phenotypically similar to natural venom glands and also exhibited similar functions as these secreted, functionally active venom components. Interestingly, the venom gland organoids exhibited cellular heterogeneity as different cell types produced different compounds of the venom [31][32]. The authors also used another 3D cell culture technology known as the organ-on-chip technology to assess the biological activity of the organoid-secreted venom peptides by exposing the murine muscle cells to the organoid supernatant. The results

revealed that the firing of the muscle cells was terminated, which simulated the paralysis condition observed upon snakebite envenoming [31].

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