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Biomarkers for simplifying HTS 3D cell culture platforms for drug discovery: the case for cytokines

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In this review, we discuss the microenvironmental cues that modulate the status of cells to yield physiologically more relevant three-dimensional (3D) cell-based high throughput drug screening (HTS) platforms for drug discovery. Evidence is provided to support the view that simplifying 3D cell culture platforms for HTS applications calls for identifying and validating ubiquitous three-dimensionality biomarkers. Published results from avascular tumorigenesis and early stages of inflammatory wound healing, where cells transition from a two-dimensional (2D) to 3D microenvironment, conclusively report regulation by cytokines, providing the physiological basis for focusing on cytokines as potential three-dimensionality biomarkers. We discuss additional support for cytokines that comes from numerous 2D and 3D comparative transcriptomic and proteomic studies, which generally report upregulation of cytokines in 3D compared with 2D culture counterparts.

Introduction

It is now a well-accepted view that traditional flat-surface cell culture dishes do not adequately represent the natural context of the cell and their use in cell-based assays might, in some cases, result in less accurate drugs' effects predictions [1–3]. Fig. 1a shows the possible cues that might affect the status of a cell, grouped along chemical, physical, and spatial and/or temporal 'dimensions' [4,5]. Recently, numerous three-dimensional (3D) cell-based assay platforms have been commercially introduced with more contexts to better mimic the complex *in vivo* microenvironment. The premise behind these products is that mimicking the *in vivo* microenvironment yields physiologically more relevant cells that provide physiologically more relevant drug responses [6].

Several studies have provided evidence in support of this premise. For example, 3D multicellular tumor spheroids (MCTS) have been shown to mimic *in vivo* situations closely [7,8]; they were also able to recapitulate fully the cell adhesion-mediated drug resistance (CAM-DR) of EMT6 tumors [9], which was induced *in vivo* but was lacking in cultured monolayers of the same cells. Also, Weaver *et al.* [10] showed the phenotypic transformation of malignant cells in a 3D collagen matrix upon treatment with integrin antibodies; however, this phenomenon, also observed *in vivo*, was not observed in traditional monolayer cultures. In another study, which highlights the relevance of 3D culture format, two tumorigenic cell lines showed protease-independent amoeboid movement within a 3D collagen, challenging the traditional screening for anti-metastatic agents against proteolytic activity with two-dimensional (2D) cultures [11]. In drug development, drug-induced liver injury or hepatotoxicity is a major deciding factor behind the approval, non-approval or limitation in the usage of the drug by the FDA. Usage of hepatoblastoma cell line (HepG2) cultured in 2D monolayers is the current gold standard in early screening of drug candidates for hepatotoxicity activity. However, cells in this format either entirely lack or express acutely low levels of many drug metabolizing enzymes [cytochrome P450s (CYPs)] and transporters found in hepatocytes *in vivo* [12]. By contrast, 3D hepatocyte-like cell cultures achieved in bioreactors have been shown to emulate *in vivo* characteristics to such a high degree that they can be utilized to fabricate a functional bioartificial organ for transplantation [13,14].

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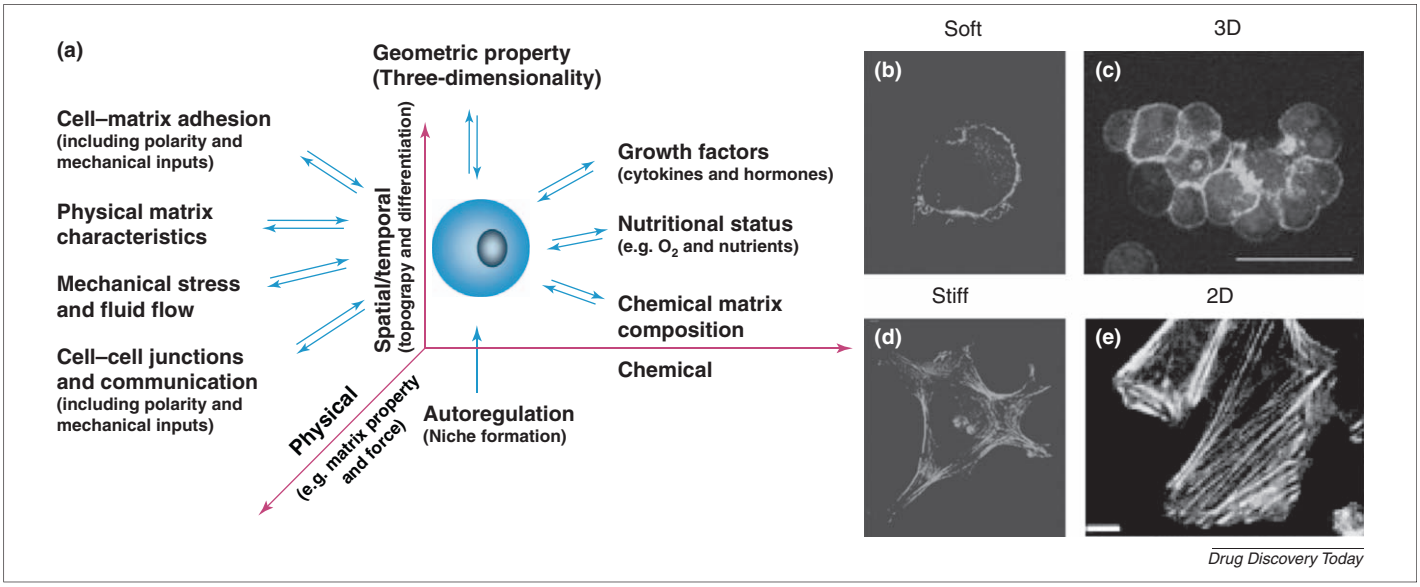

FIGURE 1

Microenvironmental cues and cellular phenotype outcomes. **(a)** The possible cues that might affect the status of a cell. The major players are spatial and/or temporal, physical and chemical cues [4,5]. **(b–e)** Phalloidin-stained F-actin. **(b, d)** Show muscle cells cultured on soft and stiff flat surfaces, respectively [20]. **(c, e)** Show SKBR malignant breast cancer cells grown in 3D lrECM [34] and 2D glass surfaces [35], respectively. Scale bars = 50 μm **(c)** and 10 μm **(d)**.

An increase in studies highlighting the physiological relevance of the 3D culture format over traditional monolayers have formed the intellectual basis behind the founding of new companies such as 3DBiotek (http://www.3dbiotek.com), KIYATEC Inc. (http://www.kiyatec.com), CellAsic (http://www.cellasic.com) and BellBrook Labs (http://www.bellbrooklabs.com), which have since launched commercial 3D products for cell-based assay high throughput screening (HTS). Examples of companies with HTS application products that have been marketed for more than at least 3 years include: Invitrogen Corporation (http://www.invitrogen.com/3D-cellculture), Glycosan BioSystems (http://www.glycosan.com) and SurModics (http://www.Surmodics.com). More products are in the pipeline, increasingly relying on developments in microfabrication and microfluidics technologies. For example, Huh *et al.* [15] successfully reconstituted organ-level lung function on a chip and Wang and Kisaalita [16] are developing a nanofibrous interconnected microwell platform for neural networks.

Although there might be exceptions, intuitively, the more complex the culture system is, the more cues it can capture and, thus, the more able it is to mimic the *in vivo* microenvironment of the cell. However, as the complexity in the culture system increases, so does the screening cost per compound. Product, cell maintenance and readout instrumentation can cost more for 3D platforms. With the industry drive to reduce screening costs, an optimal balance between cost and *in vivo* microenvironment emulation is needed. It might not be prudent to pursue a 'perfect' cell culture system that prices itself out of adoption in state-of-the-art screening laboratories. So the question that begs an answer is: among the three cue group dimensions outlined in the first paragraph, can any one of them carry more weight and, as such, constitute the major driver in designing less complex cell-based HTS platforms? To answer this question, we examine the microenvironment cues below in more detail.

Microenvironmental cues

Chemical cues can be subdivided into short- and long-range categories [3]. Short-range chemistry refers to the chemistry of the surface. To be able to emulate the *in vivo* phenotype, the approach adopted by most researchers is to present a substrate to the cell that chemically resembles the extracellular matrix (ECM). Long-range chemistry refers to diffusible chemical species that are found in the ECM that contribute to the maintenance or control of the cell behavior and/or phenotype outcome. The list includes basic nutrients, growth factors, cytokines and other morphogens, as well as metalloproteinases. Although their use in, for example, controlling differentiation is not well understood, long-range chemical cues are the easiest factor to manipulate, typically by including them in culture medium. A key attribute of chemical cues is that they can be orthogonal to the other two major cues; that is, they can be altered without affecting other properties of the platform.

Physical cues of the material or scaffolding in or on which the cells grow and differentiate have been characterized in terms of softness, stiffness, pliability or Young's modulus (terms used interchangeably to express stiffness of an isotropic elastic material). When physical properties were first investigated with hydrogels, they were intertwined with chemical cues; high water content in the hydrogel makes the hydrogel softer, but simultaneously decreases the chemical ligands density (i.e. the number of adhesion ligands for cell surface receptors per unit area of the substrate surface). To address this problem, researchers developed a novel hydrogel system in which they were able to alter the stiffness of the hydrogel without altering ligand density [17]. This type of culture system made it possible to study the effect of the physical properties of the scaffold without interfering with the chemical effects. However, most studies on substrate physical properties have been conducted on flat surfaces.

Initially, '3D cell cultures' mainly referred to culture system scaffolds that constituted complex architecture at micro and/or nano scales, which included only the spatial context. The term now has a more comprehensive meaning that entails the three 'dimensions' outlined in Fig. 1a. However, closer examination reveals that spatial cues are highly intertwined with the other cues. For example, by introducing micropores and/or nanofibers into the cell culture platform,

the surface area of the construct can increase significantly, thus increasing the amount chemical ligands that can be deposited on it. The thickness of the porous or fibrous scaffolds can also affect the concentration of the culture medium by generating a gradient of long-range chemical cues, such as oxygen and growth factors. Additionally, the porous and fibrous architecture might alter the physical properties of the material, reducing its strength and altering the anisotropism of the material. For example, the strength of fibrous scaffolds along the fiber direction is higher than the direction that is perpendicular to the fiber, and the strength in both directions is lower than the bulk property of the material [18].

Mathematical models have been used to study chemical gradients induced by 3D cell cultures (e.g. see [19]). However, few 3D cell culture studies have included physical property measurements in their studies. It is true that architecture and/or geometry have a profound impact on cell behavior. To be able to engineer a platform that more accurately emulates the *in vivo* microenvironment, it is important to isolate the contributions of the different cues to the phenotypic outcome. Consider morphology for example; the right panel of Fig. 1 shows F-actin staining from muscle cells on soft (Fig. 1b) and stiff (Fig. 1d) flat surfaces [18], SKBR malignant breast cancer cells in 3D laminin-rich extracellular matrix (lrECM) (Fig. 1c) [19] and on glass 2D surfaces (Fig. 1e) [20]. The soft surface and 3D cell cultures can both make cells more roundish and smaller in smaller size when compared with the same cells grown on rigid 2D surfaces. The F-actin organization is also similar between soft surfaces and 3D cell cultures: in both cases, the F-actin does not form stress fibers and is more abundant at the edge of the cells. Why do two different factors have similar effects on a cellular outcome? The Young's modulus for lrECM (also called Matrigel in other studies) ranges from 10 Pa to 50 Pa as concentration changes from 50% to 100% [21]. In addition, the 3D lrECM culture had a lrECM coating as thick as 500 μm (thick in comparison to the typical cell size of 50 μm). It is reasonable to speculate that the cells cultured in 3D lrECM interacted with a soft tissue-mimicking microenvironment and the morphological changes observed might have resulted mainly from pliability changes. In many 3D culture systems, cells cluster together and form multicellular aggregates [22–24]. Under such circumstances, most individual cells within the multicellular spheres are exposed to their neighboring cell surfaces, which are soft. Therefore, it is possible that the influence of the material physical properties in such cases is limited, which raises the question; if softness is all a cell requires to become round and subsequently mimic its *in vivo* counterpart, why is there a need to create a complex 3D architecture? Alternatively, the rigidity effect of the material has been bypassed by simple fabrication of micropost arrays (0.97–12.9 μm) onto a polydimethylsiloxane (PDMS) material; the rigidity of the altered material is reflected by the resulting 3D-like cellular morphology from cells cultured on the highest micropost substrate [25].

Above, we have briefly summarized current understanding of microenvironmental cues. Unfortunately, this understanding is not sufficient to guide the design of the simplest platform that provides the desired emulation of the *in vivo* situation. A common practice is the trial-and-error approach, in which the platform designer focuses on a complex physiological relevance (CPR) outcome as an indication of the capture of one or several cues sufficient for the desired outcome. CPR here means *in vitro* emulation of *in vivo* structure and/or function in 3D that is not possible in 2D cultures, such as bile canaliculi-like structures [26] and albumin secretion [27] by hepatocytes. There are many cells whose phenotypes are altered by culture in 3D structures but, unlike hepatocytes, their CPR is not readily discernible. For such cells, as the platform architecture is simplified, it is difficult to determine the simplest form that maintains the desired emulation of the *in vivo* situation. There is a need for simple technology to establish the culture status trajectory that results in formation of three-dimensionality that leads to CPR. Such a technology will enable the establishment of a balance between a simplistic architectural design and the complex microenvironmental features. As mentioned above, some cells might not require a complex platform to grow in or on; rather, they might initially need the minimal essential cues to become competent enough to produce endogenous ECM, which then provides the remaining or additional essential cues. This has been observed in several studies; for example, tumor cells grown in porous 3D poly(lactide-co-glycolide) (PLG) and Matrigel scaffolds showed upregulation of interleukin (IL)-8 compared with 2D cultures [28]. Even though both systems have a varying degree of complexity and provide different microenvironmental cues, the outcome (elevation in IL-8 level) was similar and physiologically justified as it depicts the angiogenic capability of the cells. Alternatively, IL-8 could have been added to the culture systems as a biochemical microenvironmental factor to achieve more platform complexity; however, it would have been redundant as the cells can produce it themselves if the essential initial minimal factors are present. This example underscores the need to better understand the required essential cues and their thresholds that yield CPR or *in vivo* emulation outcomes. This will enable simplification of the platform without giving up the physiologically relevant behavior of the cells. However, the task of specifying such complex microenvironments is daunting. A simpler approach is to generalize the measurement of the developmental competence that leads to CPR outcomes in terms of ubiquitous three-dimensionality biomarkers [3]. As the platform architecture design is simplified, it will be possible, with such a measure, to know when the trajectory toward CPR outcomes is being compromised by the introduced simplification(s).

To address the question of what the biomarkers might be, we reviewed the current 3D culture literature with respect to finding chemical entities that are produced differentially between 2D (minimal or no *in vivo*-like microenvironment) and 3D (more *in vivo*-like microenvironment providing CPR) systems across a variety of cells derived from different tissue types and 3D architectural platforms. This search indicates cytokines as the most probable family of compounds to provide the badly needed biomarkers. We summarize the arguments below for the case for cytokines.

Searching for three-dimensionality biomarkers

Cytokines are soluble, low molecular weight, extracellular protein mediators that usually act at short range between neighboring cells. They are crucial intercellular regulators and mobilizers of cells engaged in innate and adaptive inflammatory host defenses, cell growth, differentiation, cell death, angiogenesis, and development and repair processes. They have been assigned to various family groups based on the structural homologies of their receptors and can be broadly classified into families such as colony stimulating factors, interleukins, interferons, transforming growth factors (TGF), tumor necrosis factors (TNF), platelet-derived growth factors (PDGF) and chemokines. Although cytokines have been extensively studied in the field of immunology and oncology, they have been overlooked by tissue engineers. However, these same small proteins might have the power to revolutionize the field. Although the evidence for their existence in 3D cultures is compelling, they have not yet been looked at as candidates for potential biomarkers. However, we suggest

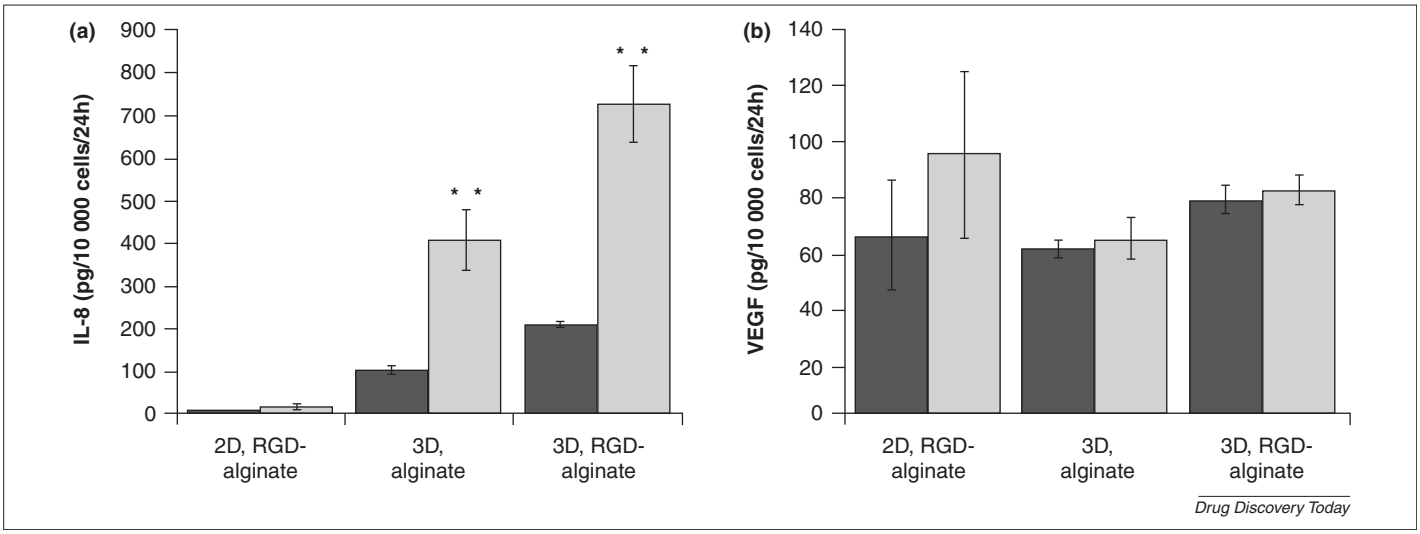

FIGURE 2

Cytokine production by tumor cells (oral squamous cell carcinoma, OSCC-3) in alginate hydrogels containing covalently coupled RDG peptides at a density that correlates to the number of RDG sites in tumors *in vivo*. The 3D system resulted in enhanced IL-8 production **(a)**; however, this was not the case for VEGF **(b)**. (*, $P \leq 0.05$; **, $P \leq 0.01$; black and gray bars represent 5 and 10 days in culture, respectively). Adapted, with permission, from [33].

that they are the ideal family to explore for identification and validation follow-up studies for the following three main reasons.

First, when cells are transitioned from a 2D monolayer to a 3D microenvironment, they become surrounded by homotypic neighbors, forming a loosely bound disorganized aggregate. *In vivo*, such a scenario is encountered only during avascular tumorigenesis or early stages of inflammatory wound healing, which are both similar in nature and regulated by cytokines [29]. Therefore, *in vitro*, the cells growing in 3D relate to any of those two models, depending on their type (malignant or primary, respectively), therefore explaining the upregulation of their cytokine levels.

Second, several 2D–3D comparative transcriptomic studies with cells from the four main tissue types (nerve, muscle, connective and epithelial) cultured on a variety of platforms, have reported the upregulation of cytokines and their receptors in 3D cultures. For example, Klapperich and Bertozzi [30] reported upregulation of seven cytokines [IL-8, chemokine (C-X-C motif) ligand (CXCL)-1, CXCL2, CXCL3, CXCL5, vascular endothelial growth factor (VEGF) and leukemia inhibitory facto (LIF)] by a human fetal lung fibroblast (IMR-90) cultured in a collagen–glycosaminoglycan (collagen/GAG) 3D mesh. The mesh was prepared by freeze drying and heat enabled cross-linking of the polymer and had an average pore size of 80–100 μm. In addition, Ghosh *et al.* [31] reported upregulation of six cytokines [CXCL1–3, IL-8, macrophage Inflammatory Protein-3 (MIP-3a) and angiopoetin-like 4] by a melanoma cell line (NA8) cultured on poly-2-hydroxyethyl methacrylate (poly-HEMA) plates when compared with 2D surfaces. The polymer coating prevented cells from attaching to the plastic surface leading to the formation of MCTS.

Third, transcriptomic findings such as those in the above examples have been supported by studies at the protein level. For example, Enzerink *et al.* [32] has shown that clustering of fibroblasts induces chemokine (CCL2-5, CXCL1-3 and CXCL8) secretion in five different fibroblast cell lines cultured in agarose. In addition, Fischbach *et al.* [33] cultured tumor cells in a 2D and 3D RGD-alginate system and reported a dramatic increase in IL-8 levels; however, no significant VEGF differences were reported between 2D and 3D cultures (Fig. 2). In the same study, cells grown in alginate gels having RGD peptides (spatial, biophysical and biochemical cues) produced a higher level of IL-8 compared with those lacking the adhesion peptide (only spatial and biophysical cues), although both produced higher levels when compared with 2D (lacking all three cues). This shows that changes in the microenvironment are conveyed directly by the difference in the level of cytokine production. In another study by the same group, the same cells also showed an upregulation of cytokines when grown in Matrigel (lrBM) compared with their 2D counterparts. This comparison is particularly important as cells grown on Matrigel have already been shown to produce a CPR outcome (formation of mammary gland acinus and milk-like secretions into the lumen) [10], which mean that Matrigel provides the relevant microenvironmental cues. Taken together, these studies suggest that the upregulation of cytokines in 3D compared with 2D cultures is not a random differential response but a potentially ubiquitous biomarker for three-dimensionality. Further studies to establish this view firmly are needed.

Cytokines are particularly attractive as biomarkers for several practical reasons. First, they are secreted in the media, making it easy for their detection to be amenable for HTS readout. Second, they are expressed in a wide range of cells from the four tissue types (muscle, connective, epithelial and nerve), suggesting the potential for their ubiquity as opposed to being cell or tissue specific. Third, their temporal expression suggests the use of profiles as opposed to single-time measurements, which increases their robustness as biomarkers.

Concluding remarks

Simplifying 3D cell culture platforms for HTS applications calls for the identification and validation of ubiquitous three-dimensionality biomarkers. Our review of comparative transcriptomic and proteomic literature strongly suggests the cytokine family as having the greatest potential to yield compounds that can serve as three-dimensionality biomarkers. However, narrowing the candidates to a practical few still remains to be done.

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