Why Not Introducing the Third Dimension in Photodynamic Therapy Research?

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Abstract

Photodynamic therapy (PDT) is a clinically approved procedure for the treatment of diseases characterized by uncontrolled cell proliferation, particularly cancer. It involves the administration of a photosensitizer (PS) that is able to produce reactive oxygen species (ROS) upon irradiation with light, leading to the selective killing of neoplastic cells. A major challenge in PDT is the development of new PSs and drug-delivery systems that improve therapy efficacy and selectivity. To succeed in drug screening, it is crucial to use cellular systems that precisely reproduce the phenotype of the target tissue in order to obtain reliable biomedical data that correlate with in vivo tests. In this way, three-dimensional (3D) cultures are particularly attractive since they integrate chemical and mechanical signals that arise from extracellular matrix (ECM) and adjacent cells. Importantly, 3D models can mimic in vivo gene expression pattern and molecular gradients. These features significantly affect the outcome of PDT, enhancing the predictive power of 3D models. Therefore, PDT research should rely on the exploitation of this third dimension, guaranteeing a custom-tailor design depending on the tissue to be modeled, an easy applicability and reproducibility. The review summarizes progress in this emerging area.

Keywords: Cancer; Photodynamic therapy

Taking A Glance at Photodynamic Therapy

Photodynamic therapy (PDT) is a clinically approved procedure for the treatment of neoplastic and other non-malignant diseases generally characterized by an abnormal cell growth [1-3]. It involves the interaction of three independent and individually non-toxic factors: a photosensitive dye called photosensitizer (PS), visible light and molecular oxygen.

The principle of PDT relies on the administration of the PS, followed by the irradiation of the diseased area with light of appropriate wavelength. In the presence of molecular oxygen, highly cytotoxic reactive oxygen species (ROS) are generated, leading to the selective destruction of neoplastic cells (Figure 1). Specifically, once the PS has absorbed a photon, an electron is promoted from the ground state (S0) to an electronically excited state (S1). This short-lived species can release its energy by emitting light (fluorescence), by internal conversion to heat or undergoing intersystem crossing to form a more stable triplet state (T1). The relatively long lifetime of the triplet state allows the interaction of the excited PS with the surrounding molecules, performing two classes of reactions, defined as Type I and Type II mechanisms. In the Type I pathway, the PS reacts directly with a substrate, transferring an hydrogen atom or an electron to form radical species. These free radicals further react with molecular oxygen, leading to ROS such as superoxide, peroxide or hydroxyl radicals. Alternatively, the Type II pathway is initiated by the energy transfer between the triplet excited state of the PS and nearby oxygen molecules, generating the singlet excited state of oxygen, referred as singlet oxygen, 1O2 (Figure 2). Both mechanisms can produce the photo-oxidation of certain amino acids residues in proteins, pyrimidine and purine bases of DNA/RNA and unsaturated lipids. This process causes DNA damage and/or cytoplasmic membrane damage, allowing leakage of cellular contents or inactivation of transport proteins or systems and, thus, triggering cellular death [4]. While it is generally accepted that 1O2 plays a key role in primary photodynamic effects, ROS generated by Type I mechanism become more important at low oxygen concentrations [5].

Figure 1: Photodynamic Therapy (PDT) principles. PDT involves the administration of a photosensitizer (PS), followed by the irradiation of the diseased area with light of appropriate wavelength. In the presence of molecular oxygen, highly cytotoxic reactive oxygen species (ROS) are generated, leading to the selective destruction of neoplastic cells.
PDT induces tumor regression by three mechanisms: direct killing of tumor cells, damage of tumor vasculature and triggering of an antitumor immune response [6,7]. The main advantage of PDT compared to other antitumoral therapies, such as chemotherapy or radiotherapy, is its dual selectivity. In particular, the PS is preferentially accumulated in the tumor tissue and its activation can be confined by restricting the illumination to a specific area, reducing undesired side effects. Paradoxically, this selectivity turns into an intrinsic limitation when having metastatic lesions, due to the impossibility of irradiating them in the whole body with appropriate doses [1].

Nowadays, there are few PS agents that have been approved for PDT treatments. Porfimer sodium, temoporfin, 5-aminolevulinic acid and verteporfin are the most commonly used [1]. However, none of them fulfills all the demands required for standardized applications in oncology. They present low selectivity between tumor and healthy tissue and a low therapy efficacy. Furthermore, porfimer sodium and temoporfin have been demonstrated to induce a pronounced and lengthy skin photosensitivity [1,8]. Based on these considerations, a concerted effort is being made to develop new PSs, with the overall aim of improving therapeutic outcomes for patients [9,10]. However, the drug development process is proved to be extremely inefficient. It is estimated that only 8% of all drug candidates that enter Phase I trials reach the bedside [11]. This high failure rate can be related with the continued use of cellular systems that altered or missed many trials reach the bedside [11]. This high failure rate can be related with the drug development process is proved to be extremely inefficient. Therefore, a challenge in drug assays, including the ones for PDT, is set on developing the appropriate cellular models that precisely reproduce the phenotype of the target tissue in order to obtain reliable biomedical data that correlate with in vivo tests.

3D Models: Capturing Tissue Physiology In Vitro

Pre-clinical assays have traditionally depended on two-dimensional (2D) cellular systems and animal models [12]. 2D cultures have provided valuable information on understanding cellular responses after early biological-activity assessment. However, cells in 2D grow in physiologically constrained conditions caused by being attached to rigid and flat substrates, which increases their surface exposed to cultured media and reduces cell-cell and cell-extracellular matrix (ECM) interactions [13]. At the opposite end of experimental platforms, animal models can display the integrated responses that result from complex interactions between tissues and organs. Nonetheless, they fail to capture important facets of human responses, are very costly, time-consuming and ethically controversial [12,14]. Therefore, there is a high demand for models that precisely recreate the complexity of human tissues while retaining the ability for high-throughput screening and cellular level imaging. As a result, three-dimensional (3D) cellular systems, which basically rely in the capacity of culturing cells in a 3D microenvironment, are currently under development [11,14] (Figure 3).

Cells in the body grow within an organized 3D ECM, surrounded by other cells. Indeed, the interactions between cell-cell and cell-ECM can determine whether a given cell undergoes proliferation, differentiation, apoptosis or invasion [11,13-15]. 3D models provide this third dimension, essential to integrate mechanical and chemical signals, mimicking the in vivo microenvironment. In particular, 3D models present two key advantages in PDT research, with a focus on screening for novel antitumoral drugs [16] and delivery systems [17,18], since they have the capacity to (i) modulate the molecular gradients that exist in tissues for any soluble component, such as oxygen and drugs [14,19] and (ii) recreate the in vivo cellular morphology and physiology, including resistance to therapy [12,20]. Furthermore, ECM components offer additional targets to enhance drug internalization and tumor penetration [18] (Table 1).

Tissues experience mass transfer phenomena for any soluble agent. This situation is worsened in tumors, since they are characterized by an abundant and dense ECM microenvironment, together with a primitive vascular network. First of all, this complex tumor microenvironment is induced by an elevated deposition and remodeling of ECM components, such as fibrillar collagen and hyaluronic acid, by cancer cells and fibroblasts of the stroma [21,22]. Secondly, tumors present a poorly organized vascular architecture, due to the imbalance between pro- and anti-angiogenic factors and the stress generated by the uncontrolled proliferation rate that forces vessels apart [19,23,24]. The concomitance...
of these two phenomena causes an inefficient delivery of drugs and oxygen (hypoxia), significantly affecting therapy efficacy. Additionally, hypoxia is aggravated by any oxygen-consuming photoreaction which rapidly lowers even more the local partial oxygen pressure [25,26]. Experimentally, conventional 2D cultures are characterized by uniformly rich oxygenation and nutrition. Instead, 3D cultures can capture these mass transfer limitations and, therefore, better predict the outcome of oxygen- and drug-dependent therapies.

The morphology and physiology of cancer cells depend strongly on tumor microenvironment. In particular, their aggressiveness is enhanced by the mechanical and chemical signals that arise from both the ECM and neighboring cells residing within a 3D architecture pattern. For instance, the communication between quiescent and hypoxic cells located at the internal core of the tumor with highly proliferative cells at the surface promotes the transmission of growth factors, which are involved in key aspects of tumor biology such as cell survival, resistance to apoptosis and metabolic reprogramming. Some of these factors are vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), placenta growth factor (PIGF) and transforming growth factor α (TGFα), among others [23,24,26-29]. Furthermore, hypoxic cells deal with a hostile metabolic environment that provides a selective pressure to favor the survival of the more malignant, aggressive and genetically unstable cancer cell types [23]. As a result, these growth conditions significantly affect RNA and protein expression profiles and, thus, cellular response to therapies, including the acquisition to drug resistance. Experimentally, 2D cultures depict a reductionist model based on a simple collection of relatively homogeneous cancer cells [28]. Instead, 3D cultures reestablish morphological and physiological properties of the tumor [11], mimicking multicellular resistance to apoptosis-inducing drugs [23,30,31] and, therefore, improving their predictive power for drug design and screening.

3D Models In PDT: Bringing PDT to the Next Dimension

The currently available 3D cell systems include tissue explants, cellular spheroids, scaffold based cultures, whole perfused organs and hollow-fiber bioreactors [11,32]. In drug discovery field, the most commonly used platforms are cellular spheroids and scaffold-based cultures. They provide tissue-specific information at different and complementary levels of complexity.

Up to date, the most popular 3D models for pre-clinical research consist in cellular spheroids. They are compact cell clusters based on the natural tendency of many cell types to aggregate. As a consequence, they contain both surface exposed and deeply buried cells, establishing zones of proliferating cells on the outside and quiescent cells on the inside due to nutrient and oxygen transport limitations. Therefore, these systems capture many aspects of the pathophysiology milieu in human tumor tissues [30,33,34]. Sutherland et al. [35,36] were pioneers in using spheroids in cancer research. Importantly, they characterized spheroids morphology and physiology through growth rate and oxygenation studies. In PDT field, the group of De Witte [37-40] used spheroids to mimic bladder carcinoma and evaluate the PDT outcome, comparing the results with classical monolayer cultures. Cells were grown until aggregates reach diameters between 450 and 500 μm. These dimensions could mimic mass transport limitations occurring in vivo, since simple diffusion typically allows for only 150-250 μm of oxygen and drug penetration [14,25]. In 2D cell systems PSSs were readily taken; whereas 3D cell systems showed a heterogeneous distribution with high concentrations of the Ps at the periphery of the structure that decreased rapidly to a steady-state situation in the inner spheroidal regions. Furthermore, spheroids showed a dramatically low phototoxicity compared to 2D. This effect was due to poor drug penetration and oxygen depletion, which created a protective microenvironment for cells [37]. The authors went a step further using spheroids to study the cellular mechanisms underlying PDT. In particular, they studied the preferential localization of Ps in tumor cells, since it was only observed in vivo. In 2D monolayer conditions the cellular uptake by tumor and normal cells was similar. They demonstrated that the accumulation of Ps in cells inversely correlated with their intercellular adhesion proteins (E-cadherin) expression. Hence, while the first layers of cells in spheroids took up Ps directly, in deeper layers passive paracellular transport was activated and modulated by adhesion proteins, since these proteins configured the histoarchitecture pattern [38-40]. Therefore, spheroids have led to major conceptual advances in understanding discrepancies in PDT mechanisms and efficacy between conventional monolayer cultures and in vivo experiments. However, they are limited by slow spontaneous aggregation and uncontrolled final size and shape, which importantly difficult a precise control of oxygen and compounds gradients [34,41].

Table 1: Comparison of cell morphology and physiology in two-dimensional (2D) cultures, three-dimensional (3D) cultures and animals.

<table>
<thead>
<tr>
<th>Tissue Architecture</th>
<th>2D cell cultures</th>
<th>3D cell cultures</th>
<th>Animals</th>
</tr>
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<tbody>
<tr>
<td>Cells are unnaturally polarized and mainly exposed to culture medium and rigid substrates</td>
<td>Cells interact with ECM and adjacent cells in 3D. They secrete and remodel ECM</td>
<td>Integrated response from interactions among all tissues and organs</td>
<td></td>
</tr>
<tr>
<td>Gene expression pattern</td>
<td>Physiological tumor properties are constricted in 2D and, thus, expression patterns</td>
<td>Key expression pattern in tumor biology is recreated (apoptosis resistance, etc.)</td>
<td>Human responses are not accurately captured (drug toxicity, metabolism, etc.)</td>
</tr>
<tr>
<td>Molecular gradients</td>
<td>Cells are submitted to uniformly rich oxygenation and drug accessibility</td>
<td>Oxygen and drug delivery is controlled by simple diffusion</td>
<td>Vascularization regulates oxygen and drug delivery, together with drug clearance</td>
</tr>
<tr>
<td>Drug discovery applicability</td>
<td>Inexpensive and simple to apply for high-throughput screening</td>
<td>More realistic drug response than 2D. Easier to apply than animal models</td>
<td>Expensive, time-consuming, poor cell-level imaging and ethically controversial</td>
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Scaffold-based cultures are considered a good alternative to spheroids models. Scaffolds are inspired on extracellular matrix in order to give cells the appropriate chemical, physical and mechanical cues and, therefore, mimic the in vivo microenvironment. They offer two important advantages compared to cellular spheroids. First of all, scaffolds can be designed depending on the tumor-tissue being modeled [28]. Secondly, scaffold architecture and dimensions can be precisely defined, controlling the spatial distribution of drug and oxygen [14]. On the basis of their origin, they can be classified as natural or synthetic materials. Natural scaffolds are extracted from animals, the most applied for cell culture are collagen and Matrigel®. Collagen is one of the main protein in ECM; whereas, Matrigel® consists in Engelbreth-Holm-Swarm (EHS) mouse tumor cell-derived basement membrane proteins [42,43]. In pioneering studies, Bissell and collaborators [44,45] established a 3D breast cancer model in which normal and malignant...
breast epithelial cells were cultured on Matrigel™. These authors observed that malignant cells maintained their invasive phenotype and formed disorganized colonies, whereas normal cells differentiated into polarized hollow spherical monolayers (acini) that resembled the original breast tissue. Therefore, they provided basic tumor biology insights into the substantial role of the extracellular context, information that could not have been gained from monolayer cultures. Within PDT research, the group of Hasan [25,46,47] developed a 3D adherent ovarian cancer (OvCa) model to mimic the avascular metastatic tumors that coat surfaces of the peritoneal cavity. In brief, OvCa cells were cultured on the surface of Matrigel™ and grown until achieving a population of nodules larger than 200 μm in diameter, relevant dimensions for being subjected to mass transport limitations. They used this model as a powerful platform to screen drug candidates capable of breaking the protective microenvironment created by hypoxia. For this purpose, they developed two different strategies. Firstly, they proposed the use of PSs that could impart cytotoxicity across Type I and II phototoxicity mechanisms and, thus, were able to treat hypoxic regions through Type I mechanism [25]. On the other hand, they also demonstrated the synergic effect of combining PDT with chemotherapy drugs to target these hypoxic-resistant cells [47]. Furthermore, these authors developed co-culture models of tumor and stromal cells, which could shed more light on stroma contribution to disease progression and treatment response. For instance, they cultured OvCa cells with endothelial cells or normal fibroblasts micropatterned on Matrigel™ and characterized these 3D systems [46,48]. The advantages of natural scaffolds are their biocompatibility, easy accessibility and capacity to provide all the spectrum of chemical and physical cues that are needed to induce morphogenesis and function from many cells. However, the diversity of these cues could be a disadvantage when trying to isolate the effects of specific factors. Moreover, the same ECM component can be very variable in its composition and mechanical properties depending on its origin and specific batch, reducing the reliability and reproducibility of the particular assay [11,14,42].

To avoid these drawbacks, fully synthetic scaffolds are under continuous development. They can be custom tailored to mimic specific ECM properties, providing reproducible cellular environments [14]. Paradoxically, this advantage makes this class of biomaterials far more challenging because a wide range of factors have to be identified and precisely recreated, including porosity, stiffness, cell-adhesion sites and growth factors binding. At the moment, the group of Semino [49] worked with a 3D model based on the self-assembling peptide hydrogel RAD16-I (commercially available as BDTM PuraMatrix™. It is composed by a sequence of 16 amino acid chain AcN-(RADA)6-CO2H, (R arginine, A alanine and D aspartic acid). This hydrogel provides a non-instructive and defined microenvironment to cells, which was previously used to promote growth and proliferation of multiple cell types, including chondrocytes, hepatocytes and osteoblasts, as well as embryonic and somatic stem cells [50-53]. Hence, it enables the analysis of the intrinsic effects of the 3D architecture and molecular gradients in PDT outcome compared to 2D models, avoiding the influence of biochemical signals. Results revealed that PDT efficacy was dramatically lower in 3D than in 2D systems. Dynamic mass transfer effects accounted for this difference, since the intrinsic mechanism of action of the therapy was maintained in both systems. In fact, total death was only observed using 20-fold higher PS concentration or when a continuous flow of oxygen was maintained during PDT treatments, despite the demonstrated presence of free-drug and/or hypoxic cells that could not undergo PDT. This apparent paradox was explained by a death-signaling cascade that could be triggered to break the protective microenvironment created by oxygen and drug limitation, inducing neighboring cell death. Importantly, the production and decay of the cytotoxic species 1O2 could be observed for the first time in a 3D culture [49]. Next action should require the incorporation of the specific cell adhesion sites and growth factor binding that characterized the tumor to be modeled.

Future Perspectives: Optimizing 3D Models

A further step towards more realistic and predictive models could be the development of co-cultures models. It is largely demonstrated that the crosstalk between cancer and stroma cells contributes to the invasive growth and metastasis of the tumor [28]. Basically, stroma cells are responsible for the synthesis, deposition and modeling of much of the ECM. Moreover, they are a source of paracrine growth factors that induce cancer cell growth [33,54]. Therefore, these cells act as active participants in tumorogenesis rather than passive bystanders. For this reason, co-cultures models could be incorporated in PDT drug screening processes, providing a better predictive outcome for therapies [46,48,55].

Another degree of complexity that can be incorporated in 3D models is vascularization. For this purpose, different strategies have been developed, including the use of perfusion bioreactors or the co-culture with endothelial cells that are stimulated with the delivery of angiogenic factors as VEGF [56]. Up today, 3D models have been designed to partially recreate mass transport limitations that characterized tumor physiology (oxygeonation and nutrition) by simple diffusion. For this reason, spheroids and scaffolds are always larger than 150-250 μm, which is the limit of simple diffusion [14,25].

Conclusions

3D models have the potential to become a fundamental research tool in PDT drug screening assays. Certainly, a big challenge in biophotonics will be the optimization and exploitation of this third dimension. These models are attractive platforms to (i) better predict the outcome of PDT; (ii) carefully study the molecular mechanisms underlying PDT and (iii) develop new drugs candidates capable of breaking the protective microenvironment triggered by drug and oxygen mass transport limitations. To fulfill all these needs, 3D models should have some essential properties. Basically, they should be customizable depending on the tumor to be modeled and easy to apply and reproducible to enable high-throughput screening applications.

References


