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J Biomol Screen 2004 9: 273

DOI: 10.1177/1087057104265040

The Use of 3-D Cultures for High-Throughput Screening: The Multicellular Spheroid Model

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Over the past few years, establishment and adaptation of cell-based assays for drug development and testing has become an important topic in high-throughput screening (HTS). Most new assays are designed to rapidly detect specific cellular effects reflecting action at various targets. However, although more complex than cell-free biochemical test systems, HTS assays using monolayer or suspension cultures still reflect a highly artificial cellular environment and may thus have limited predictive value for the clinical efficacy of a compound. Today's strategies for drug discovery and development, be they hypothesis free or mechanism based, require facile, HTS-amenable test systems that mimic the human tissue environment with increasing accuracy in order to optimize preclinical and preanimal selection of the most active molecules from a large pool of potential effectors, for example, against solid tumors. Indeed, it is recognized that 3-dimensional cell culture systems better reflect the in vivo behavior of most cell types. However, these 3-D test systems have not yet been incorporated into mainstream drug development operations. This article addresses the relevance and potential of 3-D in vitro systems for drug development, with a focus on screening for novel antitumor drugs. Examples of 3-D cell models used in cancer research are given, and the advantages and limitations of these systems of intermediate complexity are discussed in comparison with both 2-D culture and in vivo models. The most commonly used 3-D cell culture systems, multicellular spheroids, are emphasized due to their advantages and potential for rapid development as HTS systems. Thus, multicellular tumor spheroids are an ideal basis for the next step in creating HTS assays, which are predictive of in vivo antitumor efficacy. (*Journal of Biomolecular Screening* 2004:273-285)

Key words: cell-based assay, 3-D culture, spheroid, co-culture, anti-tumor drug testing

INTRODUCTION

ADVANCES IN GENOMICS AND PROTEOMICS have revolutionized drug discovery and target validation and have already started to provide researchers and the pharmaceutical industry with a rapidly increasing number of molecular targets for therapeutic intervention. Successful development and selection of the most active drug leads for a particular disease, such as cancer, requires reliable and robust test systems. Today, most pharmaceutical companies use a set of specific high-throughput screening (HTS) assays as the initial step in drug lead discovery (see Figure 1). Numerous HTS assays have been established over the past 5 to 10 years, incorporating a broad range of technological and instrument advances. This promising process is still ongoing, but the value of any individual HTS assay to predict in vivo efficacy has not been firmly established. One field of research and development for HTS focuses on automation and miniaturization issues to provide ultra–high-throughput approaches.[1,2] Another field concentrates instead on the design and adaptation of novel, more complex test systems to gain deeper insight into the effects and functions of molecules in a cellular context, that is, within the complex milieu of an intact cell. Currently available cell-based assays can be divided into 3 major categories: (a) generic cellular responses to external stimuli (eg, proliferation, viability, cytotoxicity); (b) assays to monitor signal transduction pathways (eg, ion channels, second messengers, kinase activation); and (c) cellular responses at the transcriptional/translational level (eg, reporter gene or protein,

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Received Feb 2, 2004, and in revised form Feb 20, 2004. Accepted for publication Mar 1, 2004.

Journal of Biomolecular Screening 9(4); 2004
DOI: 10.1177/1087057104265040

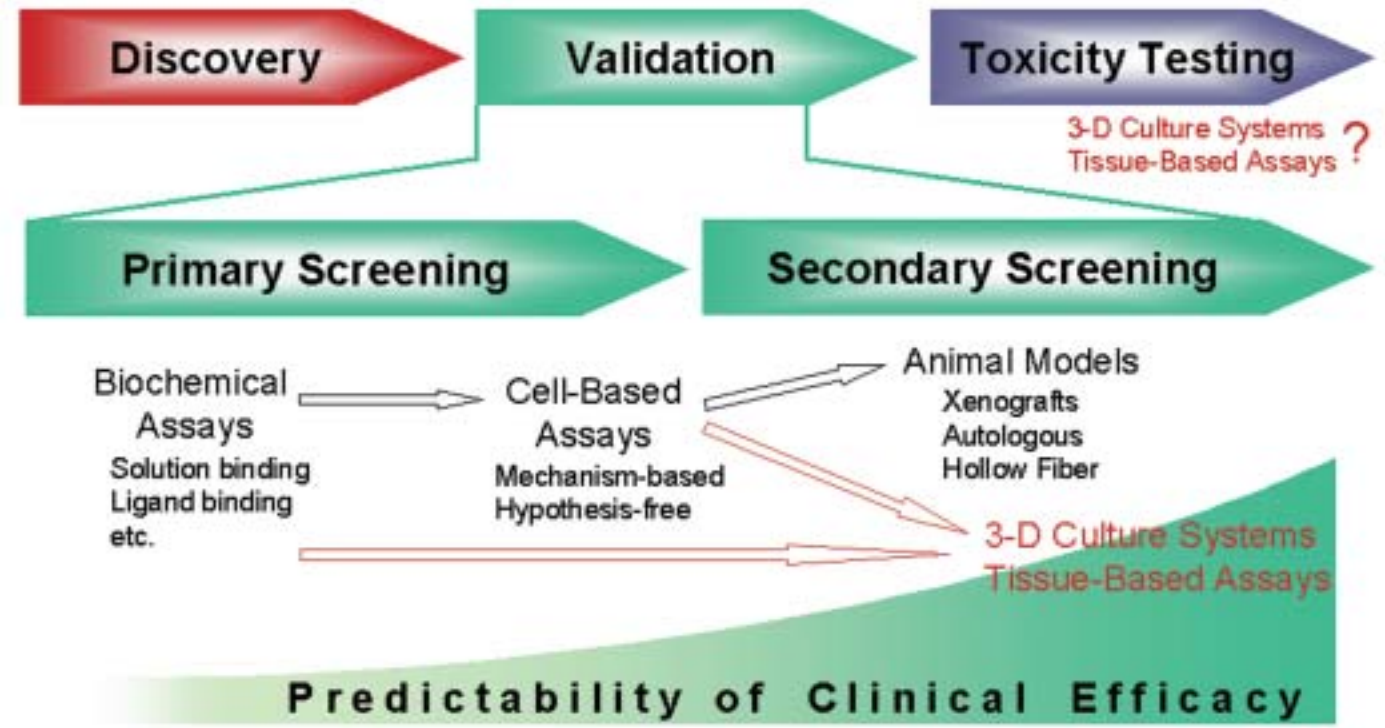

FIG. 1. Overview of the drug discovery process focusing on current strategies for the preclinical validation of antitumor candidate therapeutics via primary and secondary screening tools. Although utilization of 3-D culture assays in routine toxicity testing of candidate therapeutics is still speculative, incorporation of 3-D culture systems and tissue-based assays such as the multicellular tumor spheroid model as a secondary screening tool in drug discovery and development operations has the potential to allow increased predictability of clinical efficacy from in vitro validation and to help minimize or replace whole animal test models, thereby contributing savings in both development cost and time. This is relevant for a broad variety of drug candidates including those that affect cellular phenotype/differentiation, proliferative activity, signaling pathways, transcriptional activity, or physiological and metabolic behavior.

transcription factor activation). The latter 2 are functional, cell-based assay technologies that reflect a recent shift in the anticancer drug discovery and screening strategy from empirical to mechanistic approaches.[3-7]

Today, the common approach used by many pharmaceutical companies to screen small-molecule libraries in biochemical-based or ligand-binding assays, and to build new classes of lead compounds, is frequently complemented by a series of in vitro toxicity and functional HTS assays. However, although cell-based screening is established in the drug discovery process, particularly in the many well-described cell line models for cancer,[3] its value in predicting clinical response to new agents is limited. This lack of predictability of commonly employed 2-dimensional (2-D) cellular assays is attributable to the fact that such systems do not mimic the response of cells in the 3-D microenvironment present in a tissue, or tumor, in vivo. The focus of this review is on HTS for anticancer therapy, although the basic concepts discussed should be applicable to other disease states. We will discuss the role of 3-D in vitro culture systems in improving the ability of cell-based HTS to predict clinical effectiveness and also their potential to replace some animal models, as illustrated in Figure 1.

IN VIVO VERSUS CELL-BASED ASSAYS FOR ANTI-TUMOR DRUG TESTING

It is generally recognized that rodent test systems will remain necessary for pharmacokinetic (dosage, formulation, administration, half-life) and toxicological (systemic and organ specific) evaluation of candidate therapeutic compounds for at least decades to come. However, the number of animal models used in the initial discovery of lead compounds has already begun to gradually decline, and the same is expected in target validation. This tendency is driven not only by ethical and economic concerns but also by the fact that xenograft activity is often not predictive of clinical efficacy, particularly for a specific histological tumor type. Thus, pharmaceutical corporations often maintain 30-35 xenograft models simultaneously to identify the few that are active for a particular compound, thereby increasing the predictive value of their in vivo testing. Another strategy is reflected by the shift in the screening policy of the U.S. National Cancer Institute Developmental Therapeutic Program implemented in the late 1980s involving replacement of the P388 in vivo tumor model by an in vitro anticancer drug screen. Today, the 60-cell line screen is preceded by a 3-cell

Table 1. Summary of Three-Dimensional Cell Culture Systems Currently Employed in Tumor and Normal Cell Research

System	*Description*	*Uses*
Multicellular spheroid	Spherical aggregate of cells in static or stirred suspension culture	Tumor cell biology, therapy resistance, cell-cell interactions, invasion, drug penetration, modeling, tumor markers, nutrient gradients, tumor cell metabolism[14-16]
Cellular multilayer	Layers of cells cultured on top of a porous membrane	Drug transport and binding, therapy resistance, invasion[17,18]
Matrix-embedded culture	Single cells or aggregates embedded in a porous extracellular matrix	Tumor cell biology, cell-cell interactions, cell migration and invasion, artificial organs[19,20]
Hollow-fiber bioreactor	Cells cultured within a network of perfused artificial capillaries	Tumor cell metabolism, therapy resistance, artificial organs[21-23]
Ex vivo culture	Piece of tissue or tumor excised and cultured in vitro	Therapy resistance, cell-cell interactions, tumor markers[24,25]

The list of uses and literature citations is not intended to be exhaustive but rather to reflect the most common types of studies published.

line, 1-dose prescreen, and followed by in vivo application in a hollow fiber assay.[8] Recent reviews on the NCI-DTP experience revealed both the hollow fiber model and the in vitro human cancer cell line assay to be useful predictors of xenograft activity, with the NCI 60-cell line in vitro cancer drug screen yielding slightly higher confidence values than the hollow fiber assay.[3,8,9] However, no significant correspondence was observed between any single in vitro or in vivo preclinical model and actual clinical results. Only for compounds with activity in a large proportion of tested xenograft models was there any correlation with ultimate activity in at least some phase II trials. At first glance, this would imply that in vitro assays with monolayer cultures can already be as predictive of clinical efficacy at this stage of the drug development process as the currently used common animal test systems. Although controversy continues in this arena, several reports have emphasized the limited predictive value of routinely applied in vitro and in vivo drug screening models for clinical efficacy.[4,5,10-12] The shortcomings of these models lend further strong support to the development of complex, 3-D culture systems that better reflect the in vivo pathophysiological situation in human tumor tissues to soon replace some animal test strategies.

Indeed, cell-based models that are able to predict in vivo, clinical behavior offer obvious advantages in savings of time and money. However, as is the case with the xenograft model, many of the current in vitro systems for cell-based screening and target validation remain unreliable and nonpredictive for clinical efficacy.[4,5,10] We therefore entirely agree with Bhadriraju and Chen[13] who proposed in a recent review article that "a principle component of this failure results from our lack of understanding of, and inattention to, how to culture cells specifically so that they phenotypically represent their in vivo counterparts." It is beyond dispute that cells can behave differently depending on their environment and the culture conditions. For example, the choice of medium and medium supplements (eg, serum, growth factors, pH, oxygen), cell density, and the composition of the culture surface have a critical impact on cell proliferation, differentiation, migration, and death by affecting intracellular signal transduction. This interferes with both random and target-specific screening approaches, leading to results with high variability and questionable relevance. A better understanding of how the cellular microenvironment modulates the cellular phenotype is necessary to design more appropriate physiologic and pathophysiologic in vitro models.

Currently available cell-based assays for HTS, although retaining more cellular architectural features than cell-free biochemical test systems, still reflect a highly artificial cellular environment. For economic and ethical reasons, test systems that mimic the human tissue environment with increasing accuracy will soon need to be considered to optimize preclinical and preanimal selection of the most active molecules from the large and growing pool of drug candidates. Efforts to establish and optimize new tools for advanced cell-based in vitro screening are necessary and should be encouraged. As an example, the recent use of microfabrication technologies to control cell adhesion, cell shape, intercellular contact, and heterologous cell interactions is a promising new attempt to design better and more economic in vitro models.[13] However, it is as important to recognize the power of established sophisticated in vitro model systems that have been applied by the academic community for nonscreening purposes to better mimic the in vivo situation. Three-dimensional culture systems, either cell line or tissue based, are known to reflect the in vivo behavior of many cell types and are promising approaches for advanced drug screening. The most common 3-D cell culture systems employed in basic and applied tumor biology are described in Table 1.[14-25] A major reason that these and other 3-D culture systems have not entered the drug screening process to date is the lack of simple, controlled techniques and protocols for rapid, standardized assay of cellular responses in situ. In addition, the emphasis in improvements for in vitro drug screening has been on increasing the throughput of simple culture systems (eg, 384-well plates, microfabrication of miniaturized culture chambers, single-cell analysis methods) with little regard for whether the basic culture system being used is predictive of in vivo activity. However, as discussed in detail below, there are possibilities to adapt certain HTS methods originally

developed for monolayers to culture systems with higher complexity. This may well improve the in vivo predictability of the screening system while maintaining a high-throughput format.

RATIONALE FOR USING COMPLEX CELL SYSTEMS IN ANTITUMOR DRUG TESTING

It is well known that many cells of normal and malignant origin lose some of their phenotypic properties when grown in vitro as monolayer or suspension cultures. Current attempts to obtain, and maintain, functional differentiation of human cells similar to that seen in vivo can be divided into 3 different approaches and combinations of these: (a) culturing of primary tissues or isolated early passage primary cells, (b) application of established cell lines on specially manufactured growth surfaces and/or in controlled heterologous monolayer systems, and (c) propagation of established cell lines in 3-D culture systems.

The use of primary cells in vitro is preferable in several respects but is hampered by limited availability and high variability, as well as by ethical restrictions, at least with respect to human tissue. However, there are attempts to use such material in drug testing. Notable is the effort to establish patient-specific therapeutic test systems for specific tumor entities, such as the ex vivo ATP-based chemosensitivity assay (ATP-TCA), which is based on the 2-D culturing of tumor cells isolated from primary tissue biopsies.[26-29] Also, in parallel to improvements in the tissue engineering field, diverse culture approaches have been developed that may allow reproducible maintenance of viability and differentiation in isolated primary tissue material of different origin in a 3-D format, such as the NASA bioreactor,[30-33] various perfusion-type or gradient container systems,[34-41] or the tumor fragment spheroid model.[42-44] Some of the above-mentioned systems are still under refinement to better match specific properties of differentiated tissues.[41]

Because the availability of primary material will always be the limiting step for HTS, there is increasing demand in the pharmaceutical industry for advanced in vitro test systems based on established human cell lines. This requires restoration of morphological and functional features of the corresponding tissue in vivo. However, the formation of tissue structures is highly inhibited in monolayer culture due to the strong affinity of cells to most artificial substrates and the restriction to a 2-D space, severely limiting intercellular contacts and interaction. It is beyond the scope of this article to detail progress in engineering 2-D or pseudo-3-D microenvironments that mimic the in vivo situation,[13] but the recent advances in heterologous co-culture systems to reassemble some in vivo features of cells and tissues should be noted.[45-48] Three-dimensional heterotypic collagen assays that were primarily applied in the past few years to resemble breast epithelial morphogenesis and neoplasia are a promising approach that could also be useful for antitumor drug screening.[47,49] In the anticancer drug development arena, multiple in vitro assays are commercially available to monitor particular steps in the angiogenic cascade and the effect of antiangiogenic compounds. Most of these assays utilize multiwell plates with surfaces coated with natural or artificial ECM components and established endothelial cell lines. A promising new type of assay consisting of endothelial cells and fibroblasts in co-culture was recently shown to more closely resemble the in vivo capillary bed when compared to the commonly used Standard and Growth Factor Reduced Matrigel™ assays.[50,51] Tubule formation by endothelial cells reflects to some extent a switch from a 2-D to a pseudo-3-D structure (The term *pseudo-3-D* is used because long-term co-culturing results in a multilayered structure, but the multilayering is restricted to areas showing formation of vessel-like structures and does not exceed 3-4 cell layers). This interesting co-culture assay has the disadvantage of requiring 10-14 days of culture. Also, the system has not been made amenable to high-throughput usage so far. However, this holds true for most complex culture systems, even for some that are well established and have been used by cell biologists for decades.

Three-dimensional cultures have been utilized in biomedical research since the first half of the 20th century to gain deeper insight into the mechanisms of organogenesis and expression of malignancy. Today, it is well known that in contrast to conventional monolayer or suspension cultures, 3-D cultures can restore specific biochemical and morphological features similar to the corresponding tissue in vivo. The relevance of this observation for mechanistic studies and advanced drug testing has long been ignored by both academic researchers and the pharmaceutical industry. The National Cancer Institute program Signatures of the Cancer Cell and Its Microenvironment is a new $40 million per year effort designed to investigate the impact of the microenvironment on tumor cell behavior. This initiative will emphasize 3-D culture technologies and will clearly foster the acceptance of such approaches. Also, Alison Abbott's report "Biology's New Dimension"[52] and the corresponding commentary of the editor, "Goodbye Flat Biology," were overdue and more than welcome by scientists working in the field. In this news feature, experts in cell biology emphasize the basic necessity for 3-D culture systems before turning to whole-animal studies, for therapeutics development as well as basic research in tumor biology. Also in this article is a prediction by the chief scientific officer of a large pharmaceutical company, which has attracted major attention: "In 10 years, anyone trying to use 2-D analyses to get relevant and novel biological information will find it difficult to get funded." With respect to the utilization of relevant biological information from 3-D culture analyses for HTS approaches in drug discovery and development, there is a strong need for intercollaborations between the pharmaceutical industry and academic institutions both to provide proof of predictive value and to rapidly reach practical feasibility of such 3-D systems for sophisticated drug screening. Indeed, advances in tissue engineering, including the development of diverse bioreactor systems such as those mentioned above and of 3-D scaffolds (eg, bioactive, biodegradable, or nondegradable polymers), have improved the variety, fidelity, and quantity of models that can be utilized in cancer research. However, only a small number of 3-D model systems are sufficiently well characterized to simulate the

pathophysiological cellular microenvironment in a tumor, reconstitute a tissue-like cytoarchitecture with cell-to-cell and cell-to-matrix interactions, and present growth, differentiation, and therapy response similar to that seen in tumors in vivo.

The classical and best described 3-D tumor model system, which fulfills all of the characteristics just described, was not mentioned in the cited news feature: the multicellular tumor spheroid model (MCTS). This system was adapted for cancer research in the early 1970s by Sutherland and associates[53,54] and is in use in many laboratories throughout the world today. The spheroid culture model has not only considerably contributed to our knowledge of cellular response to a variety of treatment modalities but also has been critical in more basic studies investigating the microenvironmental regulation of tumor cell physiology.[15,16,55-58] In contrast to the other 3-D in vitro models described in Table 1, MCTS have considerable potential for application as HTS systems in anticancer drug development. This potential as well as the limitations of this unique 3-D culture system shall thus be highlighted.

MULTICELLULAR TUMOR SPHEROIDS

Over the past 30 years, the (patho)physiological behavior of diverse tumor cell types of animal and human origin has been studied in MCTS culture in detail. Today, it is well accepted in the academic community that MCTS provide an important supplement to the use of monolayer cultures and animal in vivo systems due to their intermediate complexity,[45,56] as illustrated in Figure 2. Accordingly, MCTS have served as a model for a variety of experimental therapy studies using radiotherapy, chemotherapy, radioimmunotherapy, cell- and antibody-based immunotherapy, hyperthermia, gene therapy, and photodynamic treatment.[55-71] MCTS have also been used extensively in basic studies of the microenvironmental regulation of proliferation, viability, energy metabolism, nutrient metabolism, invasion, cell-cell interactions, and extracellular matrix composition. Based on their long-term experience, experts in the field agree with Mueller-Klieser's final conclusion in a 2000 review article: "Keeping in mind the fundamental differences between monolayers and spheroids with regard to cellular sensitivity to various treatment modalities, tumor spheroids should be mandatory models in applied cancer research, for example in major programs for drug screening and development."15 This article is just one out of a review series published in the November 2000 issue of *Critical Reviews in Oncology/Hematology*, which was devoted to 3-D spheroid cultures and is recommended reading for both spheroid specialists and laymen. A general survey of previous research with the spheroid model, including technical descriptions of a variety of spheroid methods, is given in 2 rather dated but still very informative books.[72,73] More recent book chapters[46,74] and several review articles published within the past 5 years focusing on different applications of spheroids[16,45,57,75,76] provide further information to define the rationale for developing MCTS technology for HTS of chemotherapeutic drug candidates. This rationale is based on the following 4 important features of MCTS.

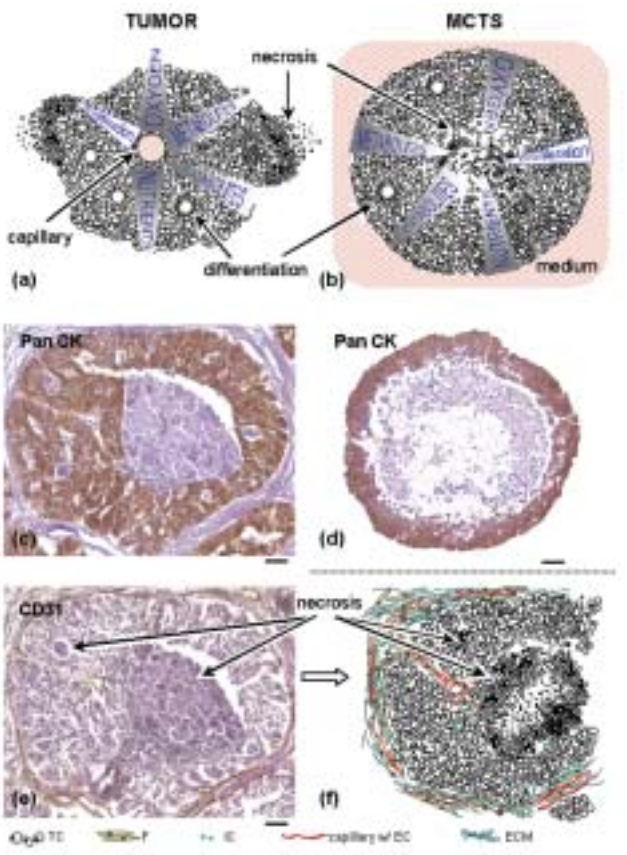

FIG. 2. Schematic illustration of the analogy between (*a*) tumor microregion and (*b*) multicellular tumor spheroid (MCTS) demonstrating the pathophysiological inside-out situation in MCTS (modified from Kunz-Schughart, 1999). (*c/d*) Labeling of tumor cells in 5 µm sections of paraffin-embedded material using a monoclonal Pan-cytokeratin (Pan-CK) antibody (clone KL1, Beckman Coulter, Fullerton, California), 2-step routine immunohistochemical staining with DAB (3'3'diaminobenzidine) as chromogen, and hematoxylin counterstain: (*c*) microregion of a colon carcinoma (G3), and (*d*) HT29 colon tumor spheroid. (*e*) Parallel section of colon carcinoma shown in (*c*) stained with the endothelial cell marker CD31 (monoclonal mouse–anti human IgG1, DakoCytomation, Hamburg, Germany) to visualize capillaries primarily located in the stromal tumor areas. (*f*) Schematic illustration of the G3 colon carcinoma shown in (*c/e*) to visualize the distribution of different cell types in this tumor microregion with TC, tumor cells; F, fibroblasts/fibroblastoid cells; EC, endothelial cells; IC, immune cells, for example, tumor-associated macrophages; ECM, extracellular matrix. Bar, 100 µm.

First, MCTS reestablish morphological, functional, and mass transport features of the corresponding tissue in vivo. In particular, tumor cells in MCTS restore a differentiation pattern similar to that observed in vivo, which is maintained for several weeks of culture.[16,55,56,77-79] These features are initiated and maintained by the tumor cell–derived extracellular matrix (ECM) assembly and a

complex 3-D network of cell-to-cell and cell-to-matrix interactions, which are largely absent from monolayer cultures. In addition, the 3-D spheroid structure is relevant for both the modeling of distribution and function of (patho)physiologically occurring factors in tissues, as well as the penetration, binding, and bioactivity of drugs.

Second, MCTS approximate many characteristics of avascular tumor nodules, micrometastases, or intervascular regions of large solid tumors with regard to both tumor growth kinetics and pathophysiological micromilieu (Figure 2). Analogous to solid tumors and metastases in vivo, spheroid growth can be mathematically modeled by the Gompertz equation as well as by several more biologically based mathematical models that have been applied to tumors.[67,80-85] A proliferation gradient is observed in spheroids, with proliferating tumor cells at the periphery, cell-cycle-arrested cells at larger distances from the surface, and a necrotic core in most spheroids larger than 400-500 μm. In parallel, inward oxygen and nutrient gradients as well as outward catabolite gradients are present, analogous (again) to the situation in poorly vascularized regions of solid tumors. These variations in cellular physiology and tumor micromilieu are associated with alterations in sensitivity to a wide variety of anticancer therapies in both tumors and spheroids.

Third, the well-defined, spherically symmetric geometry of MCTS allows direct comparison of structure to function, that is, the microenvironmental gradients that determine spheroid morphology are spatially correlated with the changes in cellular physiology. This has several advantages for the use of spheroids as in vitro tumor models. A primary advantage is that assays of cellular physiology and drug response can be directly related to location in the microenvironment, either by a variety of in situ histologic assays[16,86-89] or on cell subpopulations isolated from different spheroid regions.[16,90-94] A second advantage is that the spherical symmetry enables relatively simple theoretical analyses, for example, to predict radiation response,[95,96] drug penetration, and binding/activity[97-99] or to interpret the regulation of cellular physiology, proliferation, and viability.[67,80-82,84,100] A third advantage of the spheroid geometry is that aggregates cultured from the same cell type under the same external conditions are essentially identical in structure, morphology, microenvironment, and cellular physiology. This allows the efficient generation of a few up to literally thousands of individual, identical cultures for assay.

Finally, spheroids are amenable to the co-culture of different cell types, in particular, tumor cells and normal cells, such as stromal fibroblasts, endothelial cells, or cells of the hematopoietic/immune system.[16,45,101-106] These co-cultures retain some, if not all, of the advantages mentioned above for homologous cultures, notably the intimate and complex cell-cell and cell-matrix interactions that are present in tumors in vivo. Heterologous spheroid co-cultures have not been as extensively utilized, or even characterized, as those of pure tumor cell spheroids. However, improvements in techniques and development of more rapid assay systems promise to expand the spheroid co-culture into a very unique and multifunctional in vitro drug-testing system, as discussed in more detail below.

Due to these characteristics, MCTS have become a powerful tool to study therapeutic problems associated with metabolic and proliferative gradients, for example, the treatment sensitivity of tumor cells chronically deprived of nutrients. Thus, MCTS serve as a well-controlled, 3-D experimental, and theoretical model to better estimate in vivo antitumor effective dose ranges and treatment modalities. MCTS come into particular focus with the expectation that some lead candidates designed via modern drug discovery processes to precisely target tumor cells not only should attack the primary tumor and local relapse but also be effective against metastatic disease. As a model of avascular micrometastases (eg, diameter < 1 mm), spheroids can uniquely contribute to the understanding of, and screening for, drug penetration and the local pattern of cellular uptake and effectiveness.

EXTENSIONS TO THE USE OF THE MCTS MODEL

The reflection of additional aspects of the in vivo situation may be desired not only for cancer research but also for drug screening purposes. Thus, application of different therapy-responsive tumor cells in mixed or mosaic spheroids[61,61,107-111] or tumor and stromal host cells in spheroid co-culture[16,45,101-106] is a promising approach to further mimic the heterologous cellular environment in a solid tumor or at sites of metastasis. Indeed, in addition to the application of mixed cell populations on nonadherent surfaces, there is a wide range of strategies to co-culture different normal and tumor cell types using spheroids, each with particular advantages and disadvantages, and for specific fields of application. MCTS can, for example, be grown on monolayer cultures of fibroblasts and endothelial cells to investigate specific host-tumor cell interactions. Culturing MCTS on confluent endothelial cells with an underlying ECM is adequate to investigate aspects of tumor cell evasion and to evaluate endothelial cell injury by tumor cells. Confrontation cultures of MCTS with endothelial cell aggregates in a collagen gel or with embryonic stem cells (embryoid bodies) have been established to study endothelial cell differentiation and sprouting phenomena in vitro.[102,112-114] The application of fibroblast spheroids allows investigation of some tumor cell invasion/migration phenomena by adding tumor cell suspensions, and also enables one to study those tumor cells that do not form MCTS by themselves. Incubation of tumor or stromal spheroid cultures of defined sizes with immune cell suspensions is an interesting approach to mimic local immune responses, including migration and differentiation/activation processes.[42,101,103,105,115,116] The latter spheroid co-culture systems have been primarily used to gain deeper insight into the reciprocal interactions between tumor cells and their cellular environment, rather than for drug testing. However, it is expected that adjacent host cells can affect tumor cell drug sensitivity, and therefore spheroid co-culture systems could be utilized both for advanced in vitro

drug testing and to screen agents that affect the host-tumor interaction.

Although the attempt to routinely initiate spheroid growth directly from patient tumors for patient-specific predictive drug testing has had relatively little success, MCTS still serve as an excellent in vitro screening system in mechanistic studies of drug penetration, binding, and action (ie, with respect to new, innovative therapeutic strategies such as combination therapy, administration of drugs under defined environmental conditions, or treatment with toxin-conjugated antibodies). Bearing in mind, for example, that the heterogeneity in the binding of monoclonal antibodies depends on both cell and antibody type, as well as on cell cycle and metabolic status of the cells,[88,98,117-120] spheroid mono- and cocultures should be considered as a screening tool for local penetration, distribution, and efficacy of new drug candidates.

A final area of potential for MCTS in drug testing involves the additional culture of spheroids from normal, untransformed cells. The current literature indicates that normal cell spheroids can, with limitations, be applied to study some developmental and functional aspects of normal cells and tissues such as hepatocytes/liver,[121-125] chondrocytes/cartilage,[126-128] retinal cells forming so-called retinospheroids,[129-132] trabecular cells showing a network tissue structure in spheroid culture,[133] and pancreatic epithelial cells producing hollow spheres.[134,135] The patterns of morphological, biological, and functional development seen in these cultures are, in many respects, consistent with the differentiation seen in vivo. To date, nonhuman cell types are most frequently applied in normal spheroid culture systems; adaptation to cells of human origin would be preferable but will in most cases be limited due to availability and ethical restrictions. However, provided the limitations of the spheroid model for normal cells are recognized and respected (eg, pure normal tissue-type spheroids should not show necrosis), normal cell spheroids represent a potentially powerful tool for application in initial toxicity assays or in confrontation/mixed cultures of tumor and normal cell types[16,45] for the evaluation of drug selectivity and specificity.

DEVELOPMENT OF SPHEROIDS FOR HTS

Application of 3-D culture systems for drug screening, including homotypic MCTS, is hampered by lack of standardized, rapid analytical tools. The only systematic study using various methods for assaying cytotoxic effects in spheroids is now somewhat dated and involved only small spheroids without central necrosis,[136,137] thus limiting its relevance for evaluating drug effects on hypoxic and cell-cycle arrested tumor cell populations in nonperipheral, viable regions present in most spheroid types with a diameter of ≥ 500 μm (see Figure 2). Fortunately, one of the most widely used spheroid production techniques involves culture of a single spheroid in each well of an agar-coated multiwell plate.[16,46,72] Thus, there are several screening techniques currently applied to monolayer cultures in a multiwell plate format that should be easily adapted to spheroids, such as various assays for cytotoxicity, proliferation, drug binding, apoptosis, and ATP level. Simple measurement of the growth or shrinkage of spheroids can be accomplished by standard phase-contrast microscopy and computer image analysis, something that is very difficult to do accurately with monolayer or multilayer cell cultures. Commercial automated microscopy systems could be easily configured to provide a rapid method for screening drugs for effects on spheroid growth. A recent approach for toxicological and biomedical testing with some potential for adaptation to HTS with spheroid cultures is a so-called biohybrid sensor system, which is a new type of bioelectrical microarray for 3-D in vitro tissues on the basis of electrode/spheroid surface impedance measurements.[138-140] Although these HTS methods would be useful as an initial screen for drug activity in spheroids, the response would be an average from all the cells in the aggregate. Even though such a screening system would still incorporate the advantages of the 3-D in vitro model discussed above, any information on differential response of cells at different locations in the spheroid would be lost.

There are other possibilities to develop new screening methods that would take better advantage of the symmetrical morphology, microenvironment, and cellular physiology present in spheroids, as discussed above. As an example, work is under way in our laboratories to develop confocal microscopy methods for rapidly measuring drug penetration into individual spheroids, which could potentially be automated into a high-throughput approach. Here, the advantage of the MTCS culture system is the fact that drug candidates must penetrate a tumor-like tissue to be effective. The effectiveness of 3-D model systems for studying drug penetration effects has been established both in MCTS and in cell multilayers.[18,58,97,141-150] Cell multilayers are not easily adapted to an HTS approach; however, the use of spheroids opens up a whole new area of in vitro screening for the penetration and binding of drugs in a 3-D tissue-like structure. Analogous to the case with cellular assays, simple assays of total drug binding in individual tumor and host stromal spheroids in a multiwell format represent an easy system for screening drugs with different binding properties. Following drug uptake as a function of time in individual spheroids would allow kinetic analysis and perhaps even the extraction of effective diffusion coefficients, as has been demonstrated for bulk cultures of spheroids.[151-153] In addition, it should be possible to develop simple staining methods for measuring the extent of necrosis in intact, individual spheroids, which would then provide an assay for drug effects on the viability of the inner-region spheroid cells. There are certainly other staining techniques available that could be applied to an imaging-based screening system for MCTS, such as measuring apoptosis, proliferation, and various metabolism markers. Development of HTS techniques that measure the response of specific subpopulations in spheroids (eg, hypoxic cells, nonproliferating cells) would considerably enhance the usefulness of MCTS as a drug-screening tool, particularly in light of efforts under way to design drugs targeted against specific microenvironmental adaptations.

Another area that holds promise for HTS using spheroids is the use of microfabrication and other miniaturization techniques for cell cultures. Because most of these techniques are directed toward very small numbers of cells, or even single cells, they should be relatively easy to adapt to larger sized spheroid aggregates. One could envision a microfluidic device that contained large numbers of individual spheroids in separate culture chambers equipped with perfusive nutrient supply. Such a device could be combined with a computer-controlled imaging system to measure spheroid growth or drug penetration as discussed above. One could also potentially use the microfluidic system to introduce test compounds and measure the kinetics of spheroid response.[139,140]

One limitation to the use of spheroids for HTS is the fact that it takes a relatively longer time for the aggregates to grow as compared to monolayer cultures, especially if one wants to use spheroids of sufficient size to contain gradients in proliferation and a central necrotic core. The length of time for a typical tumor spheroid to grow from a few-cell aggregate to a large structure with proliferative and viability gradients ranges from 2 to 4 weeks. One could "jump-start" this process by forcing the aggregation of large numbers of cells into a single spheroid in a culture well, but it would still take a few days, at a minimum, for the microenvironmental and pathophysiological gradients to develop. However, for most HTS applications, only the time required to set up the assays is extended: the actual screening of drugs could be done as rapidly as with traditional monolayers once the MCTS are established. Considered in the overall context of an HTS method, the advantages of using MCTS for anticancer drug screening would appear to outweigh this time limitation.

As discussed above, the use of spheroid co-cultures of tumor and normal cells is a potentially very powerful system for advanced drug screening. Currently lacking to make this co-culture system most useful are techniques for obtaining a large population of uniformly sized spheroids with a reproducible cellular composition. One approach to improve the analysis of co-cultured spheroids would be the application of cell types expressing fluorescent markers, such as GFP or GFP analogues. One could then create large numbers of spheroid co-cultures and sort them according to size and fluorescence intensity using a modified flow sorter,[154] thereby obtaining a population with a uniform composition and size. With some developmental work in this area, it should be possible to generate a set of standard tumor:normal co-cultured spheroids that are well characterized and reproducible to serve as an HTS platform that incorporates tumor-normal cell interactions. Confocal fluorescence microscopy is an additional or alternative technical tool that could be improved to be applied for the analysis of such co-cultures.[155-157]

A final limitation to spheroids that should be mentioned is that the MCTS in vitro model will never be developed to the point that it will entirely replace the use of animals in the drug-testing process. Although the use of tumor/normal, or even purely normal cell spheroid co-cultures, is an exciting and powerful extension that will allow for a better evaluation of local bioactivity and penetration of new drug candidates, this model obviously lacks the complexity required for standard absorption, distribution, metabolism, elimination, toxicity (ADMET) screening. Considering the rapidly continuing improvements in reproducibility, throughput, and sophistication of other 3-D culture systems for nontumorous cells, for example, in tissue engineering, an increasing number of valuable complex culture models may be available in the near future for basic compound safety testing. However, as depicted in Figure 1, utilization of 3-D culture assays for routine toxicity testing of candidate therapeutics is speculative, and also pharmacokinetics still requires animal models, as there is currently no way to extrapolate these parameters from in vitro systems.

Although it is clear that some technology development work is required to bring the MCTS system into the arena of HTS, the significant advantages of this in vitro tumor model argue persuasively in favor of such an approach. Methods for simply screening, for example, cytotoxicity or growth arrest, could be quickly developed into high-throughput methods with spheroids. Techniques that would allow the rapid screening for effectiveness in tumor spheroids would be largely applicable to testing in mixed-cell spheroids and spheroids composed only of normal cells. Other limitations to HTS discussed above may preclude rapid assays for some endpoints, but it is important to recognize that these "limitations" could actually be advantages for more advanced screening techniques. For example, candidates identified by a rapid screen to be effective in killing or growth-arresting MCTS could be further screened for drug penetration, host-tumor interactions, and effects on nutrient-stressed subpopulations using the same basic in vitro model from which they were initially selected.

CONCLUSION

The fact that no preclinical study can yet replace phase II clinical trials is not disputed. However, not scientific reasoning but regulatory and legal requirements, and strong clinical tradition, remain the most important incentives for rodent model studies. The main reason and justification for routinely maintaining a large number of animal model systems for drug screening is that the predictive power for clinical efficacy is statistically poor for any single in vivo tumor model. Because the cost of drug development increases drastically at the stage of rodent tumor models, the guiding principle in the cancer drug industry has been to identify poor candidates earlier rather than later. It has therefore been proposed that increased reliance on cell-based validation early in the drug development process will prove economically advantageous. In the pathway from a simple solution-binding assay to in vivo testing, cell-based screening has proven to be a valuable stepping stone to quickly weed out toxic and nonfunctional compounds. The low cost and high speed of screening compounds in cell culture, and the obvious advantage of using intact cells as the most expedient representation of the living patient, have made cell-based screening a key component of drug discovery programs.[158] This article has reviewed the unique features of tumor spheroids as an in vitro

model system, and has also highlighted the options of heterologous tumor/stromal cell co-cultures to mimic aspects of in vivo tumor heterogeneity. The application of heterologous spheroid co-cultures in cancer research allows cellular analysis in a controlled and reproducible format. Although the complexity and limitations of these 3-D co-culture systems have to be recognized, MCTS methodology provides an important supplement to the use of animal in vivo systems, in particular, for research with human cells. Further technical developments should allow the future application of spheroids, with all of their in vivo characteristics, in HTS for anticancer drugs as well as potentially for other drug discovery and development efforts.

ACKNOWLEDGMENT

We thank Dr. Juergen Seidl for his assistance in manuscript and image preparation.

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