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Application of Cell and Tissue Culture Systems for Anticancer Drug Screening

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Abstract: A primary reason why potential anticancer drugs fail in clinical trials is the limitations of existing *in vitro* screening systems. The purpose of *in vitro* testing is to aid in the selection of efficacious compounds from a range of candidates with variable levels of the desired properties. *In vitro* cell-culture tumor models are the most widely used screening systems, however, they lack the complexity of the natural microenvironment of the host organism. Clearly, an effective screening system should possess qualities and organization similar to those of a natural tumor microenvironment. In an attempt to better simulate a tumor and its microenvironment sophisticated models have been developed, such as the three-dimensional cultures. In addition to these *in vitro* culture methods, tissue-based testing methods have been used to screen potential anticancer drugs but these methods have limited utility. This review describes the modern *in vitro* models used to evaluate cytotoxic substances that inhibit the growth of tumor cells and discusses their respective advantages and disadvantages.

Key words: Cancer · Tumor · Drug Screening · Chemotherapeutics Running title Anti-Cancer Drug Screening Systems

INTRODUCTION

In the last decade, significant advances have been made in understanding cancer biology using high-throughput methods. Through these methods, several test-systems have been developed that allow rapid and effective screening of potential anticancer compounds [1]. Indeed, such screening systems have greatly facilitated the identification of novel drugs with the potential to inhibit the growth of transformed cells *in vitro* and *in vivo*. The cellular molecule upon which an anticancer compound exerts its effect is referred to as the *drug target*. Once a potential compound with anticancer properties is identified, its pharmacokinetic characteristics should be investigated. For instance, the *in vivo* bioavailability of a compound may be substantially different from that observed in tissue culture for a number of reasons. Solubility, stability or plasma-protein binding properties are only some of the potential issues that may affect bioavailability. These and many other issues should be considered when selecting an effective method for anticancer drug screening and conducting preclinical trials. The rational approaches for primary drug screening and preclinical studies, described in this review, significantly contribute to facilitating the development of safe and effective drugs.

Tumor Classification: All tumor formation begins with cell transformation, the clinical presentation of which may vary to a significant degree. Accordingly, more than 100 types of tumors are currently described [2]. The principle method of classification divides tumors into benign and malignant. The former are bundles of cells that are typically poorly vascularized and are largely static while the latter are vascularized and are exemplified by their ability to grow and spread. For benign tumors, oxygen and nutrient delivery is limited to simple diffusion via the surface of the tumor nodule. Therefore, the cells of benign tumors consistently die off, thus limiting tumor growth. The extant tumor, which is at equilibrium with the surrounding tissue, typically does not pose a serious threat to the body. Conversely, the vascularization of

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malignant tumors commences in the primary nodule through the process of angiogenesis and accordingly, allows the delivery of nutrients and oxygen to virtually all transformed cells. As the malignant tumor grows, it gradually expands to the adjacent tissues, thus entering a stage of invasion. The malignant cells subsequently migrate, via the lymph and bloodstream, to colonize other parts of the body; this process of tumor proliferation is known as metastasis. Although the metastatic cells may migrate to other parts of the body, they frequently have the same molecular features as the initial tumor; and while some metastatic cancers can be cured, most cannot. Consequently, metastatic disease is the principal cause of cancer-related death.

Hanahan and Weinberg proposed six commonly acquired characteristics that allow cancer cells to survive, proliferate and disseminate [3]. These so-called "signs of tumors" are: 1) self-sufficiency in growth signals; 2) insensitivity to antigrowth signals; 3) evading of apoptosis; 4) unlimited proliferative potential; 5) sustained angiogenesis; and 6) tissue invasion and metastasis. The complexity of the proposed characteristics is based upon the diversity of the tumor's genetics. Therefore, even within the same tumor, transformed cells display micro-heterogeneity, making each tumor unique [2]. These "signs of tumors" can serve as useful guides when developing new anticancer compounds.

Targets and Mechanisms of Anticancer Drugs: Having an understanding of the potential molecular target and mechanism of action of a prospective anticancer compound is beneficial for a number of reasons. For instance, this information may facilitate screening of a new compound that was generated by modification of an existing one. Additionally, knowledge of the target molecule may allow one to predict potential side effects or toxicity. Cytotoxic anticancer compounds (also referred to as chemotherapeutics) are traditionally categorized into four groups according to their mechanism of action and molecular target [4]. For example, most cancers are exemplified by uncontrolled cell division; therefore, the majority of anticancer agents target molecular pathways necessary for cell propagation. The specific targets are typically nucleic acids, microtubules or the enzymes regulating and facilitating DNA replication or cell cycling. The mode of an anticancer compound's action can be subcategorized into one or more general methods of action upon each target. For instance, the class of drug, for which its mechanism of action is most thoroughly understood, is the DNA cross-linking agents. Although typically referred to as the *alkylating agents* these compounds may not necessarily contain alkyl functional groups and, therefore, are often referred to as alkylating-like agents; Cisplatin ((SP-4-2)-diamminedichloridoplatinum) is one such compound [5]. These drugs contain electrophilic functional groups that cross-link DNA by reacting with a nucleophile, such as the O-2 of cytosine or the O-6 of guanine [6]. This cross-linking prevents DNA from acting as a template for replication and transcription and ultimately triggers apoptosis [7]. The mode of action for drugs that target *microtubule function* is to block assembly of tubulin into microtubules [8]. The vinca alkaloids like Vincristine or Vinblastine are examples of drugs that block assembly of tubulin. Conversely, some agents enhance the stability of microtubules, thus preventing the separation of chromosomes during anaphase, as is the case for taxanes like Taxol and its semi-synthetic analogue Docetaxel [9].

Some drugs target the *enzymes of molecular pathways* involved in cell division; for example, the isomerase inhibitors such as Irinotecan and Topotecan can disrupt the topology of DNA, affecting both replication and transcription [10]. Other enzyme inhibitors target the synthesis of nucleic acids precursors. For instance, folic acid is essential for *de novo* synthesis of the nucleoside thymidine, a purine base required for DNA and RNA synthesis. Methotrexate competitively inhibits dihydrofolate reductase, a critical enzyme in the folic acid cycle, which facilitates the transfer of one-carbon units to amino acids, nucleotides as well as other biomolecules [11]. As a result, methotrexate inhibits cell growth at the S-phase of the cell cycle and, therefore, it is more toxic to rapidly dividing cells [12].

Less specific anti-cancer agents include the *cytotoxic antibiotics*, which have varied mechanisms of action. Possibly the earliest antibiotic approved for the treatment of cancer is Actinomycin D, which binds to the transcription initiation complex and thus prevents elongation of RNA strand by RNA polymerase [13]. The anthracycline antibiotics such as Doxorubicin and Daunorubicin exert their anticancer effect by intercalating within the DNA double helix [14]. Although a comprehensive discussion of the mechanism of chemotherapeutic drugs is beyond the scope of this review, the above examples emphasize the utility of understanding the potential mechanism when evaluating a drug for its anti-cancer properties.

Table 1: NCI60 Cell Lines

	Cell Line	NCI Panel	Description		Cell Line	NCI Panel	Description
1	CCRF-CEM	Leukemia	Acute lymphoblastic leukemia. T lymphoblast	31	M14	Melanoma	Amelanotic Melanoma
2	HL-60(TB)	Leukemia	Human promyelocytic leukemia. Myeloblast	32	MDA-MB-435	Melanoma	Adenocarcinoma, melanocyte
3	K-562	Leukemia	Chronic myelogenous leukemia, lymphoblast	33	SK-MEL-2	Melanoma	Malignant melanoma, polygonal
4	MOLT-4	Leukemia	Acute lymphoblastic leukemia. T lymphoblast	34	SK-MEL-28	Melanoma	Malignant melanoma, polygonal
5	RPMI-8226	Leukemia	Plasmacytoma; myeloma. B lymphocyte	35	SK-MEL-5	Melanoma	Malignant melanoma, stellate
6	SR	Leukemia	Lymphoma, lymphoblast	36	UACC-257	Melanoma	Melanotic melanoma; non epithelial
7	A549/ATCC	Non-Small Cell Lung	Carcinoma, epithelial	37	UACC-62	Melanoma	Melanotic melanoma; non epithelial
8	EKVX	Non-Small Cell Lung	Adenocarcinoma, epithelial	38	IGR-OV1	Ovarian	Adenocarcinoma, epithelial
9	HOP-62	Non-Small Cell Lung	Adenocarcinoma, epithelial	39	OVCAR-3	Ovarian	Adenocarcinoma, epithelial
10	HOP-92	Non-Small Cell Lung	Large Cell, epithelial	40	OVCAR-4	Ovarian	Adenocarcinoma, epithelial
11	NCI-H226	Non-Small Cell Lung	Squamous cell carcinoma; mesothelioma, epithelial	41	OVCAR-5	Ovarian	Adenocarcinoma, epithelial
12	NCI-H23	Non-Small Cell Lung	Adenocarcinoma, epithelial	42	OVCAR-8	Ovarian	Adenocarcinoma, epithelial
13	NCI-H322M	Non-Small Cell Lung	Adenocarcinoma, epithelial	43	NCI/ADR-RES	Ovarian	Adenocarcinoma, epithelial
14	NCI-H460	Non-Small Cell Lung	Carcinoma; large cell lung cancer, epithelial	44	SK-OV-3	Ovarian	Adenocarcinoma, epithelial
15	NCI-H522	Non-Small Cell Lung	Stage 2, adenocarcinoma, epithelial	45	786-0	Renal	Renal cell carcinoma, epithelial
16	COLO 205	Colon	Dukes' type D, colorectal adenocarcinoma, epithelial	46	A498	Renal	Carcinoma, epithelial
17	HCC-2998	Colon	Colorectal carcinoma, epithelial	47	ACHN	Renal	Renal cell adenocarcinoma, epithelial
18	HCT-116	Colon	Colorectal carcinoma, epithelial	48	CAKI-1	Renal	Clear cell carcinoma, epithelial
19	HCT-15	Colon	Dukes' type C, colorectal adenocarcinoma, epithelial	49	RXF 393	Renal	Renal cell carcinoma, epithelial
20	HT29	Colon	Colorectal adenocarcinoma, epithelial	50	SN12C	Renal	Renal cell carcinoma, epithelial
21	KM12	Colon	Adenocarcinoma, epithelial	51	TK-10	Renal	Renal cell carcinoma, epithelial
22	SW-620	Colon	Dukes' type C, colorectal adenocarcinoma, epithelial	52	UO-31	Renal	Renal cell carcinoma, epithelial
23	SF-268	CNS	Highly anaplastic astrocytoma; right parietal; non epithelial	53	PC-3	Prostate	Grade IV, adenocarcinoma, epithelial
24	SF-295	CNS	Glioblastoma (Multiform), glial	54	DU-145	Prostate	Carcinoma, epithelial
25	SF-539	CNS	Glioblastoma, glial	55	MCF7	Breast	Mammary Carcinoma, epithelial
26	SNB-19	CNS	Glioblastoma; left parieto-occipital; non epithelial	56	MDA-MB-231 /ATCC	Breast	Adenocarcinoma, epithelial
27	SNB-75	CNS	Glioblastoma-grade IV; non epithelial	57	MDA-MB-468	Breast	Adenocarcinoma, epithelial
28	U251	CNS	Glioblastoma, astrocytoma	58	HS 578T	Breast	Carcinoma, epithelial
29	LOX IMVI	Melanoma	Malignant Amelanotic Melanoma	59	BT-549	Breast	Ductal carcinoma, epithelial
30	MALME-3M	Melanoma	Malignant melanoma, fibroblast	60	T-47D	Breast	Ductal carcinoma, epithelial

The NCI60 Cancer Panel: The Modern Pharmaceutical Screening Protocol was adopted in 1990 by the National Cancer Institute (NCI) and is currently composed of 60 human tumor cell lines known as the NCI60 (Table 1). Presently, this is the most commonly used protocol for the preliminary screening of anticancer drugs. The major advantage of the NCI60 is that each of the 60 cell lines generates a specific response to a particular drug tested, thus providing a unique and reproducible biological pattern [15-17]. This pattern can be compared with known patterns of the NCI60 using an algorithm developed by the NCI as part of the COMPARE computer program [18]. With this algorithm, an investigator can formulate an educated hypothesis regarding the substance's mechanism of action, or, in the absence of a similar pattern in the existing database, suggest that the substance's mechanism of action is distinctive from those previously described [19-21]. During the course of NCI60-based testing, synthetic compounds or natural substances are selected for their ability to inhibit growth or promote cell death *in vitro*. At the first stage of screening, three highly sensitive human cell lines are utilized: MCF-7 (human

breast cancer), NCI H460 (human lung cancer) and SF 268 (human glioblastoma). The substance to be tested is added to the respective cultures at standardized concentrations and incubated for 48 hours. If the substance of interest inhibits growth of at least one cell line, it advances to the next phase of testing where it is subjected to the full panel comprised of all 60 cell lines; at which point the substance is tested at five different concentrations. Ultimately, the substance of interest is moved to *in vivo* screening if the results of the full 60 cell lines panel indicate that it is able to: 1) promote cell death in at least one cell line and 2) has a unique mechanism of action or 3) can inhibit cell growth at a very low concentration. For convenience, all cell lines used for testing are pre-grouped according to the types of tumors from which they were derived. Therefore, instead of analyzing responses in 60 distinct cell lines, studies can be organized into nine categories of cancer: leukemia, melanoma, tumors of the central nervous system (CNS), lung, colon, ovarian, breast, kidney and prostate. Such a system allows for the rapid screening of a compound's potential anticancer capacity within the context of a particular class of cancer. Consequently, the study is not necessarily compound oriented, but instead disease oriented, in regard to the class of tumor to which the most promising therapeutic potential is observed [1]. While over 2500 compounds are subjected to NCI60 *in vitro* testing each year, only 2% prove to be effective enough to proceed to *in vivo* testing in mice. Furthermore, even though a compound may demonstrate high effectiveness during *in vitro* testing it often displays low performance when tested *in vivo* [22-23]. This may be explained, in part, by the limitations of the NCI60 screening system. This system does not account for such factors as tissue microenvironment, cell-cell communication, autocrine and paracrine regulations and the interaction of cells within the extracellular matrix [24].

Screening Systems: the Boyden Chamber: Various *in vitro* models have been developed to simulate the complexity of intercellular interactions. One such system is the Boyden chamber, which consists of two chambers separated by a semipermeable membrane (Figure 1a). With this system, cultured cells placed in one chamber are separated from a different set of cultured cells by semipermeable membrane. Seeded in the upper compartment, cells are able to grow and migrate under the influence of a concentration gradient of biologically active substances produced by cells in the lower compartment [25]. The number of cells migrating from one compartment, through the membrane, into the other compartment is proportional to the effectiveness of the biologically active substance. Cell migration is quantified by measuring the optical density of labeled cell extracts [26-27]. The Boyden chamber membrane may contain an endothelial cell monolayer or it may be coated with a Matrigel (intracellular matrix extract) [28]. The Boyden chamber assay is easily reproduced and cell transmigration can be observed in as little as 4-6 hours [27]. However, despite its simplicity of implementation, its principal drawback is the absence of direct cell-cell interaction. This limits the ability of Boyden chamber-based models to fully simulate an *in vivo* tumor.

Microfluidic Devices: Microfluidics exhibit great potential when studying the migration of tumor cells upon exposure to a test compound. This method allows one to manipulate liquids and particles in extremely low volumes inside microscopic channels. The fluid-flow is laminar, allowing precise dosing of the chemical compound. Additionally, under laminar-flow conditions, the solute transport is dominated by local diffusion; therefore, drugs can be delivered in spatial or temporal fashion. The system consists of a chamber with two separate inlets allowing for two concentrations of chemical compound to flow into a network of microchannels, where they mix to form a stair-shaped chemical gradient (Figure 1b). Target cells seeded in growth medium or in matrix (e.g., Matrigel) are placed into the cell inlet. These cells are exposed to a concentration gradient of the chemical compound, as it flows through the chamber. Microfluidic systems minimize cell and test-agent consumption; however, they require daily growth medium change thus increasing labor expenses. Conversely, lab-on-a-chip array based microfluidic devices are currently available for simple single-cell analysis. These devices provide a microenvironment allowing 3-dimensional topographic interactions of the target cell and require only nanomoles of reagents and culture media. Additionally, microfluidic devices provide isolation of specific single cells from a complex matrices, controlled placement of target cells and precise delivery of chemical compounds [29].

Multicellular Tumor Spheroids (MCTS): In contrast to two-dimensional single-cell monolayer cultures, three-dimensional models allow cells to grow within the confines of a three-dimensional environment of supporting matrix or other biomaterial that provides a structural framework. This method allows tumor cells to expand from a monolayer into a multilayer culture, thus

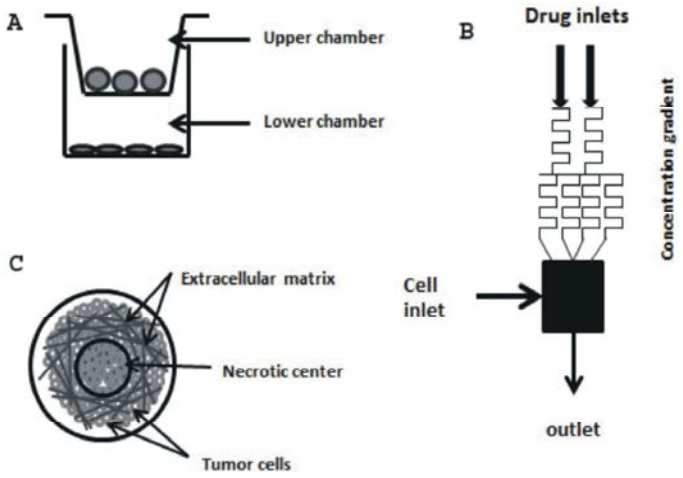

Fig. 1: *In vitro* systems for modeling human microenvironment

A. Boyden chamber
B. Microfluidic devices
C. Multicellular tumor spheroids (MCTS)

simulating the growth of an actual tumor [30]. Within this model, drug transportation to the target cells may encounter numerous impediments, similar to the physiological interactions affecting drug delivery *in vivo* [24]. The multicellular spheroid model is the most widely utilized three-dimensional screening system in that they display many features common to real tumors when studying proliferation, differentiation and metabolism and they correspond nicely with clinical findings [31]. Spheroids are self-assembled three-dimensional colonies of spherical clusters with a tissue-like architecture [32]. Cells within the spheroid become more similar to natural tumor cells, as compared to the same cells grown as a monolayer culture and more closely resemble their *in vivo* morphology [32-35]. Large spheroids (>500 microns) can be divided into three cell zones (Figure 1c): a central zone of dead cells surrounded by inner layer of living resting cells with an outer layer of actively proliferating cells [36]. Solid tumors with poor vascularization tend to display similar morphology. This tumor-structure is likely a consequence of the limited rate of nutrient and oxygen diffusion into cells of the central zone. Likewise, removal of the metabolic byproducts is restricted, creating a toxic environment for cells in the central portion of the tumor [37]. Accordingly, spheroids mimic solid tumor structures; and therefore, they make excellent models for solid tumors characterized by poor vascularization. Spheroid-like tumors form an extracellular matrix [38], as well as develop cell-cell and cell-matrix interactions [39]. Consequently, the cells in a spheroid differentiate in a similar manner to that of an *in vivo* tumor and maintain their state of differentiation for an extended period of time; often up to several weeks [40].

Not surprisingly, the expression of genes associated with cell migration and invasion is often different in spheroids when compared to that of a monolayer culture. For instance, Takagi et al. reported that gene expression patterns of the human prostate tumor cell line LNCaP were dramatically different when grown as spheroids in comparison to growth as a monolayer [32]. They additionally reported a 2 to 3-fold increase in VEGF expression in spheroids over that of monolayer cultures and also an up-regulation of genes involved in angiogenesis, immune responses and hypoxia in spheroids and a down regulation of genes involved in cell division and DNA repair [32]. Growth properties of cells in spheroids are also different in comparisons to monolayer cultures. Cells grown in spheroids tend to divide more slowly when compared to the same cells grown in a two-dimensional monolayer [33, 41] and cell survival is increased due to lower metabolic ATP and oxygen consumption when compared to that of a monolayer culture [37]. Also, the properties of *in vivo* tumors with respect to their ability to maintain changes in sensitivity and resistance to many anticancer drugs are more similar to those observed in spheroid models when compared to monolayer culture [36, 42-43]. It has been proposed that the mechanisms responsible for drug resistance may include increased diffusion distances and tightened cell-cell interactions limiting blood supply.

These physical barriers can result in an acidic and hypoxic environment, which has been shown to correlate with resistance to radiation and anticancer drug therapy [24]. Finally, similar to *in vivo* solid tumors and their metastases, spheroid growth can be mathematical modeled using equations that describe a natural tumor's growth and metastatic pattern [44].

Traditional culturing techniques for producing MCTS include growth on non-adherent surfaces; in suspension using bioreactors; and using the so-called "hanging drop" method [36, 45]. While these methods tend to be low-yield and result in spheroids that are heterogeneous in size and composition, recent improvements in the automated generation of spheroids have been developed that facilitate large-scale production of MCTS with uniform size and composition [31, 43]. For instance, microfluidic systems with precise geometric constraint allow one to create consistently sized spheroids in large amounts [24]; nevertheless, many obstacles continue to limit the utility of MCTS. For example, data collected using MCTS requires high throughput analysis techniques including automated imaging and analysis systems [43]. Additionally, the inability to track changes in real-time within the three-dimensional structure further limits the utility of MCTS. In an effort to resolve this limitation, the employment of light sheet-based fluorescence microscopy allows one to follow changes in the cell composition within MCTS [46]. A modified system of this, referred to as the "sandwich system" is often utilized which allows the microscopic observation of cells within the core of the spheroid. In this system, tumor cells are placed in a narrow space between two glass slides. Over time, a core of necrotic cells forms with two external layers of living cells resembling a cross-sectional slice of a spheroid. A principal advantage of the sandwich system is that all cells can be easily examined under the microscope, in contrast to a spheroid, where cells must be fixed and sectioned prior to visualization. In addition, by removing the upper glass of the sandwich, any layer can be isolated from the rest of the structure and used for further analysis by multiple methods [47].

Although there are many advantages in working with MTCS, spheroids grown in culture are not exposed to the immune system and they do not undergo angiogenesis; therefore, the MCTS models do not fully represent *in vivo* tissue [24, 39]. Interactions with the immune system and the adjacent healthy tissue significantly affect tumor development and the tumor's response to anticancer therapy [48-49]. For example, Olumi et al., as well as others have reported that healthy stoma cells adjacent to the tumor cells acquire genetic mutations making these cells resistant to anticancer drugs [49-50].

Three-Dimensional Cultures Grown on Matrix: The extracellular matrix (EM) provides the structural support for cells in tissue and, consequently, is an important component of the cellular microenvironment. Due to the differences in the nature and composition of the EM, many important functions including the regulation of cell-cell interactions can be investigated [51]. Consequently, incorporating EM when developing *in vitro* culture systems permits a more accurate simulation of the cellular microenvironment. A number of synthetic hydrogels have been developed using components of natural EM. They provide an environment of high water content and a framework of fibers and other structural proteins such as collagen, fibrin, laminin and hyaluronic acid. In addition, natural hydrogels, such as chitosan and alginate, can be obtained from various convenient biological sources. Another example of natural EM includes Matrigel (BD Biosciences) which is a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma [52]. In addition to the convenience such hydrogels provide, they also have the added advantage of being both biologically compatible and bioactive [53]. However, the complex nature of EM [51] limits its *in vitro* application due to the difficulty of standardizing and interpreting the biological effects of its individual components. Accordingly, synthetic EM are becoming an attractive alternative. The synthetic extracellular matrices are composed of polyethylene or polyvinyl alcohols and do not contain uncharacterized growth factors nor other contaminants. These synthetic alternatives are valuable three-dimensional *in vitro* models with many advantages in the screening of compounds with potential anticancer activity. Prestwich *et al*., was well as others, have shown that data collected using synthetic EM correlates well with results obtained using *in vivo* model [53-55]. The composition and density of a synthetic three-dimensional matrix system can be experimentally controlled, thus allowing a more accurate mimetic of the natural microenvironment. Also, the ability to modify a synthetic EM provides a valuable tool when studying cells that differ in migration pattern, morphology, function and localization within the body. However, it should be noted that synthetic EMs lack signaling molecules that are normally secreted by natural EM and these limitations must be taken into consideration [41]. Hybrid matrices

represent viable alternatives to synthetic ones, since they are composed of a synthetic scaffold containing peptides and proteins found in the natural EM. Often hybrid matrices include peptides containing the RGD (Arg-Gly-Asp) motif found in fibronectin, vitronectin and fibrinogen [56]. Their presence promotes cell-matrix cross-linking by binding to cellular integrin receptors [41, 56]. The use of these matrices, to form such three-dimensional structures, can facilitate investigations into the effect of various anticancer drugs on cell migration, invasion, metastasis and angiogenesis. The tumor microenvironment can be modeled using heterologous three-dimensional cultures, composed of tumor cells, stromal fibroblasts, osteocytes, neurons, macrophages, epithelial and endothelial cells [41-42, 57]. For cocultivation of cells, an EM scaffolds or spheroids can be used [45, 57]. Such cocultivation systems are useful to investigate the interactions between tumor and normal cells in the microenvironment. Additionally, heterologous cultures are advantageous when studying the influence of anticancer drugs on invasion and metastasis because they better preserve cell-cell and cell-matrix interactions [36, 40].

Multicellular Layers: Multicellular layers are a type of three-dimensional culture developed to directly measure drug diffusion in the tumor microenvironment [58]. In this model, cells are grown on a microporous polytetrafluoroethylene (PTFE) or Teflon membrane coated with collagen. The membrane is immersed in a large volume of growth medium with continued agitation. This leads to the development of a symmetric multicellular layer with a core of necrotic cells surrounded by living cells. Such multicellular layers can be developed for many cell lines, especially for those that are not amenable to spheroid formation [24]. An important advantage of this culture system over other models of three-dimensional structure is its utility for directly measuring diffusion or transport of drugs through a multicellular layer. To this end, the test substance is added to one side of the membrane and the kinetics of its migration is determined by measuring its appearance on the other side, using analytical methods such as high performance liquid chromatography [58]. This characteristic makes multicellular layers excellent tools for studying extravascular transport of anticancer agents such as low molecular weight drugs, pro-drugs and macromolecular agents [24].

Cocluture systems represent additional *in vitro* models that mimics cell-cell interactions in anticancer drug screening. Previously, we have demonstrated the feasibility of cell culture system whereby we cocultured human mesenchymal stem cells (MSCs) and neuro-blastoma cancer SH-SY5Y cells on differentially coated surfaces [59]. Each cancer and stem cell populations during the course of the coculture were monitored in real-time using differential live fluorescent membrane labeling. Our findings support the evidence that cancer and stem cell interactions play important roles in resistance of cancer cells to chemotherapeutics agents and additionally, demonstrate the benefit of such coculture systems.

Histological Techniques: Organotypic models, which utilize tissue explants, are a widely used method for simulating the natural tumor microenvironment. In these systems, a viable biopsy of a subject's tumor tissue is excised, sectioned on a vibratome and cultured *in vitro* for several days. This method allows one to retain the core architecture of the tissue and the individual variability of the cancer [46]. Tissue explants are used in almost all areas of biomedical research; however, as with other areas of medical research utilizing viable human tissue, many obstacles and limitations exist such as the difficulty in obtaining samples, reproducibility, scalability and ethical restrictions [36, 50].

To study tumor cells invasion in mammalian brain an *in vitro* model has been developed, which combines the Boyden chamber and the organotypic model using sections of brain tissue [60-61]. Conventional filters used in the Boyden chamber, such as Matrigel-coated filters, fail to provide the necessary signals for tumor cell invasion. These signals are generated by direct contact of tumor cells with normal brain cells. Therefore, in order to overcome these obstacles, the brain tissue itself is used as filters in Boyden chamber. This system provides a unique opportunity to study the mechanism of tumor cell migration and to test the ability of anticancer drugs to act on cell invasion in brain [60]. However, this model also has limitations including the acquisition of the tissue explants from patients, as well as the difficulties encountered when standardizing preparation of organotypic cultures. An alternative model to this system is the organotypic coculture, in which myleniated axons of a chicken embryo are cultured with tumor cells. Glioma cells are placed near the explants (two components of system are not in contact) and the degree of the tumor cell invasion is measured [62]. The two-dimensional organization of this system allows an *in vitro* real-time observation of living cell behavior using various imaging techniques. Again, as with other organotypic cultures, the problem of standardization still exists. Any system based

on organotypic cultures contains many different cell types and although such heterogeneity can accurately represent the cellular microenvironment, monitoring and analyzing the behavior of individual cell subpopulations remains a challenge.

CONCLUSION

Scientists have long searched for effective tools for the *in vitro* screening of anticancer agents. Initially, potential agents were tested only using tumor cell lines. However, three-dimensional culture models with a structural organization that is considerably more similar to that of an *in vivo* tumor microenvironment have become the systems of choice. To date, a number of additional screening systems have been developed, but none provide sufficient correlation between data collected *in vitro* and *in vivo*. Although each system has its advantages, they all possess a number of limitations; hence, using only one method to test an anticancer agent will provide an incomplete view of the agent's potential anticancer properties. To obtain reliable and comprehensive results, it is prudent to utilize various techniques. Future advances in tissue engineering combined with existing systems will likely improve the efficacy when screening novel anticancer agents. Clearly, for an accurate interpretation of biological effects there should be a compromise between the complexity of the system and the researcher's ability to analyze complex interactions among the various cellular components.

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