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Experimental anti-tumor therapy in 3-D: Spheroids – old hat or new challenge?

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Abstract

Purpose: To give a state-of-the-art overview on the promise of three-dimensional (3-D) culture systems for anticancer drug development, with particular emphasis on multicellular tumor spheroids (MCTS).

Results and conclusions: Cell-based assays have become an integral component in many stages of routine anti-tumor drug testing. However, they are almost always based on homogenous monolayer or suspension cultures and thus represent a rather artificial cellular environment. 3-D cultures – such as the well established spheroid culture system – better reflect the *in vivo* behavior of cells in tumor tissues and are increasingly recognized as valuable advanced tools for evaluating the efficacy of therapeutic intervention. The present article summarizes past and current applications and particularly discusses technological challenges, required improvements and recent progress with the use of the spheroid model in experimental therapeutics, as a basis for sophisticated drug/therapy screening. A brief overview is given focusing on the nomenclature of spherical 3-D cultures, their potential to mimic many aspects of the pathophysiological situation in tumors, and currently available protocols for culturing and analysis. A list of spheroid-forming epithelial cancer cell lines of different origin is provided and the recent trend to use spheroids for testing combination treatment strategies is highlighted. Finally, various spheroid co-culture approaches are presented that have been established to study heterologous cell interactions in solid tumors and thereby are able to reflect the cellular tumor environment with increasing accuracy. The intriguing observation that in order to retain certain tumor initiating cell properties, some primary tumor cell populations must be maintained exclusively in 3-D culture is mentioned, adding a new but fascinating challenge for future therapeutic campaigns.

Keywords: *3-D culture, multicellular tumor spheroids, anti-tumor therapy*

Introduction

Establishment and adaptation of cell-based assays which rapidly detect the most active drug candidates from large pools of potential effectors has become an integral component in many stages of anti-tumor drug development and testing. However, most cell-based assays apply monolayer or suspension cultures which present a largely artificial cellular environment with limited relevance for *in vivo* studies, and often with little value in predicting clinical efficacy of both generally cytotoxic as well as molecularly targeted drugs. Three-dimensional (3-D) cultures are gaining increased recognition as important additions to the strategic toolkit for evaluating the efficacy of therapeutic intervention. In cancer research and treatment in particular, they are becoming more and more accepted as essential research tools which ideally complement the techniques already available.

Examples of 3-D cell models employed in basic and applied tumor biology include multilayer cell systems cultured on top of porous membranes, used for example to study drug transport and binding, therapy resistance and cell invasion (Hicks et al. 1997, Padron et al. 2000). Matrix-embedded cultures of single cells or cell aggregates using various artificial and natural extracellular matrix compounds have proven helpful in investigating tumor cell-matrix interactions, tumor cell differentiation, migration and invasion processes (Casciari et al. 1994, Muralidhar et al. 2001, Debnath & Brugge 2005, Lee et al. 2007). Hollowfiber bioreactors with cells cultured within a network of perfused artificial capillaries to analyze cell metabolism and therapy resistance (Gillies et al. 1993, Legallais et al. 2001,

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Dulong et al. 2002) and more complex systems such as *ex vivo* cultures of pieces or slices from different tissues or tumors excised and cultured *in vitro* (Berglund et al. 2002, Ochs et al. 2003) have also been employed. All of these 3-D *in vitro* culture systems are of intermediate complexity, mimicking different aspects of the human tumor tissue environment, and should thus be considered as advanced models for routine anti-tumor drug testing. One of the culture systems that attracted major attention throughout the past 10 years is the model of Bissell and co-workers, who established a laminin-rich ECM 'embedded' assay, in which breast epithelial cells form either polarized, growth-arrested acinus-like or disorganized, proliferative and non-polar colonies, depending on their non-malignant or malignant status. This model system, its procedures and alternative protocols have previously been reviewed in detail (Gudjonsson et al. 2003, Nelson & Bissell 2005, Lee et al. 2007). There is also increasing effort to engineer advanced culture devices to improve and miniaturize such systems for screening purposes. Here, microfluidic devices are one of the most promising approaches (e.g., Bhadriraju & Chen 2002, Walker & Beebe 2002, Chaw et al. 2007, Kim et al. 2007, Warrick et al. 2007). For example, a recently developed tubeless microfluidics systems that can be operated by standard liquid handling automation has been successfully combined with the lrECM breast tissue architecture model (e.g., Hayes, Skoien et al, 13th Annual Conference of the Society for Biomolecular Sciences, 2007, Poster/Abstract PSTZ1H002). Fully recapitulating acinar morphogenesis, this combined platform has obvious potential as an attractive model for high-throughput screening in the near future.

The present article, however, emphasizes the potential and recent progress with the 'classical' 3-D cell system in cancer research: the multicellular spheroid model (MCS). Multicellular tumor spheroids (MCTS) reflect some pathophysiological phenomena in tumor tissues that critically affect therapeutic efficacy. They have frequently been applied to anti-tumor therapy testing throughout the past four decades, e.g., to study and/or identify mechanisms of radio- or drug resistance, as reviewed in a number of comprehensive articles and article series with different focus. But most importantly, the spheroid model is suitable to be incorporated readily into mainstream and routine drug testing operations, as suggested earlier (Mueller-Klieser 2000, Kunz-Schughart et al. 2004). In this article, the power of this *in vitro* model system for sophisticated drug testing is explained, and technical basics and recent advances with the spheroid model are briefly discussed. Spheroid-based approaches to drug screening under conditions that reflect tumor tissue conditions with increasing accuracy are highlighted in the following chapters.

Aggregates, spheres, spheroids, organoids, tumoroids: Spherical 3-D cultures reflecting tumor pathophysiology

Numerous review and book articles have summarized the history of the spheroid model, starting with its introduction in cancer research by radiobiologists in the early 1970s (Sutherland et al. 1970, 1971a, 1971b). Since then, the classical system of Sutherland and co-workers of spherical multicellular cultures of animal cell lines has been extended, e.g., by the use of a variety of human cell lines and has also been modified in other ways as detailed later. Unfortunately, much of the corresponding literature is not easily identified via standard reference searching because of varying nomenclature used by different authors. Tumoroids (Vamvakidou et al. 2007), for example, are no more or less than mixed spheroid cultures (Djordjevic & Lange 1990, Lange et al. 1992, Djordjevic et al. 1993, Kunz-Schughart 1999, Djordjevic & Lange 2004, 2006) or so-called mosaic spheroids (Boyd et al. 2002, 2004) consisting of two or more types of cells. Other synonyms describing 3-D cultures with spherical structure include aggregate, sphere or organoid. Aggregates may be more or less spherical but in most cases, the term is exclusively used to describe a loose package of cells and not the formation of compact spherical cultures (Ivascu & Kubbies 2006). On the other hand, some so-called spheroids in the literature are no more than spherical loose aggregates, may easily fall apart if advanced analytical processing is required, and may not express all pathophysiological characteristics known to be present in compact multicellular tumor spheroids. And last but not least, the term sphere is frequently used in biochemical papers, sometimes for real spheroids and sometimes for crude aggregates as well as in the field of stem/progenitor cell research which also includes the search for and study of so-called tumor stem cell populations (Ricci-Vitiani et al. 2007).

These inconsistencies have to be kept in mind when setting up spheroid-based therapeutic test systems. Here, the three-dimensional structure is mandatory to achieve the system's advantage of mimicking avascular tumor nodules or microregions of solid tumors with respect to growth kinetics, micromilieu and restoration of *in vivo* morphological and functional features of tumor cells as depicted in the cartoon of Figure 1. Cells located in the spheroid periphery reflect the situation of actively cycling tumor cells adjacent to capillaries *in vivo*. In contrast, innermost cells become quiescent and eventually die via apoptosis or necrosis to form a secondary necrotic core. The concentric arrangement of

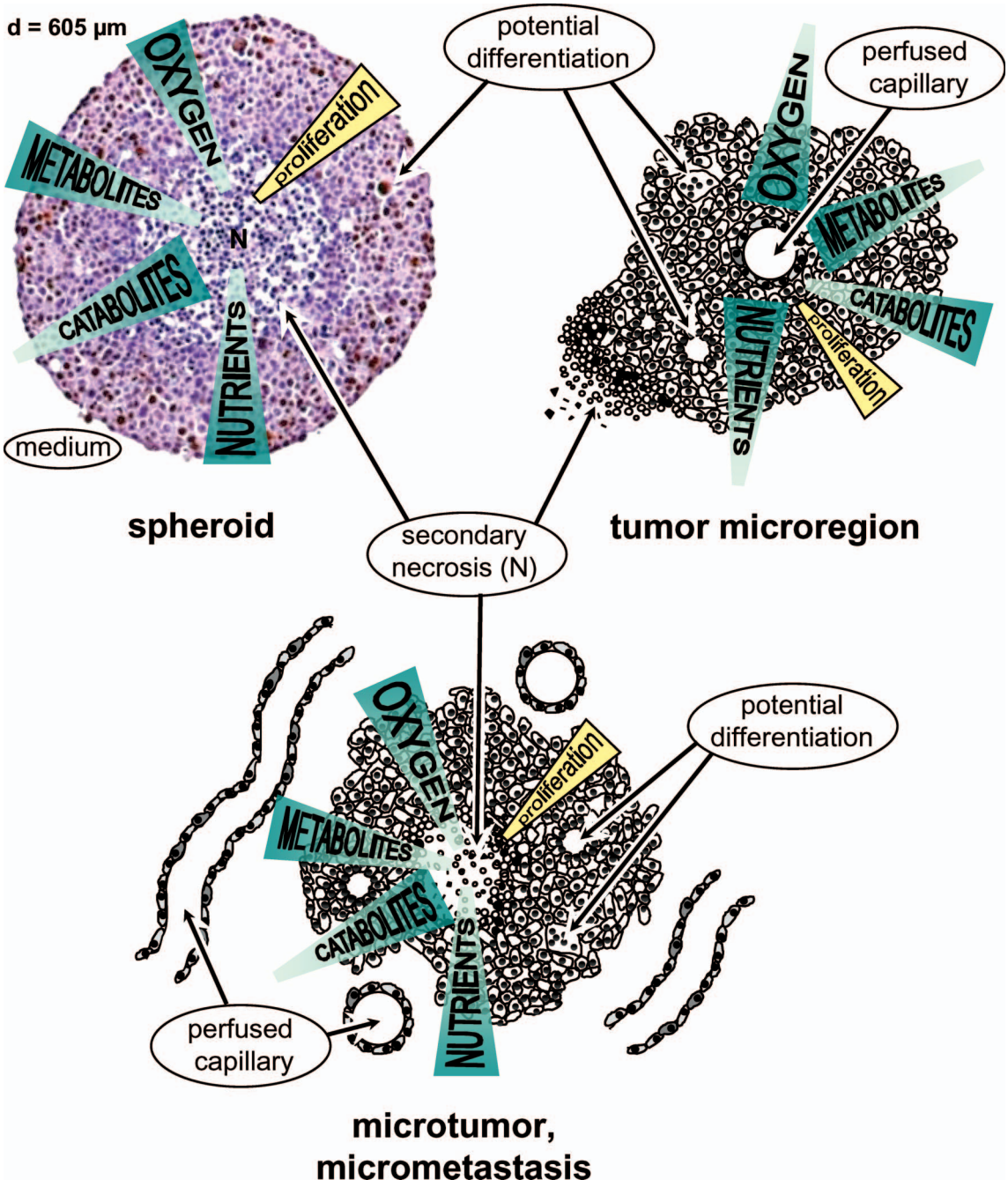

Figure 1. Multicellular tumor spheroids (upper panel left; d = diameter) display growth characteristics and numerous pathophysiological features of avascular tumor nodules and/or developing micrometastases (lower panel) and also inside out resemble the situation from the capillary towards the periphery in tumor microregions (upper panel right) (modified from Kunz-Schughart 1999).

heterogeneous cell populations in spheroids and the establishment of pathophysiological gradients is comparable to the situation in micometastases, avascular tumor microregions or inter-capillary tumor regions as detailed earlier (Mueller-Klieser 1987, Sutherland 1988). Beyond a critical size of 500 – 600 μm, central secondary necrosis develops in most but not all spheroid types from permanent cell lines. Typically surrounded by a viable rim of cells of about 100 – 300 μm, these necroses mainly relate to the limited inward and outward diffusion of positive and negative regulators which cause a lack of oxygen and/or nutrients, accumulation of waste products and low pH (Acker et al. 1987, Carlsson & Acker 1988). Studies on steady state-levels of glucose, lactate, and energy-rich phosphates as well as mitochondrial activity in spheroids indicate an adaptation of metabolism of inner spheroid regions to environmental stress, maintaining intracellular homeostasis until shortly before cell death

(Freyer et al. 1990, 1991, Walenta et al. 1990, Bredel-Geissler et al. 1992, Teutsch et al. 1995). However, it has also become evident that tumor cells can critically differ in their sensitivity to this pathophysiological stress situation, as should be expected from their highly variable capacity for energy production under both aerobic and anaerobic conditions. Multicellular tumor spheroids (MCTS) may also contain an extensive extracellular matrix (ECM) and possess a complex three-dimensional network of cell-to-matrix and cell-to-cell interactions. This not only affects the distribution and function of biological response modifiers such as hormones and growth factors but is also relevant to the penetration and action of drugs.

Technology in brief

Two major aspects in spheroid technology are relevant for integration of this model system in routine drug testing: Easy and rapid handling of both culturing and analysis. Various techniques for spheroid culturing have been described earlier and are used in routine animal cell culturing (Mueller-Klieser 1987, Lund-Johansen et al. 1992, Kunz-Schughart et al. 2000). Figure 2 details the most frequently applied methods both for the generation of large amounts of spheroids or spheres and for single spheroid monitoring. In fact, culture technology has not much changed over the past twenty years, with spinner flask cultures remaining one of the most attractive and robust approaches to obtain a large pool of spheroids of particular size-ranges under optimum, stirred nutrient supply conditions. Here, spherical aggregates of tumor cells form spontaneously in the absence of attachment to another substrate directly in the flask. Alternatively, spheroids can be obtained during an initiation interval in non-adherent, e.g. bacteriological or agar-coated, so called 'mother dishes' from which they are selectively transferred into the flasks. Comparable approaches to generate large amounts of spheroids and to avoid effects caused by stagnant medium are roller tubes or roller bottles and the gyratory shaker. An interesting newly developed technology with similar potential is the rotating wall vessel ('NASA bioreactor') that provides even more constant culture conditions due to continuous medium perfusion and nutrient flow rate. This rotary cell culture system was designed to mimic microgravity and maintain cells in suspension under very low shear stress (Ingram et al. 1997) and has been employed to also grow tumor spheroids of different complexity (e.g., Clejan et al. 2001, Song et al. 2004, Vamvakidou et al. 2007). However, while being recommended for some normal and primary cell culturing under optimized culture conditions, this technology does not provide major advantages for drug testing over the spinner flask technique due to the more cumbersome system set-up. Furthermore, the system is significantly more expensive than a spinner flask, while any advantages other than reduced sheer force have yet to be demonstrated.

One has to keep in mind that all rotating culture systems require relatively high quantities of media, conflicting with the usual demand for minimum amounts of new drug candidates to be applied in a drug test system. Therefore, it may indeed be required to use stationary spheroid culture systems at least for drug exposure. Stationary culturing technologies include the growth of spheroid cultures in 96-well plates which allows monitoring and manipulation of single spheroids, since the inoculation of aggregating tumor cells into agar-coated 96-well plates results in the formation of one single spheroid per well. The well-to-well variation in spheroid diameter after a defined initiation period is less than 5% for most spheroid types and remains extraoridinarily small throughout spheroid growth if 50% of the supernatant is renewed at least every other day. More frequent medium renewals can improve the supply conditions. High reproducibility in spheroid formation, the potential for analyzing individual spheroids before and after treatment, and the limited amount of drug required qualifies the 96-well plate approach as a practical therapy test system However, one also has to realize that some of the limitations that are inevitable in high-throughput test settings may of course complicate interpretation of some drug study data. For example, early investigations have shown that the diffusion-limited supply under stationary conditions results in significant external nutrient gradients, and these change as a function of time as the spheroid grows. Also, supply of drugs to the spheroid may be more diffusion-limited than in rotary culture, depending on the concentration and cellular uptake rate. A further consideration specifically relevant for drug testing is that drug effects themselves (e.g., cytotoxicity) can alter the metabolic rates of treated spheroids versus controls, which may result in different external (and internal) nutrient environments.

In numerous previous studies, flat bottom wells coated with 1.5–3% agar/agarose in serum-free medium to form a non-adherent, concave surface have proven to be appropriate for liquid overlay spheroid culturing (Carlsson & Yuhas 1984), but modification such as the application of round bottom wells coated with 0.5% poly-HEMA (hydroxyethyl methacrylate) in 95% ethanol are also used (Tong et al. 1992, Hoevel et al. 2004, Ivascu & Kubbies 2006). Unfortunately, individual coating of plates is still required today, since current commercially available non-adherent 96-well plates do not

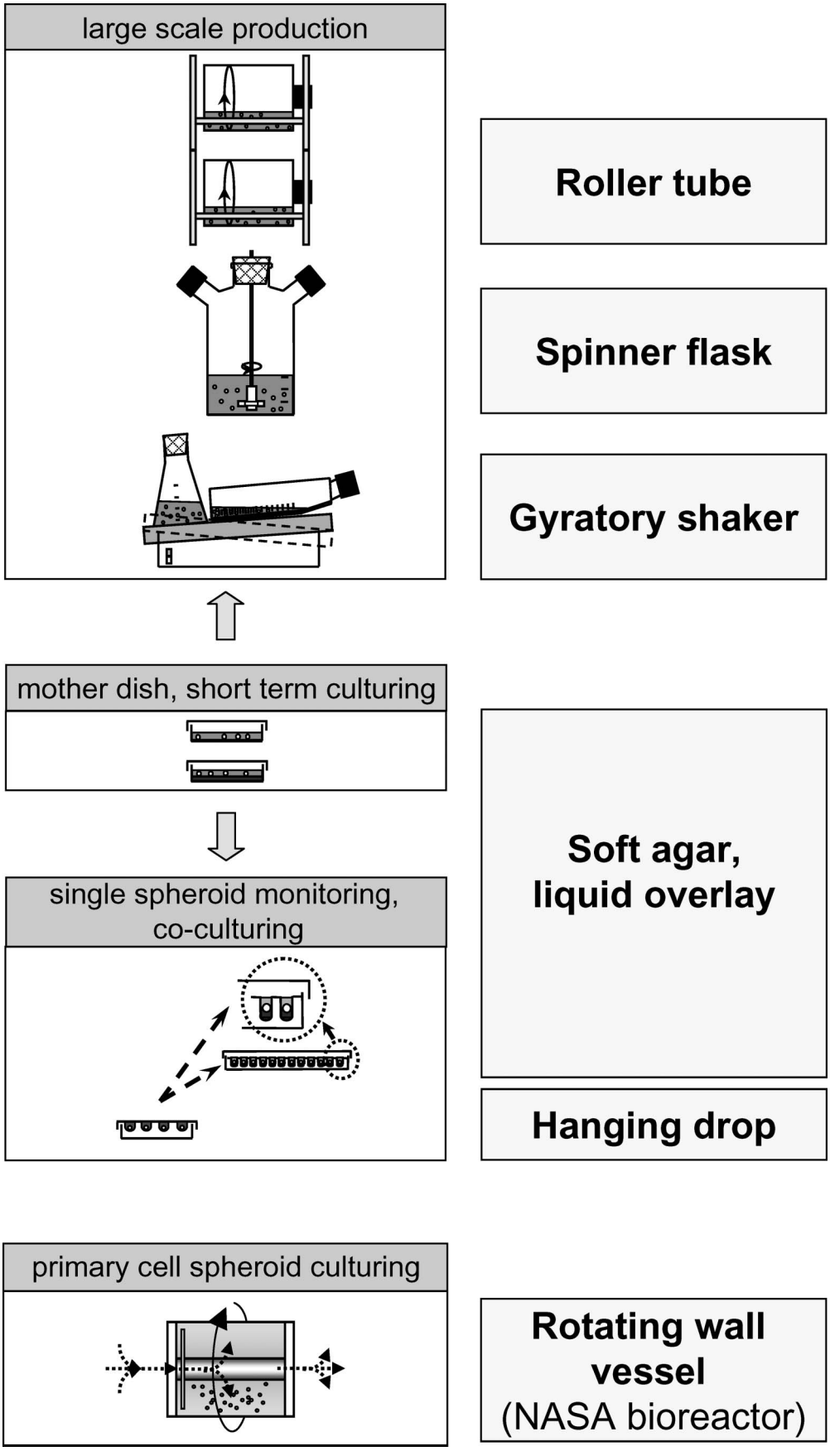

Figure 2. Techniques frequently applied to grow multicellular spheroids from cell lines or primary cells, spheroid co-cultures and fragment spheroids from tissue materials.

guarantee a reproducible formation of single spheroids per well. This drawback may also have resulted in the recent use of the hanging drop method for culturing tumor spheroids (Kelm et al. 2003, Del Duca et al. 2004), a methodology that was primarily established for culturing embryoid bodies. The technique, while avoiding the coating of plates for spheroid formation, is however only useful for short-term culturing, i.e., spheroid initiation, for as long as spheroids are not fed and/or treated.

A broad range of human tumor cell lines has been studied in spherical culture using the various culturing methods discussed. These include epithelial cell lines of different tumor entities but also brain tumors such as gliomas and glioblastomas, melanomas and sarcomas. We have been particularly interested in epithelial tumors and thus provide an overview of human carcinoma cell lines that have been shown to form spheroids in previous studies and that may be applicable for therapeutic intervention modeling (Table I). The list also contains information as to whether or not the respective cell line is included in the 60-cell line screen of the US National Cancer Institute Developmental Therapeutics Program (NCI-DTP; http://dtp.nci.nih.gov/branches/btb/ivclsp.html) which was implemented about 20 years ago. The NCI60 cell line-screen has been found to be of some value as predictor of xenograft and/or clinical drug activity (Newell 2001, Johnson et al. 2001, Monga & Sausville 2002) and has become a gold standard in drug screening (Newell 2001, Johnson et al. 2001). In the meantime, many drugs and drug candidates have been tested in these cell lines and a comprehensive database is freely available, e.g., providing parameters such as IC_{50} values in monolayer cultures. Nevertheless, tremendous controversy remains in this area with several reports stressing the limited predictive value of routine *in vitro* and *in vivo* drug screening models for clinical efficacy (Sausville 2000, Pelkonen et al. 2001, Balis 2002, Kerbel 2003, Voskoglou-Nomikos et al. 2003). Because 3-D culture systems such as spheroids better reflect the pathophysiology of human tumors, this debate lends further arguments in favor of the application of 3-D culture systems for evaluating therapeutic efficacy. Because a screening of the referenced literature shows that not all spherical cultures are compact spheroids that can easily be applied for drug testing as discussed earlier, we want to highlight a recent article from Ivascu and Kubbies (2006), who described the rapid generation of single tumor spheroids for high throughput cell function and toxicity analysis by the addition of extracellular matrix compounds during spheroid initiation. In their study, 2.5% reconstituted basement membrane was superior to fibronectin, laminin, collagen type IV, collagen type I, heparan sulphate proteoglycane, chondroitin sulphate, and diverse mixtures. Despite the fact that the addition of ECM per se may alter the expression profile and function of tumor cells, potentially affecting the sensitivity to therapeutic intervention, this is one of the few options to grow non-adherent tumor cells in spheroid culture. As an alternative, the application of methylcellulose according to the endothelial cell spheroid model introduced by Korff and Augustin (1998) may be considered.

MC(T)S and experimental therapeutics

Despite tumor cell type dependent variations in spheroid growth and histomorphology, several physiological properties determined by the spherical geometry within MCTS as described above are general in nature. Therefore, MCTS have become a powerful tool to study therapeutic problems associated with metabolic and proliferative gradients, in particular the responsiveness and effects of chronically hypoxic tumor cells and 3-D cell-cell and cell-matrix interactions, e.g., in radio- and chemo-resistance. Needless to say, studies related to radiotherapy dominated the field of spheroid work in experimental therapy for its first 20 years, mainly as a consequence of their earlier implementation in radiobiological cancer research. This work has been reviewed in diverse dated but still informative articles which also highlight and discuss the phenomenon of hypoxia and cell-cell contact related radioresistance (Carlsson & Nederman 1989, Freyer 1992, Buchsbaum et al. 1993, Olive & Durand 1994, Mueller-Klieser 1997, Santini & Rainaldi 1999). In the 1990s, the number of scientific reports on this topic decreased, although throughout several groups remained active in this area. During the same time, the spheroid technology increasingly spread into other fields of therapeutic research ranging from chemotherapy, radioimmunotherapy, cell- and antibody-based immunotherapy, hyperthermia, gene therapy and photodynamic treatment (Olive & Durand 1994, Mueller-Klieser 1997, Desoize et al. 1998, Mueller-Klieser 2000, Desoize & Jardillier 2000). The aspect of drug resistance was examined in particular detail by Olive and coworkers who have frequently used the comet assay to identify DNA-damaged cells (Durand & Olive 1992, Olive & Durand 1994).

Today, a number of classical therapeutics have been tested in spheroid culture, but the various spheroid types applied for drug exposure as well as the different analytical tools and endpoints may limit the potential for comparing individual, independent studies. Recently, Mellor et al. (2005) compared the effect of 5-fluorouracil (5-FU), vinblastine,

Table I. Overview of some epithelial tumor cell lines of different origin that form spherical cultures with representative reference list.

Entity	Cell line	NIH-DTP screen	Literature
Squamous cell carcinoma	1483	–	(Sturgis et al. 1994)
	A431	–	(Ng et al. 1987; Casciari et al. 1988; Kwok & Sutherland 1991)
	CaSki	–	(Casciari et al. 1988; Kwok & Sutherland 1991; Lohr et al. 1995)
	FaDu	–	(Stuschke et al. 1992)
	HEp-2	–	(Kohno et al. 1988)
	HN-1	–	(Schwachofer et al. 1989)
	MDA 686LN	–	(Sturgis et al. 1994)
	MDA 886Ln	–	(Sacks et al. 1989; Nishikawa et al. 1990; Sturgis et al. 1994)
	SQ5	–	(Nakayama 1991)
Lung/NSCLC	A549*	+	(Ivascu & Kubbies 2006)
	NCI-H125	–	(Schwachofer et al. 1991)
	NCI-H322M	+	(Ivascu & Kubbies 2006)
	NCI-H40*	+	(Ivascu & Kubbies 2006)
	SK-MES-1	–	(Audran et al. 1994)
SCLC	DMS53	–	(Cate et al. 1984; Douple et al. 1985)
Colon	COLO 205*	+	(Ivascu & Kubbies 2006)
	DLD-1	+	(Nicholson et al. 1997; Mellor et al. 2005)
	HCT-15*	+	(Ivascu & Kubbies 2006)
	HCT-116	+	Information by word of mouth
	HT29	+	(Lees et al. 1981; Sutton et al. 1982; Sutherland et al. 1986; Simiantonaki et al. 2002; Lowthers et al. 2003)
	LoVo	–	(Soranzo et al. 1986; Soranzo & Ingrosso 1988; Pervez et al. 1989; Dolfini et al. 2005)
	LS174T	–	(Horan et al. 1985; Langmuir et al. 1989)
	SW620*	+	(Ivascu & Kubbies 2006)
	WiDr[§]	–	(Chan et al. 1991; Banks et al. 1994; Jansen et al. 1994; Buffa et al. 2001; Mueller-Klieser et al. 2002)
Pancreas	MIAPaCa2	–	(McLeod et al. 1997)
	Panc1	–	(McLeod et al. 1997)
Liver/hepatocellular carcinoma	Hep3B	–	(Lin et al. 2006)
	HepG2*	–	(Gorlach et al. 1994; Selden et al. 2000; Kelm et al. 2003; Ivascu & Kubbies 2006; Lambert et al. 2006; Lin et al. 2006)
	Huh-7	–	(Tonary & Pezacki 2006)
Breast	BT20	–	(Muller-Holzner et al. 1996)
	BT474	–	(Kunz-Schughart et al. 2001; Seidl et al. 2002)
	MCF-7	+	(Pourreau-Schneider et al. 1984; Olea et al. 1992; Kunz-Schughart et al. 2001; Kelm et al. 2003; Ivascu & Kubbies 2006)
	MDA-MB-231	+	(Ivascu & Kubbies 2006; Muir et al. 2006)
	MDA-MB-361	–	(Hoevel et al. 2004; Ivascu & Kubbies 2006)
	MDA-MB-468	+	(Ivascu & Kubbies 2006)
	SK-BR-3	–	(Kunz-Schughart et al. 2001; Ivascu & Kubbies 2006)
	T47D	+	(Ronen et al. 1990; Kunz-Schughart et al. 2001; Kan et al. 2001; Seidl et al. 2002; Ivascu & Kubbies 2006)
	MDA-MB-435[†]	+	(Stuschke et al. 1995; Ivascu & Kubbies 2006)
	NCI/ADR^{RES}[‡]	+	(Walker et al. 2004; Ivascu & Kubbies 2006)
Ovary	IGR-OV1*	+	(Ivascu & Kubbies 2006)*
	MCAS	–	(Sonoda et al. 2003)
	OVCAR-3	+	(Bardies et al. 1992; Filippovich et al. 1996)
	OVCAR-5	+	(Casey et al. 2001)
	SKOV3*	+	(Makhija et al. 1999; Frankel et al. 2000; Casey et al. 2001; Ivascu & Kubbies 2006)*
Cervix	HeLa-S3	–	(Sommers & Alfieri 1998)
	SiHa	–	(Olive et al. 1996; Olive & Banath 1997; Durand & Sham 1998; Oloumi et al. 2000; Oloumi et al. 2002)
Kidney	786 – 0	+	(Lieubeau-Teillet et al. 1998)
	A498	+	(Heuser et al. 2003)
	(HEK)-293	–	(Talts et al. 2000; Ivascu & Kubbies 2006)
	UO-31	+	(Ivascu & Kubbies 2006)

(*continued*)

Table I. (*Continued*).

Entity	Cell line	NIH-DTP screen	Literature
Bladder	J82	–	(Lottner et al. 2004)
	MGH-U1	–	(Tannock & Kopelyan 1986; Kerr & Smart 1991; Erlichman & Wu 1992)
	MGH-U3	–	(LaRue et al. 1997; Kilani et al. 2003)
	RT-4	–	(Konur et al. 1998)
	RT-112	–	(Huygens et al. 2003; Kamuhabwa et al. 2003)
	T24	–	(Kawai et al. 2001)
	UROtsa	–	(Lottner et al. 2004)
Prostate	DU-145	+	(Essand et al. 1993; Frankel et al. 2000; Enmon, Jr. et al. 2002; Wartenberg et al. 2002; Wartenberg et al. 2003)
	LNCAP*	–	(Ballangrud et al. 1999; Ballangrud et al. 2001; Konduri et al. 2001; Enmon et al. 2003; Ivascu & Kubbies 2006)*
	PC-3*	+	(Donaldson et al. 1990; Hedlund et al. 1999; Enmon, Jr. et al. 2002; Ivascu & Kubbies 2006)*
Skin/melanoma	A375	–	(Cheng et al. 1991; Mills et al. 2002; Martiny-Baron et al. 2004)
	BEX-c	–	(Hystad & Rofstad 1994; Rofstad et al. 1996)
	BRO	–	(Schwachofer et al. 1990; Schwachofer et al. 1991)
	C32	–	(Yuhas et al. 1984)
	C918	–	(Braun et al. 2007)
	C8161	–	(Strasberg et al. 2004)
	CaCL 73–36	–	(McFadden & Kwok 1988; Kwok et al. 1988; Kwok et al. 1989)
	DC-10	–	(Feder-Mengus et al. 2007)
	HBL	–	(Feder-Mengus et al. 2007)
	HMV-I	–	(Kubota et al. 1984; Kuwashima et al. 1988; Kuwashima et al. 1993)
	Hs294	–	(Offner et al. 1996)
	IGRI	–	(Gorlach et al. 1994; Porwol et al. 1996)
	Mel108	–	(Gottfried et al. 2006)
	MelIm	–	(Gottfried et al. 2006)
	MeWo	–	(Offner et al. 1996; Jorgensen et al. 2005)
	NA8	–	(Ghosh et al. 2005; Feder-Mengus et al. 2007)
	OM467	–	(Parry et al. 1992)
	OM482	–	(Parry et al. 1992)
	SB-2	–	(Mills et al. 2002)
	SK-MEL-28	+	(Freyer 1988; Freyer & Schor 1989; Gorlach et al. 1994; Offner et al. 1996; LaRue et al. 2004)
	ST-ML-11	–	(Offner et al. 1996)
	ST-ML-12	–	(Offner et al. 1992; Offner et al. 1993; Offner et al. 1996; Simiantonaki et al. 2002)
	ST-ML-14	–	(Offner et al. 1996)
	ST-ML-25	–	(Offner et al. 1996)
	WIX-c	–	(Hystad & Rofstad 1994; Rofstad et al. 1996)
	WM9	–	(Francia et al. 2005)
	WM35	–	(Shellman et al. 2003; Francia et al. 2005; Jorgensen et al. 2005)
	WM45.1	–	(Jorgensen et al. 2005)
	WM115	–	(Fang et al. 2005; Monzani et al. 2007)
	WM164	–	(Rieber & Rieber 2006)
	WM239	–	(Francia et al. 2005; Jorgensen et al. 2005)
	WM902B	–	(Jorgensen et al. 2005)
	WM1341B	–	(Jorgensen et al. 2005)
	WM3517	–	(Fang et al. 2005)
	WM3523	–	(Fang et al. 2005)
	WM3539	–	(Fang et al. 2005)

*with reconstituted basement membrane rBM in Ivascu & Kubbies 2006.
§WiDr cells are reported to be a derivative of HT29 (Chen et al. 1987).
†MDA-MB-435 breast origin is questioned as cells express melanocyte-specific genes (Ellison et al. 2002; Rae et al. 2004).
‡NCI/ADR^{Res} cells were recently identified to be derived from the ovarian cell line OVCAR-8 (Garraway et al. 2005).

doxorubicin, and cisplatin in a proliferating and quiescent spheroid culture model of DLD-1 colon adenocarcinoma cells. Their analyses of drug efficacy included spheroid volume growth and cell survival as determined via a methylenblue staining procedure. A critical difference was documented not only between the various drugs but more importantly for the same drug in spheroids with distinct proliferation status

(Mellor et al. 2005). A similar study using HT-29 and HCT116 colon cancer cells has been performed by our group to demonstrate differences in drug efficacy and half-maximal inhibitory concentration (IC_{50}) values, respectively, of 5-FU, irinotecan and the new drug Symdax^{TM} in monolayer and spheroid culture with incubation intervals of 24 and 72 h (Friedrich et al., 2002). Orlandi et al. compared the effect of idarubicin and idarubicinol in MCF-7 breast cancer cell monolayers and MCTS, demonstrating significant activity in both culture systems with IC_{50} values (24 h exposure) that were only slightly higher in spheroids (Orlandi et al. 2005). In this study, evidence for drug resistance in spheroids seemed critically dependent on the time of exposure. It may be speculated that this finding is not due to cellular drug resistance but to an insufficient or delayed distribution of the drugs within spheroids which of course leads to a reduced efficacy. An attenuated resistance to doxorubicin in three-dimensional aggregates of the human breast carcinoma cells MDA-MB-231 was observed in a study by Muir et al. (2006) by administration of the nitric oxide mimetics diethylenetriamine/nitric oxide adduct (DETA/NO) and glyceryl trinitrate (GTN). Their data imply that the chemosensitization following NO mimetic exposure is not due to increased penetration of the drug nor associated with an increase in proliferative activity and suggest the involvement of a cyclic guanosine monophosphate (cGMP)-dependent signaling (Muir et al. 2006). It should, however, be noted that in other studies MDA-MB-231 have been described to only form loose aggregates and not compact spheroids (Ivascu & Kubbies 2006). Limitations in drug penetration are thus unlikely to occur, i.e., an effect on the penetration kinetics of a drug may not be visible. However, the study clearly reflects the present trend to use the spheroid model for testing combinatorial therapeutic strategies.

One field of interest is of course the combination of various drugs and/or radiosensitizers with single and fractionated irradiation regimens. Just briefly, supra-additive effects of cisplatin, gemcitabine or 5-Fluorouracil with irradiation were recently recorded in HepG2 hepatocellular carcinoma spheroids (Lambert et al. 2004). The same combinations were studied in glioma and glioblastoma cell line (GaMg, U87, Gli-6) and/or fragment spheroids from primary tissues, and the effect was compared with that observed in monolayer cultures (Genc et al. 2004, Fehlauer et al. 2005, Fehlauer et al. 2006). In the U87 glioma spheroid model the selective mTOR (mammalian target of rapamycin) inhibtor rapamycin and the cyclin-dependent kinase inhibitor roscovitine were shown to critically enhance the efficacy of fractionated irradiation. Interestingly, the additive effect of rapamycin was not present in two-dimensional (2-D) cultures (Eshleman et al. 2002). Radiosensitization and additive or supra-additive effects were also achieved with the xanthin derivate pentoxyfylline, studied in T98G glioma spheroids. Here, the largest impact on radiation response occurred if intermittent doses were applied (Eley et al. 2002). The Heat-shock protein Hsp-90 inhibitor 17-N-Allyl-amino-17-Demethoxy-Geldanamycin (17-AAG) was examined in LNCaP and CWR22Rv1 prostate carcinoma spheroids and showed radiosensitizing potential (Enmon et al. 2003). As a last example, the study of Khaitan et al. (2006) shall be mentioned, which compared radiomodification by 2-Deoxyglucose (2-DG) in monolayer cultures and spheroids of a human glioma cell line (BMG-1). 2-DG – also the base substance for the most frequently applied clinical radiotracer for positron emission tomography PET ([^{18}F]FDG) – enhanced the radiation-induced cell death of spheroids more drastically than that of monolayer cells. The study demonstrated that 2-DG uptake is enhanced in spheroid as opposed to monolayer cells of the BMG-1 glioma cell line. Accordingly, the radiosensitization effect leading, via different mechanisms, to transient G2 delay, apoptosis induction and late onset of DNA breaks is more pronounced in the 3-D setting. These few examples just illustrate the range of application of spheroids in the study of combination therapy and shall encourage scientists at academic institutions and in the pharmaceutical industry to use the model for future-oriented treatment strategies.

MC(T)S in drug screening: Progress and challenges

As detailed earlier, many complex methodologies have been applied to analyze intact and viable, frozen or paraffin-embedded and also dissociated spheroid cultures (Kunz-Schughart et al. 1998). Today, all modern molecular techniques can be exploited to study spheroid cells on the cellular, protein, RNA and DNA level. However, with respect to drug and therapy screening, there is still a lack of automated and easy-handling analytical tools and a need for defined, standard practice endpoints. Spheroid volume growth, which can be mathematically described in many spheroid types by a Gompertz equation (Marusic et al. 1994, Kunz-Schughart et al. 1996) or other mathematical models (Tabatabai 2005), and growth delay following treatment are just one set of parameters that should routinely be determined. This of course requires long-term measurement of spheroid diameters. Nevertheless, monitoring of these basic volume growth parameters is still the most popular technique to evaluate effects of growth-promoting or growth-suppressing factors and drugs in MCTS, is technically easy, and

provides a highly convenient endpoint for quantitative studies (e.g., Figure 3a). Simple readings of the growth or shrinkage of spheroids can be accomplished by standard phase-contrast microscopy and computer image analysis.

An interesting technique for analyzing and sorting intact spheroids is flow cytometry, first demonstrated by Freyer and colleagues (1987). To date, this technique has seen limited application (Freyer et al. 1987, 1989), primarily due to the requirement for instrument modification to handle large particles on most commercial flow cytometers. However, a recently launched commercial instrument (Union Biometrica COPASBiosorter, http://www.unionbio.com/) that is capable of both light scattering and fluorescence analysis and sorting of large particles up to 1500 μm may encourage broader use of this technology for spheroid sizing, analysis and selection. It is recognized that high throughput flow cytometric analysis may require relatively large numbers of structurally intact spheroids and may thus not necessarily be suitable for every treatment modality. However, the option of selecting intact spheroids of defined sizes remains a clear challenge. As an example, such an approach could foster increased use of MCTS pre-cultured under optimal rotary culture and nutrient supply conditions, e.g., prior to selective transfer into sufficient culture (plate) systems for testing small amounts of new drug candidates.

Since spheroid growth delay and re-growth per se do not allow an independent separation of effects on viability versus effects on growth rate or recruitment rate of quiescent cells to re-enter the cell cycle, other analytical endpoints should be considered. Parameters of interest with potential for automated analysis include cell viability and survival, respectively, outgrowth or migratory activity of spheroid cells, and the (patho)physiological status within spheroids. A multicellular spheroid-based sensor based on real-time impedimetric measurement was presented a few years ago for testing anti-cancer therapeutics. But the proposed biohybrid system, with spheroids to be positioned in a measurement capillary for monitoring impedence spectra as a measure for spheroid morphology, cell integrity and physiology has not yet entered routine analysis, probably due to the highly delicate handling and data interpretation required (Thielecke et al. 2001). The system has been used recently to study genetically altered and methotrexate treated MCF-7 spheroids (Bartholoma et al. 2005). Its applicability in drug screening, however, has yet to be validated. More importantly, there have been a number of attempts to simplify the measurement of cell number and viability in MCTS. Respiratory activity is generally regarded to reflect cellular status and to correlate with cell viability. Therefore, to verify anticancer drug sensitivity, Torisawa et al. (2005) monitored respiratory activity non-invasively in

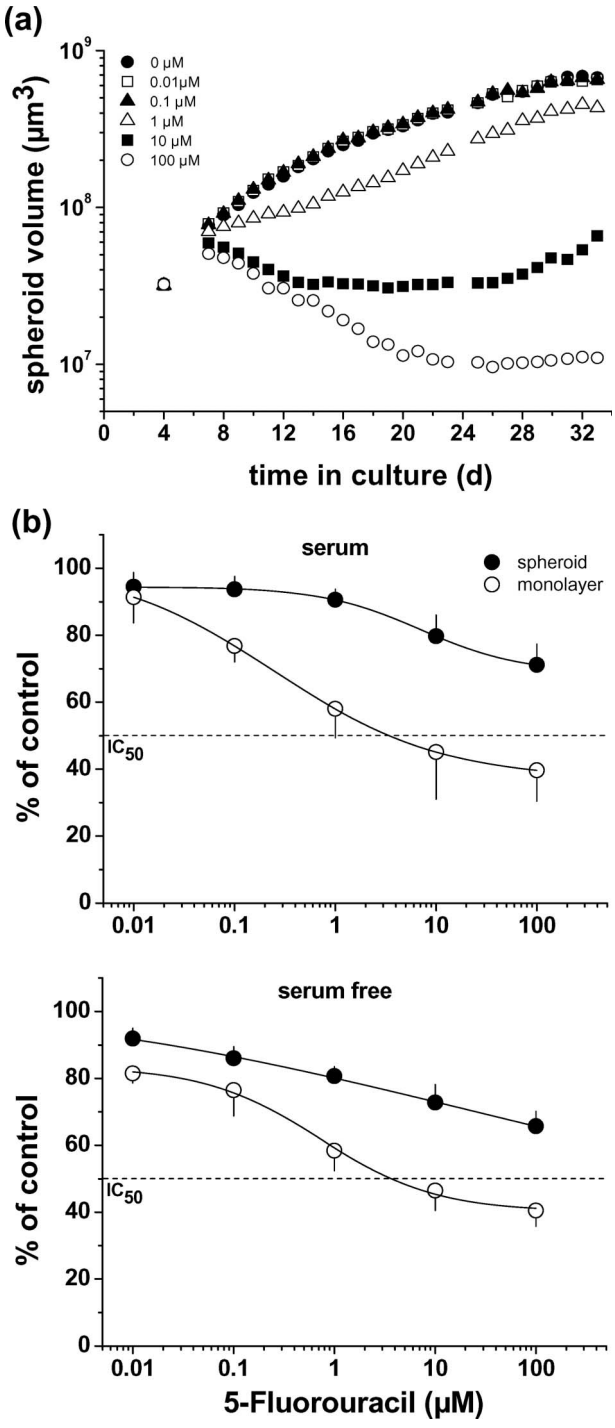

Figure 3. The effect of 5-FU treatment (72 h treatment interval) as determined by spheroid volume growth kinetics (a) and directly after treatment using the recently validated APH cell viability assay (b). The APH assay is applicable in spheroids grown under serum and serum-free conditions. The effect of 5-FU in serum-free cultured HT29 spheroids was slightly higher than for serum-conditioned spheroid cultures in this particular experiment but the prominent difference between monolayer and spheroid cells was restored.

MCF-7 MCTS by scanning electrochemical microscopy (SECM) and showed some correlation with the MTT equivalent WST-8 assay which was performed following spheroid dissociation (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; WST-8: 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium). Also, a number of researchers have applied routine cell viability assays such as the MTT or similar test systems, but in most cases their utility for spheroids larger than 200 μm has either not been well tested, or spheroid dissociation was required. This step, however, may result in artificial cell loss in particular following therapeutic intervention and should be avoided. A slightly antiquated systematic study using various methods for assaying cytotoxic effects in spheroids involved only small spheroids without central necrosis (Kosaka et al. 1996). Thus, the potential of those methods for evaluating drug effects on hypoxic and cell-cycle arrested tumor cell populations in non-peripheral, viable regions present in most spheroid types with a diameter of $\geq$500 μm is limited. In another study, cell death in spheroids was determined by monitoring of lactate dehydrogenase (LDH) activity. However, here the methodology was adapted for the location of cell death processes in spheroid sections based on image cytometry and is thus not likely to be useful as a high throughput screening tool (De Witt Hamer et al. 2005). Aside from the common classical LDH measurement in supernatants of treated vs. untreated cells, various commercially available cytotoxicity/viability assays based on absorption, luminescence or fluorescence are routinely applied in monolayer cultures as well. Measurements are performed in supernatants or on intact or lyzed cells, depending on the parameter measured, such as membrane integrity, intracellular activity or energetic status. We recently tested a number of such assay systems for their applicability to MCTS and found the Acid Phosphatase (APH) assay to be one of the most promising approaches amenable to high-throughput settings for non-dissociated spheroid cultures. We observed that the assay is reliable for monitoring cell viability in single spheroids, does not require spheroid dissociation and is linear and highly sensitive over a large cell concentration range and spheroid size, respectively, as shown for two colon carcinoma cell line spheroid types (Friedrich et al. 2007). The assay is thus simple, rapid and high-throughput compatible and has already been used to examine the effect of various drugs in spheroid culture. Moreover, our classical colon cancer spheroid model was recently adapted to serum-free conditions for highly standardized cell culturing as a prerequisite for reproducible and economical drug testing. This design also allows us to study the impact of serum on drug efficacy. The APH assay can be applied to both culture conditions as documented in Figure 3b. The reduced efficacy of 5-FU in spheroids as compared to monolayers is not critically altered by serum supplementation. The dose response curves recorded in this experiment confirmed earlier data (Friedrich et al. 2007). Comparison with the growth kinetics clearly shows that cell viability is not necessarily reflected by spheroid integrity or regrowth over a period of 32 days. The potential for easy viability assessment with the APH assay as a function of time in culture after spheroid treatment will be useful in future studies intended to also verify if cell viability is indeed maintained throughout culturing and how this relates to growth delay and regrowth capacity for various treatment regimens.

It is clearly recognized that further technological improvements and increased standardization are required for a truly high-throughput drug screening platform with MCTS. However, significant progress over the past few years including the validation of the APH assay for rapid and reproducible determination of cell viability has essentially augmented the potential of spheroids for this purpose. The application of spheroids as tumor models for monitoring local drug penetration capacity, tissue distribution and binding properties is another challenge. Indeed, one advantage of the MCTS culture system is that drug candidates *must* penetrate in order to be highly effective. However, the analytical tools for such penetration studies, be they confocal laser scanning (Martin et al. 2003, Kostarelos et al. 2004, Mellor et al. 2005) or even multiphoton microscopy based, are still awaiting more intense validation as to their practical operating conditions. At this time, they have limited potential to be automated into rapid, high through-put approaches for spheroid analysis, especially if spheroid sectioning or dissociation has to be avoided. As mentioned above, incorporation of flow cytometric analysis should aid in the measurement of additional cell parameters, such as viability or metabolic activity.

MC(T)S – from homology to heterology

Recent progress has now cleared the way for using multicellular tumor spheroids as a model of tumor pathophysiology in anti-tumor drug screening. However, at the same time it also became evident again that the spheroid mono-culture system does not reflect the tumor cell environment in all aspects. Therefore, a number of complex spheroid-based systems to mimic cellular heterogeneity in tumor tissues have been developed as illustrated in Figure 4a. While these approaches are not yet established for drug screening, they are remarkable

tools to study heterologous cell-interactions in tumors *in vitro* under well-defined conditions and are thus considered major steps to gain insight into particular drug and treatment related problems. A challenge specific to spheroid co-cultures is that the generation of spheroids with uniform fractions of the

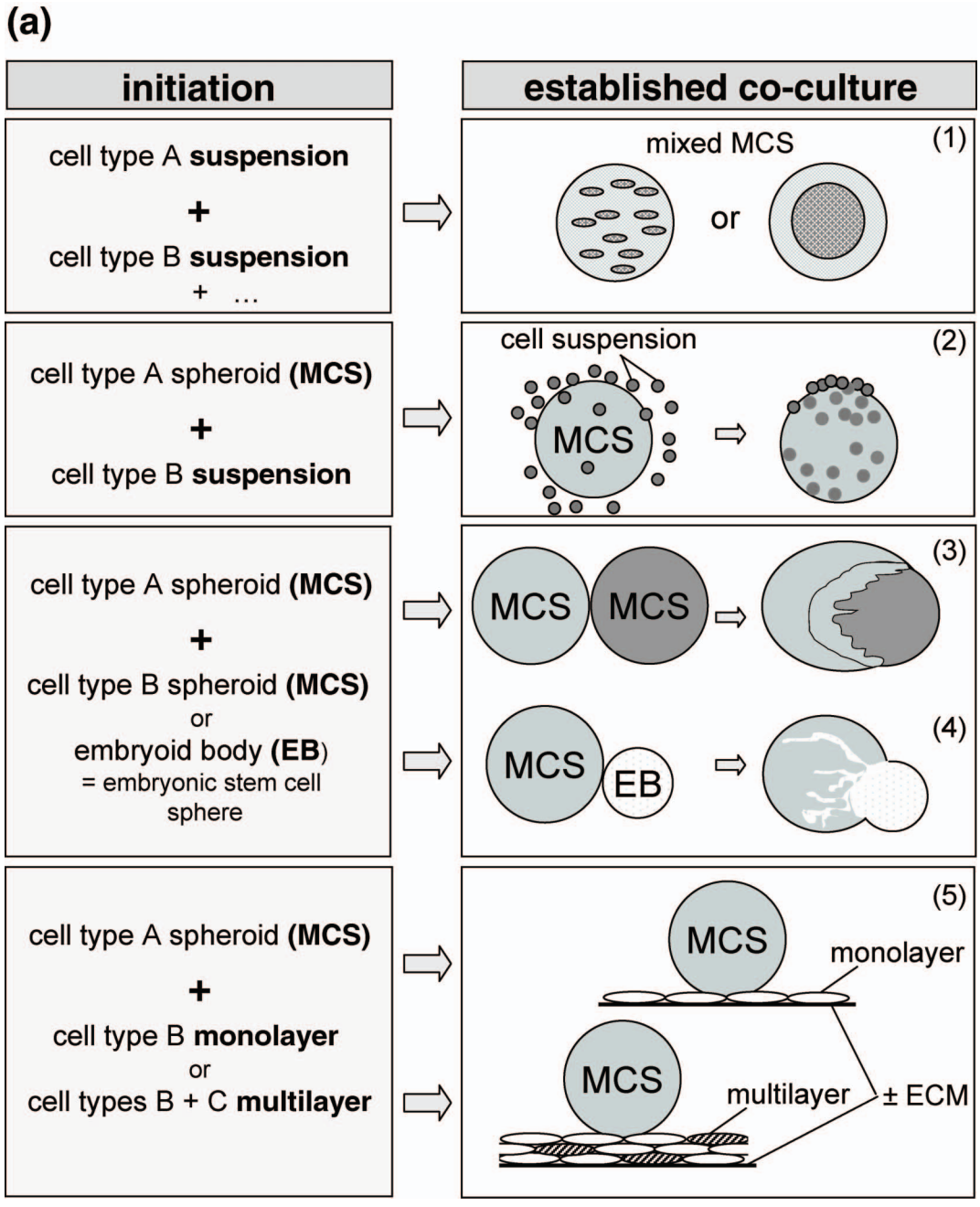

Figure 4. Overview of potential spheroid co-culturing strategies of tumor and stromal cell types to study heterologous interactions in tumor tissue (a). The most frequent cell types used for co-culturing are tumor cells (T), immune cells (I), fibroblasts (F) and endothelial cells (EC) but other cell types are also applied, in particular in non-tumor approaches. Immune cells are primarily applied in suspensions while fibroblasts and EC have been used both in suspension and in pre-cultured adherent systems (spheroid, monoloyer) for co-culturing with tumor cells and tumor cell spheroids, respectively. The combination of spheroids with multilayer co-culture systems (e.g., EC + F multilayers) is entertained but not yet realized. Representative literature for multicellular tumor spheroid co-culturing: (1) Tofilon et al. 1987, Thomsen et al. 1992, Frenzel et al. 1995, Nakamura et al. 1999, Boyd et al. 2001, 2002, Djordjevic et al. 2006, Spoettl et al. 2006; (2) Hauptmann et al. 1993, Konur et al. 1998, Siegert et al. 1999, Heimdahl et al. 2001, Olsnes et al. 2002, Furbert-Harris et al. 2003, Silzle et al. 2003, Gottfried et al. 2006; (3) Schuster et al. 1994, Kunz-Schughart et al. 1998, 2001; (4) Wartenberg et al. 2001, 2003, Wartenberg 2006, Gunther et al. 2007; (5) Brouty Boyé et al. 1994, Offner et al. 1996. Histomorphology of spheroid co-cultures is frequently documented in median sections following cryoconservation or paraffin-embedding and histological processing. Some examples of immunohistochemically or immunofluorescently stained median sections of spheroid co-cultures consisting of two or three cell types cultured with various strategies are shown in (b). Cell types and culture technique as detailed in the respective images.

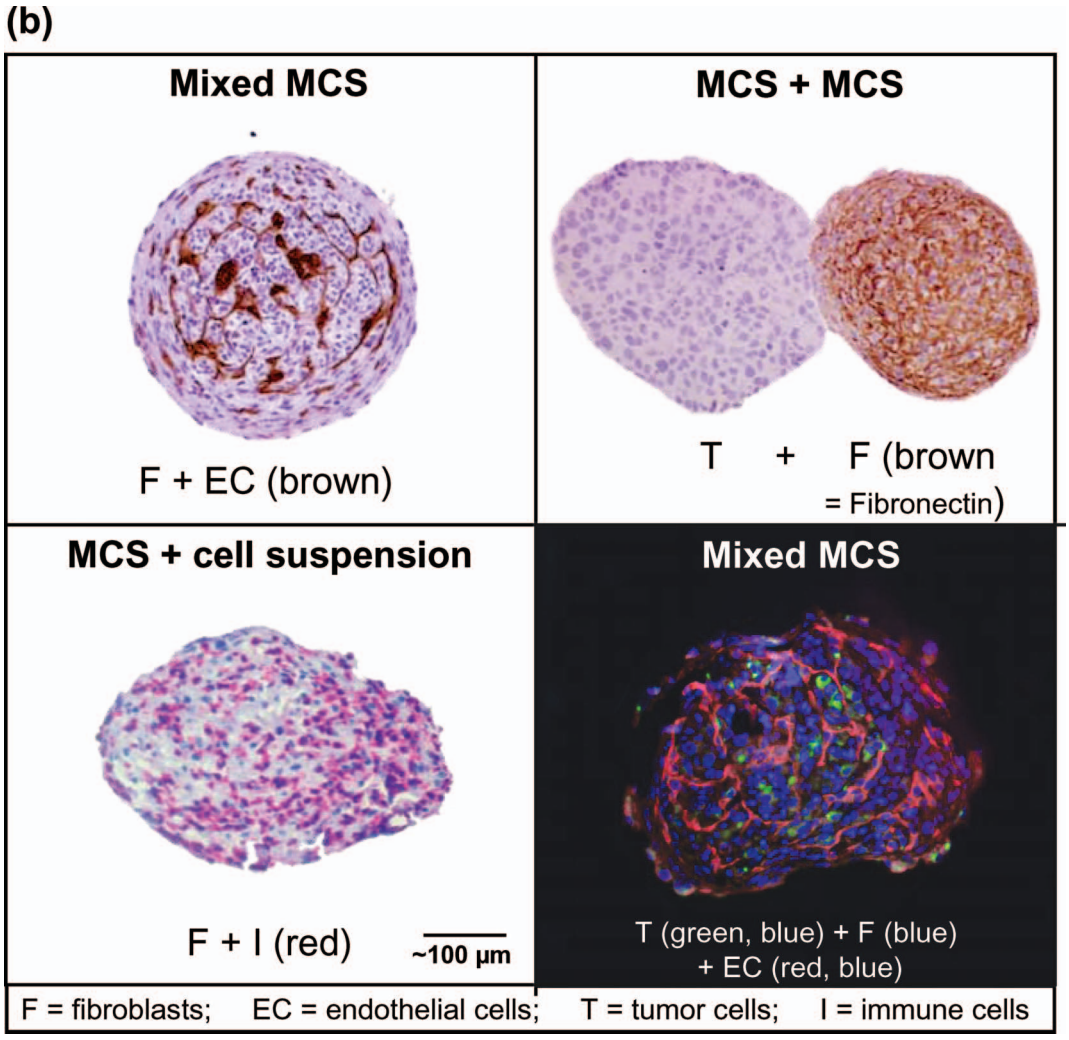

Figure 4. (*Continued*).

different cell types has in some cases been difficult. Such uniformity would in principle be needed to use the system as a drug screening platform. The problem could be minimized by careful seeding of starting cell populations and/or subsequent spheroid analyses at the onset of treatment. Flow cytometric analysis of differentially labeled cell types in intact spheroids with or without spheroid sorting has some potential to help reduce heterogeneity as well, although this has not yet been tested out for screening purposes, as discussed earlier.

Co-culturing of different therapy-responsive tumor cells is one of the strategies of interest. Mixed, mosaic, or hybrid spheroids have been developed primarily from established cell lines or cell clones, e.g. defined by their difference in sensitivity to drugs such as cisplatin or BCNU (bis-chlorethylnitrosourea), or by their differentiation pattern, as described in bladder cancer or breast tumor cells and spheroids, respectively (Tofilon et al. 1987, Bradley & Pitts 1994, Frenzel et al. 1995, Song et al. 2004, Persaud et al. 2005). Lately, a three cell line mixed spheroid system (termed tumoroid by the authors) based on three well known breast tumor cell lines, MCF-7, ZR-75-1 and MDA-MB-231, labeled with different fluorescent CellTracker Probes and cultured in the rotating wall vessel bioreactor has raised considerable attention (Vamvakidou et al. 2007). However, when using such systems, one has to bear in mind the potentially different behavior of the cell types in 3-D culture, e.g., with respect to cell attachment, survival and proliferative as well as migratory activity. These characteristics critically affect the histomorphology and cellular distribution in a mixed spheroid, even if identical cell numbers are applied for each line. The proportion of the cells not only at the time of seeding but rather at the time of treatment and their location within the spheroid are just two of the critical parameters that may modulate the

spheroid's response to drug exposure. Taking into account the historical shift in the drug development world from generally cytotoxic towards target-specific drug candidates, there may be a particular use for the 3-D co-culturing of genetically well defined tumor cell clones that differ exclusively in the gene of interest. Such a transfectant mosaic spheroid model composed of two cell populations derived from a single cell line and differing by just one gene was recently used in an interesting study series on target-specific treatment modalities, i.e. focusing on targeted radiotherapy and evaluation of the 'radiological bystander effect' conferred by a particular beta-emitting radiopharmceutic (Boyd et al. 2002, 2004).

Other approaches of spheroid co-culturing include various stromal cell types. Under investigation are immune cells such as monocytes, macrophages, dendritic cells (DC), T-lymphocytes or even NK (natural killer) cells. These latter cell types are primarily added as cell suspensions to tumor or fibroblast spheroids to monitor migration and maturation or activation processes affected by the tumor environment. With one such system, Spoettl et al. recently studied intestinal differentiation of monocytes by using a spheroid model of transfected HT29 colon cancer cells specificially overexpressing one of two chemokines of interest (Spoettl et al. 2006, 2007). One of these chemokines (MCP-1 = monocyte chemotactic protein-1) is also hypothesized to play a role in monocyte migration into breast tumor-derived stromal fibroblast spheroids (Silzle et al. 2003). The adverse impact of tumor cell lactic acid production on the differentiation of DC has been shown in spheroids of human J82 bladder and MelIm melanoma cells (Gottfried et al. 2006). The MelIm melanoma spheroid model has also been employed in a study to verify that tumor derived lactic acid critically suppresses the function of human cytotoxic T lymphocytes (CTL) (Fischer et al. 2007). Since the present article focuses on drug testing in spheroids, the numerous basic tumor-biological studies performed over the past 15 years that are based on tumor-immune cells co-cultured with spheroids cannot be comprehensively summarized. However, we want to draw attention to the recent review article 'Brave little world: spheroids as an *in vitro* model to study tumor-immune-cell interactions' by Gottfried et al. (2006). With respect to therapy, it is noteworthy that the spheroid model has been found useful in the development of mathematical models to use macrophages as vehicles for drug delivery to hypoxic tumor areas (Owen et al. 2004).

Different spheroid co-culturing techniques have also been applied to monitor interactions between tumor cells and stromal fibroblast. Experimental settings range from mixed spheroids, MCTS cultured on fibroblast monolayers (Brouty-Boye et al. 1994), MCTS co-cultured with pre-established fibroblast spheroids – also termed confrontation cultures (Schuster et al. 1994, Kunz-Schughart et al. 1998), fibroblast suspension added to MCTS, to the incubation of fibroblast spheroids with tumor cell suspensions (Brabletz et al. 2004). The latter technique was found useful to reflect several important pathological phenomena, for example the migration of non-aggregating tumor cells into fibroblastic stroma at the invasion front. It is hypothesized that fibroblasts in tumor tissues and in spheroid co-cultures, which are known to produce high amounts of ECM molecules, release numerous cytokines and growth factors and also directly interact with the tumor cells (Kunz-Schughart & Knuechel 2002, Kunz-Schughart & Freyer 2002), which may critically affect therapeutic efficacy, e.g. by altering the distribution of macromolecular drug candidates. Undoubtedly, such co-culture models may be useful to verify the impact of the stromal environment on drug efficacy. However, the practical details of their application in drug screening are not yet readily envisioned. On the contrary, their use in radiotherapy testing is more obvious, largely for historical reasons. In fact, the idea to score the effects of radiation and of other treatments on tumor cell survival and clonogenicity via the application of fibroblast-tumor cell hybrid spheroids using supralethally irradiated fibroblasts as feeder cells is not new, though the technology has only been applied in a limited number of cases (Djordjevic & Lange 1990, Lange et al. 1992, Djordjevic et al. 1998). In a recent approach, Djordjevic and co-workers (Djordjevic & Lange 2006) once more presented experimental indications for a bystander-like effect in such hybrid spheroids. Here, the presence of even highly-irradiated neighboring cells reduced clonogenic survival of (tumor) test cells in the 3D spheroid co-culture system. Clonogenicity of test cells was evaluated via growth analysis of whole spheroids composed of defined proportions of non-clonogenic feeder cells and a minority of these test cells (1 – 10%) (Djordjevic & Lange 2004, 2006). The observations with this model evidently demonstrate that using the (most) appropriate *in vitro* test system is a critical aspect for radiation sensitivity testing, and may ultimately guide the selection of predictive assays for clinical efficacy.

In addition to immune cells and fibroblasts, the tumor stroma of course contains varying amounts of endothelial cells (EC). Thus, co-culturing spheroids with EC is another field of research (Oudar 2000) which is bound to become more relevant in the near future, especially in the testing

of combined therapeutic approaches with anti-angiogenic compounds. The technique of culturing MCTS on confluent EC with an underlying ECM has been used in the past to study aspects of tumor cell evasion (Vermeulen et al. 1996). Mixed cultures of tumor and EC to study tumor angiogenesis or the application of MCTS and endothelial cell suspensions to allow EC migration, carried out over many years, have had little success. However, in 2004, Timmins et al. described an EC network in HCT116 colon cancer and even more pronounced in HepG2 hepatocellular carcinoma spheroids by using the hanging drop culturing technique (Timmins et al. 2004). It is not clear whether such EC networks may be developed in other tumor spheroid types. Observations in fibroblast spheroids co-cultured with EC suspensions indicate that at least for some tumor cell types the fibroblastic stroma may be either required or subsidiary for the formation of networking tubular structures in spheroids (Kunz-Schughart et al. 2006). In contrast to tumor or fibroblast EC co-cultured spheroids showing EC network formation, the co-culture of EC and smooth muscle cells in spheroids results in an entirely different morphology, with EC located in the spheroid periphery surrounding a smooth muscle cell core (Korff et al. 2001). This co-culture approach is based on a pure EC spheroid system developed earlier (Korff & Augustin 1998), which depends on allowing cell-cell contacts and can be maintained in culture by the supplementation of methocylcellulose (methocel). Such EC spheroids can be collected and embedded into three-dimensional ECM gels for monitoring EC migration processes and to identify modulators of angiogenesis, or they can be co-cultured with other spheroid types, even with organotypic tumor or fragment spheroids. Such approaches have gained some experimental importance in the study and manipulation of angiogenesis in gliomas and other brain tumors (Goldbrunner et al. 2000, 2004). Finally, it should be mentioned, that tumor-induced angiogenesis has also been studied in an interesting system of MCTS in confrontation culture with embryoid bodies (Wartenberg et al. 2001, 2006, Gunther et al. 2007). This *in vitro* system, however, has some limitations due to the necessary addition of non-human pluripotent, i.e. mouse, embryonic stem cells.

Some examples of immunohistochemically or immunofluorescently stained median sections from different types of histologically processed spheroid co-cultures are shown in Figure 4b. In addition to such co-cultures from cell lines, cell clones and primary cells, there is growing interest centering around the use of so-called fragment spheroids grown from tumor biopsies/tissues as already mentioned above (de Ridder et al. 2000, Heimdal et al. 2001, Kross et al. 2005, Olsnes et al. 2005). This seems of particular relevance for an individualized, patient-specific therapy testing and may be less feasible for massive parallel drug screening. When using such systems for drug testing, one has to for example consider the potential interindividual variance of multiple fragment spheroids derived from different sections of a single tumor. While such variation may in fact be representative of the tumor's real heterogeneity, it may also produce numerous analytical problems for establishing a robust drug testing platform. Having said that, tumor biopsy material may on the other hand contain so-called initiating tumor cells and tumor stem/progenitor cell populations, which are obviously of particular interest for new treatment modalities and the technique may thus have some not yet fully appreciated benefits. This hypothesis gained increased attention with the recent observations indicating that particular tumor initiating cell populations, not only of brain tumors but also of colorectal tumors, could exclusively be maintained in culture when grown as spheres (Zhang et al. 2005, Ricci-Vitiani et al. 2007). This intriguing aspect will no doubt receive thorough consideration in some future drug screening initiatives.

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