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A Multilayered Postconfluent Tumor Cell Culture System for in vitro Drug Screening

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Introduction

Current strategies for in vitro chemosensitivity testing of new anticancer drugs focus on large-scale use of human solid tumor models, in vitro. However, as with all results from in vitro assays, one should be very cautious in directly predicting an in vivo response from these assays, since many factors may have been oversimplified. For example, when the cells are growing in plastic 96-well plates as monolayers, some characteristics of solid tumors, such as a three-dimensional organization and cell-cell contacts, are missing. This can lead to overrated expectations or false-positive results [1]. The basis of our 'multilayer assay' is the formation of multiple cell layers when cell lines are cultured as postconfluent cultures, in 96-well plates with 'V'-shaped bottoms [2]. Although these multilayers are to some extent comparable with spheroids (growth in a three-dimensional way, drug penetration barriers [3]), a positive feature of the multilayer assay is the presence of a quantitative end point, which can be measured semiautomatically. Characterization and application of this model are discussed below.

Materials and Methods

Cell Culture and Chemosensitivity Testing (SRB). As model systems the human colon adenocarcinoma cell lines HT29, SW620 and the human ovarian carcinoma cell line A2780 were used. They were maintained under standard culture conditions [4]. Multilayer cell cultures were

established by seeding single-cell suspensions (15,000 cells/50 μ l) in 96-well plates with 'V'-shaped bottoms (Greiner Labortechnik, Solingen, Germany). In order to prevent medium exhaustion and acidification, medium was replaced every day [2]. Drug treatment of mono- and multilayered cultures was identical but started either at 24 h after plating (D1) or after 5 days (D5), when cells were organized as exponentially growing subconfluent monolayers or as postconfluent multilayers, respectively. After 24 h the drugs were removed and cells were cultured in drug-free medium for 96 h, after which the cells were fixed by addition of ice-cold trichloroacetic acid (TCA; 50%), and the SRB assay was performed as described [2, 4]. The cytotoxicity was defined as the concentration of drug resulting in a decrease in absorbance of 50% in treated compared to untreated wells (EC_{50}) [5]. Experiments were repeated for at least three times.

Cross Sections. On fixed time points (D5, D10) after plating, cells were fixed in situ and embedded in plastic resin (Historesin), and serial cross sections of 5 μ m thickness were made using a Jung-K Microtome (Reichter-Jung, Germany). The slices were stained with toluidine blue or haematoxylin [2].

Results and Discussion

Depending on the cell line and on the number of days (5–15) after plating, 2–15 layers of cells were formed. After exponential growth for the first days, multiple cell layers were formed after ± 5 days [2, 6]. The colon carcinoma cell line

Fig. 1. Toluidine blue-stained cross section of SW620 grown as multilayers for 5 days in a well with a 'V'-shaped bottom [from ref. 2].

Fig. 2. Cytotoxicity pattern against the colon cancer cell line HT29 of investigational drugs, such as the antimetabolite dFdC (Gemcitabine); the bioreductive alkylating agent EO9; the etherlipid HPC (miltefosine); the antitubulin RZN (rhizoxin) and the Topoisomerase I inhibitors CPT (camptothecin) and TT (Topotecan) and standard drugs such as DXR (doxorubicin); the antimetabolite FU (5-fluorouracil) and the DNA-alkylating agent DDP (cisplatin). EC₅₀ = effective concentration (see 'Materials and Methods') [adapted from refs. 5 and 6].

Table 1. Relative gene expression in multilayers¹

Gene ²	HT29	SW620	A2780
	%	%	%
Topoisomerase I	50	50	50
Topoisomerase II	75	75	60
c-myc	ND ³	83	43

¹ Adapted from ref. 6.

² Gene expression on D1 and D5 cells was assessed on total RNA by densitometry of Northern blot autoradiograms. The expression in multilayers, quantitated as percentage of monolayers is shown.

³ ND = Not detectable.

HT29 showed signs of differentiation by forming dome-like structures [2]. Various other cell lines from different histological origins, such as the colon cancer lines SW620 and SW1116 and the head and neck squamous cell carcinoma lines, were able to grow in multilayers; only some cell lines, such as the breast cancer cell line MCF7, did not form multilayers [2, 5]. In figure 1, a cross section is shown of a 5-day-old culture of SW620. The histological pattern of these cross sections showed several features comparable to that of spheroids, such as a three-dimensional organization and a low fraction of S-phase cells [2, 5].

As shown in figure 2 for HT29, monolayers were 3–10,000 times more sensitive than multilayers to almost all experimental and standard drugs tested [2, 6]. The tubulin-interacting agent Rhizoxin (RZN) which was extremely active on HT29 multilayers. A relatively small difference in sensitivity (between mono- and multilayers) was found for cisplatin (DDP) and gemcitabine (dFdC) in A2780 cells, in line with clinical activity to this tumour type. The investigational drug EO9 was one of the most active drugs on the tested cell lines. The enzyme activity of DT-diaphorase, which is related to sensitivity to EO9 [7], was comparable for mono- and multilayers [5]. Hypoxia has been reported to play a role too in the activity of EO9, which may explain the activity in multilayers.

Marked differences in gene expression of Topo I, Topo II and c-myc could be shown in mono-versus multilayers (table 1). In general, a 50% lower Topo I expression was found in multilayers, accompanied by a relative resistance to Topo I inhibitor Topotecan (fig. 2). However, this resistance also seemed to be related to a lower drug accumulation in multilayers [6].

Conclusions

This in vitro model was not developed to serve as a primary screen, but multilayers have a clear potential to serve as a secondary selectivity screening assay for compounds active in various in vitro screens. With this extra step, selective anti-solid tumor activity can be assessed before in vivo evaluation takes place. Eventually, when the predictive value of this assay is determined, compounds inactive in the multilayer assay should not be further evaluated in vivo, which will reduce the number of experimental animals. In addition, this model is well suited to perform mechanistical studies concerning enzyme targeting, drug uptake or metabolism.

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