In Vitro Chemosensitivity Testing of Multilayered Microcultures

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Abstract. A potential limitation of in vitro microtiter cytotoxicity assays as compared to in vivo antitumor studies is that the complex three-dimensional structure of the solid tumor is lost in monolayer cultures in vitro. We investigated whether more in vivo like cell-cell interactions could be easily and reproducibly obtained in an in vitro cytotoxicity assay. HT29 human colon adenocarcinoma cells were seeded in 96-well microtiter plates with «V»-shaped wells and allowed to form postconfluent multilayered cultures. Cross-sections of microcultures fixed after 2 and 3 weeks following plating revealed approximately 7 and 35 cell layers, respectively. Using a tetrazolium assay to assess cytotoxicity, the EC50 (drug concentration which gives absorbance readings 50% lower than those of non-treated wells) of multilayered cultures exposed to doxorubicin for 24 h was 12 times higher (p < 0.05) than that determined for subconfluent monolayered cells simultaneously exposed to the drug. This system offers an alternative to study the chemosensitivity of three-dimensionally organized cells using semiautomated microtiter plate technology.

The use of in vitro techniques during the preclinical evaluation of new antitumor drugs has augmented as a function of technological advances and increasing pressure limiting animal experimentation. A successful example is the use of semiautomated 96-well microtiter plate technology to provide a fast, simple and reproducible method to evaluate the chemosensitivity of tumor cells. In these tests, cells are usually cultured as subconfluent monolayers attached to the plastic substratum. Therefore, the three-dimensional cell organization that is found in in vivo neoplasms is lost in this model, as well as cell and microenvironment heterogeneity and other intrinsic characteristics of the complex tissue architecture of solid tumors, which are implicated in the outcome of radio- and chemotherapy [1]. Taking that into account, subconfluent monolayers may be seen as an unrealistic tumor model. Consequently, three-dimensionally organized cancer cells growing in collagen matrix [2], postconfluent cultures [3] or multicellular spheroids [4] have been considered, in several instances, as more valuable experimental tools due to their greater mimicry of the biology of human solid malignancies.

We have studied the organization of HT29 human colon tumor cells as multilayered supraconfluent cultures in 96-well plates with «V»-shaped bottoms. In addition, we have compared the cytotoxicity profile of doxorubicin (DXR) against mono- and multilayered cultures using the tetrazolium-MTT assay.

Materials and Methods

Chemicals and reagents. Dulbecco’s Modified Eagle’s Medium (DMEM) was purchased from Flow Laboratories (Irvine, Scotland); 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) was from Serva Laboratories (Heidelberg, Germany); fetal calf serum (FCS) was from Gibco (New York, U.S.A.); glutamine and gentamicin were from Merck (Darmstadt, Germany); trypsin, [ethylenedinitrilo]tetraacetic acid (EDTA), 3-(4,5-dimethylthiazolyl)-2-yl)-2,5-diphenyltetrazolium bromide (MTT), glutaraldehyde, osmium tetroxide, propylene oxide, trypan blue and toluidine blue were all from Sigma Chemical Co. (St. Louis, U.S.A.); DXR was purchased from Farmitalia Carlo Erba (Nivelles, Belgium); spectrophotometric graded dimethyl sulfoxide (DMSO) was from Baker Chemicals B.V. (Deventer, Holland).

Cell culture. HT29 human colon adenocarcinoma cell line was obtained from the American Type Culture Collection (Rockville, U.S.A) and grown as monolayer cultures in 20 mM Hepes-buffered DMEM supplemented with 5% heat-inactivated FCS and 1 mM L-glutamate. Cultures were maintained in 80 cm² flasks (Nunc, Roskilde, Denmark) without antibiotics in a 37°C, 5% CO₂, 95% humidified air incubator.

Standard cell culture maintenance procedures used in our laboratories were described in more detail elsewhere [5].

Microculture plating. Microculture assays were performed in 96-well plates with «V»-shaped bottoms (Greiner Laborteknik, Solingen, Germany) always using growth medium supplemented with gentamicin at a final concentration of 50 µg/ml. Exponentially growing cells were harvested with trypsin-EDTA from maintenance cultures, resuspended in antibiotic-containing medium, transferred to a plastic syringe and slowly flushed through a 0.4 × 12 mm needle. Only cell suspensions displaying...
viability rates ≥ 90%, as determined by 0.1% trypan blue dye exclusion, were subsequently counted with an automatic counter (Sysmex, CC-110, Tokyo, Japan) and diluted in order to generate the appropriate seeding density. Peripheral wells of each plate did not contain cells since they were used for background (medium and drug-containing medium) determination during the MTT assay.

Microculture cross-sections. In each well, 3×10^5 cells were plated in 50 μl (6 replicate wells/plate). One day after plating, 100 μl medium were added to all wells. Ninety-six hours after seeding, microculture medium was carefully aspirated and substituted by 150 μl fresh medium. From the first week after plating until the end of the experiments, this procedure of medium replacement was performed every 24 h.

Microcultures completing 2 or 3 weeks of incubation were fixed in situ with 1% phosphate-buffered glutaraldehyde (pH = 7.3) for 2 h, washed once with the same buffer, post-fixed in 1% osmium tetroxide and dehydrated in ethanol. Next, propylene oxide (100 μl/well) was added in order to dissolve the plastic substratum and detach cell clusters from individual wells. The isolated clusters were then rinsed in absolute ethanol and embedded in Epon. While being sectioned (thickness = 3 μm), embedded clusters were observed under a light microscope to ensure that the plane of sectioning was perpendicular to the side of the cell aggregates which was formerly in contact with the plastic bottom. Serial sections obtained during 3 independent experiments were collected on microscope glass slides and stained with toluidine blue.

Cytotoxicity tests. Seven ten-fold-different drug concentrations were prepared immediately before drug addition to triplicate «V»-bottomed wells.

A cytotoxicity test using subconfluent HT29 monolayers has been designed as follows: a) 24 h after plating of 3×10^5 cells/well, culture medium was aspirated and wells received 150 μl fresh medium without (control) or with DXR; b) following 24h of drug exposure, control and drug-exposed wells were rinsed once with culture medium and incubated in the presence of drug-free medium for another 96 h before cytotoxicity was evaluated.

In order to assess the cytotoxicity of DXR against postconfluent cultures, a second set of plates was initiated and maintained exactly as described for microculture cross-sectioning (see above). On day 14 after seeding, these plates were started on the same drug treatment protocol as that described for subconfluent monolayers.

We used a modification of the Microculture Tetrazolium Assay described by Alley et al. [6] to evaluate cytotoxicity. Briefly, at the end of the drug tests, plates were centrifuged, culture medium was substituted by 150 μl fresh medium per well and 15 μl of MTT solution (0.5 mg/ml in phosphate-buffered saline) were administered to each well. Plates were then incubated (37 °C, 5% CO2) for an additional 4 h. Following a second centrifugation step, the supernatant was aspirated and formazan crystals were solubilized after the addition of 150 μl DMSO containing 0.5% FCS. Using an ordinary plate shaker, it was very difficult to solubilize completely the large amount of crystals formed in wells corresponding to controls and low drug concentrations. Alternatively, thorough formazan solubilization was accomplished within 5-10 min using ultrasound vibration applied to water-floating plates in an ultrasonic cleaner (Branson 5200). The absorbance of each well was measured at 540 nm using a microtiter plate reader (Titertek Multiskan MCC/340; Flow Laboratories) interfaced with an Olivetti PC M19 microcomputer. The resulting dose-response curves allowed the extrapolation of EC50 values, defined as the drug concentration which yielded an absorbance reading 50% lower than that of control wells.

For data analysis, a p value (Student’s t test) of less than 0.05 was considered to be statistically significant.

Results

A few hours following seeding, plates observed under the light microscope showed that single cells were preferentially sedimented towards the very center of the «V»-shaped well bottoms, while almost no cells had sedimented on their periphery. Under these conditions, HT29 cells reached confluence around one week after plating and then started to form multilayered cultures.

The process of detachment of the cell aggregates from the plastic substratum after propylene oxide treatment was not ideal in the sense that it was not possible to preserve the multilayered microculture as a single intact structure. Despite this, it was observed, even macroscopically, that the larger fragments detached from the plastic surface had a conical shape, displaying an obvious vertice and the expected relationship with the geometry of the «V»-shaped wells. The corresponding cross-sections revealed that HT29 cells could form unpolarized, tightly structured multilayers in 96-well plates. Cell clumps derived from microcultures fixed after a two-week incubation period reproducibly showed a maximum of 7 to 10 cell layers (Figure 1A), while those fixed after three weeks displayed up to 35 layers (Figure 1B). Furthermore, in the cross-sections of three-week old cultures, it was possible to observe well delineated areas where cells were either absent or were undergoing degeneration. These areas were invariably seen in the lower half of the sections, closer to the microculture cell layer which had been in contact with the plastic substratum. The histological pattern was suggestive of a process of cellular necrosis such as that seen in the inner regions of HT29 multicellular spheroids, which has been proven to be a consequence of an inhospitable microenvironment, lacking adequate oxygen and nutrient levels [7].

Figure 2 shows the dose-response curves of DXR added to HT29 cells 1 (subconfluent monolayers) or 14 (multilayered microcultures) days after seeding in the «V»-bottom wells. Absorbance measurements of DXR-treated wells were expressed as a percentage of the readings obtained from control cultures. The profiles of the two curves were markedly different. One-log increase in DXR concentration (from 0.1 to 1.0 μM) prompted a steep reduction in MTT readings (from 83.1% to 5% of control values, respectively) from subconfluent cells, contrasting with a significantly more limited effect (from 71.9% to 59.7% of control) seen with postconfluent cultures. The EC50's±SD (means from 3 separate experiments) were 0.25 ± 0.001 μM and 3.0 ± 0.24 μM (p < 0.05), for monolayered and multilayered cultures, respectively. At a concentration of 10 μM DXR, all absorbance signal from monolayers was abolished. In multilayered microcultures, the same effect was never observed, even when 100 μM drug was used. Dose-response curves determined with the MTT were compared to those obtained by cell harvesting and counting, and the differences in EC50's found between the two assays did not reach statistical significance (data not shown).

Discussion

Our results show that it is feasible to obtain three-dimensionally organized cultures of HT29 tumor cells in 96-well plates. Due to the pattern of cell-cell interactions and the
number of cell layers observed, one may expect that this in vitro system may indeed feature some of the biochemical and biological characteristics of tumor cell deposits in vivo. The use of in vitro models with three-dimensionally organized cultures instead of monolayers to mimic in vivo cancer cell deposits is intuitively logical and has been extensively supported mainly by experiments using the multicelluar spheroid model [4]. The uniqueness of the system described in this report relies on the attempt to combine a more complex cell culture system with a semi-automated method of chemosensitivity assessment.

Our cytotoxicity evaluating tests using the MTT assay have established that HT29 cells were significantly less sensitive to DXR if they were three-dimensionally organized in multilayered cultures. Other investigators have drawn similar conclusions when HT29 and various other cell lines were tested with DXR in several in vitro systems where the cells were arranged in a three-dimensional structure [8-14]. The decreased cytotoxicity of DXR and other antitumor agents against neoplastic cell aggregates has been explained as due to limited drug penetration, the existence of hypoxic and nonproliferating cell subpopulations as well as that of biochemical and metabolic gradients.

Other studies have pointed out to some limitations of the MTT assay as a chemosensitivity test, including the loss of linearity between absorbance readings and cell numbers at high cell densities [15-17]. In our experiments, however, EC₅₀’s determined by the MTT assay or by cell counting were not significantly different. Recently, we have started the evaluation of a chemosensitivity test based on a protein-binding dye (sulfonamide B) as an alternative method to assess cytotoxicity in our system of heavily multilayered microcultures. The SRB assay, as it is also known, has shown some technical advantages over the MTT test and has been considered as a potentially more reliable cytotoxicity test, particularly when high cell densities are utilized [5, 18].

The current study has evaluated the feasibility of combin-
ing semiautomated microtiter plate technology and a multi-layered supraconfluent cell culture system. Although the necessity of daily changes of culture medium in the multilayered microcultures represented a significant increase in the working load of the laboratory personnel, this limiting factor could be overcome by: (a) the automation of these procedures; (b) the use of microtiter plates with a larger volume/well capacity (e.g., «Deep-Well Titer Plate» from Beckman Instruments Inc., U.S.A.); and/or (c) changing the experimental conditions in order to obtain multilayered cultures shortly after plating. However, any reasonable increase in labour will be rewarded if future studies show that supraconfluent cell culture systems have a better predictability of the clinical performance of the drugs tested, especially, in the case of solid tumors. Under our experimental conditions, DXR was less cytotoxic against three-dimensionally organized cells. These results are in agreement with other studies where the authors have used in vitro methods with cells structured as more in vivo-like systems. Future investigations should attempt further methodological refinement as well as the biological and biochemical characterization of this model in order to determine its possible role in preclinical chemosensitivity testing.

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