ABSTRACT

The effects of the two antitumor drugs vinblastine and 5-fluorouracil on the growth of the human tumor cell lines U-118 MG (glioma) and HTh-7 (thyroid cancer) were analyzed. The cells were cultured both as monolayers and as multicellular spheroids and exposed to vinblastine (0.1, 1.0, or 10 μg/ml) or 5-fluorouracil (10, 100, or 1000 μg/ml) for 15 min, 2 hr, or 24 hr. The drugs induced growth delays of the monolayers and delays in the outgrowth of cells from spheroids which were placed on cell-adhesive surfaces. Cell cultures exposed to sublethal drug doses showed a dose-dependent lag period followed by regrowth at normal growth rates. In all cases with vinblastine exposures, the spheroids seemed more resistant to the drug treatments than did the monolayer cultures. Much smaller differences were obtained after treatments with 5-fluorouracil. The three-dimensional arrangement of cells in spheroids giving rise to, e.g., nutrient and proliferation gradients may, to some extent, be responsible for the increased resistance. The spheroids were especially resistant to short treatments with vinblastine. This was probably due to penetration barriers.

INTRODUCTION

Cell culture techniques have been widely used to assay the cytotoxic action of antitumor drugs. Monolayer cultures of tumor cells have probably been the most used system for in vitro assays (23, 28, 31). Some discrepancies have, however, been found when drug effects have been studied in parallel experiments on monolayer cultures of tumor cells and on solid tumors in vivo (16, 29). Another in vitro system used for cytotoxicity testing during the recent years is the multicellular tumor spheroid system (26, 30, 36, 40). Spheroids are spherical aggregates consisting of cells and an extracellular matrix. The peripheral cells are proliferative and contribute to spheroid growth. Deeply-lying cells in the spheroids are mainly resting due to insufficient nutrition from the surrounding culture medium. If the spheroids are big enough, massive necrotic regions will develop in their central parts. This has been described in detail for spheroids both of rodent (27) and of human origin (1, 13, 39). Thus, the spheroids imitate growing solid tumors in many ways (25).

In this study, the cytotoxicity of 2 different antitumor drugs were tested. These were the plant alkaloid VBL3 which interferes with the formation of the mitotic spindle (37) and affects RNA synthesis (5) causing metaphase arrest, defective cytokinesis, multinucleated cells and cell death (7) and the antimitabolite 5-FU which blocks thymidylate (and thereby DNA) synthesis and interferes with RNA synthesis, leading to numerous consequences for the affected cells (15). Both drugs have their main action in the S phase of the cell cycle (7, 15). These drugs were chosen because their action is comparatively well known in the therapeutic situation, and they have quite different penetration properties (20). As a model system, 2 human tumor cell lines cultured both as monolayers and as cellular spheroids were used. Growth delays were analyzed instead of clonogenic survival because the studied cells had a low plating efficiency (21). When cultured as spheroids, they were very difficult to disintegrate (2).

The aim was to investigate whether the sensitivity of the cells was dependent on the culture technique used and to see if differences in sensitivity could be correlated to differences in penetration properties of the 2 drugs. VBL has been shown previously to have a limited penetration into cellular spheroids, while 5-FU penetrated the spheroids much more efficiently (20). A more detailed study of the penetration properties of these 2 drugs is under way. Different drug responses from monolayers and spheroids of the same cell line may also be due to other spheroid characteristics such as cell to cell contacts (8, 11), gradients in cell cycle time (4, 12, 13, 35), and the presence of chronically hypoxic cell populations (4, 6, 17).

MATERIALS AND METHODS

Cell Culture. The human glioma cell line U-118 MG (3, 13, 33) and the human thyroid cancer cell line HTh-7 (3) were used. The cells were cultured both as conventional monolayer cultures (stock cultures were passaged once per week), and as spheroids. The spheroids were cultured using the liquid overlay technique (i.e., thin-layer agarose-coated culture dishes) described previously (13, 39). All cells were cultured in Ham's F-10 medium with 10% newborn bovine serum, L-glutamine (2 mm), penicillin (100 IU/ml), and streptomycin (100 μg/ml) (Flow Laboratories Swedish AB, Stockholm, Sweden). The medium was changed 3 times weekly. The cultures were kept at 37°C and the pH was 7.3. All dishes were used for A/S Nunc, Roskilde, Denmark.

Drug Exposure. The 2 drugs used were VBL (Velbe; Eli Lilly and Company, Indianapolis, Ind.) and 5-FU (F. Hoffman-LaRoche & Co. AG, Basel, Switzerland). Immediately before use, the drugs were diluted with complete medium. The concentrations used were 0.1, 1.0, and 10 μg/ml (0.11, 1.1, and 11 μM) for VBL and 10, 100, and 1000 μg/ml (77, 770, and 7700 μM) for 5-FU.

For the drug exposure, 4 to 8 spheroids were placed in non-cell-adhesive dishes (diameter, 50 mm) along with 5 ml of complete medium containing the appropriate concentrations of the respective drugs. The diameters of the spheroids were 700 ± 150 (S.D.) μm for U-118 MG and 550 ± 100 μm for HTh-7. Degenerative processes in the central parts of the spheroids barely start in the smaller ones, while massive necrosis is well established in the larger ones (3). The monolayer cultured cells were exposed to the drugs in small culture dishes (diameter, 35 mm). Two parallel dishes with exponentially growing cells (0.2 to 0.3 confluency) were used for each combination of concentration and exposure time. Drug exposure times were 15 min, 2 hr, and 24 hr. The experimental
points (combination of cell type, drug, concentration, and exposure time) were repeated once, twice, or 3 times, each time with 4 to 8 spheroids or 2 monolayer dishes. This implies that about 10 spheroids and at least 4 monolayer culture dishes were analyzed for each point. After the drug exposures, the cells were washed 3 times with complete medium. The monolayer cells were allowed to continue to grow in the original dishes until they were denser than 0.5 confluency. The spheroids were, after washing, transferred to multidishe (24 wells, each with a diameter of 15 mm). One spheroid was placed in each well with 2 ml culture medium. The growth of the drug-treated cells was studied for at least 5 weeks after drug treatment.

Evaluation of Spheroid Cultures. The spheroids attached to the bottom of the wells after transfer to the multidishe. In a few cases, the drug treatment damaged the cells so severely that they could not attach. After some days, a monolayer culture developed at the bottom of each well. The number of outgrowing cells was measured 3 times per week (2). Growth delay was defined as the difference between the time needed for a drug-treated cell culture to reach a given number of outgrowing cells and the corresponding time for the control cell culture. The outgrowth method has been described previously by Carlsson and Nederman (2), Durand (9), Yuhas et al. (40), and Sutherland (24).

Evaluation of Monolayer Cultures. The cell density in the culture dishes was measured 3 times per week by electronic cell counting. When cultures were at least 0.5 confluency, they were trypsinized (trypsin-EDTA from Flow Laboratories); diluted 2, 4, or 8 times; and placed in new dishes. Confluency was equivalent to about 1.25 × 10^6 cells/sq cm for the 2 cell lines. The relative number of cells at any time was defined as the number of cells calculated from the absolute number of cells and the total dilution factor for the culture at this time divided by the initial number of cells. The growth delays caused by the different drug treatments were defined and measured in the same way as for the spheroid cultures. This method has been used previously to study the effects of interferon and radiation on the growth rate of cultured, human glioma cells (19).

RESULTS

Nearly all treated spheroids attached initially to the cell-adhesive surface, but subsequent outgrowth was delayed for 0 to 30 days or did not occur at all. No systematic relation between spheroid size at the time of drug treatment and rate of outgrowth could be observed. The monolayer cultures reestablished normal growth rate within 30 days, or the cells lysed or detached and disappeared at the medium changes. In a few cases, lag periods longer than 30 days (up to 55 days; Chart 1b) were observed. Examples of representative growth curves are shown in Chart 1. It could be observed that the increase in the number of cells on dishes where a spheroid had been placed was faster than the increase in the number of cells in monolayer cultures, especially during the first days after similar treatments. This was due to a combination of migration of cells from the spheroid and proliferation of the outgrown cells. Sometimes the growth rate after a drug-induced delay was a little slower or a little faster than the growth rate of the control culture. Because of this, the delays were read on 3 different levels (10^2, 10^3, and 10^4 in relative number of cells), and the mean value was calculated and used as growth delay.

The growth delays caused by different drug treatments are shown in Charts 2 and 3 for both monolayers and outgrowth from spheroids. When VBL was used, the growth delay was always longer for monolayers than for spheroids (Chart 2). This difference was much smaller, and in many cases the growth delay was about the same for monolayers and spheroids, when 5-FU was used (Chart 3). Increased drug concentrations caused, in most cases, increased growth delays in a similar manner for monolayers and spheroids; however, when 15-min treatments with VBL were used, increased concentrations of the drug caused no (or very small) increases of growth delay for spheroids compared to monolayers. The drug sensitivity in terms of growth delay seemed to be approximately the same for the 2 cell lines both when cultured as monolayers and when cultured as spheroids.

The large resistance of spheroids to short treatments with VBL could be observed more easily when the growth delays for spheroids were related to the corresponding growth delays for monolayers (Chart 4). The extra resistance to VBL seemed still to remain, however, less pronounced when the drug exposure time was increased to 2 hr, at least at the lower concentrations. However, at 24 hr of drug exposure, no differences were ob-
T. Nederman

Chart 3. Growth delays for monolayer cultures (○) and delays in the outgrowth from spheroids (□) treated with 5-FU as a function of drug concentration. U-118 MG (a to c) or HTh-7 (d to f) cells were treated with the drug for 15 min (a and d), 2 hr (b and e), or 24 hr (c and f). Points, means; bars, S.D. If the growth delay varied between the experiments from real values to infinite values, the points are located in the region for infinite values, and the ends of the bars indicate the mean of the real values. The extra scale inserted in c indicates 55 to 60 days and is valid only for the bar ending in that region (compare with Chart 1c). No bars are indicated if the growth delay was zero or infinite in all experiments.

Chart 4. Relationship between the growth delay for spheroids and the growth delay for monolayer cultures after the same drug treatments. The cells were exposed to VBL (0.1, 1.0, or 10 μg/ml) (□) or to 5-FU (10, 100, or 1000 μg/ml) (○) for 15 min (a), 2 hr (b), or 24 hr (c). ——, 5-FU points; —— ——, VBL points. In c, the broken curve falls into the solid curve. The 3 solid curves in a, b, and c fall approximately into each other. Points, means; bars, S.D. Points from both U-118 MG and HTh-7 cells are included. All drug exposures giving zero growth delay for either monolayers or spheroids were excluded. All curves were fitted by visual inspection to the experimental points. Note especially that short time exposures (a) of VBL gave dramatic growth delays in the monolayer cultures while the spheroids seemed nearly unaffected.

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DISCUSSION

The cells in the multicellular spheroids were more resistant than the monolayer cultured cells to the VBL treatments. Smaller differences were seen after 5-FU treatments. Increased resistance of spheroids to therapeutic treatments have been reported previously after both irradiation (8, 24) and exposures to antitumor drugs (10, 34). The 3-dimensional growth and the tumor-like environment of the cells in spheroids have been suggested to be responsible for the resistance. The spheroids seemed to be particularly resistant to short treatments with VBL. VBL has been reported previously to have a limited capacity to penetrate through the spheroid mass (20). Thus, it is suggested that penetration barriers played a major role in the extra resistance of spheroids treated for short time periods with VBL. This indicates that there might be suboptimal conditions for chemotherapy with VBL since the serum level of VBL has been reported to decrease very rapidly in the first phase after injection into patients (22). Limited penetration has been suggested previously to be of relevance also for resistance to other drugs (26, 32, 38).

Both VBL and 5-FU affect the cells in several ways (7, 15). However, the main action of VBL is to interfere with the formation of the mitotic spindle. HeLa cells have been shown to be most sensitive to this action during late S phase close to the S-G2 boundary (7). The main action of 5-FU is to block the DNA synthesis during S phase. Thus, it appears as if the peripheral, intensively proliferating cells (13) of the spheroids should be most sensitive to the action of the drugs. A possibility which in part might explain the spheroid resistance is the presence of deep-lying nonproliferative cells. Such cells might not, although they are exposed to the drug, be severely damaged. It is possible that these resting cells might be triggered into the cell cycle when allowed to migrate out as a monolayer in our test. However, it cannot be excluded that also deeper-lying, mainly resting cells can be affected by other, less well-investigated actions of the drugs.

The serum levels obtained at tumor therapy in vivo are in the range of 1.0 down to 0.01 μg/ml during the first 2 hours after i.v. injection of 0.2 mg/kg for VBL (22) and 50 down to 2 μg/ml during the first hr after i.v. injection of 10 mg/kg for 5-FU (14). The lethal drug doses for the spheroids were considerably higher (Charts 2 and 3). However, in vivo, several other factors may influence the therapeutic results. The metabolic state of the cells, and thereby probably also the effects of the drugs, are influenced in vivo by action of hormones and other factors. In addition, multiple doses of the drugs are usually given during therapy in
Drug Sensitivity of Monolayers and Spheroids

The spheroids were allowed to be intact after the drug treatments. The effect on spheroids and the effect on monolayer cultured cells were shown to be related to each other, and these effects could be compared for identical treatments. An alternative method for this type of comparison might have been cloning of individual cells. However, cloning requires high plating efficiency of the cells and possibilities to disintegrate the spheroids. The 2 cell lines used in this study do not comply with these requirements (2, 21). Other features with the methods used were that the spheroids were allowed to be intact after the drug treatments and that it was possible to detect regrowth also after such long times as 3 to 4 weeks (18).

This study has shown that the results from drug sensitivity tests in vitro might be influenced by the culture technique used. The multicellular spheroids were in most cases more resistant than the corresponding monolayers, especially when limited drug penetration could be expected (20). It seems reasonable to believe that the spheroid system is more representative of the in vivo situation than the monolayer cultures.

REFERENCES

Fig. 1. Paired photographs of outgrowth from U-118 MG spheroids and of U-118 MG monolayer cultures at different times (top) after treatments with drugs. The treatments were: no treatment (control); 5-FU (100 μg/ml) for 2 hr; and VBL (1.0 μg/ml) for 2 hr. The photographs from Day 0 were taken immediately after drug treatment and washings (3 times with fresh medium). Df, dilution factor; e.g., DF = 8 means that the culture has been trypsinized and diluted to 1/8 of the original cell density. The growth delays determined from this single experiment were 2.2 ± 0.5 days for the spheroids treated with 5-FU, 3.4 ± 0.4 days for the monolayers treated with 5-FU, 3.0 ± 0.1 for the spheroids treated with VBL, and 9.5 ± 0.6 days for the monolayers treated with VBL (mean ± S.D. from the values measured at the relative cell densities of 10^2, 10^3, and 10^4). Note that the exposure to 5-FU affects the monolayers and the spheroids rather equally in terms of growth delay while the exposure to VBL causes about 3 times longer growth delay for the monolayers than for the spheroids. Bottom, geometric scales.
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