Quantitative Distinction of Cisplatin-sensitive and -resistant Mouse Fibrosarcoma Cells Grown in Multicell Tumor Spheroids


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ABSTRACT

As a suitable model to study the growth behavior and therapeutic response of drug-resistant and -sensitive cells in three-dimensional culture we have established multicellular spheroids generated from both cisplatin-sensitive and -resistant cells of a murine fibrosarcoma cell line. A drug resistant clone was derived from the parent cisplatin-sensitive cells by intermittent drug exposure in vitro.

As a prerequisite for analysis of differential growth and treatment response of spheroid subpopulations, two efficient methods to discriminate between the two morphologically indistinguishable subpopulations in mixed spheroids were established. In the cisplatin-resistant cell line chosen for the present study, resistance is mainly due to an increased cellular metallothionein content and is therefore associated with increased resistance to CdCl₂. Exposure of colonies to high concentrations of CdCl₂ thus allowed selective elimination of sensitive colonies. Permanent labeling of either resistant or sensitive cells was achieved by introduction of the Escherichia coli β-galactosidase marker gene with a retroviral vector system. The transformation of an uncolored galactose derivative by this enzyme into an indigo stain allowed detection of cells carrying and expressing the marker gene. The marker gene and CdCl₂ method led to identical results when used simultaneously to distinguish quantitatively between resistant and sensitive colonies grown from plated cells of untreated or irradiated mixed spheroids. The retroviral labeling method was also used successfully in the study of intact spheroids, showing that in 1:1 mixed spheroids, cisplatin-sensitive parental cells accumulate in the spheroid periphery, outgrowing resistant cells and displacing them into the metabolically restricted spheroid center. Only when sensitive and resistant cells are initially mixed at a ratio of 1:9 are the resulting spheroids composed of equal proportions of the 2 cell types throughout 10—20 days after spheroid initiation.

INTRODUCTION

Development of drug-resistant tumor cell subpopulations which modify overall drug sensitivity is a major problem in clinical oncology (1, 2). The aim of the present study was to establish an experimental model which is suitable for analysis of growth and therapeutic response of mixed tumor populations of sensitive and resistant cells. Investigation of homogeneous or mixed populations of tumor cells in monolayer cultures is of limited relevance, since it is known that interaction of tumor cell subpopulations via cell-cell contacts contributes to their response to therapy (3). Tumor spheroids are characterized by three dimensional intercellular contacts and by defined gradients of oxygen and nutrient supply (4, 5). They therefore represent an experimental model which is intermediate in its complexity between monolayer culture and experimental tumors grown in vitro. For investigation of tumor cell growth and response to some cytotoxic treatments in mixed tumor spheroids, a quantitative distinction between drug sensitive and drug-resistant subpopulations is required.

In our laboratory, cisplatin resistance, which was shown to be stable for several passages, was induced in a mouse fibrosarcoma cell line (6) by repeated cisplatin administration. The cell line chosen for the present study is characterized by a close correlation of cisplatin resistance and cellular metallothionein concentration, i.e., CdCl₂ tolerance (6, 7). This cell line is therefore particularly suitable for testing more widely applicable techniques to differentiate tumor cell subpopulations using toxicity as a reference method. This paper presents a technique of labeling subpopulations of cells by stable infection with a retroviral vector carrying a marker gene. Identification of the marker gene product in labeled cells and their progeny allows analysis of differential growth characteristics as well as differential radiation response of untreated parental, i.e., drug sensitive, and of cisplatin-resistant spheroid subpopulations.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. The mouse fibrosarcoma SSK was originally induced by treating the skin of C3H mice with methylcholanthrene and subsequently was adapted to growth in vitro and in vivo as a cloned cell line (8). In monolayer culture the cells are grown in DMEM, supplemented with 12% newborn calf serum, 0.01% neomycin, and 0.37% NaHCO₃ in a humidified CO₂ incubator at pH 7.4 and 37°C.

Cisplatin resistance was generated in vitro by intermittent drug treatment as reported previously (6). The cells were exposed to 10 μg/ml cisplatin for 1 h and then allowed to grow up to confluence, at which time the treatment was repeated. After only three to five treatment cycles, resistant clones were isolated (6). In the present study, a resistant subline (SSK-R) was used which exhibits moderate drug resistance defined by a dose-modifying factor of 4.1 in survival curves of resistant versus sensitive cells exposed to cisplatin for 1 h.

This value is in good agreement with most clinically determined values (1).

Drug resistance was stable for at least 140—180 cell divisions.

Preparation of Spheroids. The technique for initiation and growth of spheroids described by Yuhas et al. (9) was used with slight modifications in the present study. Tumor cells from confluent monolayer cultures were trypsinized into single-cell suspensions and mixed spheroids were initiated by pipetting 2500 cells from each cell line into 24-chamber multiwell dishes coated with 2% agarose. The suspended mixed cells aggregated to form a compact spheroid within 24 h. Spheroid growth was subsequently followed by measuring the largest and the incidental spheroid diameter using an ocular grid in an inverted phase contrast microscope. Based on measurements in 48 single spheroids, the shape was assumed to conform to a rotational ellipsoid and the average spheroid volume (V) was estimated as

\[ V = \frac{4\pi}{3} \cdot \frac{a}{2} \cdot \left( \frac{b}{2} \right)^2 \]

with a designating the largest and b the incidental diameter. Measurements were carried out daily in the initial phase and then at 2—3-day intervals. Radiation experiments were performed at day 3 after initiation, at which time the spheroids had reached an average diameter of 450 μm. During the growth period of about 3 weeks the culture medium was not changed.

Cell Survival. Spheroids were exposed to graded doses of γ-rays from a 137Cs source at a dose rate of 0.8 Gy/min. Following irradiation, spheroids were disaggregated by trypsin, and defined cell numbers were incubated for another 8—10 days in tissue culture flasks. Colonies were then fixed in 70% ethanol and stained with methylene blue. Colonies comprising ≥50 cells were counted as surviving cell clones. The ratio of mean colony yield of treated to untreated cells, i.e., the surviving fraction, was calculated. Experiments were carried out in triplicate.

CdCl₂ Toxicity Assay. On the basis of differential CdCl₂ toxicity, colonies arising from cisplatin-sensitive cell populations can be selectively eliminated. A first experiment determined the drug concentration yielding the best growth in treated and untreated spheroids.
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Spheroid Growth Characteristics. While cisplatin-sensitive and -resistant cell lines showed comparable exponential growth rates in monolayer cultures, spheroids raised from sensitive cells alone were growing more quickly than purely resistant spheroids. At 10 days after initiation, spheroid volume-doubling times, calculated from growth data fitted to a power function (Fig. 1), were 7 days for sensitive as well as for 1:1 sensitive:resistant mixed spheroids and 11 days for resistant spheroids. At 3 days, when irradiation experiments were performed, spheroids consisted of viable cells. Central necrosis did not develop before day 7.

The effect of graded doses of cisplatin on cell survival, i.e., colony formation of cisplatin-resistant and -sensitive cells, was compared both in monolayer cell cultures and in homogeneous spheroids. Resistant cells were more cisplatin resistant by a factor of 4.5 when grown as monolayer cell culture and by a factor of 4.1 when grown as spheroids, indicating that cisplatin resistance was retained when cells were grown in spheroids.

Distinction of Sensitive and Resistant Spheroid Subpopulations.

In Fig. 2, the response of large colonies comprising more than 2000 cells of either sensitive or resistant cells to increasing concentrations of CdCl₂ is presented. Colony survival was assayed 2 days after CdCl₂ treatment. By this time, sensitive colonies had perished, i.e., were not develop before day 7.

The extent of irradiation was carried out in spheroids or monolayer cultures. Postirradiation, single-cell suspensions were prepared, and cells were plated and incubated for 7–10 days. At this time, surviving sensitive cell colonies were eliminated from mixed clone populations by treatment with 600 μM CdCl₂ carried out as described above. From the difference in total colony number assessed in parallel dishes with and without CdCl₂ treatment, the proportion of surviving cisplatin-sensitive cells was calculated.

β-Galactosidase Marker Gene. Labeling of cells with a marker gene was carried out before initiation of mixed spheroids. Recipient cells were infected with supernatant of a population of retrovirus packaging cells (GP+E86) (10) stably transfected with the retroviral vector pBag (11). SSK-R and SSK-S cells (n = 5 X 10⁶) were seeded in 12- × 5-cm glass flasks (Breed-Demeter & Schott, Mainz, Germany) 24 h before infection. Two ml of supernatant were harvested and filtered through a 0.45 μm filter. After addition of Polybrene (8 μg/ml) the supernatant was used to infect cisplatin-resistant or -sensitive cells. After 4 h, the Polybrene concentration was reduced to 2 µg/ml by adding 6 ml of normal medium. Cells were then incubated for 2 days, corresponding to a minimum of 2 or 3 cell generation times. For identification of successfully infected cells, single-cell suspensions were then exposed to selective conditions. Since the vector contained the neomycin resistance gene, cells were incubated with DMEM with 12% FCS and 800 mg/ml G418 (neomycin analogue) added. After 3 days the G418-containing medium was changed. Following a selection period of 7–10 days, G418-resistant colonies were clearly visible and could be culled. In monolayers arising from single G418-resistant colonies, the clones with the most intensive β-galactosidase enzyme reaction were selected.

The galactosidase staining was carried out according to the method of Price et al. (12). Briefly, cells were washed with ice-cold PBS, fixed with 2% paraformaldehyde in PBS for 20 min, and washed again in three changes of PBS for at least 10 min. Cells were exposed to staining solution, consisting of X-Gal (Sigma) dissolved at 40 mg/ml in dimethylformamide (Sigma) and diluted to a final concentration of 1 mg/ml in 20 mM K₄Fe(CN)₆, 20 mM K₃Fe(CN)₆·3H₂O, and 2 mM MgCl₂ in PBS. The blue indigo stain appeared after a 4–8 h incubation period at 37°C. β-Galactosidase-negative cells were counterstained with nuclear fast red for 2 min.

In order to identify galactosidase-positive cells in mixed spheroids, the same staining procedure was used with intact spheroids except that the incubation time in the X-Gal-staining solution was extended overnight to allow penetration of the spheroid to obtain equally intensive X-Gal staining of β-galactosidase-positive cells in the spheroid center as in the periphery. For histological examination, spheroids were embedded in glycol acrylate (Technovit 8100; Heraeus Kulzer, Wehrheim, Germany), sectioned, and counterstained with nuclear fast red. Plastic embedding was preferred to conventional histological techniques because it provides better structural preservation of fragile spheroids and retains enzyme activity.

RESULTS

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survival of sensitive colonies at 2 days to about 0.04% while survival of resistant colonies is hardly affected. Based on these results a concentration of 600 μM was chosen to eliminate sensitive colonies in mixed populations to quantify the proportions of subpopulations. The discrimination of sensitive and resistant cells is therefore based on acute CdCl₂ toxicity and does not assess clonogenic cell survival.

Both sensitive and resistant cells could be successfully infected and selected in G418 medium. Resulting colonies were picked and tested for β-galactosidase expression. The best performing clones were expanded and used to generate mixed spheroids with noninfected cells. Infection of either resistant or sensitive cell lines with the galactosidase marker gene yielded 3000–5000 colony-forming units/ml of infectious medium. It should be stated that the vector is infectious but replication deficient after incorporation in the target cell genome.

The detection of infected cells in situ is based on the conversion of X-Gal into a blue stain by β-galactosidase. Fig. 3, A and B, shows a histological section through a 4-day-old mixed spheroid, where the sensitive cells have been infected with the retroviral vector and are galactosidase positive. After staining with X-Gal, the transfected cells appear blue and can be readily distinguished from X-Gal-negative resistant cells. The morphological appearance of resistant and sensitive cells in the spheroid is indistinguishable.

**Comparison of CdCl₂ Toxicity Assay and Labeling with the Galactosidase Marker Gene.** A comparison of the CdCl₂ toxicity and gene labeling method was carried out in colonies grown from a mixture of defined numbers of sensitive and resistant cells. In these experiments, alternatively the sensitive or the resistant subpopulation was used as the labeled species. Colonies were subjected to CdCl₂ or to G418 selective culture medium or were stained for galactosidase activity. The number of colonies surviving CdCl₂ treatment must represent cisplatin-resistant cells, while colonies surviving G418 selection medium as well as those staining positively for β-galactosidase must represent the cell population infected with the marker gene. X-Gal staining allows simultaneous visualization and identification of the progeny of both β-galactosidase-positive and -negative colonies. The quantitative results are summarized in Table 1. Although the plating efficiency is different in the two parallel experiments, there is
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Table 1 Comparison of distinction of cisplatin-sensitive and -resistant cell colonies by differential CdCl₂ toxicity and by infection with the galactosidase marker gene

<table>
<thead>
<tr>
<th>Plated no. of cells</th>
<th>Total no. of colonies</th>
<th>CdCl₂</th>
<th>G418</th>
<th>X-Gal</th>
</tr>
</thead>
<tbody>
<tr>
<td>S° β-gal+</td>
<td>201.5 ± 16.3</td>
<td>117 ± 12.8</td>
<td>102 ± 7.4</td>
<td></td>
</tr>
<tr>
<td>R β-gal-</td>
<td>PE = 67.2%</td>
<td>92 ± 6.7</td>
<td>88 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>S β-gal-</td>
<td>297 ± 17</td>
<td>136 ± 8.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R β-gal+</td>
<td>PE = 99%</td>
<td>129 ± 14.8</td>
<td>126 ± 10.5</td>
<td>149 ± 7.1</td>
</tr>
</tbody>
</table>

*S*, sensitive; *R*, resistant; PE, plating efficiency.

...a very good correlation between the different methods used to identify the subpopulations.

Development of Sensitive and Resistant Subpopulations in Growing Mixed Spheroids. Fig. 4 illustrates the growth of resistant and sensitive cells in mixed spheroids initiated with various starting proportions based on determination using the CdCl₂ toxicity assay. While sensitive cells grow continuously until stationary phase, resistant cells grow less regularly in the initial phase and in spheroids with starting proportions of 1:1 or 1:2 they become stationary or begin to decline after day 8, as a result they are eventually entirely overgrown by the sensitive cells. In spheroids, which were initiated at a starting proportion of 1:9, a balance between sensitive and resistant cells is reached around day 8 and is then approximately maintained throughout the remaining observation time. In general, these growth patterns are similar for small (i.e., 500) and large (i.e., 5000 or 9000) absolute cell numbers initially inoculated (Fig. 4).

In Fig. 5 a histological section through a 10-day-old mixed spheroid with β-galactosidase-positive sensitive cells is demonstrated. There is an accumulation of blue-staining cells in the vial indicating that the unlabeled resistant cells have been outgrown and displaced toward the metabolically compromised spheroid center, bordering on necrosis. When sensitive and resistant cells are seeded in a ratio of 1:9, the resulting spheroids show a 1:1 ratio of the 2 subpopulations at day 10.

Comparison of β-galactosidase staining intensity in Figs. 3a and 5 also demonstrates that enzyme expression has stayed constant over multiple cell generations.

Radiation Response of Subpopulations of Mixed Spheroids. Fig. 6 demonstrates survival of cisplatin-sensitive and -resistant cells grown in mixed spheroids and exposed to graded doses of irradiation. Spheroids were trypsinized immediately after treatment, and after colony formation the survival of subpopulations was determined. Sensitive and resistant cells were identified by CdCl₂ toxicity, by incubation with G418 selective medium, or by β-galactosidase staining. Again there is very good agreement between the different assays (Fig. 6). From the averaged survival curves, the radiation response appears to be similar for the two lines. When fitted by the single-hit multitarget model (13), the *D₀* values, designating the terminal slope of the survival curve as a measure of intrinsic radiosensitivity, were 1.9 and 2.3 Gy for the drug-resistant and drug-sensitive cells, respectively. The extrapolation numbers, which are an indirect measure of the width of the initial shoulder of exponential survival curves, were 2.7 and 1.8, respectively. These values are in agreement with those of other murine tumor cell lines (14).

DISCUSSION

The presence of drug-resistant cell populations in a tumor and their possible interaction are important subjects of study. Mixed tumor spheroids are an experimental model suitable for investigating such interactions, provided that subpopulations and their progeny can be quantitatively differentiated in a spheroid exposed to treatment. In the present study a cisplatin-resistant mouse fibrosarcoma cell line and its cisplatin-sensitive parent cell line were used to grow mixed spheroids.

Cisplatin-resistant tumor cells started growing in spheroids only after a lag phase of at least 4 days. Thereafter, their growth rate was comparable to those of sensitive cells. Miller et al. (3) report mixed tumors composed of thioguanine-sensitive and -resistant mouse mammary carcinoma cells. In their model a pronounced growth inhibition of sensitive cells is induced by contact with resistant cells. Even when the initial cell number of sensitive cells exceeds resistant cells by a factor of 100, the population will eventually be predominated by resistant cells. Thus the differential growth behavior of drug-sensitive and drug-resistant subpopulations in a spheroid is very variable and appears to be cell line specific.

During spheroid growth, the initial proportions of sensitive and resistant cells are likely to change. In addition, the localization of subpopulations within the spheroid with respect to nutrient gradients influence their treatment response. Thus interpretation of observations in mixed spheroids requires quantitative and topographic identification of tumor cell subpopulations at all growth stages as well as after treatment. One aim of the present study was therefore to define growth properties and therapeutic response of the two populations when cocultured in mixed spheroids. This requires reliable methods to discriminate between morphologically indistinguishable subpopulations, both in situ, i.e., in spheroids, and in cell suspension.

This study presents and compares two different techniques of differentiating sensitive and resistant cells. CdCl₂ toxicity serves as a reference technique and is limited in its applicability to the particular cell line used in the present study. A variety of molecular mechanisms...
Fig. 5. Photomicrograph of a histological section through a 10-day-old spheroid, grown from a 1:1 mixture of cisplatin-sensitive and -resistant cells. The cisplatin-sensitive cell population was labeled with the β-galactosidase marker gene and appears bright blue after enzyme reaction with X-Gal. Sensitive cells accumulate in the viable outer layers of the spheroid. They have outgrown resistant cells and displaced them to the more central layers bordering necrosis. × 300.

has been associated with cisplatin resistance, including increased DNA repair (15–17), enhanced drug detoxification by protein or nonprotein thiols (S18, 7), or reduced drug accumulation (19, 20). In the SSK cell line used in the present study, an increase in drug-inactivating metallothioneins was recently recognized as the overriding mechanism of cisplatin resistance. The high metallothionein content of cisplatin-resistant cells is associated with resistance to the cytotoxic effect of CdCl₂.

Comparison of various sensitive, resistant, as well as reverted resistant cells, which have at least partly regained their cisplatin sensitivity, showed a good correlation between CdCl₂ toxicity and cisplatin resistance in some but not all cell lines (6, 7, 21). The technique of induction of cisplatin resistance appears to modulate the mechanism of resistance and hence the correlation between cellular metallothionein content and cisplatin resistance. In monolayer cultures of the cell line used in the present study, the effect of CdCl₂ on clonogenic cell survival was tested. A 600 μM concentration of CdCl₂ can be expected to reduce clonogenic survival to 0.01 and 2% in sensitive and resistant sublines, respectively (6). This implies that a large number of cells, even in resistant colonies which are nearly unchanged at 2 days after CdCl₂ treatment, will perish in the following period. However, both the acute toxicity assay used in the present study for distinction of cell colonies surviving irradiation and the clonogenic survival after CdCl₂ tested in monolayer cultures show a difference in response of sensitive and resistant cells by a factor of several orders of magnitude. This cell line is therefore particularly well suited for the experiments reported because differential CdCl₂ toxicity can be used to effectively eliminate sensitive cells from a mixed population and can thus serve as a reference method. A similar approach of differentiating sensitive and resistant spheroid subpopulations by identifying factors associated with the specific mechanism of resistance was presented by Tofilon et al. (22). In rat brain tumor cells, 1,3-bis(2-chloroethyl)-1-nitrosourea-induced DNA damage is correlated to sister chromatid exchange; i.e., 1,3-bis(2-chloroethyl)-1-nitrosourea-resistant cells are characterized by the absence of such changes. Using this assay, resistant and sensitive spheroid subpopulations could be quantified. Similar to the CdCl₂ toxicity assay, this technique does require disaggregation of spheroids and therefore does not retain topographic information. Furthermore, it is also limited in its applicability to the experimental model and drug as presented.

In contrast to these two assays, topographic information can be retained by labeling subpopulations of tumor cell with a membrane-binding fluorescent dye (23). During cell division the label is transmitted to daughter cells and can be detected by fluorescence-activated cell-sorting analysis or histologically in cryostat sections. The number of marker molecules per cell, however, is halved with every cell division; therefore the signal intensity is diluted below detectability within 5 or 6 cell divisions. In our own pilot experiments we found in addition that the marker became partly liberated during spheroid disaggregation, with the result of contaminating primarily unlabeled cells.3

Infection of subpopulations with a retroviral vector carrying a marker gene provides not only important topographic information but also because of the stable integration of the vector in the host genome, the marker gene is fully transferred to daughter cells over a theoretically unlimited number of generations. The technique of infection

3 K. R. Frenzel, unpublished data.
proved to be effective and stable. Even after multiple cell doublings, infected cells could be easily recognized with the simple galactosidase enzyme reaction in histological sections. Thus the changes in proportions of resistant and sensitive cells during growth of mixed spheroids, their respective localization within the spheroid, as well as their differential response to radiation could be quantitatively determined on a single-cell basis. Since this technique is largely independent of specific characteristics, i.e., origin or mechanism of drug resistance, it offers a wide range of applications beyond the specific aim of the present study and opens new perspectives of investigating cell interactions in the spheroid model.

REFERENCES
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