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Evaluation of a Tetrazolium-based Semiautomated Colorimetric Assay: Assessment of Radiosensitivity[1]

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ABSTRACT

Radiation survival curves were generated for V79 Chinese hamster and two human lung cancer cell lines (NCI-H460 and NCI-H249) with doubling times of 10, 20, and 85 h, respectively, using a standard clonogenic assay, a dye exclusion assay, and a semiautomated colorimetric assay utilizing a tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide. Comparable results for D_0 and extrapolation number (n) were observed for all assays in the lines with doubling times of 10 and 20 h. In these instances the tumor cell lines had undergone seven or more doublings after radiation. For the tumor line (H249) with an 80-h doubling time the D_0s were comparable between the assays while the extrapolation number was increased in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide assay, a result probably related to the lower number of doublings (<4) after radiation. We then tested the ability of the assays to detect radiation protection and sensitization using known agents. We found that cysteamine treatment resulted in radioprotection (by a factor of 8 at 8 Gy) while 5-bromo-2-deoxyuridine incorporation caused enhancement of radiation sensitivity in all three assays. We conclude that, while optimal conditions for each cell line (cell number plated and doubling time) must be established, using characterized tumor cell lines, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide assay could be automated and thus be of great value in screening large numbers of potential radiosensitizers or protectors.

INTRODUCTION

Radiation sensitization has been assessed in numerous cell lines over the past 20 years, with clonogenic survival generally considered the optimal assay method. Although evidence was forthcoming that information on radiation survival could be obtained by back extrapolation of growth curves (1), the clonogenic assay has remained the investigation of choice following the original description of the technique in 1955 (2). Although short and intermediate term nonclonogenic assays have been tested in the assessment of drug sensitivity (3–7), many of these would be considered inadequate to measure radiation sensitivity. This mainly relates to the short duration of the assays. Following a dose of radiation, cells destined to die undergo one or more cell divisions (8, 9). Therefore, a considerable period of time may elapse before these sterilized cells express their damage via a mitotic linked death (10–12) and are subsequently lost from the population. During this time interval, lethally irradiated cells appear to function normally with respect to metabolism (13) and maintenance of intact cell structure (membranes). Many nonclonogenic assays quantitate cell number based on certain metabolic functions, such as tritiated thymidine incorporation (14, 15), glucose utilization via the pentose phosphate cycle (16), reduction of various drugs (17), and automated image analysis of crystal violet-stained cells (18). If assays such as these are used for radiation survival assessment, it is important that adequate time following the radiation exposure elapse, such that sterilized cells express their damage and are lost from the population prior to performing the assay. We chose to investigate whether the nonclonogenic assays described in the previous study (19) could be modified to predict radiation sensitivity. In addition, because there has been great interest recently in the study of radioprotectors and radiosensitizers, we wondered whether these assays could provide a rapid, simple, but reliable method of detecting radioprotection or radiosensitization.

MATERIALS AND METHODS

Cell Lines. The cell lines used in this study were Chinese hamster V79 cells and 2 human lung cancer cell lines, NCI-H249, a small cell carcinoma, and NCI-H460, a large cell carcinoma. Cell cultures were maintained as described previously (19) with growth rates and plating efficiencies as shown.

Clonogenic Assay. Single cell suspensions were obtained as described previously and cells were counted using an Elzone particle counter (Particle Data, Inc., Elmhurst, IL). Cells were then aliquoted into test tubes at a concentration of 10^5 cells/ml, with irradiation carried out at room temperature over a dose range of 0–14 Gy in V79 cells, 0–10 Gy in NCI-H460 cells, and 0–5 Gy in NCI-H249 cells. After irradiation, cells were recounted and plated on Petri dishes (V79 and NCI-H460) or in soft agar (NCI-H249) using previously described methods (20). Individual survival points were plated in triplicate, with experiments repeated 3 times. V79 cells were inoculated, using 100 cells for control plates, with higher cell numbers for treated plates, and incubated for 6 days. Control NCI-H460 plates were inoculated with 250 cells, with higher cell numbers for irradiated plates, following which plates were incubated for 10 days; the colonies were fixed, stained, and counted. Control NCI-H249 dishes were plated with 10,000 cells/dish, following which plates were incubated for 18 days in agar, prior to counting of colonies.

MTT[3] Assay. A similar modification of the MTT assay was used, as described previously (19), with mineral oil used to resolubilize V79 and NCI-H460 plates and dimethyl sulfoxide used for NCI-H249. Following irradiation, cells were plated in microwell plates at the appropriate inoculum. Seeding cell number varied between cell lines to ensure adequate absorbance readings in control wells, with 100 cells/well used for V79, 250 cells/well for NCI-H460, and 2000 cells/well for the NCI-H249 cell line. Where possible, assays were run for a minimum of 6 cell-doubling times; times for each cell line were 3 days for V79 cells (7 doubling times), 7 days for NCI-H460 cells (7 doubling times), and 14 days for NCI-H249 cells (4 doubling times). Plates containing NCI-H249 were centrifuged at 450 $\times g$ for 5 min on day 8, 100 μl of medium were removed from each well, and 100 μl of fresh medium were added. Optimally, an incubation time of 21 days would have been appropriate for NCI-H249 cells. However, refeeding of cells can lead to problems of contamination and loss of cells during aspiration of medium, with evaporation of medium also a problem with prolonged incubation. For these practical considerations, incubation was limited to 14 days in this cell line. A minimum of 16 wells per survival point were plated for each cell line, with cells plated in 0.2 ml of culture medium. Following the appropriate incubation period, 0.1 mg MTT (50 μl of 2-mg/ml solution)

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[3] The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan bromide (thiazolyl blue); BrdUrd, 5-bromo-2-deoxyuridine.

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was added to each well, followed by a further 4-h incubation. The medium from culture plates containing adherent cells was then aspirated using a fine-point Pasteur pipet and the crystals were solubilized using mineral oil. NCI-H249 plates were centrifuged at 450 × *g* for 5 min, the majority of the medium was removed carefully, and the crystals were solubilized in dimethyl sulfoxide as described in Part 1. All experiments were repeated a minimum of 3 times. Multiwell plates were assayed during a scanning multiwell spectrophotometer (enzyme-linked immunosorbent assay reader; Biotek Instruments, Inc., Burlington, VT) at wavelengths of 540 nm for dimethyl sulfoxide and 570 nm for mineral oil.

Dye Exclusion Assay. Following irradiation in test tubes at a concentration of 10^5 cells/ml, cells were diluted appropriately and seeded into conical polypropylene tubes as described previously (19). One ml of cell suspension was added to each tube. In control tubes cell numbers of 500/ml for V79 and NCI-H460 cells and 2000/ml for NCI-249 cells were used. Higher cell numbers, up to 10^5 cells/ml, were used at higher radiation doses to define a surviving fraction. The tubes were incubated at 37°C for 3 days for V79 cells, 7 days for NCI-H460 cells, or 14 days for NCI-H249 cells. In the latter assay, 1 ml of fresh medium was added to each tube on day 8. As with the MTT assay, an incubation time of 21 days would have been more appropriate. However, a 14-day incubation was used so that both nonclonogenic assays could be directly compared. Following incubation, cytospin specimens were prepared as described previously (19), following addition of the aldehyde-fixed duck RBC. Slides were counted using a light microscope at ×400. The viable tumor cell/duck RBC ratio was determined for each radiation dose and compared to control values.

Radiation. Cell suspensions were irradiated at room temperature using a 15-MeV photon beam from a linear accelerator at a dose rate of 2 Gy/min.

Drugs. Studies involving radiosensitization and radioprotection were limited to the V79 cell line. Cysteamine (Sigma Chemical Co., St. Louis, MO), 10 mM, was used as a radioprotector. For these studies cells were suspended at a concentration of 10^5 cells/ml in test tubes with or without 10 mM cysteamine, and cells were irradiated within 10 min of drug addition. Following irradiation, test tubes were centrifuged at 250 × *g* for 5 min, the supernatant was aspirated, and the cells were washed twice in phosphate-buffered saline. Cells were counted and then plated in all three assays simultaneously. BrdUrd (Sigma) was used as a radiosensitizer. For these studies, exponentially grown cultures were exposed to 10^{-5} M BrdUrd for 15 h (approximately 2 cell doublings). This dosage of BrdUrd caused approximately 15% thymidine replacement (21). Immediately prior to radiation cells were trypsinized, washed twice in phosphate-buffered saline, resuspended in Ham's F-12 medium, and counted using a particle counter. Cells were suspended at a concentration of 10^5 cells/ml, irradiated, and then plated in all three assays. Triplicate assays were performed at all radiation doses, and the experiment was repeated on three occasions.

Statistical Methods. Standard errors were calculated for radiation survival curves according to the method of Bevington (22). Error bars are shown on the figures when larger than the diameter of the symbol.

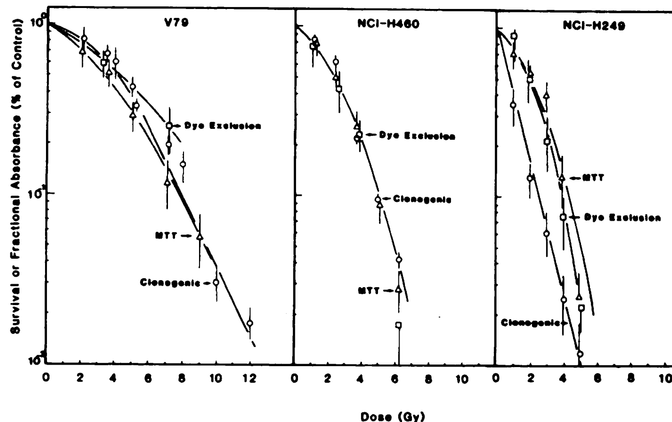

Fig. 1. Assessment of survival (percentage of control) using clonogenic, MTT, and dye exclusion assays for three different cell lines as a function of radiation dose. *Bars*, SE.

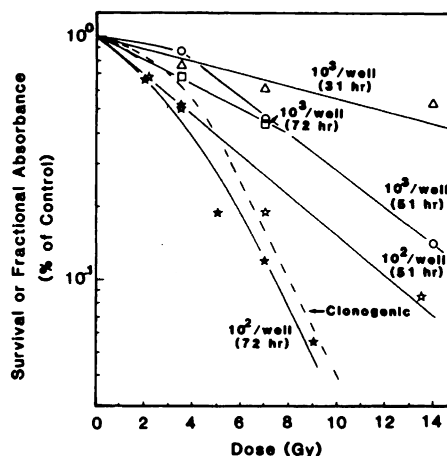

Fig. 2. X-ray survival curves (V79 cells) for the MTT assay where the time of assay and number of cells/well are varied. ----, a clonogenic X-ray survival curve replotted from Fig. 1.

RESULTS

Radiation survival curves derived from all three assays for V79 cells are illustrated in Fig. 1, *left*. No significant difference was seen between the MTT and clonogenic assays at any radiation dose, with D_0s of 1.9 and 2.0 and extrapolation numbers $n = 4.5$ and $n = 2.5$, respectively. Unfortunately, insufficient data were generated to obtain D_0 and n values from the dye exclusion assay data. Radiation survival curves for NCI-H460 cells with all three assays were virtually identical, exhibiting a D_0 of 1.5 Gy and extrapolation number $n = 3.0$. These results are illustrated in Fig. 1, *center*. Greater variance was observed with NCI-H249 cells; however, as shown in Fig. 1, *right*. D_0s were similar between assays, but extrapolation number was higher as depicted by the nonclonogenic assays. The difference may well be related to inadequate assay time, because this cell line is relatively slowly growing, with the clonogenic assay optimally read at day 18. D_0s were 1.0, 0.85, and 0.94 using clonogenic assay, dye exclusion, and MTT assay, respectively. The corresponding extrapolation numbers were 1.0, 4.5, and 4.0 with these results depicted in Fig. 1, *right*.

The MTT results described thus far were achieved following the definition of optimal seeding cell number and assay duration time. Fig. 2 illustrates the effect of altering these parameters on the radiation survival curve of V79 cells. As can be seen, by seeding too many cells or by shortening the duration of the assay the results can be potentially invalidated. At 31 and 52 h this may be due to persistent dehydrogenase activity in cells destined to die, whereas at 72 h seeding 1000 cells/well, confluence of control wells is a major factor.

The effect of cysteamine and BrdUrd on V79 radiation survival curves generated by all three assays is shown in Fig. 3. As can be seen in Fig. 3, *left*, using the clonogenic assay, marked radioprotection was observed for cysteamine treatment. Similar results were achieved using both the dye exclusion assay and the MTT assay. Cysteamine treatment resulted in an increase in survival of 9-, 8-, and 6.5-fold at 8 Gy for the clonogenic, MTT, and dye exclusion assays, respectively. Likewise, BrdUrd treatment resulted in enhancement of radiation effect. Enhancement ratios (control radiation dose divided by BrdUrd dose at 10% survival) were 1.9, 1.9, and 1.3 for clonogenic, MTT, and dye exclusion assays, respectively.

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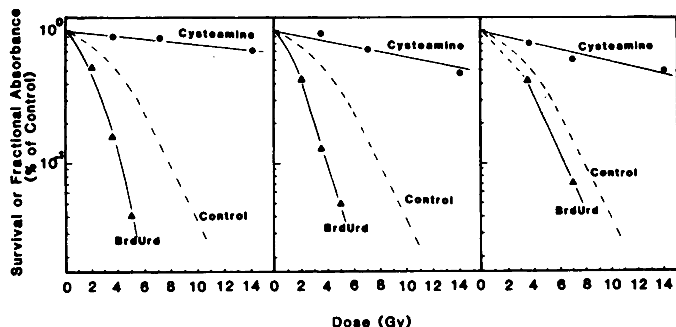

Fig. 3. X-ray survival curves (V79 cells) assessed by clonogenic (*left*), MTT (*middle*), and dye exclusion (*right*) assays. ●, 10 mM cysteamine, 10 min prior to X-ray exposure; ▲, cells were grown approximately 2 cell doublings in the presence of 10^{-5} M BrdUrd; ——, clonogenic X-ray survival curve replotted from Fig. 1.

DISCUSSION

The nonclonogenic assays used in this study give reproducible and comparable results to the clonogenic assay. Better correlations between nonclonogenic and clonogenic assays were observed for the adherent cell lines NCI-H460 and V79. Although the D_0s among the assays for NCI-H249 were similar, a significant increase in extrapolation number was observed using the nonclonogenic assays. This difference in extrapolation number is almost certainly due to inadequate incubation time for nonclonogenic assays, using NCI-H249 cells, where only 4 population-doubling times were allowed. However, there are practical problems that limit the duration of the MTT assay. Evaporation of medium can be problematical with prolonged incubation, particularly in the outer wells, and refeeding should be performed at least once, but optimally twice weekly. This can cause problems of contamination, and also because medium must be removed prior to the addition of fresh medium, cells can be lost, particularly with floating cell lines. There are a number of potential causes for the increase in extrapolation number. The incubation time of these experiments allowed only 4 cell divisions, insufficient time to allow full mitotic linked death, particularly at low radiation doses, where a great number of divisions are required (23). Using the dye exclusion assay cells destined to die require several days to lose their membrane integrity. Similarly, it is possible that dehydrogenase activity is maintained for some time after cells have been killed. These factors could certainly result in an artifactual shoulder on the radiation survival curve.

Using the MTT assay, as shown in Fig. 2, optimal seeding cell number and assay duration require elucidation for each cell line. It is essential that sufficient time is allowed for cell death and loss of dehydrogenase activity and that all treatment groups, particularly control cells, remain in exponential growth up to the point of assay. Similarly, using the dye exclusion assay cell number plated is critical, in that 5×10^4–10^5 viable cells/tube in control samples at the time of reading the experiment is optimal. Obviously, similar conditions apply to the clonogenic assay, but these have previously been established.

The degree of enhancement observed with BrdUrd incorporation, a well-known radiosensitizer (24, 25), was quantitatively and qualitatively similar using all three assays. Likewise, cysteamine, a known radioprotector (26), was shown to exhibit similar degrees of radioprotection in all three assays with the degree of protection similar to that previously reported (27). Thus both nonclonogenic assays could be used for the assessment of radiation sensitivity, but the dye exclusion assay is unlikely to be utilized for this purpose, as it is fairly labor intensive, particularly at the time of reading the cytospin preparations. In contrast, the MTT assay is semiautomated, and could potentially test a large number of samples over a short time period. Of particular interest would be the use of this assay to screen potential radioprotectors and radiosensitizers. For these studies we would recommend the use of V79 cells as used in this study, although should a human cancer cell line be considered advantageous, a rapidly growing cell line such as NCI-H460 would be acceptable. This assay could also be used in the screening of potential hypoxic cell sensitizers.[4]

In conclusion, nonclonogenic assays give reproducible and comparable results to those for clonogenic assays in the assessment of radiation survival. Minimal variation in values was observed for adherent cell lines, with the standard deviation generally less than 10%. The MTT assay offers a rapid, simple method for the assessment of radiation sensitivity in selected cell lines, with the potential for use in the screening of compounds for radiosensitization and radioprotection.

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[4] J. M. Brown, personal communication.

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