

Tumor Spheroid Model for the Biologically Targeted Radiotherapy of Neuroblastoma Micrometastases¹

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Abstract

Neuroblastoma is a pediatric malignancy with a poor prognosis at least partly attributable to an early pattern of dissemination. New approaches to treatment of micrometastases include targeted radiotherapy using radiolabeled antibodies or molecules which are taken up preferentially by tumor cells. Multicellular tumor spheroids (MTS) resemble micrometastases during the avascular phase of their development. A human neuroblastoma cell line (NBI-G) was grown as MTS and incubated briefly with a radiolabeled monoclonal antibody (¹³¹I-UJ13A) directed against neuroectodermal antigens. Spheroid response was evaluated in terms of regrowth delay or proportion sterilized. A dose-response relationship was demonstrated in terms of ¹³¹I activity or duration of incubation. Control experiments using unlabeled UJ13A, radiolabeled non-specific antibody (T2.10), radiolabeled human serum albumin, and radiolabeled sodium iodide showed these to be relatively ineffective compared to ¹³¹I-UJ13A. The cell line NBI-G grown as MTS has also been found to preferentially accumulate the radiolabeled catecholamine precursor molecule *m*-[¹³¹I]iodobenzylguanidine compared to cell lines derived from other tumor types. NBI-G cells grown as MTS provide a promising laboratory model for targeted radiotherapy of neuroblastoma micrometastases using radiolabeled antibodies or *m*-iodobenzylguanidine.

Introduction

Neuroblastoma is a pediatric malignancy which derives from neuroectodermal cells of the sympathetic nervous system. Early dissemination is the rule and accounts for the poor prognosis of this tumor, which is usually sensitive to both radiation and cytotoxic drugs (1). Treatment of neuroblastoma micrometastases is an important clinical problem and one for which targeted radiotherapy, the selective delivery of radionuclides attached to molecules with preferential affinity for tumor cells, provides a promising approach.

Usually, targeted radiotherapy is considered in terms of radiolabeled antibodies, but neuroblastoma provides an example of a tumor for which radionuclide delivery might be achieved by radiolabeled molecules which are precursors of molecules synthesized by tumor cells. Neuroblastoma cells often synthesize catecholamines, as do normal cells of sympathetic nervous type, and preferentially accumulate catecholamines in cytoplasmic organelles (neurosecretory granules). The radiolabeled catecholamine precursor *m*-[¹³¹I]iodobenzylguanidine is presently receiving attention as a possible means of selectively targeting radiotherapy to neuroblastoma cells (2). It is not yet known whether monoclonal antibodies or tumor product precursors like mIBG² will prove superior as delivery vehicles for biologically targeted radiotherapy.

It would be very useful if it were possible to develop a laboratory *in vitro* model for targeted radiotherapy of neuroblastoma micrometastases. Although some problems are amenable to study only in the clinical setting, others may be capable of solution in a simpler context. Multicellular tumor spheroids

represent a tumor model the complexity of which is intermediate between that of conventional monolayer culture and *in vivo* models such as xenografts. MTS are essentially growing aggregates of tumor cells in suspension culture. These may be grown from clumps of a few cells to visible structures of 1–2 mm diameter with cell content up to 10⁶ cells. Beyond this, growth is limited by diffusion of oxygen and nutrients leading to an inner necrotic core within which death comes to balance new cell production by continuing mitosis in the rim of the spheroid. The biological structure and range of applications of MTS have been reviewed recently by Sutherland (3). Of importance here is the resemblance to micrometastases during the avascular phase of their development and the opportunities to study phenomena such as penetration of cytotoxic agents and dependence of treatment effectiveness on spheroid size. Applications of the MTS model in the context of targeted therapy have, however, only begun to be explored. We report, in this paper, the suitability of a human neuroblastoma cell line (NBI-G) grown as MTS to provide an *in vitro* model for targeted radiotherapy using radiolabeled antibodies or mIBG.

Materials and Methods

Cell Lines. NBI-G is a cell line derived from human neuroblastoma the characteristics of which have been described previously (4). Briefly, NBI-G cells have been confirmed as human neuroblastoma by cytogenetic and immunological methods. The human oncogene *N-myc* was found to be amplified 20–24 times. The cells are moderately radiosensitive as is typical for neuroblastoma cell lines. Other cell lines were used for comparison with NBI-G. Cell line A2780 was derived from human ovarian carcinoma.³ Cell line B008 was derived from human malignant melanoma (5). Each cell line was multiply passaged in conventional monolayer culture (Eagle's minimum essential medium) supplemented with 15% fetal calf serum, 2 mM L-glutamine, and antibiotics at 5% CO₂, 37°C, and 100% humidity) prior to spheroid culture. All media and supplements were obtained from Flow Laboratories, Rickmansworth, Hertfordshire, United Kingdom.

Spheroid Culture. MTS were initiated from dispersed monolayer cultures by the method of Yuhas *et al.* (6) (B008) or in magnetic stirrer flasks (Techne, Cambridge, United Kingdom; NBI-G and A2780). Following treatment (see below) MTS were transferred individually into agar-coated wells of 24-well test plates (Linbro) and spheroid growth was quantified by regular measurement of cross-sectional area of individual spheroids.

Antibodies and Chemicals. UJ13A, a neuroectodermal specific murine monoclonal antibody, has been shown to bind to neuroblastoma cells by indirect immunofluorescence staining (7). The murine monoclonal antibody T2.10, used as control, has been shown not to bind to neuroblastoma cells. Radioiodination of both antibodies was by the Iodo-Gen method. Some experiments were carried out using ¹³¹I-human serum albumin (also radioiodinated by the Iodo-Gen method) and Na¹³¹I. Diagnostic grade [¹³¹I]mIBG was obtained from Amersham International.

Treatment Procedures. In antibody experiments, aliquots of about 40 spheroids (diameter, 200 μm) were transferred into universal containers and incubated in 5 ml medium containing 15% fetal calf serum and 44 mM NaI. To the spheroids were added ¹³¹I-UJ13A, ¹³¹I-T2.10, ¹³¹I-

³ R. Ozols, personal communication.

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² The abbreviations used are: mIBG, *m*-iodobenzyl-guanidine; MTS, multicellular tumor spheroids.

human serum albumin, or Na¹³¹I in various additives, (specific activity, ~0.3 MBq/μg protein) and the spheroids were incubated at 37°C for up to 2 h. Following the incubation period, the spheroids were washed by allowing them to sediment and draining off the incubation medium. This was replaced with 5 ml of fresh medium containing 44 mM NaI. This procedure was repeated 6 times in total. Spheroids were then transferred individually to multiwell test plates and their growth was monitored as described above.

In mIBG experiments, spheroids 300 μm in diameter were placed individually in 1-ml sterile Eppendorf microfuge tubes, with Eagle's minimum essential medium. To each spheroid, 37 kBq (1–5 μCi/mg) [¹³¹I]mIBG was added. After incubation at 37°C for 2 h, the spheroids were washed with Eagle's minimum essential medium until the activity of the supernatant approached background levels. The spheroids were then transferred to fresh Eppendorf tubes and the retained activity was determined by gamma counting using a well-type scintillation counter.

Results

Fig. 1 shows in summary form the relative effects of similar activities of ¹³¹I-UJ13A and Na¹³¹I following incubation for 2 h, in terms of delaying the growth of NBI-G spheroids. Growth delay was defined as median time to reach 10 times the treatment volume. The results for unlabeled UJ13A, ¹³¹I-T2.10, and ¹³¹I-human serum albumin were identical to those for Na¹³¹I and are omitted for clarity. Up to an activity of almost 30 MBq, Na¹³¹I had no detectable effect on growth of NBI-G spheroids, the time to reach 10 times the original treatment volume (7 days) not differing significantly from that for untreated controls. By contrast, as the activity of ¹³¹I-UJ13A was increased from 0 to 28 MBq the regrowth delay increased from 9 days to about 25 days.

Fig. 2 shows the loss of radioactivity from three lines of tumor spheroids (NBI-G, A2780, B008) following 2 h incubation with [¹³¹I]mIBG as a function of number of washings postincubation. It can be seen that activity is more greatly retained by NBI-G spheroids. By the sixth (usually final) wash, NBI-G spheroids retained 3 times more activity than A2780 spheroids and 10 times more activity than B008 spheroids.

Discussion and Conclusions

These experiments demonstrate targeted radiotherapy of NBI-G spheroids in the laboratory setting. The dose-response

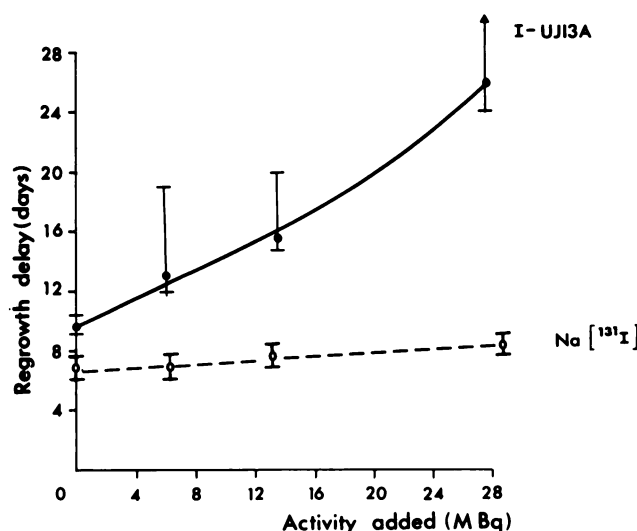


Fig. 1. Time taken for median volume of NBI-G spheroids to reach 10 times the original volume and estimated 95% confidence limits as a function of activity of ¹³¹I-UJ13A or Na¹³¹I. Arrow, point at which an upper confidence limit could not be calculated inasmuch as it exceeded the duration of the experiment.

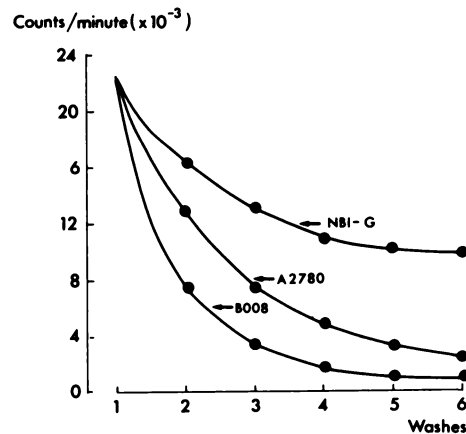


Fig. 2. Loss of unbound [¹³¹I]mIBG from three spheroid lines (NBI-G, A2780, B008) initially exposed to similar activities during the washing procedure.

relationship for ¹³¹I-UJ13A confirms the effectiveness of the antibody in delivering the radionuclide to the tumor cells and effecting a degree of cell kill (as evaluated by growth delay) in proportion to the activity added. Experiments of this sort may be useful in providing biological dosimetry for targeted radiotherapy of micrometastases. By comparison of the regrowth delay produced in the same experimental system by targeting and by external beam irradiation, operational estimates may be made of radiation dose delivered by targeting. For example, it may be seen (Fig. 1) that exposure of NBI-G spheroids to 14 MBq ¹³¹I-UJ13A for 2 h results in a regrowth delay of about 14 days. Previous studies using external beam irradiation (8) have shown that a regrowth delay of 14 days is caused in NBI-G spheroids by a dose of 2 Gy. This provides a reference point which could be used in quantitative analysis of models of cell killing in spheroids by radiolabeled antibodies. Such experimental comparisons are likely to be especially useful as studies are made of radionuclides (e.g., ²¹¹At, ⁶⁷Cu) yielding short-range emissions, for which heterogeneity of dose within larger spheroids is likely to be an important consideration.

The mIBG preliminary studies suggest that NBI-G spheroids should provide a useful *in vitro* model for this mode of targeting also. Experiments are now in progress to relate retained activity in spheroids to resultant growth delay in the different cell lines. These are likely to be complicated by differences in the radiobiology of these cell lines necessitating experiments using external beam irradiation to proceed in parallel. Experiments directly comparing the effectiveness of [¹³¹I]mIBG and ¹³¹I-UJ13A as a means of targeted radiotherapy of spheroids of differing size are also in progress. These will be of interest in view of likely differences in penetrating capacity of antibody and mIBG in larger spheroids. The relative efficacies of antibody fragments and of various proposed pharmacological manipulations to alter antibody or mIBG uptake or loss rate should also be amenable to study in this system. Further studies, using NBI-G spheroids as an *in vitro* model for targeted radiotherapy of neuroblastoma micrometastases, seem warranted.

Acknowledgments

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