CONF
FINAL

ANL/CP--73541

DE91 015199

EVALUATION OF DIFFERENT IN VITRO ASSAYS OF INHERENT SENSITIVITY AS PREDICTORS OF RADIOTHERAPY RESPONSE

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JUL 1 1991

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INTRODUCTION

Radiotherapy is an important component of cancer therapy, achieving local control and regional cures in a number of different diseases. While radiotherapy has had some dramatic successes in the treatment of certain malignancies, its overall success rate varies with tumor type, stage, or grade. Variation in response can be found even with tumors of similar type, stage, and grade. Understanding the basis for these different responses would be clinically important, suggesting new alternative therapies to overcome radiation resistance and predictive assays of response to identify those patients who would benefit from conventional therapies.

Both the tumor microenvironment and the inherent sensitivity of the cells within the tumor have been suggested to play an important role in tumor response to radiotherapy (1-3). The tumor microenvironment influences blood flow which in turn will define local oxygen tension and pH. These parameters will modulate the radiation response of tumor cells. The presence of inherently radioresistant or repair-proficient tumor cells might also play an important role in radiotherapy failure in some patients.

RESULTS AND DISCUSSION

Radioresistant Tumor Cells in Radiotherapy Failures and Recurrences:

To investigate the potential importance of inherent cellular radiosensitivity in tumor response to radiotherapy, the radiation sensitivity of human squamous cell carcinoma cells derived from in-field tumor failures (nonresponders and in-field recurrences) was compared to that of similarly derived lines from tumors samples prior to any therapy (4). If inherent sensitivity plays a major role in radiotherapy response, then the cells derived from in-field radiotherapy failures should be more resistant to radiation than those from tumors sampled before any radiotherapy. Radiation sensitivity was determined by *in vitro* clonogenic survival assays of cell lines derived from head and neck cancers. For head and neck cancers, the tumors are usually accessible for sampling, and local control is an important component of the cure in head and neck cancer (5).

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Cell lines were initiated as previously described (4,6) from 34 tumors sampled prior to radiotherapy and 17 tumors sampled after radiotherapy (in-field failures). Radiation sensitivity was determined by clonogenic survival assay of lines in passages 3 to 20. Microscopic analysis insured that greater than 99% of the cells in culture were tumor cells and not normal fibroblasts. Experimentally derived data points were the mean of 2-6 independent measurements. The plating efficiencies ranged from 0.01 to 0.25. From the survival curves, a mean inactivation dose ($\bar{D}$) was calculated as previously described (7,8). Results are presented as a cumulative frequency of tumor cell lines (in percent) at a given $\bar{D}$ value (Figure 1A).

Figure 1. Distribution (percent cumulative frequency) of radiosensitivities ($\bar{D}$) in cell lines derived from head and neck cancers. A) Before radiotherapy (O) and after in-field failures (●). B) Before radiotherapy; NED (O), in-field failures (●).

The distribution of radiation sensitivities was broad. Values of $\bar{D}$ ranged from 1.1 to 3.63 Gy, with a mean of 2.29 ± 0.08 Gy. This distribution is much broader than seen in normal fibroblast cell lines (9) and extends more into the resistant range than normal fibroblast cell lines. There appear to be tumor-type specific ranges of radiation sensitivities. Human sarcoma cell lines have a much narrower range of $\bar{D}$ values (1.36-2.05 Gy) with a mean (1.66 ± 0.07 Gy) in the more radiosensitive end. Ovarian adenocarcinomas have a broad range (1.75-3.54 Gy) with a mean (2.41 ± 0.15 Gy) similar to that of the squamous cell carcinomas (10).

The distribution of radiation sensitivities for the post-radiotherapy samples (in-field failures) was different than that for the pretreatment samples. The range of $\bar{D}$ values was smaller for the failures (1.5-2.9 Gy)

than for the pretreatment samples (1.1-3.63 Gy). There was also a shift in radiation sensitivities to the more resistant range for the in-field failures (Figure 1A). The mean $\bar{D}$ value for the in-field failures was 2.64 ± 0.12 Gy, whereas that for the pretreatment samples was 2.34 ± 0.08 Gy.

These results suggest that radiotherapy selects for tumors that contain inherently radioresistant cells and that therefore inherent radiation sensitivity is an important component of radiation response. Furthermore, these studies suggest that measurement of radiation sensitivity in tumor cell lines *in vitro* might serve as a predictive assay of tumor response *in vivo*.

In Vitro Radiation Sensitivity and Patient Outcome:

The utility of *in vitro* clonogenic measurements of tumor cell radiosensitivity as predictive measures of tumor response was investigated by following the 34 patients used for the pretreatment samples to determine their radiotherapy response. All patients have been followed with routine examination at regular intervals for at least 1 year and in many cases for 2-5 years after radiotherapy. Of the 34 patients in the study, 13 have had either persistent disease or local in-field recurrences (in-field failures).

The analysis of *in vitro* sensitivity in the tumor cell lines derived from these patients is shown in Figure 1B. The radiosensitivity measure $\bar{D}$ did not distinguish the radiotherapy failures from those patients with no evidence of disease (NED); the distribution was similar for both groups. The mean $\bar{D}$ for cell lines derived from patients who suffered in-field failures was 2.33 ± 0.14 Gy; the mean $\bar{D}$ for lines derived from patients with NED was 2.35 ± 0.11 Gy. Although the sample numbers are small and follow-up is not yet complete for all the patients, the results suggest that measurement of inherent radiation sensitivity by *in vitro* clonogenic assay is not sufficient to predict tumor response *in vivo*.

Clinical Complications:

The failure to predict tumor response with the *in vitro* survival assays probably reflects the complex clinical picture seen in head and neck cancer, and the need to look at multiple variables to predict response. For example, the staging of the tumor is likely to play an important role in response. In general, large tumors are more difficult to control than smaller tumors. The sample size in this study was too small to factor in staging. Many of the patients in this study received multiple treatments including surgery and chemotherapy in addition to radiotherapy. Surgery and chemotherapy will obviously influence therapy response and therefore they need to be considered in interpreting predictive assays. Surgery was an important component underlying response in this study, with 73% of the patients showing NED and only 31% of the failures receiving both radiation and surgery.

Induction/Selection of Resistant Cells *In Vivo*:

It is unlikely that the failure to distinguish radiotherapy failures on the basis of *in vitro* sensitivity in the pretreatment samples is due to the induction of radioresistance by radiotherapy *in vivo*. The distribution of sensitivities in the pretreatment samples (Figure 1A) suggests that radioresistant cells exist in tumors prior to therapy. Heterogeneity with respect to radiation sensitivity has been reported in human tumors (11), and radiotherapy may select for these radioresistant subsets within a tumor. The importance of selection as a complicating factor for predictive assays will require analysis of cell lines derived from the same patient prior to and after radiotherapy.

Selection of Resistant Clones *In Vitro*:

The clonogenic survival assay requires the establishment of cell lines. Our success rate in establishing cell lines is about 70%. There may be intrinsic cellular features that favor establishment in culture which might tend to skew the population of tumors and cells that are analyzed. Most of the failure in establishing cell lines, however, is due to contamination of the culture with either bacteria, fungi, or normal fibroblasts. There is no evidence that cell lines can be established from only head and neck tumors of a specific radiobiological character.

Figure 2. Survival analysis (individual experiments) of four tumor cell lines in passages 3 to 5 (O) and passages 15 to 18 (●).

As analysis is done on cells in passages 3 to 10, *in vitro* selection for better growing clones will obviously occur. Radioresistant cell lines tend to grow better and faster than radiosensitive cell lines (8). Thus, the use of later-passage cell lines might not be representative of the tumor cells *in vivo*. A comparison of radiation sensitivity as a function of passage

number in these tumor cell lines (Figure 2) suggests that radiation sensitivity is a relatively stable trait. There is no obvious selection for more radioresistant cell lines.

Alternative *In Vitro* Assays:

There is a close correlation between radiation sensitivity as measured by clonogenic assay and rates of DNA double-strand break rejoining as measured by DNA filter elution or pulse-field gel electrophoresis (12,13, manuscript in preparation). Measurement of DNA double-strand break rejoining by either filter elution or pulse-field gel techniques do not require establishing cell lines or measuring clonogenic growth. In a recent study (13), a close correlation was seen between elution measurements, made on cells in first passage after sampling, and clonogenic survival measurements, made on the same cells at a much later passage. Measurement of DNA strand break rejoining might provide a rapid and inexpensive alternative to clonogenic survival analysis.

Difference in tumor cell radiosensitivity reflect alterations in chromosome structure (14,15). These alterations can be detected in biopsy material by flow cytometric analysis (16). Chromosome structure (nucleoid expansion) alterations were analyzed in tumor cells derived from 28 patients with muscle invasive transitional cell carcinoma of the bladder prior to radiotherapy (16). Chromosome changes associated with *in vitro* radiation resistance (14,15) were found in 12 out of the 14 samples from patients who suffered persistent disease 3 months after radiotherapy. These changes were found in only 4 of the 14 samples from patients with NED three months after radiotherapy. This rapid and powerful approach to predictive assay measurement is now being applied to head-and-neck cancers.

SUMMARY

The inherent sensitivity of cells within a tumor plays an important role in the response of the tumor to radiotherapy. Clonogenic assays show that cells established from in-field radiotherapy failures are significantly more resistant to radiation than cell lines established from pre-treatment samples. Clonogenic assays fail to predict tumor response to radiotherapy, however. The failure might be due to (1) the small sample size in this study, (2) the complicating factors of staging, surgery, and chemotherapy, and (3) *in vivo* selection by radiotherapy for resistant tumor cells. *In vitro* selection for resistant cell lines does not appear to be a complicating factor. Nonclonogenic assays such as those that measure DNA strand break rejoining rates (filter elution, pulse-field gel electrophoresis) or chromosome structure (flow cytometric analysis) show promise as alternative rapid assays of radiation sensitivity and possibly tumor response.

This work was supported by grant CA42596 from the NCI, The Center for Radiation Therapy; a Faculty Research Award from the American Cancer Society; and by the U. S. Department of Energy, Office of Health and Environmental Research (Contract No. W-31-109-ENG-38).

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