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Radiation Sensitivity and Study of Glutathione and Related Enzymes in Human Colorectal Cancer Cell Lines*

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Abstract—*A panel of* 13 *human colorectal cell lines was studied, with these lines exhibiting a histological profile similar to that observed in clinical practice. In the five lines tested, variable sensitivity to radiation was observed, from the relatively sensitive NCI-H716 to the highly resistant line NCI-H630, with the latter cell line derived from a patient who had previously received radiation treatment. Glutathione levels and glutathione related enzyme activity varied widely between all* 13 *cell lines, showing no relationship to radiation sensitivity. The variability observed suggests that some colonic tumours may be responsive to radiation, although their identification remains difficult. However, this may prove possible by incorporation of recently developed cell adhesive matrix assays using survival following a* 2 Gy *radiation dose as a parameter of radiation sensitivity. This panel of human cancer cell lines offers an ideal model for the study of parameters affecting the radiosensitivity and chemosensitivity pattern of colorectal cancer cells.*

INTRODUCTION

COLORECTAL CANCER is a very common malignant tumour, second only to lung cancer in incidence. In 1986 it is estimated that approx. 140,000 new cases will be diagnosed and that 60,000 of these will die of their disease [1].

Surgery is effective when the disease is localized; however, in patients with rectal cancer whose disease is not controlled by surgery alone, approx. 25% will die of local disease progression in the absence of metastatic disease [2]. On dissemination the therapeutic approaches are limited. Radiotherapy has been of limited value in the management of patients with advanced colorectal cancer, due both to primary resistance of the tumour [3] and that gastrointestinal toxicity may be dose limiting. In the presence of metastatic disease, chemotherapy has been singularly unsuccessful, with 5-fluorouracil the most widely used agent [4–6].

Recently a panel of human colorectal cell lines has been established [7], offering an excellent model for the investigation of human colorectal cancer [8]. The chemosensitivity profile of these cell lines has been established, with 5-fluorouracil proving to be the only agent with significant activity [8]. In view of the problem of local disease progression, we studied the radiosensitivity pattern of a selection of these cell lines. In addition, as glutathione and glutathione-related enzymes have been shown to be important in the cellular response to radiation [9], these enzymes were measured in this panel of 13 cell lines in an attempt to identify differences between lines, and potentially to predict sensitivity or resistance to radiation or to cytotoxic drugs.

METHODS AND MATERIALS

Cell lines

Details of the cell lines have previously been recorded [7, 8]. All cell lines were maintained in RPMI 1640 medium supplemented with 10% (v/v) foetal calf serum, with added penicillin and streptomycin. The cells were kept in a humidified

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atmosphere with 7% CO_2/93% air. Two cell lines grew as floating aggregates, in contrast to the remainder which grew as adherent cultures.

Clonogenic assays

Exponentially growing cells were harvested from 75 cm^3 plastic flasks. Following irradiation in liquid suspension, the floating cell lines SNU-C1 and NCI-H716 were cloned in soft agarose 0.3%, over an 0.6% agarose underlayer. Cells were incubated for 14–21 days, then cultures were examined using an inverted phase microscope, with colonies of greater than 50 cells counted. The remaining cell lines grew as adherent cultures in liquid medium, using 60 mm Petri dishes for seeding cell densities of up to 5×10^4 cells, with 100 mm dishes used for larger cell inoculae. These cultures were incubated for 10–21 days, then fixed in acetic acid and methanol 1:3 (v/v), stained with 0.1% crystal violet and colonies of greater than 50 cells counted under a microscope at 8 × magnification.

Irradiation

Cells were irradiated as floating cultures in test tubes at 1–10 Gy with the use of a 6 MeV photon beam from a Mevatron VI linear accelerator. The dose rate was 2 Gy/min.

Glutathione assay

Cells from all 13 cell lines were seeded at the same density in 5 × 100 mm Petri dishes and were harvested during exponential growth, having been fed with fresh medium 48 h prior to harvest. They were washed twice in ice-cold phosphate buffered saline (PBS), with the cells from three plates lysed using 0.6% sulfosalicyclic acid at 4°C. The supernatant was then aspirated from each dish and assayed individually for total glutathione content as previously described [10]. The remaining two dishes for each cell line were assayed for total protein content using Bradford's solution [11].

Enzyme assays

Exponentially growing cells were harvested from 175 cm^3 plastic flasks and were washed twice in PBS. The cells were then lysed by sonication following resuspension in 2 ml PBS with 0.005 M EDTA, and the samples stored at −80°C until use. All experiments were perfomed in triplicate, with protein estimations performed using Bradford's solution.

Glutathione-S-transferases. Glutathione transferase activity was estimated using the method described by Habig *et al.* [12], using 1-chloro-2,4-dinitrobenzene as substrate with the activity monitored by spectrophotometric absorbance at 340 nm.

Glutathione reductase. Glutathione reductase activity was measured using the method described by Massey and Williams [13], followint coupling of the substrate glutathione at an absorbance of 412 nm.

γ-Glutamyl transpeptidase. γ-Glutamyl transpeptidase was estimated using L-γ-glutamyl-*p*-nitroanilide as a substrate as described by Szasz [14], monitoring the reaction by measurement of absorbance at 412 nm.

RESULTS

The *in vitro* radiation survival curves for the 5 cell lines tested are shown in Figs 1 and 2, with extrapolation numbers (n), D_0 values and surviving fraction values following a 2 Gy (S_2) radiation dose listed in Table 1 for each cell line. NCI-H630 was by far the most radio-resistant, as indicated by an S_2 of 0.89. In contrast, two cell lines, NCI-H716 and SNU-C1, were relatively radiosensitive with surviving fractions of 0.26 and 0.32 respectively following a 2 Gy radiation dose.

Glutathione and glutathione related enzyme activities are listed in Table 2, for all 13 colorectal

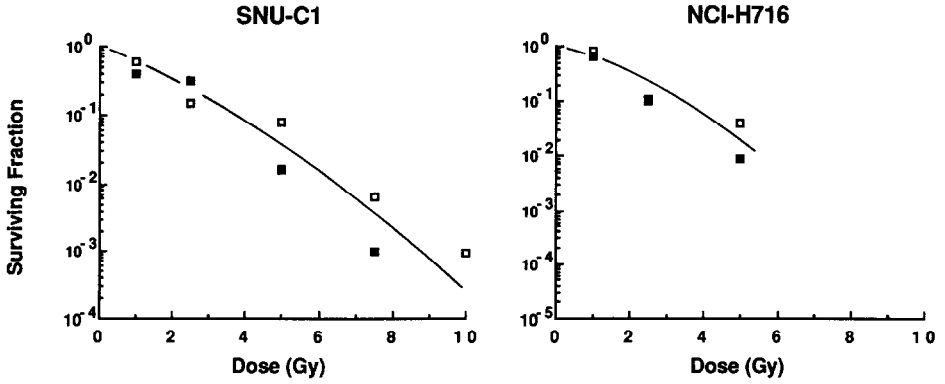

Fig. 1. Radiation survival curves of two human colorectal cell lines performed using a standard clonogenic assay technique. The curves represent the best fit of two repeat experiments, with each data point the mean of three determinations.

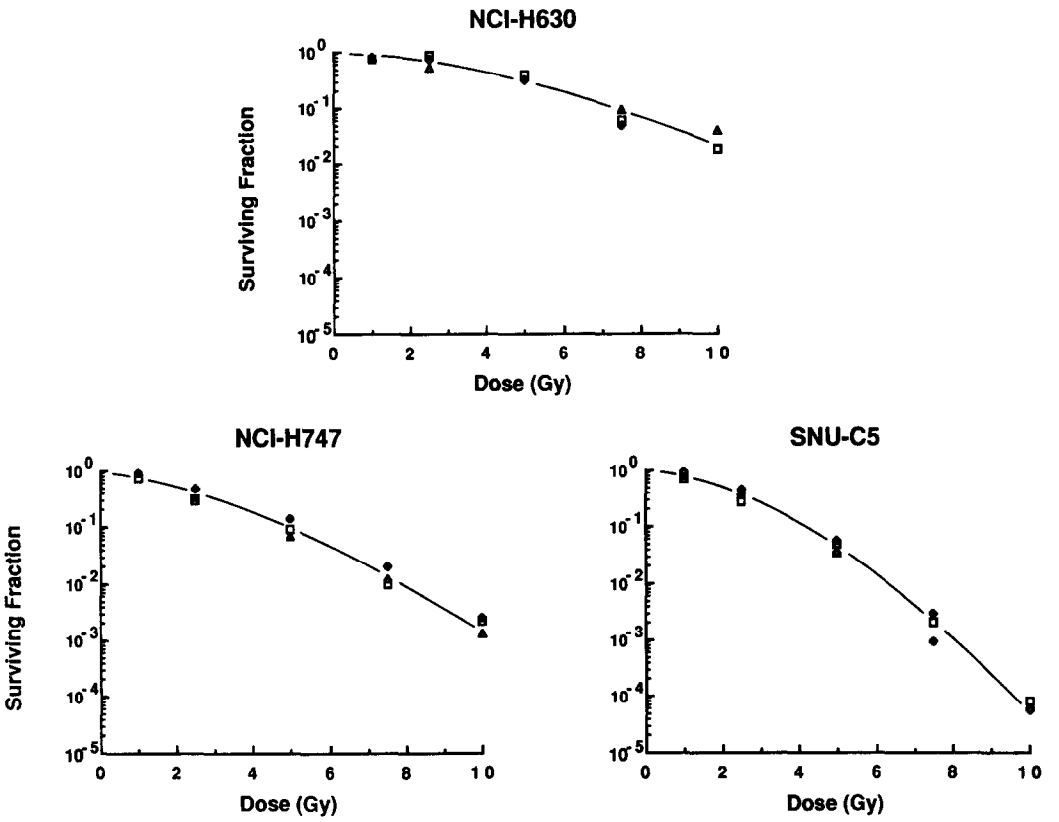

Fig. 2. Radiation survival curves of three human colorectal cell lines performed using a clonogenic assay. The survival curves represent the best fit of three repeat experiments with each data point representative of the mean of three determinations.

cell lines, with these lines grouped according to histological sub-type. Glutathione levels varied from 19.3 to 207.5 nmoles mg protein and glutathione transferase activity from 1.63 to 237.4 Units/mg protein. Likewise, glutathione reductase activity ranged widely from 23.5 to 122 Units/mg protein and γ-glutamyl transpeptidase activity from 6.8 Units in the NCI-H958 cell line to 2205 Units per mg protein in SNU-C4, with this variability bearing no apparent relationship to histological sub-type.

DISCUSSION

An interesting spectrum of radiation sensitivity was observed in the five colon cancer cell lines, as illustrated by Figs 1 and 2, and by extrapolation numbers, D_0 and S_2 values shown in Table 1. Of particular interest was the wide variation in S_2 values observed among the five colorectal cell lines. Conventional radiotherapy is delivered commonly over 5–7 weeks in small fractions of 2 Gy to a total dose of 50–70 Gy. The extent of cell killing from a 2 Gy dose is therefore important, with the expectation that 25–35 fractions of 2 Gy will be sufficient for tumour sterilization. Small differences in S_2 values could relate to significant differences in tumour response over the course of 25–35 fractions. S_2 values of cell lines derived from different types of human tumours have been shown to correlate with the clinical responsiveness of the respective tumours, with higher S_2 values relating to relative resistance [15, 16]. Using this parameter the cell line NCI-H630 was the most radio-resistant, and interestingly, this cell line was derived from a recurrent liver metastases in a rectal cancer patient who had previously received a fractionated course of radiotherapy of 30 Gy to the liver. In contrast, the two cell lines NCI-H716 and SNU-C1 were found to be relatively radiosensitive, as reflected by their low S_2 values. These differences in the radiation response among the colorectal cell lines studied are consistent with other reports of heterogeneous radiation sensitivities among *in vitro* [17] and *in vivo* xenograft [18] sub-populations of human colon carcinomas. In the present study, unfortunately no relationship between the extent of radiation

Table 1. Radiobiological parameters of five colorectal cancer cell lines, derived from the radiation survival curves

Cell line	Extrapolation number (n)	D_0 (Gy)	Surviving fraction at 2 Gy
SNU-C1	2.0	1.15	0.32
SNU-C5	6.2	0.91	0.52
NCI-H630	5.5	1.8	0.89
NCI-H716	1.9	1.04	0.26
NCI-H747	5.3	1.24	0.69

Table 2. Glutathione levels and glutathione-related enzyme activity of 13 human colorectal cancer cell lines

Cell line	Diff^n	Glutathione (nmoles/mgprotein)	Glutathione transferase (Units)	Glutathione reductase (Units)	γ-Glutamyl transpeptidase (Units)
SNU-C2A	Poorly	207.5 ± 15	1.63 ± 0.1	122 ± 45	205 ± 100
SNU-C2B	Poorly	63.5 ± 1.3	197.3 ± 29	58 ± 11	180 ± 25
SNU-C4	Poorly	87.3 ± 6.7	86.6 ± 18	69 ± 32	2205 ± 605
SNU-C5	Poorly	97.5 ± 8.3	76.7 ± 21	40 ± 5.6	115 ± 10
NCI-H716	Poorly	36.8 ± 0.9	45.5 ± 9.9	46.5 ± 8	61.2 ± 9
SNU-C1	Mod	31.8 ± 2.3	103.4 ± 13	23.5 ± 3.5	206 ± 17
NCI-H508	Mod	32.0 ± 8.5	218.2 ± 23	61 ± 12	765 ± 43
NCI-H747	Mod	66.9 ± 52	43.3 ± 6.7	41 ± 42	866 ± 475
NCI-H548	Well	62.7 ± 10	237.4 ± 39	41 ± 12	413 ± 4
NCI-H630	Well	51.1 ± 3.4	118.7 ± 78	75 ± 7	930 ± 49
NCI-H684	Well	81.2 ± 16	184.6 ± 62	43.5 ± 5	948 ± 49
NCI-H958	Well	13.8 ± 1.0	107.5 ± 13	27 ± 5.6	6.8 ± 4
NCI-H498	Mucinous	19.3 ± 2.2	215.4 ± 1.5	51 ± 4.2	1959 ± 92

Glutathione transferase activity 1u = 1 nanomole CDNB conjugated per minute.
Glutathione reductase activity 1u = 1nanomole NADPH utilized per minute.
γ-Glutamyl transpeptidase activity 1mu = 1 nanomole L-γ-glutamyl-*p*-nitroanilide metabolized per minute.

sensitivity and tumour cell differentiation was found.

Glutathione has previously been shown to be important in the cellular response both to radiation [9, 19] and to certain cytotoxic drugs [20, 21]. Elevation in glutathione levels has been observed in drug resistant cells [22], and reduction of glutathione levels by numerous agents, such as buthionine sulfoximine, has been shown to sensitize cells to the effects of both radiation and certain cytotoxic drugs [9, 19–23]. A wide range of glutathione levels was observed between these cell lines, however, and great variability was also seen in glutathione reductase and γ-glutamyl transpeptidase activity, although no correlation was seen with the degree of radiation sensitivity. Likewise glutathione transferase levels ranged from a very low level in the cell line SNU-C2A to relatively high levels in a number of cell lines including NCI-H548. These levels did not correlate with radiation sensitivity in these cell lines, although numbers were very small. Unfortunately, the isoenzyme distribution of the glutathione transferases was not determined, as an acidic transferase has recently been proposed as a tumour marker for colonic carcinoma [24] and gastric carcinoma [25] and this may have revealed interesting differences between the cell lines.

The treatment of advanced colorectal cancer remains unsatisfactory. Although improvements in the treatment and prognosis of colorectal carcinoma have recently been observed [26], many patients will die both of widely metastatic disease, and uncontrolled local disease. This study shows that there may be a variation in radiation response of these tumours as indicated by these cell lines. Should it become possible to identify these patients whose tumour cells are more sensitive to radiation, then they could potentially derive great benefit from therapeutic radiation. Measurement of glutathione and related enzymes failed to identify patients sensitive to radiation, although these measurements may be of more value in predicting response to cytotoxic drugs. Measurements of other parameters of drug resistance in tumour tissue, may also help to identify chemo-sensitive patients. Enhanced expression of the multi-drug resistance gene (mdr-1) [27], has been shown to be elevated in a number of tissues such as kidney, colon, liver and adrenal, with tumours originating in these tissues generally resistant to chemotherapy. Expression of this gene has likewise been shown to be increased in renal cancer tissue compared to normal kidney [28], and it is possible that colorectal carcinoma tissue may have very high levels of this gene. Previous studies looking at other variables such as DNA ploidy [29] have been unsuccessful in detecting patients with good prognosis.

One possible way to identify radiosensitive patients would be by use of the cell adhesive matrix assay [30]. X-Ray dose–response curves have been reported from a variety of different human tumours [31, 32] using this assay. These survival curves based on growth rate differences between control and irradiated cultures can be analysed in a similar manner to clonogenic survival curves. Survival curve parameters including S_2 values could be determined in less than 2 weeks following the biopsy [30]. This assay could prove to be useful in determining the extent of heterogeneity in radiation response within a given colon tumour and perhaps

in the selection of those tumours best suited for radiotherapy.

However, in conclusion, this panel of 13 colorectal cancer cell lines offers great potential for the study of the biology of and factors influencing the response of colon cancer cells both to radiation and to cytotoxic drugs.

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