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Effect of Single Dose Ionizing Radiation on the Cellular Proliferation and Apparent Radiosensitivity of Melanoma

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Abstract. *An important variable in determining in vitro cell survival is the time interval between treatment and assay. One manifestation of radiation damage is the introduction of a division delay in which the post-radiation division rate is less than the pre-radiation division rate. We have examined the influence of length of incubation on radiosensitivity of murine and human melanoma cells in a soft-agar assay. In general, the longer the incubation period the larger was the median colony diameter within control or experimental groups. Hence apparent radiosensitivity decreased with increased length of incubation.*

Discrimination between radiation sensitivity and resistance *in vitro* has conventionally been based on the level of cell survival at a designated period of time (1, 2). One manifestation of radiation damage is the loss of proliferative capacity (3-6). Another is that division delay occurs after radiation damage (1, 5, 7). The length of division delay and loss of proliferative capacity are determined by cell type, physiological condition of the cells at the time of irradiation and the radiation dose (1). After irradiation some cells lyse before cell division, others do not divide, and the remainder divide after the initial division delay. Some cells are capable of dividing only a limited number of times and, when assayed for colony formation, these cells give rise to smaller colonies consisting of cells with limited proliferative capacity.

Treated cells which proliferated to form colonies of 50 or more cells have traditionally been identified as resistant clones (1). Heterogeneity in size of colonies at any particular time point has been recognized for some time (1, 3, 7). For human tumors which frequently contain many slowly growing clones, distinguishing slowly growing from abortive colonies using only a single time determination is problematic. Recently, time course studies of cells from ovarian, breast, melanoma, and small cell carcinomas have found that multiple time points were necessary to distinguish the proliferative capacity of colony-forming cells (8-10).

In addition to time course studies, we have carried out replating experiments. In these experiments cells were irradiated by a dose of 6 Gy and allowed to incubate. Colonies of specified sizes were plucked from the agar, pooled, thoroughly mixed and monodispersed cells were replated and further incubated. Any differences in median colony diameter allows one to distinguish slow-growing survivors from abortive colonies. Our experiments are unique in that both time course studies and replating experiments have been done to illustrate the importance of adequate incubation for the determination of *in vitro* radiation survival of melanoma.

Materials and Methods

Maintenance of CCL murine melanoma cell line. Cloudman S91 murine melanoma clone CCL 53.1 was obtained from the American Type Culture Collection, Rockville, MD, and maintained by serial transplantation in DBA/2J mice. The tumors were harvested, and single-cell suspensions were obtained as previously described (11). The cells were cultured in Ham's F-10 medium with supplements.

Preparations and culture of cells from patient biopsies. The general approach to the preparation of cell suspensions has been described elsewhere (11). Cells were grown in Ham's F-10 medium with supplements.

Culture of human melanoma cell lines and cell strains. Cell strains, which were subcultured less than 10 times, were maintained in Ham's F-10 medium with supplements. Cells were checked for mycoplasma contamination periodically. The long-term human cell line M1RW5, developed in our laboratory, was passed through nude mice. M1RW5 tumors were aseptically removed from mice, single cell suspensions were obtained and cells were cultured as above. Human karyology was confirmed by Dr. J. Trent in our instution.

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Soft-agar bilayer assay. The soft-agar assay has been described elsewhere (12). A modification of this assay using a microtiter culture dish is described by Thomson *et al* (10). The optimal number of cells to be plated was determined for each cell line, strain and patient biopsy prior to radiation studies. The single-cell nature of the plated cells was assured by checking for cellular aggregates one hour after plating. Six replicates and 500 cellular units per replicate (randomly selected 6.25 mm^2 areas) were examined for each experiment. Cells had less than one aggregate per replicate. Cells were incubated in a well-humidified 5% CO_2 and 95% air atmosphere at 37° C for one or more weeks, as specified for each experiment.

Counting and grouping of colonies. An automated colony counter was utilized for counting and grouping of colonies. The Omnicon model FAS II optical image analyzer (Bausch and Lomb, Rochester, NY) has been described elsewher (13). A vital stain, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride, was utilized to determine the viability of cells within colonies (> 99 percent).

Examination for presence of enlarged cells. Individual colonies were removed aseptically by placing agar plates and an inverted microscope and micromanipulator inside an isolation box (14). Colonies of different diameters were removed after 14 or more days of growth. The colonies were expelled onto a micrsocope slide. Slides of individual colonies were prepared and the cell diameter of individual cells was measured. For all cell lines, strains and patient biopsies, colonies that contained from 25 to 100 cells were plucked from non-irradiated and irradiated plates and individual cell diameters measured. The average cell diameter was determined by measuring cells from 100 colonies of each of four size classes ≥ 25 cells, ≥ 50 cells, ≥ 75 cells and ≥ 100 cells. The average S.E. was 9%. Mean cell diameter did not vary from one another within the S.E. of control *versus* experimental groups. Enlarged cells were not present in colonies and only observed as single cells at the higher doses.

Quantitation of cell numbers within colonies. Quantitation of number of cells within clusters or colonies has been delineated for several tumor types grown in soft agar (15) and was determined for each cell described here.

Radiation. Cells were irradiated by a single dose of X-rays generated by a Varian Associates 18 MeV linear accelerator operating at 10 MeV and yielding a dose rate of 5.0 Gy per minute. The cells were irradiated at ambient temperature under normal atmospheric conditions. All radiation dosages and dosimetry readings were provided by the Department of Radiation Oncology of the University Medical Center.

Preparation of survival curves and statistical analysis. Survival data were calculated according to standard radiobiological statistical methods (1, 4, 6). There were 12 replicates per control and 6 replicates per experimental dose. Experiments were repeated at least twice. D_o values were calculated from the line fitted by least-squares linear regression analysis of survival points, determined by visual inspection to be off the shoulder region of the curve. The extrapolation number, n, was the y-intercept of the line fitted by linear regression. Ninety-five percent confidence intervals were calculated for the D_o values. The degree of fit to the linear equation is estimated by the correlation coefficient, R (see Results and Table I).

Results

Influence of length of incubation on radiobiological parameters. The length of incubation influenced determination of measured radiosensitivity. As the length of incubation increased, there was a trend toward an increase in the slope of the survival curve, the D_o value, and/or and increase in the extent of the shoulder region, the D_q value, over time (see Figure 1 and Table I). For example, in Figure 1 note the difference in D_q values between two and four weeks of incubation and the difference in D_o values between four and six weeks. For murine melanoma CCL 53.1 the D_o value shifted from 160 cGy to 485 cGy and the D_q value shifted slightly form 48 to 71 cGy following one to three weeks of incubation. There was a similar shift towards higher D_o and/or D_q values for the other human melanoma cells.

There appeared to be a time at which the survival curve stabilized. For example, the survival curves for CCL 53.1 and C8442 stabilized after three weeks of incubation. The survival curve for M1RW5 stabilized after two weeks. In contrast, for other cells the survival curves did not stabilize until after four of more weeks.

Influence of length of incubation on cellular proliferation and on the frequency distribution. Both control and irradiated plates showed an increase in number of colonies over time, although there was a reduction in activity of cellular division in irradiated cells. There was a shift towards greater median colony diameter with time.

Frequency histograms of colonies of selected sizes *versus* total colonies reveals that there was a component of small clusters of cells (< 42 μm) which proliferated and formed larger colonies over time as evidenced by the increase in total colonies over the same time period. In replating experiments, cells were irradiated by a dose of 6 Gy and allowed to incubate for two weeks. Colonies were plucked from agar, pooled, thoroughly mixed and monodispersed cells were replated in microtiter plates. Colonies of 25, 50, 75 or 100 cells were replated by the above protocol. Frequency histograms of the colonies formed from replated cells had the same median colony diameter for cells replated from the smallest (25 cells) to the largest colonies (100 cells).

For those cells in which D_o values increased over time, the frequency histograms revealed a shift towards a greater proportion of colonies (> 50 cells) per each experimental treatment *versus* control. For those cells in which D_q values increased over time, cells irradiated at the lowest doses recovered and proliferative capacity approached that of the nonirradiated controls.

Discussion

The length of incubation influences the measurement of radiosensitivity. As the length of incubation increases, there was a trend toward an increase in the slope of the survival curve, *i.e.*, an increase in the D_o value, and/or an increase in the extent of the shoulder, D_q, over time. The conventional two week incubation period may not allow one to distinguish between cells which have been irreversibly inhibited compared to those which have not. An underestimation of survival for slowly growing cells will occur as these cells do not have adequate time to proliferate and form colonies of 50 or more cells. The component of the slowly growing survivors

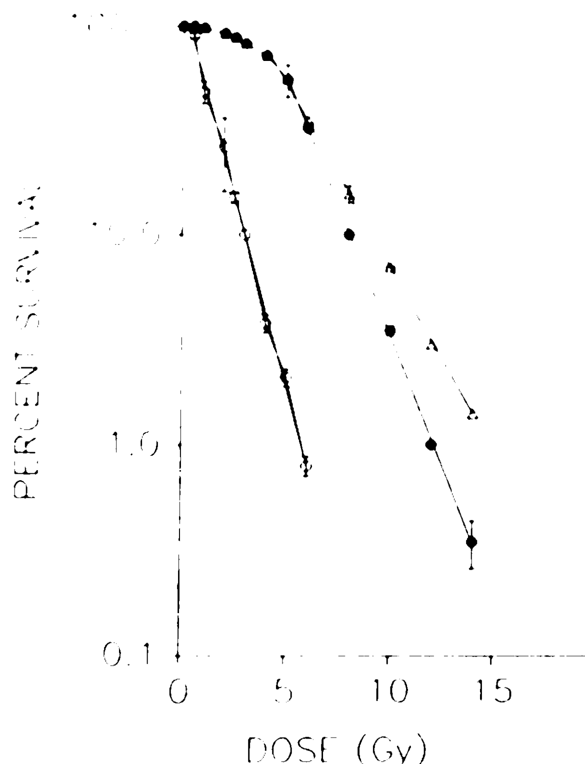

Figure 1. *Influence of length of incubation on apparent radiosensitivity of human melanoma 83-4. Mean of six replicates +/− S.E. are shown; no error bars means S.E. was smaller than symbol. Open circles, two weeks of incubation; closed circles, four weeks of incubation; open triangles, six weeks of incubation.*

Table 1. *Influence of length of incubation on radiobiological parameters of murine and human melanoma cells.*

	Length of incubation (wks)	Radiobiological parameters			Coorelation coefficient
		D_o value (cGy)[a]	D_q value (cGy)	n number	
Murine cell line					
Cloudman S91	1	160+/−11	48	1.35	0.95
CCL 53.1	2	222+/−23	61	1.33	0.95
	3	485+/−34	71	1.16	0.96
Human cell line					
M1RW5	1	171+/−15	92	1.71	0.92
	2	250+/−34	40	1.12	0.95
Human cell strains					
C8146A	2	70+/−18	60	2.35	0.96
	4	73+/− 5	122	5.40	0.96
	6	124+/− 8	120	2.64	0.97
C8146C	2	170+/−20	40	1.20	0.88
	3	155+/−17	121	2.18	0.89
	4	169+/−23	351	8.00	0.89
C8161	2	183+/−15	180	2.68	0.95
	3	209+/−12	132	3.03	0.97
	4	382+/−27	153	1.50	0.95
C8442	2	100+/−15	20	1.20	0.88
	3	302+/−15	331	3.00	0.98
	4	309+/−17	330	2.90	0.97
R83-4	2	269+/−30	239	2.44	0.93
	3	326+/−14	121	1.45	0.98
	4	330+/−13	380	3.15	0.98
Patient biopsy					
83-4	2	130+/−50	39	1.25	0.87
	4	165+/−16	378	9.90	0.95
	6	237+/−20	329	4.00	0.95

[a] Mean +/− ninety-five percent confidence interval.

is evident by the increase in total colonies over time. After two weeks of incubation, some of the smaller colonies (< 50 cells) were capable of forming larger colonies with longer incubation periods.

Replating experiments were used as an alternative approach to define an adequate length of incubation for measuring survival. The frequency histograms showed no significant differences in median colony diameter between irradiated cells derived from the smallest (25 cells) to the largest (100 cells) colonies examined. This would suggest that many of the smaller colonies had extensive proliferative capacity, although at two weeks incubation this was not readily apparent. This also provides further evidence for the presence of slowly growing survivors.

There appeared to be a time after which the survival curves stabilized, *e.g.*, the survival curves for CCL 53.1 and C8442 appeared to stabilize at three weeks. Three weeks incubation may be the preferred length of time between treatment and assay for these cells. For other cells, further incubation may be necessary to allow the slowly growing clones to express their capacity for proliferative ability, *i.e.*, cell survival.

Since the first reports of the formation of giant cells after radiation (16), the presence of enlarged "giant" cells has made the interpretation of radiobiological data difficult (17). We have checked for the presence of enlarged cells in colonies after two weeks incubation by plucking individual colonies and measuring individual cell diameters. Enlarged cells are not present in colonies and are evident only at the highest doses as single cells.

Although there are many studies reporting survival of melanoma cells to radiation (18-25), the basic definition of colony size has been infrequently examined as a variable. The influence of length of incubation on radiation survival has been examined by other investigators (1, 7), and more recently by Kirkels *et al* (8) and Verheijen *et al* (9) for human tumor cells in soft agar. It is clear that adequate length of incubation is important in reporting cellular radiosensitivity. Adequate incubation allows slow-growing survivors to express their ability to proliferate and, therefore, enables the researcher to distinguish between slow-growing survivors and abortive colonies. Replating experiments are helpful in delineating adequate length of incubation. Replating studies have been reported for human tumors (26) and for non-treated melanoma (27). These experiments are important for optimizing the assay for other treatments.

In summary, murine and human melanoma cells appear to be more radioresistant with longer incubation periods after single doses of ionizing radiation. Incubation at a defined time period may underestimate survival of cells. After adequate incubation (which allowed the more slowly growing survivors to grow into larger colonies (≥ 50 cells), the radiation curve stabilized. Further incubation did not alter the shape of the survival curve. These results should help

define proliferation and apparent radiosensitivity of tumor colony growth in semisolid medium and alter the interpretation of survival curves which measure sensitivity to agents using this or a similar assay.

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