Int. J. Radiation Oncology Biol. Phys., Vol. 13, pp. 1725–1733
Printed in the U.S.A.

0360-3016/87 $3.00 + .00

● *Original Contribution*

EFFECT OF TUMOR COLONY DEFINITION ON IONIZING RADIATION SURVIVAL CURVES OF MELANOMA-COLONY FORMING CELLS

KARIN H. YOHEM, PH.D., MARVIN D. BREGMAN, PH.D. AND FRANK L. MEYSKENS JR., M.D.

Department of Internal Medicine and Cancer Center, University of Arizona, Tucson, AR 85724

Definition of survival and measurement of colony size in soft agar assays is important in establishing *in vitro* radiation survival curves. Conventionally, survival is assessed according to colony-forming ability. The distinction between small colonies that are abortive and those that are viable often involves a difficult and arbitrary choice for the investigator. We have examined the effect of different minimum colony sizes (≥25, ≥50, ≥75, and ≥100 cells) on ionizing radiation survival curves for cells from established murine (CCL 53.1) and human (M1RW5) melanoma cell lines as well as from short-term human melanoma cell strains (C8146A, C8146C, C8161, C83-2C, C82-7A1, and C8442) and patient biopsy (83-4). Single cell suspensions were plated in the upper layer of the agar bilayer and cells were irradiated by single dose X rays. Giant cells did not form in colonies containing 50 or more cells. D_0 values were highest (D_0 values, from 390 to 100 cGy) for cells forming smaller colonies (≥25 cells, ≥4–5 doublings) and lowest (D_0 values, from 190 to 50 cGy) for cells forming larger colonies (≥100 cells, ≥6–7 doublings). Therefore, apparent radiosensitivity was dependent on colony size selected for analysis. Precise measurement of colony size was important in establishing radiation survival curves because errors in determining the colony size will alter apparent radiosensitivity of cells. These results should help define the biological meaning of tumor colony growth in semisolid medium, and alter the interpretation of survival curves which measure sensitivity to agents using this assay.

Metastatic melanoma, Soft agar assay, Human tumor clonogenic assay.

INTRODUCTION

For *in vitro* studies, survival has been assessed according to colony-forming ability. Many investigators have observed that in mammalian cell experiments not all surviving colonies are alike in size or appearance.[11,12,39,44] The distinction between small colonies that are abortive and those that are viable often involves a difficult choice for the investigator.[37,42] Colonies are defined by using either a minimum cutoff of number of cells per colony or a minimum colony diameter. To test the validity of a clonogenic assay, Rockwell[29] suggested that survival curves to ionizing radiation should be generated. One objective of our research was to validate the Hamburger-Salmon soft agar assay by generating radiation survival curves for human melanoma cells. To date, few ionizing radiation survival curves have been published using this method.[20,21,52]

The first *in vitro* radiation survival curves for human tumor cells were published in 1956 by Puck and Marcus[28] and were calculated from the proportion of colony-forming survivors with 50 or more cells from single HeLa cells plated 11–13 days prior to counting. Growth units containing less than 50 cells were designated as non-survivors, that is, abortive colonies with limited proliferative capacity. Most investigators have used the Puck and Marcus definition of cell survival based on colony size (≥50 cells) for establishing radiation survival curves for a variety of tumor cell types.[3,12,34,43,52] A few investigators have not used the conventional 50-cell definition of cell survival, choosing other indices of survival.[27,32] Investigators using the soft agar assay to study the effect of chemotherapeutic agents on cells from patient biopsies often define survival based on a minimum colony diameter.[7,8,13,35]

A soft agar assay is advantageous in that colony counts are reproducible,[17,19,36] large numbers of replicates can be counted allowing for better statistical analysis of data, simultaneous counts of different-sized colonies can be made, and the number of cells per colony in different-

This work was supported in part by grants from the National Institute of Health (CA 27502; CA 34689) and a Cancer Biology Training Grant (CA09213-07) to K.Y.

Reprint requests to: Frank L. Meyskens Jr., M.D., Department of Internal Medicine, Cancer Center, Room 3945, University of Arizona, Tucson, AR 85724.

Acknowledgements—We thank S. Thomson, S. Salmon, and E. Gerner for their helpful comments, Maggie Pulliam for excellent secretarial assistance and the staff of the Radiation Oncology Department for their technical assistance.

Accepted for publication 22 May 1987.

sized colonies can be defined.[25] Two major disadvantages of the soft agar assay are that only cell types that grow in agar can be studied, and although it is possible to obtain good single cell suspensions from cell lines, it is often difficult to obtain good single cell suspensions from human tumors.[1] Most criticisms of the soft agar assay have emanated from reports based on too little growth or initial plating of clumps of tumor cells.[16,29,37,48]

We have previously reported radiation survival curves for melanoma cells from human biopsies grown in soft agar.[20] Some cells from patient biopsies appeared to have marked radioresistance, but since then it has been shown that these unusual radiation survival curves were based on the presence of initial cellular aggregates.[21,23] We have extensively refined and quantitated the soft agar system for melanoma colony-forming cells.[25,46]

Our second major objective of this research was to elucidate information to help others using this soft agar assay to select appropriate criterion for generation of survival curves. Several requirements must be met including initial good single cell suspensions, adequate growth, and a linear relationship between the number of cells plated and the number of colonies formed. In addition, relevant colony size and adequate length of incubation to distinguish slowly-growing survivors from non-survivors are important. The influence of the former on interpretation of survival curves has been a main focus of prior reports.[11,12,27]

In this report we have shown that factors which can distort survival curves can be eliminated with appropriate experimental design. We have examined the proliferative capacity of murine and human melanoma cells to single dose ionizing radiation. For this study we used the conventional 50 cell size cut-off established for monolayer experiments and described our data by conventional radiobiological D_0 and n values. In addition, we have studied the effect of other minimum cell size cut-offs on determination of D_0 values. Small differences in colony size selected for analysis markedly affected the shape of the survival curves and the apparent D_0 values and, hence, altered the apparent radiosensitivity of murine and human melanoma cells.

METHODS AND MATERIALS

Maintenance of CCL murine melanoma cell line

The Cloudman S91 murine melanoma clone CCl 53.1 was obtained from the American Type Culture Collection, Rockville, MD, and has been maintained by serial transplantation in DBA/2J mice. The tumors were harvested, and single-cell suspensions were obtained as previously described.[7] The cells were added to a flask containing Ham's F-10 medium supplemented with 10% horse serum and 2% heat-inactivated fetal bovine serum,* gentamicin (10 mg/ml),† penicillin (100 mg/ml), and streptomycin (100 units/ml).‡ CCL 53.1 cells readily formed a monolayer and were subsequently subcultured. All experiments were performed on cells that had been subcultured no more than 10 times after isolation from mouse melanomas.

Preparation and culture of cells from patient biopsies

The general approach to the preparation of cell suspensions has been extensively described elsewhere.[8,10,22,33] Technical modifications have been made to improve yield and viability. Briefly, subcutaneous nodules of metastatic melanomas were obtained under aseptic conditions from patients. The tumor samples were secured as part of routine diagnostic or therapeutic procedures (protocol approved by the University of Arizona Committee on Human Subjects). Tumor tissues were cut free of necrotic and normal tissue and minced into 1-sq-mm pieces or less by extensive slicing with scissors. The tissue was placed into a 50-ml plastic conical tube containing Ham's F-10 medium supplemented with 10% heat-inactivated fetal bovine serum, L-glutamine (0.8 mg/ml), gentamicin (10 mg/ml), penicillin (100 mg/ml), and streptomycin (100 units/ml) and inverted several times. Tumor pieces and macroscopic clumps were allowed to settle to the bottom of the tube for 5 to 10 minutes at unit gravity, and the supernatant containing the single cells aspirated. Tumor pieces were resuspended in media, and the process repeated several times until the supernatant was clear. No enzymatic procedures were used. Cells were pooled, counted, tested for viability by exclusion of 0.4% trypan blue§ and stored in the liquid phase of liquid nitrogen in 10% dimethylsulfoxide** in Ham's F-10 medium containing 10% heat-inactivated fetal bovine serum, L-glutamine (0.8 mg/ml), gentamicin (10 mg/ml), penicillin (100 mg/ml), and streptomycin (100 units/ml). Recovery of single cell suspensions of melanoma biopsies that were cryopreserved has been routinely excellent and primary colony formation and self-renewal capacity was preserved.[45]

Establishment and culture of human melanoma cell lines and cell strains

Tumor cells were plated in 35 mm-diameter Petri plates in the upper layer of an agar bilayer.[22,45] Cells were allowed to grow in agar for 2 to 4 weeks, at which time the entire contents of the plates were aseptically transferred to 100-mm-diameter Petri plates to which 5 ml of media were added. Cells were further incubated with addition of fresh media as needed. After sufficient time,

* Grand Island Biological Co., Santa Clara, CA.
† Irvine Scientific, Santa Ana, CA.
‡ Eli Lilly, Indianapolis, IN.
§ Sigma Chemical Co., St. Louis, MO.
** Aldrich Chemical Co., Milwaukee, WI.

contents of plates were aseptically transferred to flasks. At confluency, cells were either subcultured or cryopreserved. Cell strains were checked for mycoplasma contamination periodically. Cell strains that were used in these experiments were subcultured less than 10 times. The long-term human cell line M1RW5, developed in our laboratory, was passed through nude mice.[6] M1RW5 tumors were aseptically removed from mice, single cell suspensions were obtained, and cells were cultured or cryopreserved as above. Human karyology was confirmed for all cells by Dr. J. Trent in our institution.

Soft-agar bilayer assay

The soft-agar assay has been described extensively elsewhere.[22,45] In addition to the usual supplements neutral human platelet sonicates were added (80 mg/ml; 96 ml/100 ml media) to the upper layer to stimulate growth of tumor cells from patient biopsies.[40] Sonicates were not added to cell strains and cell lines.

The optimal number of cells to be plated was determined for each cell line, strain, and patient biopsy prior to the radiation studies.[24,46] The number of cells plated for optimal growth was determined for each cell type from the linear portion of the growth curve. In our studies, cell lines CCL 53.1 and M1RW5 were plated at 5000 cells per dish, cell strains C8146A, C8146C, C8161, C82-7A1, C83-2C, and C8442 at 10,000 cells per dish, and patient cells 83-4 at 33,000 viable tumor cells per dish, respectively. As described elsewhere, a non-optimal number of cells per plate can have a profound effect on interpretation of results.[24] In determining plating efficiencies for tumor cells from patient biopsies, the number of viable tumor cells provided a better estimate of the cells that can form colonies than the number of total nucleated cells. Enumeration of live melanoma tumor cells has been described extensively elsewhere.[45] Only patient biopsies that grew well in agar and formed good single cell suspension by mechanical dissociation were used for these studies. (Not all melanoma biopsies are amenable for study because many do not grow well in agar or do not form good single cell suspensions). The single-cell nature of the plated cells was assured by checking for cellular aggregates 1 hour after plating. Since the automated counter can count only clusters of cells ≥ 42 μm in diameter we also manually inspected the plates for smaller clusters of cells. Six replicates (35 mm-diameter Petri dishes) 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 except for cell strain C82-7A1 which had doublets (0.4%) in some plates. Cells were incubated in a well-humidified 5% CO_2 and 95% air atmosphere at 37°C for 2 weeks except for CCL 53.1 which was incubated for 1 week.

Counting and grouping of colonies

An automated colony counter was used for counting and grouping of colonies. The Omnicon model FAS II optical image analyzer† has been described elsewhere.[17,19,36] The automated colony counter is highly reproducible and can group colonies into different size categories based on diameter in the horizontal axis. In these experiments cumulative size classes of ≥42, ≥50, ≥60, ≥72, ≥86, ≥104, ≥124, ≥149, ≥179, ≥214, ≥256, and ≥309 μm were used. Small differences in size are very difficult to determine accurately by visual counts, but are reliably counted by the colony counter. In addition to the visual counts of Day 0 plates, sodium azide control plates were counted by the image analyzer along with the experiment itself. These controls were used to confirm the single cell nature of the original cell suspension and to assess whether cellular debris was present. A vital stain, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride,[2] was used to determine the viability of cells within colonies (>99%). Plating efficiencies were determined for colonies of 50 or more cells. Plating efficiency (P.E.) of viable tumor cells from patient biopsy was 1.4% for 83-4. P.E. of cell strains C8146A, C8146C, C8161, C82-7A1, C83-2C, and C8442 were 3.1, 12.3, 10.5, 1.9, 1.3, and 6.6%. For human melanoma cell line M1RW5 the plating efficiency was 16.0%. The murine melanoma cell line CCL 53.1 had the highest P.E., 27.8%.

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 and has been described in detail elsewhere.[25] Cell diameter was measured for each cell type to determine minimum colony size from counts based on colony diameter as used in either visual or automated counts. The number of cells contained within a colony of specified diameter depends on cell diameter (which are fairly uniform within colonies from any one cell strain or biopsy). The relationship is described in the equation:

$$\text{number of cells/colony} = 2.40\,\frac{(\text{colony diameter})^{2.378}}{(\text{cell diameter})^{2.804}}.$$

From this equation, it follows that colonies of the same diameter can contain different amounts of cells depending on the diameter of the cells. We use colony size as a shorthand term for number of cells per colony. Table 1 details the number of cells per colony for colony diameters from ≥50 to ≥124 μm. The number of cells per colony of other size classes can be determined by using the formula previously cited. M1RW5 contained large diameter cells (20.0 μm). A 50 μm-diameter colony contained 6 cells (2–3 doublings) and a 124 μm-diameter colony, 51 cells (5–6 doublings). This is in contrast to

† Bausch and Lomb, Rochester, NY.

Table 1. The number of cells within each melanoma growth unit and the number of growth units per size class*

	Horizontal diameter of growth unit					
	≥50 μm	≥60 μm	≥72 μm	≥86 μm	≥104 μm	≥124 μm
	Number of cells within growth units					
Cell line						
Murine CCL	≥18	≥27	≥43	≥66	≥104	≥158
Human M1RW5	≥6	≥9	≥14	≥21	≥34	≥51
Cell strain						
C8146A	≥6	≥10	≥15	≥23	≥36	≥54
C8146C	≥15	≥24	≥37	≥56	≥88	≥134
C8161	≥10	≥16	≥24	≥37	≥59	≥89
C82-7A1	≥6	≥9	≥14	≥21	≥34	≥51
C83-2C	≥18	≥27	≥43	≥66	≥104	≥158
C8442	≥15	≥23	≥35	≥53	≥83	≥126
Patient cells						
83-4	≥6	≥10	≥15	≥23	≥37	≥56

* The number of growth units per size class was determined using an automated image analysis as described previously[36]. The number of cells within growth units of different size was calculated using previously described equations[25].

colonies that had small diameter cells (12.7 μm) such as CCL 53.1, which contained 18 cells (4–5 doublings) in a 50 μm-diameter colony and 158 cells (7–8 doublings) in a 124 μm-diameter colony.

Radiation

Cells were irradiated by single dose X rays generated by a Varian Associates 18 MeV linear accelerator operating at 10 MeV and yielding a dose rate of 5.0 gray (Gy) per minute. A 2.0 cm thick bolus was placed on top of the 35 mm-diameter plastic Petri dishes containing the cells. A source to target distance of 100 cm was used, and 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. We have chosen to irradiate cells in agar plates because we can avoid using cell suspensions that contain initial cellular aggregates or clumps of cells. This was especially important in selecting suitable patient biopsies for study. Cells were carried to/from the Radiation Oncology Department in an air-tight modular incubator chamber containing 5% CO_2 and 95% air.

Examination for presence of enlarged cells

In agar individual colonies can be plucked from the Petri dish. 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 mean cell diameter was determined by measuring individual cell diameters from 10 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. These enlarged cells did not approach the size of the smallest colony diameter (42 μm) counted by the image analyzer. Therefore, enlarged "giant" cells within colonies did not alter the interpretation of our results.[12,29,47]

Preparation of survival curves and statistical analysis

Survival data were calculated according to standard radiobiological statistical methods.[12,28,43] There were 12 replicates per control and 6 replicates per experimental dose. D_0 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 slope of the line fitted by linear regression. From values thus obtained, 95 percent confidence intervals for D_0 and D_q values were calculated for colonies containing 50 or more cells. The degree of fit to a linear equation is estimated by the correlation coefficient, R (see results and Table 2).

Preparation of frequency histograms and statistical analysis

Various sizes of colonies formed in agar. Frequency histograms of the various colony diameters are presented in Figure 1. The relative frequency of colonies is expressed as a percentage of the total control colonies. Number of cells per colony equivalents are presented for each experiment. The median of the frequency distribution of colony size (diameter) was analyzed using the

Table 2. Radiobiological parameters and statistical analysis of survival of murine and human melanoma colonies (≥50 cells) grown in bilayer soft agar*

	D_0 value (cGy)	D_q value (cGy)	n number	R†
Cell line				
Murine CCL (1 wk)	160 ± 5 (11)	48 ± 9 (19)	1.4	−0.96
Human M1RW5	250 ± 16 (34)	40 ± 9 (19)	1.1	−0.95
Cell strain				
C8146A	70 ± 8 (18)	60 ± 10 (21)	2.3	−0.96
C8146C	170 ± 9 (20)	40 ± 8 (17)	1.2	−0.88
C8161	350 ± 5 (11)	170 ± 20 (43)	1.6	−0.95
C82-7A1	180 ± 26 (56)	40 ± 11 (23)	1.2	−0.75
C83-2C	160 ± 39 (84)	50 ± 19 (40)	1.4	−0.85
C8442	100 ± 7 (15)	20 ± 1 (02)	1.2	−0.88
Patient biopsy				
83-4	130 ± 23 (50)	40 ± 9 (19)	1.2	−0.87

* The number of cells within a colony was calculated using previously described equations[25]. The colony diameter (colony = ≥50 cells) varied for different cell lines because cell diameters are different.
† Correlation coefficient.
Standard error (95% confidence interval).

nonparametric 2-sample Wilcoxon test to compare the median frequency distribution of control and irradiated cells. Six replicates of each control and irradiation dose were compared.

RESULTS

Irradiation in liquid culture versus agar plates

In these studies, cells were either irradiated in media-containing 4 ml snap-top plastic tubes or in agar in plastic 35 mm-diameter Petri dishes. We found no difference between survival curves generated under the two conditions; however, clumping of cells sometimes occurred for cells irradiated in suspension then plated in agar (data not shown) and, therefore, we have irradiated cells after plating in all subsequent experiments.

Intra- and inter-experimental variability

There is intra-experimental reproducibility for the same experiment counted twice. Survival curves for colonies with ≥50 cells generated by each experiment were the same. The survival curves for different experiments have the same or very similar slope although mean ± S.E. values of survival per dosage and 95% confidence intervals vary for individual experiments.

Conventional survival curves (≥50 cells/colony)

For all melanoma cells we have examined, standard radiation survival curves with initial shoulder regions followed by exponential survival were observed (Fig. 2). D_0, D_q, and n values for these curves are listed in Table 2. In addition, 95% confidence intervals for D_0 and D_q are presented. D_0 values ranged from 70 to 350 cGy, D_q

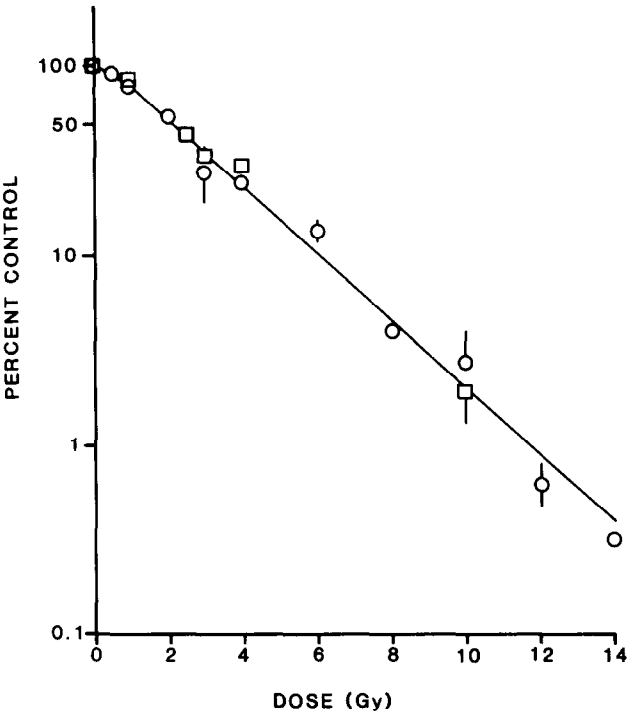

Fig. 1. Frequency distribution of colonies by diameter (percent of control colonies). Human cell line M1RW5. Arrow indicates median colony diameter.

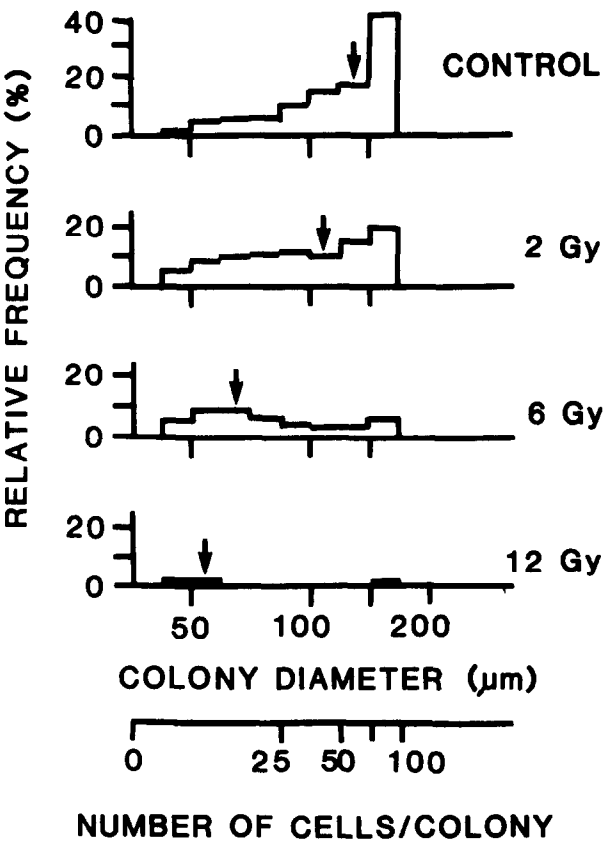

Fig. 2. *In vitro* radiation survival curve for melanoma colonies (≥50 cells). Human cell line M1RW5. Mean of six replicates ± SE are shown; no error bar means SE was smaller than symbol. Symbols: ○, □ denote different experiments.

values from 20 to 170 cGy, and n from 1.1 to 2.3 for human melanoma. Murine melanoma had a D_0 value of 160 cGy, D_q value of 48 cGy and an n number of 1.2. The correlation coefficient, R is listed for each curve in Table 2. R values varied from −0.96 to −0.75.

Effect of colony size on apparent radiation survival

We observed that small differences in colony size, markedly affected the shape of the survival curves in some cases. D_0 values are listed in Table 3 for all curves. For uniform comparison, survival curves for colonies with ≥25 cells (≥4–5 doublings), ≥50 cells (≥5–6 doublings), ≥75 cells (≥6–7 doublings), and ≥100 cell (≥6–7 doublings) per colony are presented. Survival curves for smaller colonies (≥25 cells) had higher D_0 values. Survival curves for intermediate colony sizes had intermediate D_0 values while survival curves for larger colonies had the lowest D_0 values. D_0 values for murine melanoma cell line CCL 53.1 were 168, 160, 152, 140, respectively for colonies with ≥25, ≥50, ≥75, and ≥100 cells. Survival curves for human melanoma cell line M1RW5 also illustrates the dependence of apparent radiosensitivity on colony size scored as survivors. Cells that formed smaller colonies had higher D_0 values than cells that formed larger colonies. D_0 values were 290, 250, 220, and 190 cGy for colonies with ≥25, ≥50, ≥75, and ≥100 cells respectively. Human cell strains had D_0 values that depended on colony size and ranged from 100 to 390, 70 to 350, 70 to 170, and 50 to 160 cGy respectively for colonies with ≥25, ≥50, ≥75, and ≥100 cells. Of these cell strains, C8146A had the lowest D_0 values, from 100 to 50 cGy for colonies with ≥25 to ≥100 cells and C8161 had the highest D_0 values, from 390 to 350 cGy for colonies with ≥25 and ≥50 cells. Cells from patient biopsy 83-4 had D_0 values, ranging from 150 to 130 cGy for colonies with ≥25 and ≥50 cells respectively.

There was wide variation in the D_0's, and, hence, variation in apparent radiosensitivity depended upon the minimum colony size chosen for survival. In experiments in which cells did not uniformly give rise to large colonies (much greater than 50 cells), determination of colony size was critical for reporting precise survival data. This is further illustrated in the histograms of Figure 1. M1RW5 is representative of the other melanoma cells. Note that, many colonies were within the 25 to 100 cell per colony size range. Median colony diameter is indicated by an arrow.

The median colony size of control cells, derived from frequency distribution data of individual plates was compared to the median colony size of irradiated cells by using the non-parametric 2-sample Wilcoxon test. Medians of colony size of control and irradiated cells were significantly different from one another (<0.03) with the following few exceptions: (a) control and 2 Gy from murine cell line CCL and (b) control and 2 Gy from cell strains C8146A and C8161.

Table 3. The D_0 (cGy) values and 95% confidence interval for radiation survival of murine and human melanoma colonies (≥25 cells, ≥50 cells, ≥75 cells, ≥100 cells) grown in bilayer soft agar*

Number of cells per colony	≥25 cells	≥50 cells	≥75 cells	≥100 cells
Cell line				
Murine CCL 53.1	168 ± 11	160 ± 11	152 ± 11	140 ± 12
Human M1RW5	290 ± 21	250 ± 34	220 ± 26	190 ± 24
Cell strain				
C8146A	100 ± 16	70 ± 18	90 ± 35	50 ± 38
C8146C	260 ± 20	170 ± 20	170 ± 20	160 ± 41
C8161	390 ± 11	350 ± 12	N.D.†	N.D.
C82-7A1	200 ± 51	180 ± 56	70 ± 59	N.D.
C83-2C	180 ± 83	160 ± 84	N.D.	N.D.
C8442	120 ± 15	100 ± 15	90 ± 16	80 ± 25
Patient biopsy 83-4	150 ± 29	130 ± 50	N.D.	N.D.

* The number of cells within growth units of different sizes was calculated using previously described equations[25].

† Value not determined, insufficient time for adequate growth of larger colonies.

DISCUSSION

In the current study using precise methodology, we have obtained conventional radiation survival curves with initial shoulders for all murine and human melanoma cells, whether those cells were from established cell lines, short-term cell strains, or from patient biopsies. These results lend considerable support to the notion that growth of cells to form colonies in semisolid medium is a valid measure of clonogenic melanoma tumor cells.

This study defines apparent radiation survival curves for murine and human melanoma cells for colonies containing ≥25, ≥50, ≥75, and ≥100 cells per colony in soft agar. Since the first reported survival curves for human tumor cells,[28] it has been demonstrated that following ionizing irradiation cells do not uniformly give rise to large colonies above the selected survival definition (≥50 cells). Our analysis of colony frequency histograms in response to radiation shows that, at least for murine and human melanoma cells grown in soft agar, minimum colony size must be precisely determined because not all survivors are alike in size. In fact many colonies are at or below 50 cells per growth unit especially at the higher doses of irradiation. If smaller growth units (≥25 cells) are scored as survivors, survival curves are more shallow and have higher D_0 values. For literature using colony diameter as the minimum cutoff for survival, it is possible that these smaller growth units are scored as survivors and hence cells would appear to be radioresistant. There

would be an overestimation of survival because aberrant radiation-damaged cells are able to undergo up to four or five cell divisions. If larger colonies (≥75 cells, ≥100 cells) are scored as survivors, survival curves thus generated would be steeper than for conventional curves and would have lower D_0 values. If by selecting survival based on a minimum colony diameter the larger colonies were scored as survivors, there would be an underestimation of survival, that is, cells would appear to be more radiosensitive. Small variations in colony diameter can markedly affect apparent radiosensitivity. For the murine and human melanoma cells examined, colony diameter varies from approximately 60 to 90 μm for colonies containing ≥25 cells. In contrast, for colonies containing ≥75 and ≥100 cells, the colony diameter is ≥90 μm. Colony diameters and cell diameters must be accurately determined for precise measurement of survival in the soft agar assay.

As with other tumor cell types in the melanoma radiation literature, there was variation in reported survival to ionizing radiation.[4,9,14,15,30–34,38,41,50,51] Reported D_0 values for human melanoma range from 58 to 247 cGy and n values ranged from 1.7 to 40. Part of this variation is due to the range of ionizing radiation sensitivity exhibited by melanoma. Other variation may be due to inaccurate determination of relevant colony size, differences in assay and length of incubation period. We feel that the conditions of the assay should be clearly stated to avoid confusion as to interpretation of the results. In addition to establishing the basic definition of colony size, the influence of length of incubation on radiation survival is an important variable and must be considered when selecting appropriate criteria for generation of survival curves. The length of incubation is so important that data elucidating its effect on the interpretation of survival curves will be presented in a separate manuscript. The influence of length of incubation on radiation survival has been previously examined by other investigators[12,27,44] and more recently by Kirkels *et al.*[18] and Verheijen *et al.*[49] for human tumor cells in soft agar. Adequate incubation allows slow-growing survivors to express their ability to proliferate and, therefore, enables the researcher to distinguish between slowly growing survivors and abortive colonies. Inadequate length of incubation will overestimate radiosensitivity and long periods of incubation may underestimate radiosensitivity. This phenomenon has important implications for any researcher that must select appropriate criteria for generation of survival curves.

Although radiation curves for human melanoma cells have been published,[4,9,14,15,30–34,38,41,50,51] the effect of colony size on the scoring of cell survival has not been closely examined. In our experiments, colonies with ≥25 cells, ≥50 cells, ≥75 cells, and ≥100 cells have been scored as survivors and curves have been generated. Cells forming smaller colonies (≥25 cells) had higher D_0 values; those that formed the largest colonies had lower D_0 values. Small deviations in measurement of colony size changed D_0 values and, hence, the apparent *in vitro* sensitivity. From our results, it follows that measurements of colony diameter and number of cells per colony are crucial in obtaining precise survival curves. We feel that the number of cells per colony is a better designation of survival than is the minimum colony diameter. For those researchers using this assay, cell diameter and colony diameter should be reported for survival curves that are based on a minimum colony diameter so that other researchers can determine the number of cells per colony.

Criticism of the clonogenic assay and radiosensitivity studies have recently focused on the presence of "giant" cells, cells which are up to 10 times the diameter of control cells.[29] In these melanoma soft agar assays, enlarged "giant" cells do not readily form. By careful measurement of single colonies removed from the agar, we found that enlarged single cells were not formed in colonies that contained 25 or more cells. Enlarged cells were present as single cells at the highest doses of radiation, but these enlarged cells did not approach the size of the smallest colony diameter (42 μm) counted by the image analyzer. Therefore this criticism does not hold for melanoma radiation survival and possibly for other tumor types. We agree that this is an important issue and other tumor types should be evaluated.[12,29,47]

Colony survival in the literature has been based on an a designated minimum cutoff of 50 cells for monolayer experiments. It is often difficult to distinguish between small, slowly-growing colonies and abortive colonies. Longer incubation times will be studied and replating experiments will be used to determine proliferatively impaired and nonimpaired small colonies. Experiments will be allowed to incubate until all cells which have the ability to produce a 50 cell colony can do so, and therefore, survivors that grow slowly can be ascertained from non-survivors. In addition to these experiments, replating experiments will be done to confirm the proliferative capability (self-renewal) of irradiated melanoma cells. Self-renewal is an important indicator of proliferative capacity of clonogenic cells[5,26] and may be useful to determine a more biologically relevant definition of survival. Recently, we have demonstrated that self-renewal of human melanoma cells was not limited to cells in large colonies and suggested that the minimum cutoff for self-renewal may be below 16 cells per growth unit.[26] In the future, we will examine the self-renewal capacities of irradiated cells from different sized colonies which should define a biologically relevant size for colony growth. Self-renewal and radiation studies have important implications for chemosensitivity *in vitro* testing since effects of ionizing radiation on cell survival would be expected to be more straight-forward than effects of cytotoxic or cytostatic chemotherapeutic drugs.

REFERENCES

1. Agrez, M.V., Kovach, J.S., Lieber, M.M.: Cell aggregates in the soft agar "human tumour stem-cell assay." *Br. J. Cancer* **46:** 880–887, 1982.
2. Alley, M.C., Uhl, C.B., Lieber, M.M.: Improved detection of drug cytotoxicity in the soft agar colony formation assay through use of a metabolizable tetrazolium salt. *Life Sci.* **31:** 3071–3078, 1982.
3. Alper, T.: *Cellular Radiobiology.* Cambridge, Cambridge University Press, 1979.
4. Barranco, S.C., Romsdahl, M.M., Humphrey, R.M.: The radiation response of human malignant melanoma cells grown *in vitro. Cancer Res.* **31:** 830–833, 1971.
5. Bizzari, J.P., MacKillop, W.J.: The estimation of self-renewal in the clonogenic cells of human solid tumors: A comparison of secondary plating efficiency and colony size. *Br. J. Cancer* **52:** 189–195, 1985.
6. Bregman, M.D., Meyskens, F.L., Jr.: *In vitro* modulation of human and murine melanoma growth by prostanoid analogues. *Prostaglandins* **26:** 449–456, 1983.
7. Bregman, M.D., Peters, E., Sander, D., Meyskens, F.L., Jr.: Dexamethasone, prostaglandin A, and retinoic acid modulation of murine and human melanoma cells grown in soft agar. *JNCI* **71:** 927–932, 1983.
8. Bregman, M.D., Sander, D., Meyskens, F.L., Jr.: Prostaglandin A_1 and E_1 inhibit the plating efficiency and proliferation of murine melanoma cells (Cloudman S91) in soft agar. *Biochem. Biophys. Res. Commun.* **104:** 1080–1086, 1982.
9. Courdi, A., Gioanni, J., Lalanne C.M., Fischel, J.L., Schneider, M., Ettore, F., Lambert, J.C.: Establishment, characterization, and response to cytotoxic and radiation treatment of three human melanoma cell lines. *In Vitro* **19:** 453–461, 1981.
10. Courtenay, V.D., Selby, P.J., Smith, I.E., Mills, J., Peckham, M.J.: Growth of human tumour cell colonies from biopsies using two soft-agar techniques. *Br. J. Cancer* **38:** 77–81, 1978.
11. Dewey, W.C., Humphrey, R.M., Cork, A.: Comparison of cell-multiplication and colony-formation as criteria for radiation damage in cells grown *in vitro. Int. J. Radiat. Biol.* **6:** 463–471, 1963.
12. Elkind, M.M., Whitmore, G.F.: *The Radiobiology of Cultured Mammalian Cells.* New York, Gordon and Breach, 1967.
13. Endresen, L., Tveit, K.M., Rugstad, H.E., Pihl, A.: Chemosensitivity measurements of human tumour cells by soft agar assays are influenced by the culture conditions. *Br. J. Cancer* **51:** 843–852, 1985.
14. Fertil, B., Malaise, E.P.: Intrinsic radiosensitivity of human cell lines is correlated with radioresponsiveness of human tumors: analysis of 101 published survival curves. *Int. J. Radiat. Oncol. Biol. Phys.* **11:** 1699–1707, 1985.
15. Good, M., Lavin, M., Chen, P., Kidson, C.: Dependence on cloning method of survival of human melanoma cells after ultraviolet and ionizing radiation. *Cancer Res.* **38:** 4671–4675, 1978.
16. Hager, T.: Cancer drug sensitivity test has problems. *JAMA* **248:** 3082–3084, 1982.
17. Herman, C.J., Pelgrim, O.E., Kirkels, W.J., Verheijen, R., Debruyne, F.M.J., Kenemans, P., Vooijs, G.P.: In-use evaluation of the Omnicon automated tumor colony counter. *Cytometry* **3:** 439–442, 1983.
18. Kirkels, W.J., Pelgrim, O.E., Hoogenboom, A.M.M., Aalders, M.W., Debruyne, F.M.J., Vooijs, G.P., Herman, C.J.: Patterns of tumor colony development over time in soft-agar culture. *Int. J. Cancer* **32:** 399–406, 1983.
19. Kressner, B.E., Morton, R.R.A., Martens, A.E., Salmon, S.E., Von Hoff, D.D., Soehnlen, B.: Use of the an image analysis sytem to count colonies in stem cell assays of human tumors. *Prog. Clin. Biol. Res.* **48:** 179–193, 1980.
20. Meyskens, F.L., Jr: Human melanoma colony formation in soft agar. *Prog. Clin. Biol. Res.* **48:** 85–99, 1980.
21. Meyskens, F.L., Jr.: Radiation sensitivity of clonogenic human melanoma cells. *Lancet* **2:** 219, 1983.
22. Meyskens, F.L., Jr., Soehnlen, B.J., Saxe, D.F., Casey, W.J., Salmon, S.E.: *In vitro* clonal assay for human metastatic melanoma cells. *Stem Cells* **1:** 61–72, 1981.
23. Meyskens, F.L., Jr., Thomson, S.P.: Biology and chemosensitivity of clonogenic human tumor cells. *Cancer Treat. Res.* **9:** 133–150, 1984.
24. Meyskens, F.L., Jr., Thomson, S.P., Hickie, R.A., Sipes, N.J.: Potential biological explanation of stimulation of colony growth in semisolid agar by cytotoxic agents. *Br. J. Cancer* **48:** 863–868, 1983.
25. Meyskens, F.L., Jr., Thomson, S.P., Moon, T.E.: Quantitation of the number of cells within tumor colonies in semisolid medium and their growth as oblate spheroids. *Cancer Res.* **44:** 271–277, 1984.
26. Meyskens, F.L., Jr., Thomson, S.P., Moon, T.E.: Similar self-renewal properties for different sizes of human primary melanoma colonies replated in agar. *Cancer Res.* **45:** 1101–1107, 1985.
27. Nias, A.H.W., Gilbert, C.W., Lajtha, L.G., Lange, C.S.: Clone-size analysis in the study of cell growth following single or during continuous irradiation. *Int. J. Radiat. Biol.* **9:** 275–290, 1965.
28. Puck, T.T., Marcus, P.I.: Action of X-rays on mammalian cells. *J. Exp. Med.* **103:** 653–666, 1956.
29. Rockwell, S.: Effects of clumps and clusters on survival measurements with clonogenic assays. *Cancer Res.* **45:** 1601–1607, 1985.
30. Rofstad, E.K., Brustad, T.: Broad-shouldered survival curves of a human melanoma xenograft. *Acta Radiol. Oncol.* **20:** 261–265, 1981.
31. Rofstad, E.K., Brustad, T.: Radiation response *in vitro* of cells from five human malignant melanoma xenografts. *Int. J. Radiat. Biol.* **40:** 677–680, 1981.
32. Rofstad, E.K., Brustad, T.: Radiosensitivity of the cells of an established human melanoma cell line and the parent melanoma xenograft. *Int. J. Radiat. Biol.* **44:** 447–454, 1983.
33. Rofstad, E.K., Brustad, T.: Tumor growth delay following single dose irradiation of human melanoma xenografts. Correlations with tumour growth parameters, vascular structure and cellular radiosensitivity. *Br. J. Cancer* **51:** 201–210, 1985.
34. Rofstad, E.K., Wahl, A., Brustad, T.: Radiation sensitivity *in vitro* of cells isolated from human surgical specimens. *Cancer Res.* **47:** 106–110, 1987.
35. Sakamoto, K., Komatsu, K., Matsumoto, Y.: The *in vitro* and *in vivo* antitumor effects of pepleomycin alone or in combination with radiation. *Radiat. Res.* **102:** 224–231, 1985.
36. Salmon, S.E., Young, L., Lebowitz, J., Thomson, S.P., Einsphar, J., Tong, T., and Moon, T.E.: Evaluation of an automated image analysis system for counting human tumor colonies. *Int. J. Cell Cloning* **2:** 142–160, 1984.
37. Selby, P., Buick, R.N., Tannock, I.: A critical appraisal of the "human tumor stem-cell assay." *N. Engl. J. Med.* **308:** 129–134, 1983.

38. Selby, P.J., Courtenay, V.D.: *In vitro* cellular radiosensitivity of human malignant melanoma. *Int. J. Radiat. Oncol. Biol. Phys.* **8:** 1235–1237, 1982.
39. Sinclair, W.K.: X-ray-induced heritable damage (small-colony formation) in cultured mammalian cells. *Radiat. Res.* **21:** 584–611, 1964.
40. Sipes, N.J., Bregman, M.D., Meyskens, F.L., Jr.: Stimulation of human metastatic melanoma colony-forming cells by an acid-sensitive factor in human platelet sonicate. *Cancer Res.* **42:** 6268–6272, 1985.
41. Smith, I.A., Courtenay, V.D., Mills, J., Peckham, M.J.: *In vitro* radiation response of cells from four human tumors propagated in immune-suppressed mice. *Cancer Res.* **38:** 390–392, 1978.
42. Steel, G.G.: *Growth Kinetics of Tumors: Cell Population Kinetics in Relation to Growth and Treatment of Cancer.* Oxford, Clarendon Press, 1977.
43. Steel, G.G., Courtenay, V.D.: The radiobiology of human tumour cells. In *The Biological Basis of Radiotherapy* G.G. Steel, G.E. Adams, and M.J. Peckham (Eds.). New York, Elsevier Science Publishers. 1983, pp. 123–137.
44. Thompson, L.H., Suit, H.D.: Proliferation kinetics of X-irradiated mouse L cells studied with time-lapse photography. I. Experimental methods and data analysis. *Int. J. Radiat. Biol.* **13:** 391–397, 1967.
45. Thomson, S.P., Meyskens, F.L., Jr.: Method for measurement of self-renewal capacity of clonogenic cells from biopsies of metastatic human malignant melanoma. *Cancer Res.* **42:** 4606–4613, 1982.
46. Thomson, S.P., Moon, T.E., Meyskens, F.L., Jr.: Kinetics of clonogenic melanoma cell proliferation and the limits on growth within a bilayer agar system. *J. Cell Physiol.* **121:** 114–124, 1984.
47. Tolmach, L.J., Marcus, P.I.: Development of X-ray induced giant HeLa cells. *Exp. Cell Res.* **20:** 350–360, 1960.
48. Umbach, G., Spitzer, G., Drewinko, B.: "Clumpogenic" versus clonogenic assay. *Lancet* **2:** 628, 1983.
49. Verheijen, R.H.M., Feitz, W.F.J., Kenemans, P., Vooys, G.P., Herman, C.J.: Time course of ovarian tumor growth in soft agar culture. *Br. J. Cancer* **52:** 707–712, 1985.
50. Wiechselbaum, R.R., Nove, J., Little, J.B.: X ray sensitivity of human cells *in vitro*. *Int. J. Radiat. Oncol. Biol. Phys.* **6:** 437–440, 1980.
51. Weininger, J., Guichard, M., Joly, A.M., Malaise, E.P., Lachet, B.: Radiosensitivity and growth parameters *in vitro* of three human melanoma cell strains. *Int. J. Radiat. Biol.* **34:** 285–290, 1978.
52. West, C.M.L., Sutherland, R.M.: A radiobiological comparison of human tumor soft-agar clonogenic assays. *Int. J. Cancer* **37:** 897–903, 1986.