Using Computerized Video Time Lapse for Quantifying Cell Death of X-irradiated Rat Embryo Cells Transfected with c-myc or c-Ha-ras

Helen B. Forrester, Charles A. Vidaire, Norman Albright, Clif C. Ling, and William C. Dewey

Radiation Oncology Research Laboratory, University of California San Francisco, San Francisco, California 94103-0806 [H. B. F., C. A. V., N. A., W. C. D.]; and Department of Medical Physics, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [C. C. L.]

ABSTRACT

Rat embryo fibroblasts that had been transfected with the c-myc or c-Ha-ras oncogene were X-irradiated, after which individual cells and their progeny were followed in multiple fields for 5–6 days by computerized video time lapse microscopy to quantify the lethal events that resulted in loss of clonogenic survival. The loss of clonogenic survival of X-irradiated (9.5 or 2.5 Gy) REC:myc cells was attributed almost entirely to the cells dying by apoptosis, with almost all of the apoptosis occurring after the progeny had divided from one to four times. In contrast, the loss of clonogenic survival of X-irradiated REC:ras cells was attributed to two processes. After 9.5 Gy, ~60% of the nonclonogenic cells died by apoptosis (with a very small amount of necrosis), and the other 40% underwent a senescent-type process in which some of the cells and their progeny stopped dividing but remained as viable cells throughout the 140 h of observation. Both processes usually occurred after the cells had divided and continued to occur in the cells’ progeny for up to five divisions after irradiation. Furthermore, the duration of the apoptotic process was shorter for REC:myc cells (0.5–1 h) than for REC:ras cells (4–5 h). By using computerized video time lapse to follow individual cells, we were able to determine the mode of cell death. This cannot be determined by conventional clonogenic survival experiments. Also, only by following the individual cells and their progeny can the true amount of apoptosis be determined. The cumulative percentage of apoptosis scored in whole populations, without distinguishing between the progeny of individually irradiated cells, does not reflect the true amount of apoptosis that occurs in cells that undergo postmitotic apoptosis after irradiation. Scoring cell death in whole populations of cells gives erroneous results because both clonogenic and nonclonogenic cells are dividing as nonclonogenic cells are apotosing or senescing over a period of many days. For example, after 9.5 Gy, which causes reproductive cell death in 99% of both types of cells, the cumulative percentage of the cells scored as dead in the whole population at 60–80 h after irradiation, when the maximum amount of cumulative apoptosis occurred, was ~60% for REC:myc cells, compared with only ~40% for REC:ras cells.

INTRODUCTION

Studies have indicated that REC:myc rat embryo cells transfected with c-myc are more susceptible to radiation-induced apoptosis than REC:ras cells transfected with c-Ha-ras (1). However, the clonogenic survival after irradiation is almost the same for the two cell types (2). The same results were obtained with rat-1 fibroblasts transfected with c-myc or c-Ha-ras oncogenes (3). Therefore, one might conclude that radiation-induced apoptosis plays a different role in these two cell lines in the loss of reproductive integrity after irradiation. One might hypothesize that the same level of lethal damage is sustained but expressed differently, as a considerable amount of apoptosis in REC:myc cells but as some other mode of cell death in REC:ras cells.

Recently, by constructing pedigrees of individual irradiated REC:myc cells by time lapse video microscopy (4), we demonstrated that, after 9.5 Gy of irradiation, almost all reproductive cell death was attributed to apoptosis, with at least 96% of the events occurring after one to four cell divisions. Thus, for this cell line, postmitotic apoptosis appears to be the main mode of radiation-induced death. Possibly, the REC:ras cells also undergo a reproductive cell death that is expressed not as apoptosis but instead as necrosis and/or giant cell formation, as discussed recently in a review (5).

In the past, time lapse studies have demonstrated that HeLa cells (6, 7) and mouse L cells (8) undergo postmitotic death that is associated with trapping in mitosis, fusion of daughter cells shortly after division, giant cell formation, and death during interphase that was classified as pyknosis or necrosis. When these studies were conducted, apoptosis had not been identified. Also, with the aerial photographic camera that was used (8), individual cells could not be adequately visualized to discern morphological alterations that could be related to the ultimate fate of the cells. The individual HeLa cells were followed with sufficient magnification, but the fields contained too many cells to distinguish between pedigrees derived from clonogenic or nonclonogenic cells. Attempts have been made to develop time lapse systems using multiple fields (9, 10) so that progeny from one or two cells in each field at the time of irradiation could be followed as a colony of ~50 cells developed. However, such approaches have not been reported for quantifying the different types of lethal events occurring in pedigrees derived from clonogenic or nonclonogenic cells after irradiation. Here, we have used a CVTL system, which has allowed us to carry out such a study to characterize and quantify the lethal events that result in reproductive cell death in irradiated REC:myc and REC:ras cell populations.

MATERIALS AND METHODS

Cells, Set-Up for CVTL Analysis, and Irradiation. Rat embryo cell lines expressing either the c-myc oncogene (REC:myc) or the c-Ha-ras oncogene (REC:ras; Refs. 1 and 2) were cultured in DMEM with 10% FCS (Hyclone Laboratories Inc., Logan, UT). Oncogene expression was confirmed during the course of these experiments. Cells growing exponentially at 37°C and at a pH of 7.4 were plated into a T-25 Costar tissue culture flask (104 cells/flask). The flask was incubated for ~4–6 h to allow the cells to attach and for the pH to adjust, and then the top was screwed down tightly. Next, the flask was placed into a temperature-controlled (37°C) incubation chamber enclosing an inverted phase-contrast microscope shielded from ambient light. The cells were incubated for 23–26 h while images of cells in up to 50 different fields were being obtained at 10-min intervals with a CVTL system (described below). Because the generation times of REC:myc and REC:ras cells are ~10 and 12 h, respectively, the cells divided one to three times before they were irradiated. Then, a small area of the flask, delineated with a 2.5-mm-diameter lead collimator, was irradiated (2.5, 4.0, or 9.5 Gy) with a Phillips 250 kVp X-ray machine at a dose rate of 1.1 GY/min and a dose uniformity of 2.3%. The portion of the flask outside the collimator was covered with a 2-mm-thick lead shield above and below the flask such that the dose rate was 0.2–0.7% that of the exposed area. After irradiation, CVTL images were acquired over a period of 5–6 days from the same fields acquired before irradiation. About 40 and 10 fields were imaged from the irradiated and unirradiated sections, respectively. In one experiment, cells were irradiated at a dose rate of 4 Gy/min without a collimator (4).

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2 To whom requests for reprints should be addressed, at Radiation Oncology Research Laboratory, University of California San Francisco, MCB 200, 1855 Folsom Street, San Francisco, CA 94103-0806. Phone: (415) 476-9062; Fax: (415) 476-9009; E-mail: dewey@rosl.ucsf.edu.

The abbreviation used is: CVTL, computerized video time lapse.
Description of CVTL System. With the CVTL system (Compix, Inc., Cranberry Township, PA), images were obtained at ×200 with a Nikon Diaphot 300 inverted phase contrast microscope connected to a charged coupled device camera. The data acquisition station consists of a Pentium 90-MHz system with 96 megabytes of memory and two 540-megabyte disk drives with the Windows 95 operation system. The analysis workstation consists of a Pentium Pro 200-MHz system (64 megabytes of memory and two 2-gigabyte hard drives) operating with Windows NT 4.0 and an additional 30-gigabyte storage array. This system enables us to analyze two sets of data simultaneously or to analyze one set of data while other data are being acquired. Images are taken every ~11 s, under computer control of the autofocus of the microscope and movement of the stage from one field to the next. For ~1000 images of each of 50 fields observed every 10 min during ~5 days, 15 gigabytes of memory are required. Each field can be played back at ~10 images/s or as individual images. Later, 6–12 fields that contained from two to eight cells at the time of irradiation were selected for analysis of the irradiated cells and their pedigrees. The images from each experiment were stored permanently on tape.

Identification and Quantification of Apoptotic Cells in Irradiated Populations. For a valid comparison with previous studies, cells were plated and irradiated as described (4, 11). Specifically, REC:myc or REC:ras cells were plated at a relative high density (~2.5 × 10^4 cells/T25 flask), and the whole flask was irradiated (9.5 Gy) at a dose rate of 4 Gy/min (4). For data illustrating and quantifying the staining characteristics of the cells, the cells were incubated at 37°C for 40 h after irradiation (0 or 9.5 Gy) and then stained in the flasks by adding propidium iodide and Hoechst 33342 (both at 10 μg/mL). Cells were incubated at 37°C for 40 h after irradiation (0 or 9.5 Gy) and then stained in the flasks by adding propidium iodide and Hoechst 33342 (both at 10 μg/mL) directly to the medium. Within 10–20 min of staining, the cells were scored and quantifying the staining characteristics of the cells, the cells were incubated at 37°C for 40 h after irradiation (0 or 9.5 Gy) and then stained in the flasks by adding propidium iodide and Hoechst 33342 (both at 10 μg/mL). Cells were incubated at 37°C for 40 h after irradiation (0 or 9.5 Gy) and then stained in the flasks by adding propidium iodide and Hoechst 33342 (both at 10 μg/mL) directly to the medium. Within 10–20 min of staining, the cells were scored and photographed by fluorescence microscopy (12) using filters (exciter, D 360/40; emitter, gg420; and beamsplitter, 400DCLP supplied by Chroma Technology Corp., Brattleboro, Vt.) and Kodak 400 ASA slide film, Elite II.

RESULTS

Morphological Characteristics of the Cells. The unirradiated REC:myc and REC:ras cells are different in appearance although they were derived from the same parent cell. The REC:myc cells are flat, and colonies of cells take on a cobblestone-like appearance, which makes it easy to follow individual cells. In contrast, REC:ras cells are spindle-like in shape, are not always completely flat, and have a tendency to move over and under each other. Also, they were more likely to move out of the visual field than the REC:myc cells. Therefore, we derived an equation to predict the outcome of the lost cells based on the outcome of the related cells that remained within the field. We refer to these derived results as “corrected for lost cells” (see Appendix).

To support the classification of apoptosis, irradiated cells (and unirradiated control cells) were scored in populations by fluorescence microscopy (Figs. 1 and 2). This analysis indicated that practically all of the cell death that was observed as rounded cells in progeny of both REC:myc and REC:ras cells was due to apoptosis. Furthermore, electrophoresis of DNA extracted from rounded loosely attached cells demonstrated DNA laddering (1).4 Note that the amount of apoptosis was scored in the populations at 40 h after irradiation before many of the cells had apoptosed. Therefore, the percentage of the cells that had apoptosed by 40 h (Fig. 2) was much lower than the percentage that apoptosed by 120 h, i.e., ~40% versus 99% for REC:myc cells and ~25% versus 55% for REC:ras cells (data shown later).

Identifying Pedigrees as Clonogenic or Nonclonogenic and Fate of Cells within Pedigrees. Approximately 5% of the unirradiated REC:myc and REC:ras cells that appeared healthy at 4 h after plating resulted in progeny that had a high frequency of spontaneous cell death or abnormal cell divisions, when followed by CVTL for two to three generations. These cells were eliminated from the study. In most experiments, unirradiated cells also were followed in the same flask as the irradiated cells to assure that the conditions in the flask could maintain normal cell division. Some cell death did occur in the progeny of unirradiated cells, but the frequency of cell death was only ~0.01 per generation (data not shown). Also, the cells were followed before irradiation to estimate their phase in the cell cycle at the time of irradiation (time of irradiation after birth of the cell, i.e., division of the cell’s mother).

Individual cells and their progeny were followed for 5–6 days after irradiation and were characterized as normal interphase cells, large or giant cells, fused cells, multinucleated cells, dividing cells, and dying cells. The times of these events were noted so family trees (pedigrees), presented on a time scale, could be developed (Fig. 3). An example of a pedigree from an unirradiated REC:ras cell is shown in Fig. 3B. Binucleated cells occurred either when cells fused together or when cells rounded and appeared to attempt division before flattening into binucleated cells (Fig. 3 and data not shown). Cell death was classified as apoptosis when the cells rounded followed by vigorous membrane blebbing for 30–60 min in the case of REC:myc cells (illustrated in Ref. 11) and for ~4–5 h in the case of REC:ras cells (Fig. 4). Because the prolonged blebbing process exceeded 3 h only when cells started blebbing more than 8 h after they had divided (data not shown), these cells may have traversed the cell cycle and been trapped as rounded cells in mitosis. When the rounded cells stopped moving, they were classified as being dead, but they remained loosely attached to the substratum for the duration of the experiment.

Individual irradiated cells were classified as either clonogenic or nonclonogenic. A cell was clonogenic if it divided and produced progeny that formed a colony (40–50 normal cells) with generation times similar to the control cells. Examples of clonogenic colonies formed from a REC:myc cell irradiated with 2.5 Gy, an unirradiated REC:ras cell, and a REC:ras cell irradiated with 9.5 Gy are shown in Fig. 3, A, B, and C, respectively. A cell is defined as nonclonogenic if it died without dividing, or if its progeny did not form a colony because of cell death or prolonged generation times. Examples of the nonclonogenic pedigrees of irradiated REC:ras cells (9.5 Gy) are shown in Fig. 3, D–G.

We also observed lethal sectoring within clonogenic colonies derived from irradiated cells (examples shown in Fig. 3, A and C). Lethal sectoring is defined as a cell division that results in only one of the daughter cells producing a clonogenic subpopulation (8). In one of seven clonogenic colonies from 17 REC:myc cells that received 2.5 Gy (Fig. 3A), there were four lethal sectoring events leading to death by apoptosis. No lethal sectoring was observed in four clonogenic colonies from 20 REC:ras cells irradiated with 2.5 Gy (data not shown). In the one clonogenic colony from 38 REC:ras cells irradiated with 9.5 Gy, lethal sectoring was observed as a senescent-like process in which one of the daughter cells produced progeny that consisted of three cells that remained viable for >140 h without dividing (Fig. 3C).

Fig. 5 summarizes the fate of the irradiated REC:myc and REC:ras cells (9.5 Gy) and their progeny, when followed by CVTL for 140 h. Fig. 5A shows that, for both cell types, only a small fraction (0.05–0.09) died by apoptosis without division. None of the 49 irradiated REC:myc cells formed colonies, only one remained alive without dividing (senescence), and the remainder produced progeny that underwent apoptosis. In contrast, of the 38 REC:ras cells, 60% were nonclonogenic due to apoptosis only in the progeny, 16% were nonclonogenic due to a senescent-like process only in the progeny, 16% had progeny that manifested both apoptosis and “senescence,” and only one was clonogenic. Insufficient data were available to determine how these percentages depended on the phase of the cell cycle irradiated.

Cell death and senescence can occur after the irradiated cells have divided several times. Fig. 5B summarizes the frequency of these events for the irradiated REC:myc and REC:ras cells in each generation. Most of the REC:myc cells died in generation 1, and the

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4 Unpublished data.
remainder died in the next generation. Interestingly, one REC:myc cell survived until the end of the video without dividing. In contrast, death, division, and division followed by cell fusion continued to occur in the REC:ras cells for five generations after irradiation. About 5–10% of the divisions were followed by fusion of the daughter cells for both REC:myc and REC:ras cells. All of the fused REC:myc cells that could be followed apoptosed (before or after another division), whereas five of the six fused REC:ras cells that could be followed remained alive without further division. The other REC:ras cell that divided and fused, divided again into three cells before fusing again into one cell; then, the cell died by apoptosis (data not shown). Another two REC:ras sister cells, which were irradiated with 9.5 Gy in G₁ ≈ 1.7 h after they were born, fused after irradiation; these two cells were excluded from Fig. 5B because this event would be classified as a fusion between two irradiated daughter cells instead of a fusion after division of an irradiated cell. Also, there were three REC:ras cells that did not divide or die but were lost ≈ 94 h after irradiation; these cells were included in Fig. 5 as senescent cells.

Times of Cell Divisions and Generation Times. Fig. 6 shows the time postirradiation (9.5 Gy) when the cells divided. For both REC:ras and REC:myc cells, the times of division for the different generations often overlapped (Fig. 6, A and B). Therefore, cells from different generations were present at the same time, which is caused by the variation in generation times between individual cells in the pedigrees.

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Fig. 1. Fluorescence photomicrographs of cells treated with Hoechst 33342 and propidium iodide. Blue, DNA has been stained only with Hoechst 33342 because the PI was excluded by the intact cell membrane. Pink, nucleus has been stained with both dyes because a defective cell membrane has allowed PI to enter the cell. The unirradiated cells and normal looking irradiated cells have flat oval-shaped nuclei that fluoresce uniformly blue; the nuclei of early apoptotic cells contain several lobular or fragmented blue bodies; and necrotic nuclei contain pink nuclei that are round or oval in shape. Micronuclei appear as smaller blue dots along side a flattened nucleus. A, unirradiated REC:myc cells that have normal-looking blue nuclei plus two cells in mitosis. B, REC:myc cells stained 40 h after 9.5 Gy. Several normal blue nuclei are seen, along with cells in early and late apoptosis. The flattened blue nuclei are slightly out of focus as they are in a different focal plane than the “rounded” apoptotic cells. C, unirradiated REC:ras cells that have normal-looking blue nuclei plus one cell in mitosis. D, REC:ras cells stained 40 h after 9.5 Gy. Several normal blue nuclei are seen, along with cells in early and late apoptosis, plus one possible necrotic cell. Also, a couple of small micronuclei are seen. Note that the apoptotic REC:ras cells appear in the same focal plane as the flattened cells.

Fig. 2. REC:ras and REC:myc cells were stained with Hoechst 33342 and propidium iodide 40 h after irradiation with 9.5 Gy, and 500 cells were scored in each group as live normal cells or as dead apoptotic or necrotic cells (as in Fig. 1). Many of the nonclonogenic REC:myc and REC:ras cells that will apoptose are still alive at this time point (see Figs. 8A and 9). Note that for both REC:myc and REC:ras cells, most of the cell death was due to apoptosis, with very little due to necrosis. Two separate experiments are shown for REC:ras cells. For the unirradiated populations, fewer than 4% of the cells were apoptotic or necrotic (data not shown).
Section of a document discussing the effects of irradiation on cell division and cell death. The text describes pedigrees of individual cells, showing how cells divide and die over time, and how different cell lines respond to radiation. The figures illustrate the family trees of cells, with generations marked by dashed lines and time intervals between cell division indicated by solid lines. The text explains that some cells die by apoptosis, while others enter senescence, and that the generation times for the progeny of irradiated cells are longer than those of unirradiated cells. The results are compared with conventional studies to highlight the advantages of analyzing individual cell pedigrees. The overall goal is to understand the kinetics of cell death and the fate of irradiated cells in vitro. 

As illustrated in Fig. 6C, the generation times for REC:myc and REC:ras cells, respectively, increased from 10–12 h for generation −1 (prior to irradiation) to 15–20 h for generation 0, in which the cells were irradiated. In the following generation 1, the generation times for both the irradiated REC:myc and REC:ras cells returned to approximately the same time as for the unirradiated cells. The remaining REC:myc cells died in generation 2, so there were no generation times beyond generation 1. However, the generation times for the REC:ras cells gradually increased, until by generations 5 and 6, they were almost three times that of the unirradiated cells (see Fig. 3F for a specific example). Even the generation times for the progeny of the irradiated clonogenic REC:ras cell (Fig. 3C) were longer than those for the unirradiated REC:ras control colony (Fig. 3B). This lengthening of generation times was not caused by deterioration of growth conditions in the flasks having irradiated cells; i.e., the unirradiated REC:ras cells in the same flask as the irradiated cells maintained the same 10–12 h generation times for 1–6 generations (Fig. 6C). 

Fates of Populations of Irradiated Cells: Analysis of Pedigrees Compared with Analysis of Populations as a Whole. From the pedigrees of individual cells we can obtain information for an irradiated population that can be compared with results obtained from conventional studies involving an analysis of the population as a whole (5, 11, 13). Fig. 7, A and B, shows the relative number of live cells (relative to the number of irradiated cells) remaining attached to the surfaces of the flasks as a function of time after irradiation with 9.5 Gy, and Fig. 7C illustrates the kinetics of cell death by the cumulative percentage of the nonclonogenic cells that had died by detaching from the surface. The data show that, after 9.5 Gy, there was much more...
for cells lost from the fields (see Appendix), the one clonogenic cell by the curve (apoptotic death that occurred more rapidly for REC:myc cells than for REC:ras, 7 after 2.5 Gy, and 54 after 9.5 Gy; and for REC:ras, 7 after 2.5 Gy, and 54 after 9.5 Gy; bars, SE). These durations did not depend on the time after irradiation when the cells died (Ref. 4 and data not shown). Columns, numbers of cells scored, as follows: for REC:myc, 18 after 2.5 Gy, 219 after 4 Gy, and 96 after 9.5 Gy; and for REC:ras, 7 after 2.5 Gy, and 54 after 9.5 Gy; bars, SE.

Several studies have been reported in which cell death by apoptosis was quantified by scoring whole populations for the percentages of cells that were apoptotic at various times after irradiation (Refs. 1, 11, and 13; and see Ref. 5 for other examples). Therefore, we have presented our data in this manner and compared the results with what was observed when the amount of cell death (apoptosis) was determined by following the pedigrees derived from individual irradiated cells. These comparisons have been made in Fig. 8A for REC:myc cells irradiated with 9.5 Gy (data from Ref. 4) or 2.5 Gy and in Fig. 9 for REC:ras cells irradiated with 9.5 Gy. In these figures, curves for the cumulative percentage of the cells that had died are plotted versus time after irradiation. Curves are also plotted for the percentages of the live cells in the progeny being analyzed that were attributed to progeny derived from the clonogenic cells.

Fig. 8A shows that by 120 h after irradiation of REC:myc cells with 9.5 Gy, ~98% of the progeny of nonclonogenic cells (Non-clono.) had apoptosed and that essentially only progeny of clonogenic cells persisted (% of live cells from clono.). In this experiment (4), the progeny apoptosed rapidly, but they did not apoptose as rapidly as the progeny analyzed in the experiment shown in Figs. 6A and 7, possibly because the cell density at the time of irradiation was ~4 times higher than that for the results illustrated in Figs. 6A and 7. In the experiment illustrated in Fig. 8A, the proliferation of the progeny derived from the one clonogenic cell was largely responsible for the decrease after 80 h postirradiation in the cumulative percent of cells dead (with clono.), i.e., from a maximum of 60% to ~20% at 130 h. When the REC:myc cells were irradiated with a lower dose of 2.5 Gy (Fig. 8F), the maximum cumulative apoptotic percentage (with clono.) occurred earlier, with a value of ~15% at 40–50 h after irradiation, compared with the true apoptotic percentage of ~60% (CVTL % Non-clono.). Also, curves similar to the cumulative percentage of cells dead (with clono.) were obtained when the cells in the field were quantified, without any regard to pedigree analysis, for the percentage of the cells that appeared rounded and loosely attached (field analysis); these values from the field analyses for 9.5 and 2.5 Gy are almost the same as the cumulative apoptotic values obtained from the analyses of the populations as a whole (1). For 9.5 Gy and, presumably, for 2.5 Gy, almost all of the nonclonogenic cells would have apoptosed by 120 h after irradiation. However, by this time, the proliferation of surviving clonogenic cells caused the cumulative apoptotic percentages to drop to ~20% and ~5% for 9.5 and 2.5 Gy, respectively, values that are much lower than the true apoptotic percentages of 98% and 60%, respectively. Therefore, when cells apoptose after division, the fraction of the irradiated cells, including their progeny, that apoptose can be determined accurately only by following pedigrees of individual cells to account for the proliferation of clonogenic and nonclonogenic cells.

Fig. 9 plots the cumulative percentage of the progeny that were dead (apoptotic) after REC:ras cells received 9.5 Gy. The maximum percentage dead by field analysis (30%) was much lower than both the true percentage of the original population that became nonclonogenic (~98%) and the percentage of the nonclonogenic progeny that apoptosed (~60%). Note that for the field analysis, the percentage dead at 40 h (25%) was not much different from the percentage (13%) scored immediately after irradiation; the relatively high initial value is probably attributed to rounded cells that were either in mitosis or had just completed mitosis at the time of irradiation or to interphase cells that were not completely flat. In previous studies of whole populations (1, 11), the amount of apoptosis was determined by the percentage of cells that could be rinsed off the flask. This was reported to be even less than what we observed in the field analysis, and this difference may be due either to apoptotic cells remaining on the flask (see Fig. 2D, which illustrates that apoptotic cells adhere to the surface) or to the apoptotic cells breaking up (which may account for the percentage of apoptotic cells from the field analysis being somewhat less than the percentage from the pedigree analysis; Fig. 9, with clono.). Thus, just by observing the population of irradiated cells in a field of a flask or from counting the cells that could be rinsed off, one could reach the conclusion that the REC:ras cells undergo very little cell death by either apoptosis or necrosis, whereas by following individual cells by CVTL, one can see that, by 140 h after irradiation, ~96% of the original cells were nonclonogenic, with ~60% of the nonclonogenic progeny dying by apoptosis and ~40% of them remaining as nonclonogenic senescent-like cells.

In summary, the percentage of cells scored by Hoechst 33342 and propidium iodide fluorescence as being apoptotic at 40 h after 9.5 Gy (40% for REC:myc and 25% for REC:ras in Fig. 2) was about the same as the cumulative percentage of cells dead as obtained from the CVTL field analysis and the CVTL analysis of the pedigrees (Figs. 8 and 9, with clono.). However, by using the CVTL to distinguish the pedigrees of nonclonogenic cells from the pedigrees of proliferating clonogenic cells, we determined that by 120 h after irradiation, ~98% of the nonclonogenic REC:myc cells had apoptosed, whereas only ~60% of the nonclonogenic REC:ras cells had apoptosed.

**DISCUSSION**

Here, we have demonstrated that both REC:myc cells and REC:ras cells usually die by a postmitotic process after irradiation (Figs. 1, 5B, and
This has been demonstrated for many cell types for many years (8, 14, 15) and has been attributed primarily to unrejoined and misrejoined DNA double strand breaks that are observed as exchanges and fragments in interphase cells with prematurely condensed chromosomes (16). After the cells traverse the cell cycle and enter division, these exchanges and fragments are observed in mitotic cells as chromosomal aberrations (16–18). Then, after division, these aberrations appear as micronuclei that result in cell lethality apparently from the loss of genetic information in daughter cells (18, 19). We have now demonstrated for both REC:myc and REC:ras cells that postmitotic death is manifested as apoptosis of the progeny during the generations after irradiation (Figs. 3; 5, A and B; 6; and 7). For REC:ras cells, but not REC:myc cells, 40% of the postmitotic death in the progeny of the nonclonogenic cells are senescent-like events (Figs. 5A and 9), similar to that reported from time lapse studies.

Fig. 5. The fates of 49 REC:myc and 38 REC:ras cells and their progeny were tracked for 140 h after irradiation with 9.5 Gy. A, the fraction of irradiated cells classified in the following categories are shown: (a) clonogenic with and without lethal sectoring (clonogenic), (b) nonclonogenic without cell death (senes.), (c) nonclonogenic with cell death and live cells (senes. and death), (d) nonclonogenic due to cell death (death), and (e) nonclonogenic cells that died without dividing after irradiation (death without div.). B, the fraction of nonclonogenic cells in each generation that: (a), divided; (b), divided with the daughter cells fusing; (c), died; and (d), did not divide again but remained alive. Generation 0 is the generation during which irradiation occurred. No REC:myc cells survived beyond generation 2. Numbers above columns, numbers of cells scored.

Fig. 6. The times at which cells irradiated with 9.5 Gy completed their first, second, third, and so on divisions after irradiation. A, REC:myc cells, all of which were nonclonogenic. B, REC:ras cells; , the progeny of a cell that was clonogenic; △, the progeny of cells that were nonclonogenic. C, mean generation times for both the unirradiated REC:myc and REC:ras cells (9.5 Gy) are shown for the generation before the cells were irradiated (generation −1), the generation in which irradiation occurred (generation 0), and the following 1–6 generations. None of the irradiated REC:myc cells survived beyond generation 2. Unirradiated REC:ras cells located in a shielded area of the irradiated flask were also followed, and the mean generation times for these cells are also presented. Bars, SE.
Fig. 7. A, the number of cells including their progeny still alive at various times after irradiation (9.5 Gy) relative to the original number of live cells present at the time of irradiation are shown separately for REC:myc cells (■) and REC:ras cells (○ and △); 49 nonclonogenic REC:myc cells, 37 nonclonogenic REC:ras cells, and 1 clonogenic REC:ras cell were followed. For REC:ras, data are shown for nonclonogenic cells only (○) and for the whole population (△) that included the one clonogenic cell. B, the same data shown in A, except that a correction was applied for cells lost from the fields (see Appendix). For REC:ras cells, the correction made a large difference particularly for the population after the cells were born. For example, REC:myc cells irradiated with 9.5 Gy early in the cell cycle apoptosed later after undergoing more divisions than when they were irradiated late in the cycle.\(^3\) In addition, genomic instability should be related to the amount and type of HeLa and mouse L cells (6–8, 20–22). The cells sustain a delay in the cell cycle in which they are irradiated, followed by very little, if any, delay in the next generation but with increasing delays in the subsequent generations (Fig. 6C). Similar observations have been reported for other cell types (6, 23).

Senescent-like events have been observed for years (14, 15), as frequently as nonclonogenic progeny that stop dividing and then form giant and/or multinucleated cells that persist for days. Also, senescent-like events manifested during the irradiated G1 phase (24, 25) or during the second G1 phase after division (26) have been reported for human cell lines that express wild-type p53. Although we have referred to this phenomenon as senescent-like or, for brevity, as senescence, we do not imply that the underlying molecular process is the same as senescence that occurs as cells age.

Here, we have made two major new observations pertaining to radiation-induced apoptosis and senescence. (a) The characteristics of postmitotic death are modified by transfecting different oncogenes. Essentially all of the nonclonogenic REC:myc progeny die by a postmitotic apoptotic process that has a duration of 30–60 min once the apoptotic process is initiated. In contrast, nonclonogenic REC:ras progeny undergo postmitotic death that is manifested both as senescence and as an apoptotic process that has a duration of 4–5 h (Fig. 4). (b) We have demonstrated that, to quantify the amounts and types of events associated with postmitotic death, individual cells and their progeny must be followed for several generations after irradiation. This can be accomplished by using CVTL to discriminate between proliferating clonogenic and nonclonogenic descendants as apoptosis and/or senescence are occurring in nonclonogenic descendants during several days and generations after irradiation. In fact, the cumulative percentage of apoptosis assessed in the population as a whole will often give misleading results. For example, the analysis of a whole population of irradiated REC:ras cells gave the impression, as reported (1), that no more than ~20% of the nonclonogenic cells would apoptose after 9.5 Gy (Figs. 2 and 9), whereas the CVTL analysis of pedigrees revealed that ~60% of the nonclonogenic progeny apoptosed (Figs. 7C and 9). Such observations can explain why different cell lines manifesting greatly different amounts of apoptosis as quantified in irradiated populations as a whole can have the same clonogenic survival (3, 27–29). However, for cells that apoptose shortly after irradiation without dividing, as lymphocytes and lymphomas, a reasonably accurate assessment of the amount of apoptosis should be possible by quantifying apoptosis in the irradiated population as a whole (5).

Finally, senescent-like cells that we have observed in vitro for REC:ras may play an important role in vivo. For example, for tumors grown from two different isoegenic cells lines in severe combined immunodeficient mice, the radiation-induced decrease in tumor volume and subsequent delay in regrowth was less for the cell line that had a lawn of senescent-like cells present for as long as 14 days after irradiation of the same tumor cells cultured in vitro (29). The role that apoptosis plays is unclear (29–31), but the data suggest that, in vivo, an increase in radiation-induced apoptosis relative to senescence results in an increase in tumor shrinkage shortly after irradiation. The effect on tumor cures (the dose to cure 50% of the tumors, TCD-50), however, remains to be determined.

By following individual cells, we can determine the times and types of events that result in loss of reproductive integrity when cells are irradiated in different phases of the cell cycle, i.e., at different times after the cells were born. For example, REC:myc cells irradiated with 4 or 9.5 Gy early in the cell cycle apoptosed later after undergoing more divisions than when they were irradiated late in the cycle.\(^3\) In addition, genomic instability should be related to the amount and type of...
of lethal sectoring, i.e., apoptosis or senescence, that occurs in progeny that develop from clonogenic cells irradiated in different phases of the cell cycle. Also, senescent-like cells should be followed long enough to determine their ultimate fate. Finally, the amount of radiation-induced apoptosis should be modulated to determine the molecular pathways that are important when apoptosis occurs either before the cells divide, as they divide, or after they divide. For example, ~90% of L5178Y and Molt-4 cells that received 4 Gy apoposed during prolonged abortive mitoses; therefore, alterations in molecular processes associated with mitosis should be investigated (see sections 4, 8, and 9 on p. 793 in Ref. 5 for a detailed discussion of this approach).

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APPENDIX

Equations Correcting for Lost Cells. The fate of a lost cell was predicted to be the same as the fate of the other cells within the same pedigree. The equations used to calculate the number of live (y) and dead (z) cells present (corrected for lost cells) at time n are: $y(n) = y(n-1) + a(n) + b(n)\alpha\delta(n-1)$ (Eq. K) and $z(n) = z(n-1) + y(n-1)\alpha\delta(n-1) + e(n-1)$ (Eq. L), respectively. These equations, which are defined and derived below, were used to correct for the lost cells within a pedigree every 10 h after irradiation (i.e., (time at $n+1$) − (time at $n-1$)) = 10 h.

Notations. Notations are as follows: $a(n)$ = number of cells alive and present on the video at time n; $e(n)$ = number of dead cells present at time n; $r(n)$ = number of live lost cells at time n; and $s(n)$ = number of dead lost cells at time n. $y(n)$, which is the number of cells alive at time n (corrected value), is then:

$$y(n) = a(n) + r(n)$$ (A)

$z(n)$, which is the number of dead cells at time n (corrected value), is then:

$$z(n) = e(n) + s(n)$$ (B)

The remaining terms are as follows: $t(n)$ = the total number of cells, dead and alive, at time n (corrected value); $b(n)$ = the number of live and present cells lost (from $a(n-1)$) between time n − 1 and n; and $d(n)$ = the number of live and present cells that died (from $a(n-1)$) between n − 1 and n.

Fractions. Also, the following fractions are introduced: $P_M = \frac{a(n)}{a(n-1)}$ that divide between n − 1 and n, and $P_f = \frac{a(n)}{a(n-1)}$ that fuse...
between \( n - 1 \) and \( n \), \( P_D \), which is the fraction of \( a_{n-1} \) that die between \( n - 1 \) and \( n \), is:

\[
P_D = \frac{d_{n}/a_{n-1}}{P_{L}}
\]

and substituting Eq. C for \( P_D \):

\[
Z(n) = z(n-1) + y(n-1) \cdot d_{n}/a_{n-1}
\]

Then the fraction of cells present at \( a_{n-1} \) that do not divide, fuse, die, or become lost between time \( n - 1 \) and \( n \) would be:

\[
1 - P_M - P_F - P_D - P_{L}
\]

\( P_L \), which is the relative increase in \( a_{n-1} \) excluding death and loss of cells, is:

\[
P_L = (1 + P_M - 0.5P_F)
\]

or, in words, the relative increase would be the fraction of the cells that divide (as one cell becomes two) minus half the fraction that fuse (as two cells become one).

Because the number of cells present at time \( n \) (\( a_n \)) will be the number of cells present at time \( n - 1 \) (\( a_{n-1} \)) multiplied by the relative increase (excluding death and loss) minus the cells that are lost and die, then:

\[
a(n) = P_L \cdot a_{n-1} - P_D \cdot a_{n-1} - P_{LL} \cdot d_{n-1}
\]

Because values of \( a_{n-1} \) and \( d_{n-1} \) and \( b_{n-1} \) are the values determined from the CVTL, \( P_L \) can be calculated:

\[
P_L = (a(n) + d_{n-1} + b_{n-1})/a_{n-1}
\]

If we assume that the cells that are alive and lost lose the same as the cells that are alive and present in the same pedigree (progeny from the same irradiated cell):

\[
r(n) = P_L \cdot r_{n-1} - P_D \cdot r_{n-1} + P_{LL} \cdot a_{n-1}
\]

\[
s(n) = s(n-1) + P_P \cdot r_{n-1}
\]

Substituting Eqs. E and G into Eq. A:

\[
y(n) = P_1(a(n) + r_{n-1}) - P_0(a(n) + r_{n-1}) + r_{n-1}
\]

with:

\[
y(n-1) = a(n-1) + r_{n-1}
\]

\[
y(n) = (P_L - P_0) \cdot y(n-1)
\]

To determine the corrected for lost cell value for the number of live cells, substituting Eqs. C and G into Eq. J:

\[
y(n) = [(a(n) + d_{n-1} + b_{n-1})a(n-1) - d_{n-1}a(n-1)] / y(n-1)
\]

\[
y(n) = y(n-1) \cdot (a(n) + b_{n-1})a(n-1)
\]

To determine the value of all of the dead cells (corrected value for lost cells), substitute Eqs. F and I into Eq. B:

\[
z(n) = e(n-1) + s(n-1) + P_F \cdot (a(n-1) + r_{n-1})
\]

and with:

\[
z(n-1) = e(n-1) + s(n-1)
\]

\[
z(n) = z(n-1) + P_D \cdot y(n-1)
\]
Using Computerized Video Time Lapse for Quantifying Cell Death of X-irradiated Rat Embryo Cells Transfected with c- myc or c-Ha- ras

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