Apoptosis Induced by X-Irradiation of rec-myc Cells Is Postmitotic and not Predicted by the Time after Irradiation or Behavior of Sister Cells

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Abstract

Rat embryo cells expressing the c-myc oncogene (rec-myc) were studied by time-lapse microscopy to determine whether radiation-induced apoptosis occurred before or after mitosis. Following X-irradiation with 9.5 Gy, cells were imaged every 3 min for 6 days. Episodes of apoptotic blebbing were very consistent from cell to cell, lasting 30–60 min, followed by cessation of movement and cell death. In contrast, the time of initiation of apoptotic blebbing was unpredictable. At least 96% of the apoptotic episodes were postmitotic, after one to four cell divisions and 2–97 h after a given division. Sister cells often behaved differently from one another, with apoptosis in one sister occurring many h or several divisions after apoptosis in the other. Thus, the onset of radiation-induced apoptosis in rec-myc cells is not strictly programmed but may result from the segregation of chromosome aberrations in the postirradiation generations.

Introduction

Apoptosis is currently being evaluated for its importance as a pathway of radiation-induced cell killing (1). Both premitotic (2, 3) and postmitotic (4, 5) apoptosis have been reported following X-irradiation. However, the methods used in those studies could not provide quantitative data on the fraction of cells apoptosing before versus after mitosis or on the behavior of sister cells resulting from cell divisions following irradiation. For nonapoptotic cell lines, their behavior in the generations following X-irradiation provided valuable clues that helped focus attention on chromosome damage as the primary lethal event (6–8). Therefore, we undertook a study of the apoptosis-sensitive rec-myc cell line (9) to determine whether irradiated cells apoptosed before or after mitosis and to see whether the pattern of apoptotic death could provide information about underlying mechanisms.

Materials and Methods

Cells, Culture Conditions, and Irradiation. The rec-myc cell line used in this study was originally established by transfecting the human c-myc gene into rat embryo cells as described (9). Cells were cultured in DMEM with 10% iron-supplemented calf serum (HyClone). Cells growing exponentially in T-25 plastic tissue culture flasks were irradiated at room temperature with a Westinghouse Quadrofocus X-ray machine at 250 kV (peak) and 15 mA at a dose rate of approximately 4 Gy/min. Time-lapse microscopy commenced 0–2 h later.

Time-Lapse Microscopy. Cells in sealed T-25 tissue culture flasks were placed into a temperature-controlled incubation chamber built enclosing an inverted phase-contrast microscope. The chamber was shielded from ambient room light. Every 3 min, the microscope light was switched on for 12 s, during which time an image was captured by an imaging system from Compix, Inc. (Mars, PA). Image sequences were analyzed with software from the same company.

Results

Radiation induces a large amount of cell death by apoptosis in this c-myc-transfected cell line (9). The cells detaching from the substratum during the first 48 h following irradiation showed the following hallmarks of apoptosis: (a) failure to exclude trypan blue; (b) chromatin condensation; and (c) low molecular weight DNA “laddering” (9). Time-lapse imaging of irradiated cells enabled us to see the dynamic morphological events that accompanied cell detachment. For 140 h following a radiation dose of 9.5 Gy, 96% (90 of 94) of the detaching cells behaved in the following way. The first recognizable change was a rapid retraction of the cell from the plastic substratum, usually in less than 3 min, and cell rounding. This was accompanied by vigorous blebbing consisting of rapid extrusion and retraction of membrane buds over the entire surface of the cell. The duration of each episode of active blebbing was very consistent from cell to cell, averaging between 20 and 30 min (Table 1). A similar duration for apoptotic blebbing was seen after serum withdrawal from the Rat-l cell line transfected with c-myc (10). In addition, this interval was not altered when apoptosis occurred after the first, second, or third mitosis after irradiation (the increase after four mitoses may be due to the small number of events recorded). No episodes of apoptotic blebbing were observed in 87 complete cell cycles of unirradiated controls (data not shown). Following the blebbing interval, all movement ceased. By this time, the cell was usually fully rounded, phase bright, and loosely attached to the substratum. Such cells were followed for several days to substantiate that death had occurred. In no instance did such cells reattach and/or divide, although lysis and fragmentation was often seen. The fraction of detached cells in the microscope field was determined every 12 h (data not shown). The kinetics of cell detachment measured in this way were quite close to those measured by Chen et al. (9). Taken together, these observations allow us to conclude that the cells observed by time-lapse imaging to undergo vigorous membrane blebbing for 20–30 min, followed by cessation of movement, correspond to the apoptotic cells.

As shown in Table 1, episodes of apoptosis were postmitotic. Fig. 1 illustrates this fact in greater detail. For all three pedigrees, apoptosis occurred only after 1 to 3 rounds of cell division. Fig. 1A shows that normal generation times could precede apoptosis, illustrating that perturbations in cell cycle kinetics in the generations preceding apoptosis were not required for induction of apoptosis (cell cycle times of unirradiated cells were 10–12 h). Fig. 1, B and C, illustrates the often dissimilar behavior of sister cells. In Fig. 18, following the first mitosis, one sister apoptosed, whereas the other completed another cell cycle, yielding sisters with widely different times to apoptosis (20.5 versus 80 h). In Fig. 1C, one branch of the pedigree yielded a second mitosis after irradiation, whereas the other branch yielded a third mitosis. This third mitosis produced two sister cells, one of
which died by apoptosis, whereas the other remained metabolically viable up to the end of the imaging. We think it unlikely that this cell was clonogenic, because the pedigree of the lone clonogenic cell of the total of 43 that were imaged in this experiment did not contain any apoptosing cells (data not shown). Thus, these pedigrees illustrate the highly unpredictable behavior of these cells following irradiation with respect to the number of mitoses prior to apoptosis, the time of apoptosis, and the behavior of sister cells.

Fig. 2 shows when apoptosis occurred for the entire population. Apoptosis was 96% (86 of 90 cells) postmitotic. However, we think it likely that this figure was really 100% for the following reasons: (a) imaging did not commence until 2 h after irradiation, allowing time for some cells irradiated late in the cell cycle to divide prior to imaging; (b) for another experiment with 9.5 Gy and one with 2.5 Gy, 87 of 87 apoptotic events were postmitotic (data not shown); and (c) the four cells that apparently apoptosed prior to mitosis were located in two pairs in the microscope field, consistent with the idea that each pair resulted from a mitosis completed between irradiation and imaging. Fig. 2 also shows a trend for apoptosis following many mitoses to occur later after irradiation than apoptosis following few mitoses; however, there were many exceptions. Thus, both the time from irradiation to apoptosis and the number of mitoses prior to apoptosis showed great variability.

Last, we measured the time from mitosis to apoptosis and categorized these data according to the number of mitoses occurring prior to apoptosis (Fig. 3). We did this to determine whether cells might be undergoing apoptosis at a particular point in the cell cycle. Most cells exhibited delays between mitosis and apoptosis that were much longer than the cell cycle times of unirradiated cells (10—12 h) or even irradiated cells (Fig. 1), suggesting that such cells must either be cycling very slowly or delayed in part of the cycle. In contrast, other cells apoptosed within a few h of mitosis, indicating that apoptosis in G1 was likely in those instances. These data show that, unlike the interval required for cell detachment and blebbing (Table 1), the time from mitosis to apoptosis was highly variable.

### Discussion

Discriminating between premitotic and postmitotic apoptosis is important for understanding mechanisms of radiation-induced apoptosis. Both patterns have been described (2—5). In general, premitotic apoptosis tends to be rapid and occurs in cell lines with increased sensitivities to radiation killing compared with nonapoptotic cell lines (1). That postmitotic apoptosis after radiation is much more difficult to assess is illustrated by the data presented here. As the cells destined to die by apoptosis divided up to four times after 9.5 Gy (Fig. 2) and five times after 2.5 Gy (data not shown), proliferation of clonogenic and nonclonogenic cells was also taking place (data not shown). In addition, lengthening of cell cycle times (Fig. 1) and the often long intervals between mitosis and apoptosis (Fig. 3) tended to place the apoptotic event many h following irradiation, thus allowing clonogenic cells to multiply and become an ever larger fraction of the population. Unless the population is monitored continuously, it is

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**Table 1** Average length of episodes of apoptotic blebbing in X-irradiated (9.5 Gy) rec-myc cells

<table>
<thead>
<tr>
<th>No. of mitoses prior to blebbing</th>
<th>Average length of blebbing [min (±SEM)]</th>
<th>No. of events</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19 ± 2</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>25 ± 2</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>23 ± 1</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>36 ± 3</td>
<td>6</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Three pedigrees of irradiated (9.5 Gy) rec-myc cells. Numbers, h after X-irradiation. Numbers to the left of two branched lines, times of entry into mitosis (cell rounding). Numbers to the left of *apop*, times of the beginning of apoptotic blebbing.

**Fig. 2.** Timing of the death of rec-myc cells following X-irradiation (9.5 Gy). Each datum point represents a cell. O, apoptotic blebbing and death; and •, necrotic death (rounding without blebbing).
Thompson and Suit (7). Using apparently nonapoptotic cell lines, they predicted by comparing the behavior of sister cells. Preliminary time-lapse studies with rec-ras cells, which do not undergo apoptosis after irradiation (9), demonstrate patterns of cell death similar to those seen in the study by Thompson and Suit (7). Preliminary experiments at 2.5 Gy suggest that lethal sectoring does occur in rec-myc cells (data not shown); however, such experiments must be extended for longer periods to verify that the nonapoptosing cells really were clonogenic. If chromosome aberrations are able to trigger apoptosis, then this could explain the finding that the apoptotic-sensitive rec-myc cells are only slightly more sensitive to killing by radiation compared with the apoptosis-refractory rec-ras cells (15). Thus, modulating cell killing in tumors containing cells that apopitise after mitosis may be best accomplished by modulating the production and repair of chromosome aberrations rather than apoptosis.

The time-lapse data shown here demonstrate that apoptosis in rec-myc cells is a postmitotic event. Furthermore, although the apoptotic program of vigorous membrane blebbing followed by cell detachment was quite constant from cell to cell, the time of commencement of each blebbing episode was highly unpredictable with respect to the time after irradiation, number of divisions between irradiation and apoptosis, and behavior of sister cells. Such unpredictable segregation of the expression of radiation damage in the postirradiation generations was documented in 1969 by Hurwitz and Tolmach (6) and Thompson and Suit (7). Using apparently nonapoptotic cell lines, they described mitotic abnormalities (such as mitosis followed by fusion and trapping in mitosis) as well as cell death by necrosis (pyknosis). In both studies, these events were postmitotic and could not be predicted by comparing the behavior of sister cells. Preliminary time-lapse studies with rec-ras cells, which do not undergo apoptosis after irradiation (9), demonstrate patterns of cell death similar to those described in the 1969 studies (6,7). In the years since those earlier studies, evidence has mounted supporting a causal role for chromosome aberrations in the killing of apoptosis-refractory cell lines by radiation (8–13). Chromosomes suffering radiation-induced aberrations can replicate and persist for many generations prior to cell death (12). Furthermore, Carrano (14) showed that the fraction of cells with chromosome aberrations (asymmetric exchanges resulting in dicentrics and acentric fragments) correlated with the fraction of cells that died during the first eight generations following irradiation. Specifically, he showed that the increase in survival and reduction in the frequency of cells with chromosome aberrations that occurred as a population of cells traversed from one division to the next could be completely accounted for by the cells that were arrested or died between successive divisions. Therefore, we think it reasonable to propose that in rec-myc cells, segregation of chromosome aberrations in postirradiation generations is sufficient to trigger the apoptotic program. If this hypothesis is correct, we would expect lower doses of radiation to yield instances of lethal sectoring (pedigrees with mixed lineages of both clonogenic and nonclonogenic/apoptotic cells), as seen in the study by Thompson and Suit (7).

Fig. 3. Intervals from the last completed mitosis to apoptotic blebbing and death for irradiated (9.5 Gy) rec-myc cells. Each datum point represents a cell.

### References


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1. C. A. Vidair, unpublished data.
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