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The Radiation-Induced Bystander Effect for Clonogenic Survival

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It has long been accepted that the radiation-induced heritable effects in mammalian cells are the result of direct DNA damage. Recent evidence, however, suggests that when a cell population is exposed to a low dose of α particles, biological effects occur in a larger proportion of cells than are estimated to have been traversed by α particles. Experiments involving the Columbia University microbeam, which allows a known fraction of cells to be traversed by a defined number of α particles, have demonstrated a bystander effect for clonogenic survival and oncogenic transformation in C3H 10T½ cells. When 1 to 16 α particles were passed through the nuclei of 10% of a C3H 10T½ cell population, more cells were unable to form colonies than were actually traversed by α particles. Both hit and non-hit cells contributed to the outcome of the experiments. The present work was undertaken to assess the bystander effect of radiation in only non-hit cells. For this purpose, Chinese hamster V79 cells transfected with hygromycin- or neomycin-resistance genes were used. V79 cells stably transfected with a hygromycin resistance gene and stained with a nuclear dye were irradiated with the charged-particle microbeam in the presence of neomycin-resistant cells. The biological effect was studied in the neomycin-resistant V79 cells after selective removal of the hit cells with geneticin treatment. © 2002 by Radiation Research Society

INTRODUCTION

Interaction of radiation with DNA (*1, 2*), either by direct ionization or by the production of hydroxyl radicals in water molecules close to the DNA, has been widely assumed to be the mechanism of radiation-induced heritable damage. Over the past decade, considerable evidence has emerged for the existence of a phenomenon that has been termed the bystander effect. The bystander effect is defined as a biological response in cells that do not themselves receive any energy deposition from ionizing radiation but respond to signals produced by cells that do. The phrase "bystander effect" has been borrowed from the gene therapy field, where it usually refers to the killing of several types of tumor cells by targeting only one type of cell within a mixed population (*3*). This phenomenon was first reported in 1992 by Nagasawa and Little (*4*), who exposed cells to a low dose of α particles; 30% of the cells showed an increase in sister chromatid exchanges even though less than 1% were calculated to have undergone a nuclear traversal. Since then, reports of apparently the same phenomenon have appeared with biological end points including increases in sister chromatid exchanges (*4, 5*), up-regulation of TP53 expression (*6, 7*), up-regulation of oxidative metabolism (*8*), chromosomal instability (*9*), protein modulation in bystander cells (*7*), and mutation induction (*10*). More recently we demonstrated bystander effects of ionizing radiation for clonogenic survival and oncogenic transformation in C3H 10T½ cells using the Columbia Single Cell Microbeam System (*11, 12*). When the single-cell microbeam delivered from 1 to 16 α particles through the nuclei of 10% of a C3H 10T½ cell population, more cells were inactivated than were actually traversed by α particles. The magnitude of this bystander effect increased with particle number per cell. Both the hit and the bystander cells contributed to the outcome of the experiments. The goal of the present work was to assess the bystander effect of radiation in only non-hit cells by using selectively transfected Chinese hamster V79 cells.

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MATERIALS AND METHODS

All media, sera and reagents were from GIBCO BRL unless otherwise noted. V79 cells (ATCC) were cultured in Dulbecco's MEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum. All cultures were maintained at 37°C in 95% air/5% CO_2.

Transfection

The day before transfection, cells were trypsinized and counted. Approximately, 10,000 cells were plated in 100-mm dishes. On the day of transfection, the cell cultures were 50% to 80% confluent. LipofectAMINE PLUS™ Reagent (Life Technologies) was used to transfect the V79 cells, and the transfections were conducted according to the manufacturer's recommendations. Briefly, lipofectAMINE Reagent and pCDNA3.1 or pCDNA3.1/Hygro (Invitrogen) DNA were diluted sepa-

rately in serum-free medium. PLUS Reagent was added into the DNA solution and incubated at room temperature for 15 min. The DNA-PLUS Reagent was mixed with diluted lipofectAMINE Reagent and then incubated at room temperature for 15 min. The DNA-PLUS Reagent-lipofectAMINE Reagent complex was added to the cells, then incubated at 37°C for 3 h. At the end of the 3-h incubation period, the transfection medium was replaced with fresh complete medium. Cells were harvested 24 h after transfection and replated in complete medium. At 48 h after transfection, clone selection was initiated. Selection for growth in the presence of geneticin or hygromycin antibiotic continued for 2 to 10 days. Individual colonies were then picked and expanded in the medium containing appropriate antibiotic.

Microbeam Irradiation

The Columbia microbeam system and the irradiation procedure have been described in detail elsewhere (*13, 14*). Briefly, 1000–1200 cells were attached at low density to the thin bases (3.8 μm polypropylene) of 6.3-mm-diameter mini-wells. At this density, 50–60% of the cells are touching at the time of irradiation. The average stopping power of the α particles traversing the cells was 90 keV/μm. Individual nuclei were identified and located with an optical image analysis system. For each dish, a computer/microscope-based analysis system first automatically locates and records the *x, y* coordinates of all the cells and their nuclei on the dish. Next, the dish is moved sequentially under computer control such that the first cell nucleus is positioned over a highly collimated α-particle beam. The beam shutter is opened until the required numbers of α particles are detected (with a transparent gas-filled ionization detector mounted on the microscope objective) to have passed through the nucleus. Alpha particles (5.4) accelerated by a Van de Graaff accelerator were used for the irradiations. The shutter is then closed, and the next cell is moved under the beam. The overall spatial precision of the beam, including positioning and beam spread, is about ±3.5 μm. Parallel experiments were performed in which cells were sham irradiated, i.e. were handled in an identical fashion except that the beam shutter was not opened. The search-and-irradiate software can be instructed to expose any given proportion of the cells, selected at random, to any desired number of α particles. In this case, all the stained (hygromycin-resistant) cells were exposed to 1 to 16 α particles through the nucleus.

Cell Culture

Before irradiation, V79 cells (both hygromycin- and neomycin-resistant) from passage 15 were grown in complete DMEM. Approximately 6 h prior to exposure, the exponentially growing hygromycin-resistant V79 cells were stained for 0.5 h with an extremely low concentration (50 n*M*) of the vital nuclear dye Hoechst 33342, enabling individual nuclei to be identified and located (see Fig. 1) with the optical image analysis system (*13*). Excess stain was removed by washing the cells with complete medium for 10 min. Cells resistant to hygromycin (stained) or neomycin (unstained) were trypsinized. Cells were counted and added at the proportion of 1:9; for each hygromycin-resistant cell, there were nine neomycin-resistant cells. Approximately 1000–1200 mixed cells were plated into the center of each of a series of 6.3-mm-diameter mini-wells. In parallel, about 1,000–1,200 hygromycin-resistant cells stained with the nuclear dye were plated into the center of each microbeam dish. Cells were incubated for 5–6 h at 37°C; then the stained (hygromycin-resistant) cells were irradiated through the nucleus with an exact number of α particles. Prior to irradiation, cells were washed with serum-free medium to avoid fluorescence from serum components, and irradiations were carried out in the presence of a thin film of serum-free medium surrounding the cells. After irradiation, the cells were trypsinized from the irradiation container and replated at a low density of about 100 viable cells per 100-mm culture dish in 10 ml of complete DMEM. After 24 h incubation at 37°C, the culture medium was replaced with fresh medium containing 200 μg/ml of geneticin and cells were incubated for 2 weeks. After incubation, cell colonies were stained to determine the plating efficiencies and the surviving fractions of the control and irradiated cells.

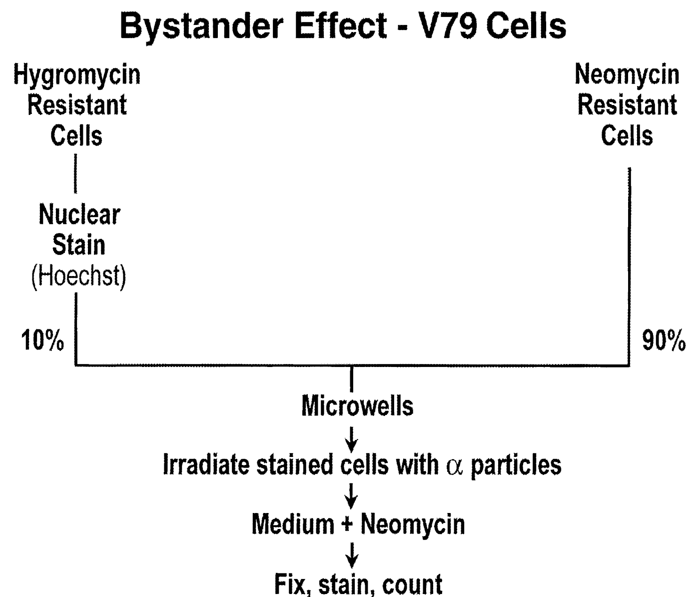

FIG. 1. Schematic showing clonogenic cell survival protocol.

RESULTS AND DISCUSSION

The use of the Columbia Single-Cell Microbeam Facility has made it possible to define precisely what proportion and which compartment (nucleus or cytoplasm) of cells are traversed by an exactly defined number of α particles, rather than relying on estimates of probabilities. Using the Columbia Single Cell Microbeam as a means of localized energy delivery, we have demonstrated the bystander effect of radiation for cell survival (*11, 12*). When the single-cell microbeam delivered from 1 to 16 α particles through the nuclei of 10% of a C3H 10T½ cell population, more cells were inactivated than were actually traversed by α particles. The magnitude of this bystander effect increased with particle number per cell. In these experiments, both the hit and the non-hit cells formed colonies that were accounted for by calculating the surviving fraction. The goal of this study was to eliminate the hit cells from the population after irradiation so that the surviving fraction would be calculated based on colonies formed by the non-hit or bystander cells. V79 cells transfected with plasmid DNA containing either the hygromycin- or neomycin-resistance gene were used for the present study. Hygromycin-resistant cells were stained with low concentrations of a nuclear dye and then mixed with neomycin-resistant (nonstained) cells at the proportion of 1:9 before they were plated on microbeam dishes. All stained cells on the microbeam dishes were irradiated with an exact number of α particles, and the mixed cell population was replated in 100-mm cell culture dishes. The hit cells (hygromycin-resistant cells) were selectively killed in the mixed culture by incubating them in the presence of

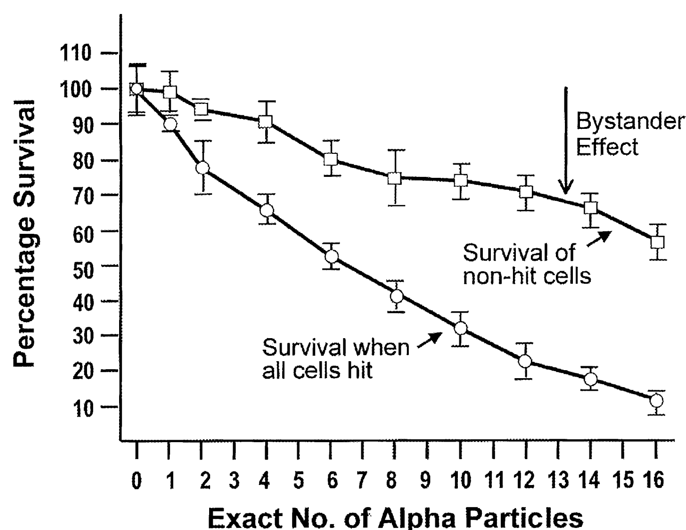

FIG. 2. The bystander effect for cell survival in V79 cells. Each data point (mean ± SE) on the line with circles refers to the survival of cells when all cell nuclei on each dish were exposed to the same exact numbers of α-particle traversals using the microbeam system. The squares show survival for various numbers of α particles, from 1 to 16, traversing 10% of the cell population. The extent to which this falls below the 100% survival for non-hit cells is an indication of the magnitude of the bystander effect. Each data point represents the mean ± SD of the clonogenic survivals from three culture plates.

geneticin. Therefore, the cell colonies formed at the end of 2 weeks of incubation were derived from the non-hit (neomycin-resistant) cells, the bystander cells.

The results of the experiment evaluating the effect of radiation on the survival of bystander cells are shown in Fig. 2. The lower line in Fig. 2 shows the observed survival when all cells on the microbeam dish are hit by various numbers of α particles. In the absence of a bystander effect, removal of hit cells (hygromycin-resistant cells) from the mixed cell culture after irradiation would be expected to result in a survival of almost 100% in all groups. The upper line shows the actual results of irradiating 10% of the cells (which were subsequently removed using medium containing geneticin). In contrast to the expected 100% survival, the survival falls progressively as more α particles traverse each nucleus, eventually reaching 60%. These findings strongly support our previous demonstration of a radiation-induced bystander effect for cell survival. The magnitude of the bystander effect in these studies is much greater than that reported by investigators at the Gray Institute for Cancer Research, where only 5 to 10% lethality is seen in non-hit cells, using protons or soft X rays in a microbeam (B. Michael, personal communication, 2001). The difference is probably accounted for by the cell density. In the Gray Institute studies, only about 200 cells were seeded in an area of 10 × 10 mm. The average distance between cells therefore would be some hundreds of micrometers, so it is likely that communication through gap junctions would not contribute to the effect observed in that study. By contrast, in the studies reported here, 1000 to 1200 cells are plated in mini-wells 6.3 mm in diameter, so that 50 to 60% are in contact, allowing gap junction communication, which has been demonstrated to be important in mutation studies with the microbeam (*15, 16*). Therefore, the current study also supports the need for gap junction communication as a mediator of bystander effects in relation to radiation-induced cell killing.

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REFERENCES

1. R. E. Zirkle and W. Bloom, Irradiation of parts of individual cells. *Science* **117**, 487–493 (1953).

2. T. R. Munro, The relative radiosensitivity of the nucleus and cytoplasm of Chinese hamster fibroblasts. *Radiat. Res.* **42**, 451–470 (1970).

3. T. L. Cheng, S. L. Wei, B. M. Chen, J. W. Chern, M. F. Wu, P. W. Liu and S. R. Roffler, Bystander killing of tumour cells by antibody-targeted enzymatic activation of a glucuronide prodrug. *Br. J. Cancer* **79**, 1378–1385 (1999).

4. H. Nagasawa and J. B. Little, Induction of sister chromatid exchanges by extremely low doses of alpha particles. *Cancer Res.* **52**, 6394–6396 (1992).

5. A. Deshpande, E. H. Goodwin, S. M. Bailey, B. Marrone and B. Lehnert, Alpha-particle-induced sister chromatid exchange in normal human lung fibroblasts: Evidence for an extranuclear target. *Radiat. Res.* **145**, 260–267 (1996).

6. A. W. Hickman, R. J. Jaramillo, J. F. Lechner and N. F. Johnson, Alpha-particle-induced p53 protein expression in a rat lung epithelial cell strain. *Cancer Res.* **54**, 5797–5800 (1994).

7. E. I. Azzam, S. M. de Toledo, T. Gooding and J. B. Little, Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. *Radiat. Res.* **150**, 497–504 (1994).

8. P. K. Narayanan, K. E. A. LaRue, E. H. Goodwin and B. E. Lehnert, Alpha particles initiate biological production of interleukin-8 in human cells. *Radiat. Res.* **152**, 57–63 (1999).

9. S. A. Lorimore, M. A. Kadhim, D. A. Pocock, D. Papworth, D. L. Stevens, D. T. Goodhead and E. G. Wright, Chromosomal instability in the descendants of unirradiated surviving cells after α-particle irradiation. *Proc. Natl. Acad. Sci. USA* **95**, 5730–5733 (1998).

10. H. Zhou, G. Randers-Pehrson, C. A. Waldren, D. Vannais, E. J. Hall and T. K. Hei, Induction of a bystander mutagenic effect of alpha particles in mammalian cells. *Proc. Natl. Acad. Sci. USA* **97**, 2099–2104 (2000).

11. S. G. Sawant, G. Randers-Pehrson, C. R. Geard, D. J. Brenner and E. J. Hall, The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells *in vitro* can be initiated in the unirradiated neighbors of irradiated cells. *Radiat. Res.* **155**, 397–401 (2001).

12. S. G. Sawant, G. Randers-Pehrson, N. F. Metting and E. J. Hall, Adaptive response and the bystander effect induced by radiation in C3H 10T1/2 cells in culture. *Radiat. Res.* **156**, 177–180 (2001).

13. G. Randers-Pehrson, C. R. Geard, G. Johnson, C. D. Elliston and D. J. Brenner, The Columbia University Single-ion Microbeam. *Radiat. Res.* **156**, 210–214 (2001).

14. C. R. Geard, D. J. Brenner, G. Randers-Pehrson and S. A. Marino,

Single-particle irradiation of mammalian cells at the Radiological Research Accelerator Facility: Induction of chromosomal changes. *Nucl. Instrum. Methods* **B54**, 411–416 (1991).

15. H. Zhou, G. Randers-Pehrson, C. A. Waldren, D. Vannais, E. J. Hall and T. H. Hei, Induction of a bystander mutagenic effect of alpha particles in mammalian cells. *Proc. Natl. Acad. Sci. USA* **97**, 2099–2104 (2000).

16. H. Zhou, M. Suzuki, G. Randers-Pehrson, D. Vannais, G. Chen, J. E. Trosko, C. A Wldren and T. K. Hei. Radiation risk at low doses may be greater than we thought. *Proc. Natl. Acad. Sci. USA* **98**, 14410–14415 (2001).