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Source: Radiation Research, 169(4) : 460-468

Published By: Radiation Research Society

URL: https://doi.org/10.1667/RR1212.1

RADIATION RESEARCH **169**, 460–468 (2008)
0033-7587/08 $15.00

TECHNICAL ADVANCE

Refinement of the Dichlorofluorescein Assay for Flow Cytometric Measurement of Reactive Oxygen Species in Irradiated and Bystander Cell Populations

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Hafer, K., Iwamoto, K. S. and Schiestl, R. H. Refinement of the Dichlorofluorescein Assay for Flow Cytometric Measurement of Reactive Oxygen Species in Irradiated and Bystander Cell Populations. *Radiat. Res.* 169, 460–468 (2008).

Reactive oxygen species (ROS) have been implicated in many ionizing radiation-related phenomena, including bystander effects. The oxidation of 2′7′-dichlorofluorescin (DCFH) to fluorescent 2′7′-dichlorofluorescein (DCF) is commonly used for the detection of radiation-induced ROS. The DCF assay was adapted for efficient, systematic flow cytometry quantification of low-linear energy transfer (LET) γ-radiation-induced ROS *in vitro* in Chinese hamster ovary (CHO) cells. This method is optimized for increased sensitivity to radiation-induced ROS and to discriminate against measurement of extracellular ROS. This method can detect a significant increase in ROS in cells exposed to γ radiation at doses as low as 10 cGy. The antioxidants *N*-acetyl-cysteine and ascorbic acid (vitamin C) significantly reduced the amount of ROS measured in cells exposed to 5 Gy ionizing radiation. This method was used to measure the intracellular ROS in unirradiated CHO bystander cells co-cultured with low-LET-irradiated cells. No increase in ROS was measured in bystander cell populations co-cultured with the irradiated cells beginning 9 s after radiation exposure.

INTRODUCTION

Approximately 65% of intracellular damage caused by low-LET ionizing radiation such as X rays and γ rays occurs indirectly through radiolytic production of ROS (*1*). Oxidative damage to DNA can result in a variety of lesions, including base damage, inter- and intrastrand crosslinks, DNA-protein crosslinks, single-strand breaks and double-strand breaks (*2, 3*). Fixation of such damage has been implemented in numerous diseases such as diabetes, heart failure, neurodegeneration, aging and cancer [for review, see ref. (*4*)]. Likewise, scavenging of ROS has become an important target for radioprotectors (*5*) and general anticancer agents (*6*).

ROS have recently been implicated in multiple radiation-related phenomena including bystander effects, delayed effects and adaptive response. Unirradiated HFL1 normal human diploid lung fibroblasts demonstrated increased intracellular production of H_2O_2 when cultured with medium previously used to culture α-particle-irradiated cells 24 h after irradiation (*7*). Similarly, bystander phenotypes have been alleviated by addition of antioxidants to bystander cells in both medium transfer (*8*) and co-culture systems (*9*). It has also been suggested that radioadaptation is at least partially due to an increased cellular ability to scavenge ROS (*10*) and repair oxidative DNA lesions (*11*) inducible by small priming doses of radiation. ROS may also be involved in delayed radiation effects and radiation-induced genomic instability, because elevated levels of oxygen radicals have been detected in the progeny of γ-irradiated cells up to 29 days postexposure (*12–14*).

Many methods have been reported for quantifying intracellular ROS generated either endogenously or from radiation exposure. A plethora of fluorescent probes have been described, each characteristically able to efficiently detect specific radical species [for review, see ref. (*15*)]. 2′7′-Dichlorofluorescin diacetate (DCFH-DA) has been used previously to measure radiation-induced ROS. DCFH-DA is permeable to the cellular membrane and once inside the cell is rapidly hydrolyzed by cellular esterases to non-fluorescent DCFH (*16*); oxidation of DCFH by hydrogen peroxide or other ROS produces the fluorescent indicator DCF (*15*). Measurement methods using DCFH-DA to quantify radiation-induced ROS have been reported using fluorescence microscopy (*17*), video microscopy (*18*), flow cytometry (*19*), and microtiter plate analysis (*20*).

Previous studies using flow cytometry to quantify radi-

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ation-induced DCF fluorescence have commonly had low sensitivity, especially when compared to microscopy and microplate detection methods, which have exhibited sensitivity to as low as 2 cGy (*20*). For example, in one study using flow cytometry, no significant increase in DCF fluorescence was measured in T-lymphoma cells exposed to 10 Gy (*21*). In another study, no increase in DCF fluorescence was detected at 10 Gy and only a small increase was measured between 20–60 Gy (*22*). In another study, a DCF fluorescence dose response was observed in mouse macrophage cells, yet the lowest dose that induced a significant increase in measurable ROS was 100 Gy (*23*).

MATERIALS AND METHODS

Cell Lines and Culture

AA8 cells, a wild-type Chinese hamster ovary (CHO) cell line, were acquired from the American Type Culture Collection (Manassas, VA). Cells were cultured in α-MEM supplemented with 9% fetal bovine serum, 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were repassaged at ~85% confluence by washing twice with PBS and detaching with 0.05% trypsin-EDTA. AA8-rfp cells were derived from AA8 cells by transfection with *Aat*II-linearized pCMV-DsRed-Express vector (Clontech) with lipid reagent Lipofectamine 2000 per the manufacturer's instructions. After transfection, cells were grown in the presence of 4 μg/ml G418 for 4 weeks; red-fluorescing cells were then sorted by fluorescence-activated cell sorting (FACS) and cultured continuously in α-MEM supplemented with 4 μg/ml G418 as above.

γ Irradiation

All irradiations were done using a Mark I ^{137}Cs γ irradiator (J. L. Shepherd and Associates, Glendale, CA). Cells were irradiated in aerobic conditions at room temperature. A dose rate of 4.98 Gy/min was used for all DCF dose–response experiments between 0.5–10 Gy; 2.37 Gy/min was used for doses between 5–25 cGy. A dose rate of 33.3 Gy/min was used for bystander experiments.

Flow Cytometry Analysis

All cell samples were analyzed using a BD FACSCalibur flow cytometer with CellQuest software. For quantification of DCF fluorescence, at least 10,000 events were used for each measurement. Cells were excited at 488 nm and DCF fluorescence was read on FL1 (530 ± 15 nm) in log scale with FL1 gain set to 443. The histogram for cell fluorescence was fitted with the best least-squares fit Gaussian:

$$f(x) = \frac{A}{\sqrt{2\pi}\sigma} \exp\left[\frac{-1}{2}\left(\frac{(x - x_0)}{\sigma}\right)^2\right], \quad (1)$$

where x is FL1 fluorescence (RFU), A is the number of cells, σ is the full width at half maximum (FWHM), and x_0 is the mean fluorescence of the Gaussian, which was taken as the cell population mean fluorescence. For bystander experiments, unirradiated AA8-rfp cells mixed with irradiated AA8 cells were gated by FL3 fluorescence and DCF fluorescence of AA8-rfp cells was measured on FL1.

Optimization of DCF Assay

Different methods were tested for optimizing FACS measurement of radiation-induced DCF fluorescence. In the first experimental scheme, AA8 cells attached to T-25 culture flasks were incubated with 50 μ*M* DCFH-DA in medium for 30 min while in the dark. Then DCFH-DA was aspirated, and cells were washed with PBS, detached with trypsin, resuspended in medium, centrifuged for 5 min at 1000 rpm (167*g*), resuspended in PBS in 5-ml tubes, sham-irradiated or exposed to 7 Gy γ radiation, put on ice, and assayed by FACS. In variations of the above protocol, cells were incubated with DCFH-DA in PBS rather than in medium or the centrifugal force was varied from 17.2*g* to 167*g*. In the second experimental scheme, cells attached to T-25 culture flasks were incubated with 50 μ*M* DCFH-DA in PBS in the dark for 30 min. Then attached cells were exposed to sham or 7 Gy γ radiation. After irradiation, PBS was aspirated, cells were washed with PBS, detached with trypsin, resuspended in medium, centrifuged for 5 min, resuspended in PBS, put on ice, and assayed by FACS. In the third experimental scheme, attached AA8 cells were trypsinized, resuspended in medium, centrifuged 5 min at 17.2*g*, resuspended at 1×10^6/ml in 50 μ*M* DCFH-DA in medium, incubated for 30 min in the dark, exposed in suspension to γ radiation, put on ice, and read by FACS. In a variation of the third experimental scheme, cells were resuspended in DCFH-DA dissolved in PBS as opposed to medium.

Measurement of Cellular Kinetics of DCFH-DA

The rate at which DCFH-DA, when incubated with cells, was removed from the extracellular medium was measured as follows: 1×10^6 AA8 cells were incubated in 50 μ*M* DCFH-DA dissolved in 1 ml PBS in the dark. At times between 0.5–40 min, cells were filtered through 0.12 g of Kimwipe (roughly one-fourth of a 11.4 × 21.5-cm Kimwipe) using a 3-ml syringe (BD); filtered DCFH-DA in PBS was collected in microcentrifuge tubes. Time 0 was established by filtering DCFH-DA in PBS before mixture with cells. DCFH-DA was deacetylated to DCFH *in vitro* by addition of 20 m*M* NaOH. DCFH was oxidized to DCF by exposing microcentrifuge tubes to 10 Gy γ rays, and three to five 100-μl aliquots were dispensed into microwells of a 96-well plate (Corning). Fluorescence was measured using a Molecular Devices SpectraMax M5 microplate reader with 485 nm excitation and 528 nm emission.

The rate at which DCF-DA is absorbed and deacetylated by cells was measured as follows: 500 μ*M* DCFH-DA dissolved in PBS was oxidized *in vitro* to DCF-DA by exposure to 10 Gy γ rays. Nonfluorescent DCF-DA was added at 50 μ*M* to 1×10^6 AA8 cells in 1 ml PBS and samples were assayed by flow cytometry to quantify intracellular DCF fluorescence at 0.3–40 min. Time 0 was determined by reading endogenous cellular 530 nm fluorescence just prior to addition of DCF-DA. In both cases, probe concentration was kept at 50 μ*M* to be consistent with other experiments.

DCF Radiation Dose Response and Antioxidant Treatment

A variation of the third experimental scheme was used to quantify the ROS dose response to γ rays in AA8 cells. Upon reaching ~80% confluence in T-75 flasks, cells were detached with trypsin, resuspended in medium, and centrifuged for 5 min at 17.2*g*. Cells were then resuspended at 1×10^6/ml in 50 μ*M* DCFH-DA in PBS and incubated in 5-ml tubes for 20 min in the dark at room temperature. Cells were then exposed to γ radiation and put on ice, and DCF fluorescence was assayed by FACS between 8–12 min after γ-ray exposure. Experiments evaluating the effect of antioxidants on radiation-induced ROS were done exactly as above except that cells were incubated with either 10 μ*M* ascorbic acid (Sigma) or 100 μ*M* *N*-acetyl-cysteine (NAC) (Sigma) in addition to DCFH-DA for 20 min prior to and during γ irradiation. Sham-irradiated cell cultures were always run in parallel with γ-irradiated samples.

Analysis of ROS in Bystander Cell Cultures

Upon reaching ~80% confluence, AA8 and AA8-rfp cells were detached with trypsin, resuspended separately in medium, and centrifuged 5 min at 17.2*g*. AA8 cells were resuspended at 2×10^6/0.5 ml in PBS containing 1% BSA while AA8-rfp cells were resuspended at 2×10^6/0.5 ml in 50 μ*M* DCFH-DA in PBS supplemented with 1% BSA. Cells

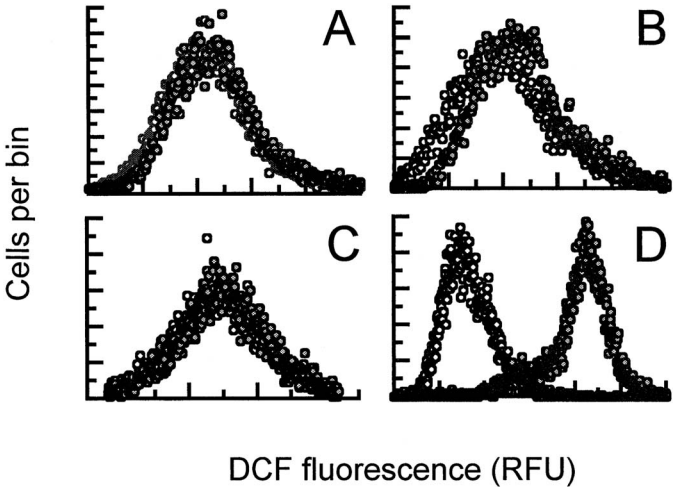

FIG. 1. Ionizing radiation-induced DCF fluorescence as determined by flow cytometry for four different experimental methods. The number of cells per fluorescent bin is plotted for sham-irradiated cells (○) and 7-Gy irradiated cells (●). Panel A: Attached cells were incubated with DCFH-DA for 30 min, washed, collected, irradiated and assayed by FACS. Panel B: Same as panel A except cells when collected were centrifuged at weaker force. Panel C: Attached cells were incubated with DCFH-DA for 30 min, irradiated, washed, collected and assayed by FACS. Panel D: Attached cells were washed, collected, incubated with DCFH-DA for 30 min, irradiated and assayed by FACS. The details of each protocol are given in the Materials and Methods.

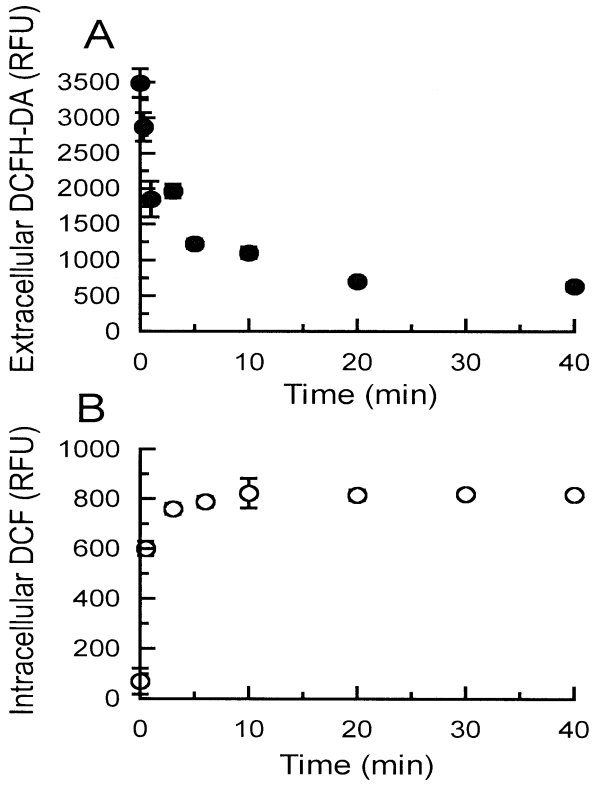

FIG. 2. Panel A: Amount of DCFH-DA remaining in medium after incubation with AA8 cells. Points are means ± SD for three or more experiments. Panel B: The amount of DCF-DA absorbed by AA8 cells. Points are means ± SD for three independent measurements.

were incubated in parallel for 20 min in 5-ml tubes, after which AA8 cells were γ-irradiated. Approximately 9 s after irradiation, AA8-rfp cells were added and mixed with irradiated AA8 cells. Cells were incubated together for 30 min, after which the DCF fluorescence of gated AA8-rfp cells was assayed by FACS. Sham-irradiated AA8 cells were always run in parallel with γ-irradiated cells.

Statistical Analysis

Statistical analysis of paired measurements was done using the nonparametric Wilcoxon signed rank test. KyPlot software was used for all statistical analyses.

RESULTS

Optimization of DCF Flow Cytometry Assay

Three separate experimental schemes, each with multiple variations, were used to find the procedure maximizing the sensitivity of radiation-induced DCF fluorescence measured by flow cytometry. In the first set of experiments, attached cells were incubated with 50 μ*M* DCFH-DA for 30 min, after which DCFH-DA was washed off and cells were trypsinized and resuspended in medium (to inactivate trypsin). Cells were then centrifuged and resuspended in PBS, and separate aliquots were sham-exposed or exposed to 7 Gy γ rays before being assayed by FACS. Such a procedure produced a very minor shift in fluorescence between irradiated and sham-irradiated populations (Fig. 1A) of about 6 RFU (relative fluorescence units). This was improved upon to an extent when cells were incubated with DCFH-DA in PBS rather than medium and when cells were centrifuged at the lowest force able to pellet cells (17.2*g*). Under these conditions, 7 Gy induced a shift of 17 RFU in DCF fluorescence compared to sham exposure (Fig. 1B).

In a second set of experiments, cells in PBS were incubated with 50 μ*M* DCFH-DA for 30 min while attached in culture and were then exposed to γ radiation in the presence of DCFH-DA. Cells were then collected with trypsin, resuspended in medium, centrifuged and resuspended in PBS, and assayed by FACS. This procedure (Fig. 1C) and its variations resulted in a similar small shift in fluorescence of <10 RFU.

In a third set of experiments, cultured AA8 cells were detached and collected in medium, centrifuged and resuspended in medium containing 50 μ*M* DCFH-DA for 30 min. Cells in suspension were then exposed to γ radiation and read by FACS. A greater increase in fluorescence was observed than with the previous experimental schemes, but a variation in which cells were incubated with DCFH-DA in PBS rather than medium yielded an even greater increase in cellular DCF fluorescence by 7 Gy (Fig. 1D). A method to measure γ-ray-induced DCF fluorescence was then developed from this experimental scheme to minimize artifacts that could arise from extracellular oxidation of DCFH and DCFH-DA.

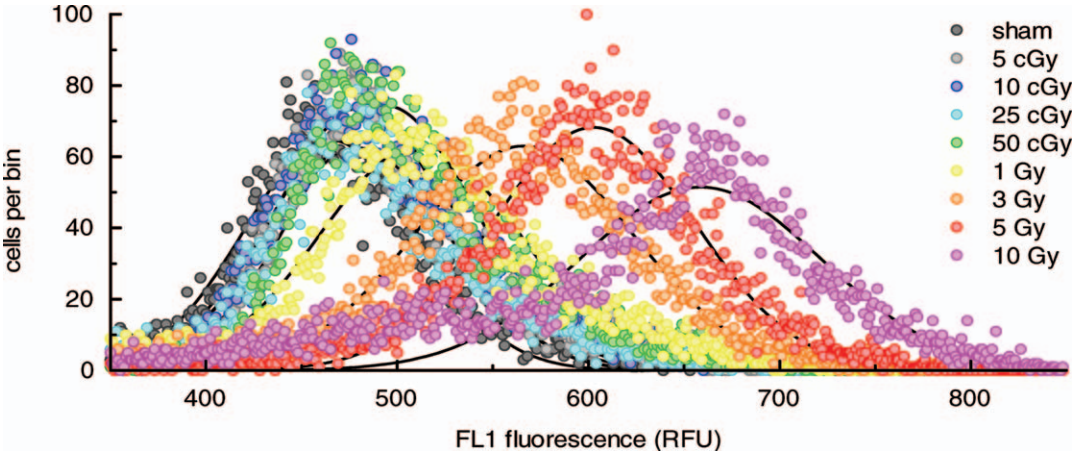

FIG. 3. Ionizing radiation-induced DCF fluorescence as assayed by flow cytometry is using the optimized experimental conditions. A dose-dependent shift in fluorescence can be seen for each radiation dose.

Measurement of Kinetics of Cellular Uptake of DCFH-DA

The experimental method that provided the most sensitive measurement of DCF oxidation was used to determine the kinetics of cellular DCFH-DA uptake. The rate at which DCFH-DA is removed by cells from extracellular medium was determined first. Cells were incubated with 50 μ*M* DCFH-DA in PBS, and at subsequent times the amount of DCFH-DA remaining in the PBS was quantified by collecting extracellular PBS, deacetylating the dissolved DCFH-DA *in vitro* with 20 m*M* NaOH, oxidizing the resultant DCFH with 10 Gy γ rays, and measuring DCF fluorescence with a microplate reader. The amount of DCF fluorescence measured in extracellular PBS decreased rapidly after incubation with cells (Fig. 2A). After 20 min, a drop of greater than 80% in DCF fluorescence was measured, indicating that most DCFH-DA is accumulated within cells after a 20-min incubation. No further change was seen after 40 min.

The kinetics of the uptake of 2′7′-dichlorofluorescein diacetate (DCF-DA) was determined by measuring accumulation within the cells. Like DCFH-DA, DCF-DA is nonfluorescent and is permeable to cells, but when DCF-DA is taken up in cells, deacetylation by cellular esterases converts it into the fluorescent product DCF. Cells were incubated with 50 μ*M* DCF-DA in suspension, and intracellular DCF fluorescence was measured at different times by flow cytometry. DCF-DA accumulated rapidly and was deacetylated in cells (Fig. 2B). After 10 min, cellular DCF fluorescence saturated and did not increase significantly at longer incubation times.

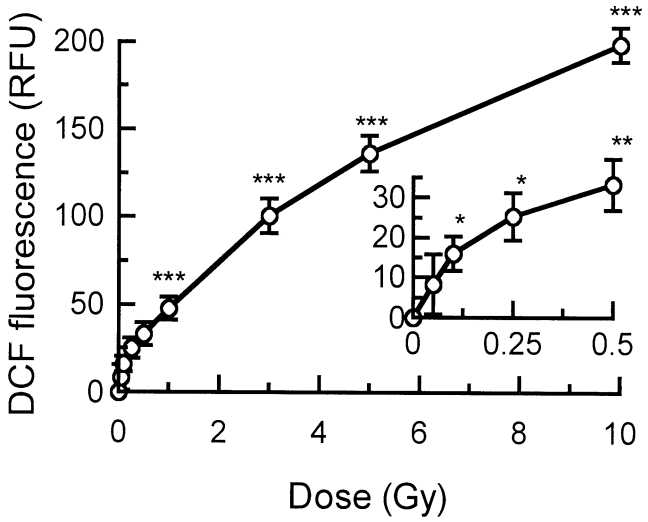

FIG. 4. Mean cellular ROS as measured by DCF fluorescence as a function of radiation dose. Results are means ± SD from five independent measurements. ***$P < 0.005$; **$P < 0.01$; *$P < 0.05$.

Measurement of Ionizing Radiation-Induced ROS

The optimized DCF flow cytometry method was used to measure the dose response for ROS generated intracellularly by ionizing radiation. AA8 cells in suspension were incubated with DCFH-DA for 20 min prior to γ-ray exposure. Immediately after radiation exposure, cells were placed in ice to slow down the normal oxidative metabolic production of radicals, because cells on ice were found to oxidize DCFH at a rate of 0.42 RFU/min compared to 1.68 RFU/min of cells at room temperature (data not shown). FL1 fluorescence was assayed between 8–12 min after radiation exposure. The histograms were fitted with the best-fit Gaussian according to Eq. (1) (Fig. 3). The mean fluorescence bin of the Gaussian, x_0, fitted to each population of irradiated cells was taken as the value of DCF fluorescence for that dose. A dose response in radiation-induced DCF fluorescence was observed, and the lowest dose observed to induce a significant increase was 10 cGy (Fig. 4).

Antioxidants were tested for their ability to reduce radiation-induced intracellular oxidative stress. Cells were incubated with 10 μ*M* ascorbic acid or 100 μ*M* NAC for 20 min before and during both sham and 5-Gy exposure. Both

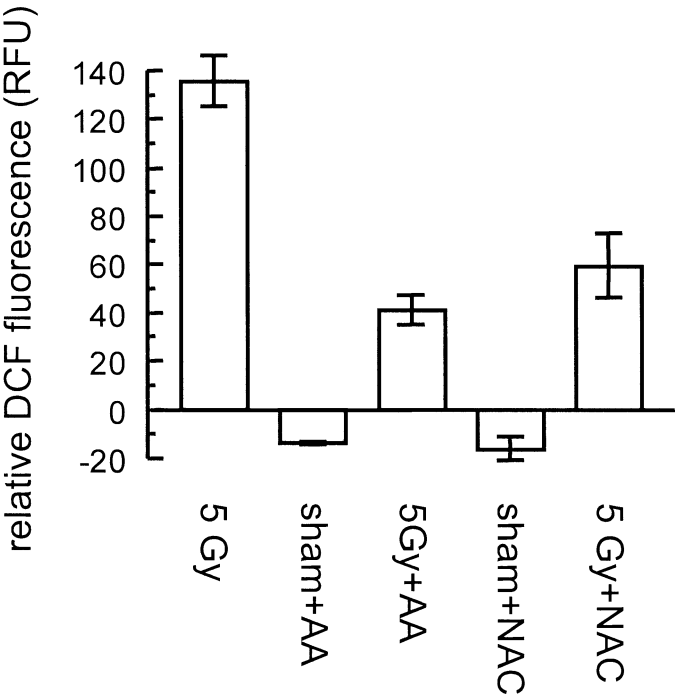

FIG. 5. The effects of incubation of cells with 10 μ*M* ascorbic acid (AA) and 100 μ*M* *N*-acetyl-cysteine (NAC) on radiation-induced ROS. Cells were cultured with antioxidants for 20 min prior to and during sham irradiation or 5-Gy radiation exposure. Results are presented as changes from control cells; means ± SD from at least three independent measurements.

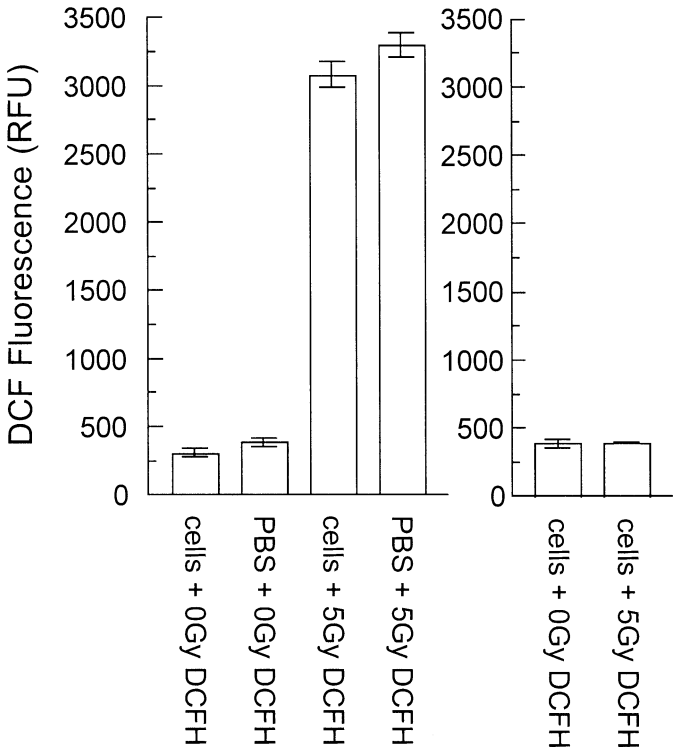

FIG. 6. Panel A: DCFH was sham-irradiated or irradiated with 5 Gy and then added to microwells containing cells in PBS or to control wells with PBS but no cells. Fluorescence was measured using a microplate reader. An increase in fluorescence was observed in wells with and without cells when DCFH was irradiated. Panel B: DCFH was sham-irradiated or irradiated with 5 Gy and added to cells in 5-ml tubes, and fluorescence was measured by flow cytometry. No increase in fluorescence was observed in cells incubated with irradiated DCFH, demonstrating that flow cytometry can discriminate against DCFH oxidized extracellularly. Results are means ± SD from at least three independent measurements.

ascorbic acid and NAC reduced the amount of DCF oxidized by ionizing radiation as well as to that produced in sham-irradiated cells through normal oxidative metabolism (Fig. 5). Both of these observations are in agreement with previous reports (*20, 28*) and serve as a validation of the optimized DCF flow cytometry method.

The optimized DCF flow cytometry method was also tested for its ability to discriminate between intracellular and extracellular radiation-induced DCF fluorescence. Because the method involves irradiating cells in suspension in the presence of DCFH-DA, DCFH may exist extracellularly through either cellular leakage (*29*) or extracellular hydrolysis of DCFH-DA (*30*). Extracellular DCFH may be a target for radiation-induced fluorescence. DCFH was prepared *in vitro* by 20 min incubation of 100 μ*M* DCFH-DA with 20 m*M* NaOH. DCFH was oxidized by exposure to 5 Gy γ rays. When irradiated DCFH was added to microwells (final concentration 50 μ*M*), the DCF fluorescence was significantly greater than for sham-irradiated DCFH (Fig. 6A). This confirms that DCFH is oxidized to DCF by irradiation *in vitro* (*20*). When irradiated DCFH was added to microwells containing cells (~100,000 cells in 100 μl PBS, final concentration of DCFH 50 μ*M*), significantly more DCF fluorescence was seen than for cells incubated with sham-exposed DCFH. This suggests that microplate measurements do not discriminate between extracellular and intracellular DCF fluorescence. When irradiated DCFH was added to cells and fluorescence was measured by flow cytometry, no difference was observed between irradiated and nonirradiated DCFH samples (Fig. 6B). This is presumably due to the fact that extracellular DCF and DCFH do not diffuse into cells (*31*).

Measurement of ROS in Bystander Cell Cultures

A mixed suspension system using AA8 and AA8-rfp cells was developed to measure ROS generation in bystander cell populations. AA8-rfp cells are distinguishable from AA8 cells using flow cytometry by their red fluorescence assayed on FL3 (670 nm).

AA8 cells were irradiated and 9 s afterward were mixed with AA8-rfp cells preloaded for 20 min with DCFH-DA. Mixed populations were incubated for 30 min, after which DCF fluorescence in gated AA8-rfp cells was quantified. Control experiments in which AA8-rfp cells were mixed with sham-irradiated AA8 cells were run in parallel. The difference in the fluorescence of AA8-rfp cells mixed with irradiated cells compared to those mixed with control cells was measured for doses of 50 cGy, 2 Gy and 10 Gy. These results are tabulated in Table 1. Bystander cells showed no

TABLE 1
Measurement of ROS in Bystander Cells

Dose	ΔF (RFU)	Standard deviation (RFU)	Independent measurements	*P* value
50 cGy	3.53	2.48	6	0.37
2 Gy	1.41	5.48	7	0.33
10 Gy	4.27	5.62	7	0.53

Notes. Nonirradiated AA8-rfp cells were mixed with irradiated AA8 cells 9 s after exposure and DCF fluorescence in AA8-rfp cells was assayed 30 min later. Control experiments in which AA8-rfp cells were mixed with sham-irradiated AA8 cells were run in parallel for each experiment. ΔF is the difference in fluorescence of AA8-rfp cells mixed with irradiated cells compared to AA8-rfp cells mixed with sham-irradiated cells. The *P* value was calculated using a Wilcoxon signed rank test for paired data. No significant increase in DCF fluorescence in bystander cell populations was observed at any doses tested.

significant increase in DCF fluorescence when mixed with irradiated cells for 30 min after irradiation with any of the doses tested.

DISCUSSION

Here the DCFH method is adapted and refined for flow cytometry measurement of radiation-induced ROS. Consistent with other reports of the low sensitivity of measurement of radiation-induced DCF fluorescence by flow cytometry (*21, 23*), many experimental procedures tested here yielded little measurable difference in cellular DCF fluorescence between irradiated and control cell populations (Fig. 1). Only when the measurement was make on cells in suspension in the presence of DCFH-DA was a substantial increase in radiation-induced DCF fluorescence observed. Under these conditions, the assay was sensitive to 10 cGy radiation, and the increase in radiation-induced intracellular DCF fluorescence was found to correlate with dose up to 10 Gy.

The method for analyzing radiation-induced ROS presented here has both benefits and drawbacks in comparison to other DCF-based methods. A comparison can be made to the microwell plate method reported by Wan *et al.* (*20*) and used later to quantify ROS induced by low- and high-LET radiation (*28, 32*). This microwell method can detect ROS induced by as few as 2 cGy, surpassing the 10-cGy sensitivity observed here with flow cytometry. The microwell method is also completely immune to the artifact resulting from extracellular oxidized DCFH-DA entering cells because DCFH-DA is washed off cells prior to irradiation (*20*). A possible shortcoming of the microwell assay reflects DCFH leakage. In certain cell lines, DCFH can leak out of DCFU-DA-loaded cells into the extracellular medium that lacks the probe (*29, 33*). This extracellular DCFH can be oxidized by ROS induced by radiation (as shown in Fig. 6A). Microwell plate analysis is unable to distinguish such DCF fluorescence from DCF fluorescence occurring intracellularly. The total fluorescence assayed in microwells therefore does not accurately depict intracellular oxidation events for cells that leak DCFH, especially those that do so rapidly. When DCF fluorescence is scored by flow cytometry, extracellular fluorescence is not measured (Fig. 6B).

The optimized flow cytometry method offers additional benefits for measuring radiation-induced cellular DCF fluorescence. The flow cytometry and microplate methods both require a background subtraction correction to normalize data, but the microwell method requires a cell type-specific correction factor for cellular density (*34*) that is automatically accounted for by flow cytometry, in which uniform cell numbers are used in all measurements. Flow cytometry is also uniquely suited for taking multiple measurements with different fluorescent markers simultaneously across a population of cells. The method reported here could be used to correlate radiation-induced ROS with a number of other biologically relevant end points reportable by nonoverlapping fluorescent probes. Here, for example, flow cytometry was used to measure rfp fluorescence concurrently with DCF fluorescence to quantify ROS induction in bystander cells.

Recent reports have indicated that the bystander effect may be due at least in part to the transfer of ROS induced in irradiated cells to neighboring unirradiated cells. Cells cultured *in vitro* have been shown to exhibit an increased rate of oxidative ROS production immediately after exposure to radiation (*20, 35*); this effect was observed to last for 3–4 min in CHO cells after exposure and to be dependent on mitochondria in osteosarcoma cells (*18*). In an elegant experiment by Wang and Coderre, an increase in micronuclei was seen in bystander cells co-cultured during α-particle exposure to irradiated cells; however, if an antioxidant (DMSO) was added to medium during co-culture exposure, the bystander phenotype was not seen. If bystander cells were added to α-particle-irradiated cells ~1 min after exposure, no bystander effect was observed (*9*). Thus it is reasonable to hypothesize that ROS produced by irradiated cells may diffuse and oxidize in unirradiated bystander cells promptly after irradiation. Here we tested this hypothesis using CHO cells, in which radiation bystander effects have been observed previously (*24–27*).

We measured ROS levels by DCF fluorescence in bystander cells mixed with γ-irradiated cells as quickly as possible after exposure. AA8 cells in suspension were exposed to γ rays; 9 s after exposure, AA8-rfp cells were added and the cells were cultured together for 30 min, at which time DCF fluorescence was quantified in gated AA8-rfp cells by FACS. The DCF fluorescence in bystander AA8-rfp cells mixed with cells irradiated with 0.5, 2 or 10 Gy were the same as that in bystander cells mixed with unirradiated cells. These results suggest that ROS generated in irradiated CHO cells do not enter and/or induce ROS in neighboring bystander cells between 9 s–30 min after irradiation.

Due to the limitations of the irradiation device used here, bystander cells could not be added to irradiated cultures until 9 s after irradiation. Previous studies narrowed the

time during which ROS could be implicated in the bystander effect to <1 min after α-particle exposure (*9*). If ROS mediate the bystander effect, they may be expressed for just seconds after radiation exposure and thus would have been undetectable in our experiments. It is also plausible that ROS may partially mediate bystander effects within the time frame measured here but that their effect is too small to assay.

Care should be taken to avoid artifacts that can arise from flow cytometry measurement of DCF fluorescence. DCFH-DA can be photo-oxidized by UV and visible light (*36*); thus incubation, irradiation and FACS measurement should be done in the dark. ROS produced by normal oxidative metabolism can produce artifacts if DCF fluorescence is measured when the times after exposure are significantly different; such artifacts can be minimized if samples are kept on ice after exposure and are assayed at as nearly at the same time as possible (*37*). DCFH-DA present in the medium can also be oxidized extracellularly to DCF-DA after irradiation and then enter the cell to become fluorescent after deacetylation to DCF (see Fig. 2B), consequently providing a false measurement of intracellular ROS events. Although artifact is minimal because DCFH-DA is substantially more resistant to oxidation than the deacetylated DCFH (*38*), cells here were irradiated only after they had been fully loaded with DCFH-DA substrate. After 20 min of incubation, over 80% of extracellular DCFH-DA was absorbed by AA8 cells (Fig. 2A), and between 20 and 40 min of DCF-DA incubation, no additional DCF-DA accumulated intracellularly (Fig. 2B). Although substrate diffusion is likely dependent on concentration and cell type, the DCFH-DA loading kinetics measured here correlates well with previous reports that intracellular DCFH-DA concentration stabilized after 10 min in cardiomyocytes (*39*) and 15 min in endothelial cell cultures (*29*), and that the concentration remained stable for over 1 h. To avoid artifacts, cells in suspension with DCFH-DA should not be exposed to radiation prior to substrate saturation.

When using flow cytometry to analyze ROS fluorescence using the DCF method, care must be taken to avoid conditions that lead to probe leakage (*29, 33*). DCFH and DCF are most likely to leak from cells if the cells are washed after DCFH-DA incubation with fresh medium not containing the probe. Interestingly, the experimental variations tested here that resulted in low radiation-induced DCF fluorescence (Fig. 1 A–C) and the previous studies that failed to demonstrate sensitive radiation-induced ROS measurement using FACS (*21–23*) were done using conditions most susceptible to probe leakage. A recent report (*37*) was highly critical of studies using DCFH-DA for quantifying intracellular radicals formed by radiation, especially the studies of Wan *et al.* (*20, 28, 32, 34*), and proposed that the DCF method is subject to an artifact due to an influx of extracellularly generated hydrogen peroxides after irradiation. We subsequently sought to measure the contribution of this artifact hypothesized by Korystov *et al.* (*37*) and found that its contribution to DCF fluorescence in CHO cells is negligible (*40*).

Additional assay refinements may increase sensitivity in both low and high dose ranges outside those tested here. The assay's sensitivity to low-dose radiation can be increased through normal optimization of cytometry, such as decreased cell flow rate and increased excitement laser intensity, which offer more sensitive fluorescence measurement [for review, see ref. (*41*)]. However, simple cell-size gating also may increase the sensitivity of the assay. Cells contain varying amounts of fluorescent probe based on cell size (*42*); this leads to a broadening of the DCF fluorescence Gaussian when cells are measured by FACS. Because resolution and thus sensitivity worsen with increased FWHM (*43*), both can be improved by gating cells for size when scoring DCF fluorescence. In our studies, DCF fluorescence correlated well with dose between 5 cGy and 10 Gy, but the response was not linear (Fig. 4). This response is consistent with the probe saturation inherent in detection systems that contain a finite number of probes (*44, 45*). Whereas the dynamic range under the current conditions likely extends beyond 10 Gy, the effects of probe saturation at higher doses can be offset by increasing the concentration of DCFH-DA, which was previously used successfully *in vitro* up to at least 200 μ*M* (*46, 47*).

Here the DCFH method has been adapted for measurement of radiation-induced ROS using flow cytometry. An optimized method detected low-LET radiation-induced ROS at doses as low as 10 cGy. The antioxidants ascorbic acid and NAC abrogated radiation-induced oxidative stress when present during cellular radiation exposure. This flow cytometry method is both sensitive to radiation-induced ROS and capable of simultaneously taking other fluorescence measurements in relation to DCF fluorescence. This is demonstrated in bystander co-culture experiments in which DCF fluorescence was measured in unirradiated AA8-rfp cells mixed with irradiated AA8 cells. We suggest that this flow cytometry method is useful for correlating radiation-induced intracellular oxidative stress in cells with other end points reportable by fluorescent markers.

ACKNOWLEDGMENTS

KH was partially funded by a National Institute of Biomedical Imaging and Bioengineering training grant. This research was supported in part by project 1 to RHS of NIH grant 1 U19 AI 67769-01 to William McBride.

Received: August 31, 2007; accepted: November 2, 2007

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