ABSTRACT

Rat embryo cells (REC) transformed by the H-ras oncogene plus the cooperating oncogene v-myc are highly resistant to ionizing radiation as compared with the nontransformed parent cells, REC, or immortalized REC. In an attempt to understand the potential mechanism of resistance in these cells, the induction and repair of double strand breaks (dsb) in DNA were measured in a H-ras plus v-myc transformed (3.7) and an immortalized REC (mycREC) line using pulsed field gel electrophoresis. Cells were irradiated in the exponential phase of growth, and the amount of DNA dsb present was quantified by measuring the fraction of DNA activity released from the agarose plugs in which cells were embedded. Similar values of the fraction of DNA activity released were measured for both cell lines at equal X-ray doses, after correction for differences in cell cycle distribution, suggesting a similar induction of DNA dsb per Gy. Repair of DNA dsb measured after exposure to 40 Gy of X-rays was similar in both cell lines and displayed a fast and a slow component. The fast component had a 50% repair time of approximately 12 min, and the slow component, 50% repair time of about 3 hr. These results suggest that the relative radioresistance of 3.7 cells is not conferred by a decrease in the amount of DNA dsb induced per Gy per dalton or by alterations in the capacity of the cells to repair DNA dsb. It is hypothesized that alterations in the expression of potentially lethal damage underlie this phenomenon.

INTRODUCTION

Evidence accumulates that expression of certain types of oncogenes in cells of various origins can confer resistance to ionizing radiation. Thus, Chang et al. (1) reported that normal fibroblasts from skin biopsies of patients with the Li-Fraumeni syndrome showed overexpression of the myc and activation of the raf oncogene and displayed at the same time increased resistance to ionizing radiation. Furthermore, Kasid et al. (2) demonstrated that a cell line developed from a clinically radioresistant human squamous cell carcinoma was also characterized by an activation of the raf oncogene. FitzGerald et al. (3) observed increased radioresistance in NIH3T3 cells containing the human N-ras gene. Increased radioresistance in NIH3T3 cells transfected with various forms of the ras oncogene has also been observed by Sklar (4). More recently, evidence has been presented that the effect of oncogenes such as H-ras in conferring radioresistance can be augmented in REC if cotransfected with the oncogene myc (5, 6). These findings correlate genetic alterations with modifications in the intrinsic radiosensitivity of a cell and may have implications for the treatment of human tumors by ionizing radiation. The effect of the activated ras oncogene on radiation resistance may be of great clinical significance, since 90% of human pancreatic carcinomas (7), 50% of colon carcinomas (8, 9), and 20% of lung cancers (10) have oncogenic mutations in ras genes. The myc oncogene family is often amplified or overexpressed in a wide variety of tumors (11). It is possible that the presence of certain types of oncogenes in human tumors reduces their radiosensitivity, thus inadvertently affecting the outcome of treatment by radiation.

Although radioresistance mediated by oncogenic transformation has been demonstrated in a number of cell lines and the associated complications for the treatment by radiation of human tumors discussed (see previous paragraph for references), little is known about the mechanism that underlies the phenomenon. Yet elucidation of the mechanism underlying reduced radiosensitivity in cell lines transfected with certain families of oncogenes may help devise strategies for overcoming problems in the radiotherapy of malignancies that happen to express these genes.

There is compelling evidence suggesting that the target of ionizing radiation-induced cell killing is the DNA (12), and that DNA double strand breaks are the lethal lesions. For example, a direct correlation between unrepaired DNA dsb and cell killing was established in DNA dsb repair-deficient mutants of yeast (13–15), and radiosensitive mutant cell lines have been isolated that are partially defective in their ability to repair these lesions (16–19). It was, therefore, queried whether the reduction in radiosensitivity observed in ras-transfected cell lines is associated with modulations in either the induction or the repair of DNA dsb.

A radiation-resistant cell line obtained by cotransfection of REC with the oncogenes H-ras and v-myc (3.7) was used for the experiments (6). The results were compared to those obtained with a cell line immortalized by transfection of REC with c-myc (mycREC) that displayed normal sensitivity to ionizing radiation exposure (6). Induction and repair of DNA dsb were assayed by a recently developed pulsed field gel electrophoresis technique, the AFIGE (20, 21). Pulsed field gel electrophoresis, originally devised for the separation of DNA molecules up to several million base pairs (22–26), has recently been shown to be a powerful assay for the measurement of induction and repair of DNA dsb in mammalian cells. Thus, clamped homogeneous electric field gel electrophoresis (27), transverse alternating field electrophoresis (28–30), and AFIGE (20, 21, 31, 32) have been successfully used for the measurement of dsb in the DNA of mammalian cells.

Here, we report experiments designed to study induction and repair of DNA dsb in irradiated 3.7 and mycREC cells, using AFIGE. The results obtained indicate similar induction and similar rates of repair for DNA dsb in both cell lines and...
suggest that the mechanism that confers radiation resistance in 3.7 does not rely on modulation of these parameters.

MATERIALS AND METHODS

Cell Culture. Cell lines 3.7 and mycREC derived from primary rat embryo fibroblasts as previously described (6), were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, at 37°C in a humidified incubator in an atmosphere of 5% CO₂ and 95% air. Cells were kept in a quiescent continuous culture by subculturing every second day at an initial density of 5 x 10⁴ cells/flask (80 cm² tissue culture flask, 20 ml of medium). For experiments with 3.7 cells, 1 x 10⁶ and 10⁷ cells were seeded in 80 cm² tissue culture flasks (20 ml of medium) and were used 2 days later (cells were still in the exponential phase of growth); different numbers of cells were seeded per flask, within the above given range, in order to obtain cultures with different distributions of cells throughout the cycle (see "Results"). In general, high initial cell numbers per flask resulted in cell populations enriched in G₁, cells after 2 days of growth. MR cells were seeded at 5 x 10⁶ cells per 150 cm² dish (20 ml of medium) and were used after 2 days of growth. Cells were labeled with 0.74 kBq/ml of [³H]thymin plus 5 μM thymidine, added at the time of culture preparation. Distribution of cells throughout the cell cycle was measured in each experiment using a mercury arc lamp flow cytometer (Partec, PAS II). For this purpose, cells were stained by direct suspension (about 5 x 10⁶ cells) in a solution (1 ml) containing 0.1 M Tris, 0.1 M NaCl, 5 mM MgCl₂, 0.05% Triton X-100, and 2 μg/ml of 4’,6-diamidino-2-phenyl-indole (DAPI). Data were collected in a computer equipped with the necessary software to calculate the fraction of cells in G₁, S, and G₂ + M phase. The cell cycle distributions thus obtained were used for the interpretation of the results at the DNA level. Compared with previously published data (6), small differences in the distribution of cells through the cycle were observed. These differences are probably due to slight differences in the growth protocols or the serum lot used; however, alterations in the growth characteristics of the cells cannot be excluded. The clonogenic assay was used to measure cell survival after exposure to various doses of X-rays; the parameters of the resulting survival curves were derived as described previously (6).

Measurement of Induction and Repair of DNA dsb. Dose-response curves for the induction of DNA dsb were measured using a pulsed field gel electrophoresis technique, the AFIGE (20, 21, 31, 32). For this purpose cells were trypsinized, spun down, and suspended in 1% agarose at a concentration of 3 x 10⁶/ml (FMC InCert) at 37°C. This suspension was pipetted into glass tubes, was allowed to cool in ice until solidification, and was then cut into 3 x 5 mm pieces containing about 1 x 10⁵ cells. Cells were irradiated on ice, using a therapeutic Siemens X-ray machine operated at 250 kV, 15 mA with a 2 mm Al filter. Immediately after irradiation cells were lysed for 20 h at 50°C in a solution containing 0.5 mM EDTA, 0.01 mM Tris, 2% N-lauryl sarcosine (NLS), and 0.1 mg/ml of proteinase K at pH 8.0. Subsequently, samples were washed for 1 h in 0.1 M EDTA, 0.01 M Tris, pH 8.0, and were then treated for 1 h at 37°C with 0.1 mg/ml of RNase A (boiled 10 min to eliminate DNsase contamination) dissolved in the same solution.

To measure repair of DNA dsb, cells were suspended in fresh growth medium, irradiated with 40 Gy, and held in suspension in glass tubes at 37°C plus 5% CO₂ to allow repair (6 x 10⁶ cells/ml). Aliquots were taken after various times, and cells were mixed quickly with melted agarose (37°C, some repair is possible during this step) and cooled by submersion in ice. Subsequently, plugs were made, and cells were lysed as described above. Results obtained with CHO cells suggested that incubation of cells in suspension at this concentration did not affect their ability to repair as compared with cells kept in monolayer.

Asymmetric Field Inversion Gel Electrophoresis. Electrophoresis was carried out for 40 h in half-strength 45 mM Tris, 45 mM boric acid, 1.5 mM EDTA, pH 8.2, at 10°C, in BRL (H4) horizontal boxes, in 0.5% agarose gels (FMC SeaKem GTG) cast in the presence of 0.5 g/ml of ethidium bromide. AFIGE was performed by applying cycles of 1.25 V/cm for 900 s in the direction of net DNA migration, and 5 V/cm for 75 s in reverse direction, using a power supply, an IBI (Minipulse) switching unit and a custom-made device with two rheostats to reduce the voltage in forward direction. After the end of the electrophoresis time, gels were photographed under UV and subsequently cut to separate the plug from the lane for each sample. The ¹⁴C activity of each piece was measured in a scintillation counter and the FAR was calculated as lane DPM divided by total (plug + lane) DPM. The FAR in cells that were not exposed to radiation (termed background) was determined in each experiment (1 to 3%) and was subtracted from the FAR values obtained in irradiated cells before plotting. There was no change in the FAR of unirradiated cells kept in suspension for repair up to 4 h.

Biphasic repair kinetics was observed throughout this work, and repair time constants for the fast (a) and the low (b) component were calculated by fitting the results obtained to the equation

FAR = A x 10^-a + B x 10^-b

using a two-step procedure (A and B represent the fraction of DNA dsb repaired with fast and slow kinetics, respectively). In the first step, data points between 60 and 240 min were used to determine B and b by fitting to the equation FAR = B x 10^-a. In the second step, A and a were determined by fitting the data between 0 and 30 min to the equation FAR = B x 10^-a = A x 10^-a. The findings presented here have been reproduced in 3 to 7 independent experiments, and the results are shown as the resulting mean ± standard error.

RESULTS

In Fig. 1, recent survival curves are shown that were obtained with exponentially growing mycREC (A) and 3.7 (●) cells. The data demonstrate a marked resistance to ionizing radiation exposure for 3.7 cells and confirm previously published observations (6).

In Fig. 2, dose-response curves are shown for the induction of damage in the DNA, as measured by AFIGE in the range between 0 and 70 Gy. Plotted in the figure is the FAR, measured as described under "Materials and Methods," as a function of the radiation dose for 3.7 and mycREC cells grown under various conditions. Fig. 2, A, depicts results obtained with exponentially growing mycREC cells. In the range of doses examined, the results obtained could be adequately fitted by a straight line. It has been pointed out elsewhere (31, 32), how-

Fig. 1. Clonogenic radiation survival curves for mycREC (A, D₀ = 1.1 Gy) and 3.7 (●, D₀ = 2.2 Gy) cells. Fitting of the data to a linear quadratic equation gave the following results: MycRec cells: a = 0.16 Gy⁻¹, β = 0.74 Gy⁻²; 3.7 cells: a = 0.0 Gy⁻¹, β = 0.59 Gy⁻²;
ever, that this way of fitting is only a first approximation that is valid in the low-dose region; it does not assume or imply a linear dependence of the FAR on dose in the entire range of doses where measurements are possible. The results obtained with 3.7 cells seeded at an initial density of approximately $10^6$ cells per flask are shown in Fig. 2. Once again, the results obtained could be fitted by a straight line. For a given dose of radiation, the FAR in 3.7 cells was slightly lower than that of mycREC cells. This difference could, in principle, be attributed to differences between the two cell lines in the induction of DNA dsb per Gy per dalton. However, experiments performed with synchronized populations of CHO cells (31, 32) suggest that such conclusions are only valid when the distribution of cells throughout the cycle in the two cell lines is identical. Flow cytometry, carried out in parallel to the experiments shown in Fig. 2, indicated that MR cells had a lower percentage of S-phase cells (20 to 23%) than 3.7 cells when seeded at approximately $10^6$ cells/dish (33 to 42%).

Since it has previously been shown that S-phase cells show a lower FAR per Gy than cells irradiated in $G_1$ or $G_2$, for the same induction of DNA dsb per Gy per dalton (31, 32), experiments were carried out with 3.7 cells grown under conditions that reduced the percentage of S-phase cells in the population. Reduction in the fraction of S-phase cells in 3.7 cells was effected by preparing cultures at higher initial cell concentrations (1.5 to $3 \times 10^6$ cells/dish) and allowing cells to grow for 2 days. Under these conditions cell populations were obtained with a fraction of S-phase cells (20 to 29%) closer to that measured in mycREC cells. Fig. 2, depicts results obtained for DNA damage induction in these cultures. The FAR for a given dose of radiation was slightly higher than that measured in mycREC cells. Taken together, the set of results presented with 3.7 and mycREC cells in Fig. 2 suggests that only small differences exist in the DNA damage induction dose-response curves which can be attributed to changes in the FAR per Gy caused by differences in the distribution of cells through the cycle. Although the available results appear consistent with a slightly higher induction of damage (DNA dsb) in 3.7 cells, the effect is small and cannot be confirmed without experiments with synchronized cell populations. Within the uncertainties of this type of experiments, these results are consistent with similar induction of DNA dsb in mycREC and 3.7 cells.

In Fig. 3, repair kinetic values are shown for mycREC and 3.7 cells exposed to 40 Gy in the exponential phase of growth. Plotted in the figure is the FAR as a function of the postirradiation incubation time at $37^\circ$C. Cell populations similar to those used in the experiments described in Fig. 2 were used. 3.7 cells grown at high initial concentrations gave larger initial $t = 0$ values for the FAR than did the corresponding mycREC cells, in agreement with the results shown in Fig. 2. However, the difference between mycREC and 3.7 cells grown after seeding at an initial density of $10^6$ cells was smaller than that shown in Fig. 2, probably due to experimental variation. Despite the small differences in the initial levels, the FAR rapidly decreased as a function of time according to biphasic kinetics. The reduction in the FAR with increasing postirradiation incubation time is caused by a restoration of the molecular size of the DNA to values closer to those obtained with nonirradiated cells and reflects repair of DNA dsb (21, 27).

To facilitate comparison of the repair kinetics measured in the different cell lines used after growth under various conditions, the FAR values of Fig. 3 were normalized to the values of FAR measured at $t = 0$, and the results obtained are shown in Fig. 4. To prevent congestion in the graph and to facilitate comparison of the different sets of data, a 1-h shift was introduced for each set during plotting. The results indicate biphasic repair kinetics for radiation-induced DNA dsb for both cell lines. Regression analysis was carried out using these data, assuming two exponential functions, in order to calculate the $t_{50}$ values for the slow and the fast repair components, as well as the fraction of DNA dsb repaired with slow kinetics. The results obtained from this analysis are summarized in Table 1. No significant differences were observed in the repair time constants or in the fraction of DNA dsb repaired with slow kinetics between mycREC and 3.7 cells. The fast component...
had $t_{50}$ values of about 12 min, whereas the slow component had $t_{50}$ values of about 3 h for both cell lines, and 25 to 35% of the DNA dsb were repaired with slow kinetics in every case examined.

DISCUSSION

The results presented in the previous section indicate that the increased radioresistance of 3.7 cells, as compared with that of mycREC cells, cannot be attributed to a decrease in the induction of DNA dsb per Gy per dalton. This conclusion is based on the observation that the small differences found in the dose-response curves for DNA dsb induction in the two cell lines could be explained by the small differences in the distribution of cells throughout the cell cycle. It is important to point out that comparisons of dose-response curves for the induction of DNA dsb obtained with different cell lines need to be made with great caution, since differences in the distribution of cells through the cycle affect the factor that correlates the generated (FAR in our experiments) with the number of dsb present (31,32). Specifically, experiments performed with synchronized populations of CHO cells demonstrated large fluctuations in the FAR per Gy throughout the cell cycle, with a minimum in the middle of S phase. These results are the mirror image of those obtained with ataxia telangiectasia cells (35) and may suggest mechanisms by which increased resistance to radiation is conferred in 3.7 cells. Of importance also is the observation that the difference in radiosensitivity between mycREC and 3.7 cells is significantly reduced by postirradiation incubation with caffeine, that is known to sensitize cells to radiation by fixing potentially lethal damage (36–39).

In summary, the results presented here indicate that the increased resistance to ionizing radiation of 3.7 cells may not be due to modulations in the induction and repair of DNA dsb. Reduction in potentially lethal damage fixation, mediated by alterations in the delays induced by radiation in the progression of cells through the cycle, probably underlies the observed differences in radiosensitivity between mycREC and 3.7 cells.

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REFERENCES


$^a$ G. Liakakis et al., unpublished observations.

$^b$ Unpublished results.
DNA dsb REPAIR IN ONCOGENE-TRANSFORMED CELLS

Induction and Repair of DNA Double Strand Breaks in Radiation-resistant Cells Obtained by Transformation of Primary Rat Embryo Cells with the Oncogenes H-\textit{ras} and \textit{v-myc}

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