Inhibition of Ionizing Radiation Recovery Processes in Polyamine-depleted Chinese Hamster Cells

Eugene W. Gerner, Margaret E. Tome, Steven E. Fry, and G. Timothy Bowden

Department of Radiation Oncology and Cancer Center, The University of Arizona Health Sciences Center, Tucson, Arizona 85724

ABSTRACT

Polyamines are involved in many cellular processes, including DNA structure and function. Since DNA, or some DNA-containing structure, is known to be the target for cell killing induced by ionizing radiation and a number of chemotherapeutic agents, we investigated the effects of polyamine depletion on cytotoxic responses of Chinese hamster cells to X-irradiation. Colony formation ability after single, acute radiation exposures of cells growing under oxic conditions was minimally affected by endogenous putrescine and spermidine depletion, achieved after treatment with α-difluoromethylornithine. Survival of cells rendered hypoxic and then irradiated was unaffected by α-difluoromethylornithine treatment. With a-difluoromethylornithine. Survival of cells rendered hypoxic and endogenous putrescine and spermidine depletion, achieved after treatment with α-difluoromethylornithine. Survival of cells rendered hypoxic and then irradiated was unaffected by α-difluoromethylornithine treatment. However, cellular recovery processes were nearly completely suppressed in polyamine-depleted cells, including sublethal damage recovery, as evidenced by split-dose irradiations in log phase cultures, and potentially lethal damage recovery, observed when growth-inhibited cultures were allowed time to repair radiation damage prior to being plated for colony formation. Both these recovery processes were restored by exogenous putrescine treatment. Reaccumulation of intracellular spermidine content closely correlated with restoration of potentially lethal damage recovery. Depletion of putrescine and spermidine pools had little effect on either single or double strand DNA break production or rejoining. These data demonstrate that both sublethal and potentially lethal damage recovery are polyamine-dependent processes in Chinese hamster cells, and imply that the mechanisms by which hamster cells recover from these types of radiation damage are unrelated to their ability to rejoin DNA strand breaks, at least during the first hour after irradiation. Finally, these results suggest that the depletion of tumor polyamine content may be an effective method of enhancing the sensitivity of human tumors to fractionated radiotherapy.

INTRODUCTION

It is widely documented that inhibitors of ODC, the first enzyme in the polyamine biosynthetic pathway, are cytostatic agents due to their ability to deplete endogenous putrescine and spermidine pools (see References 1 and 2 for review). Depletion of putrescine and spermidine contents also affects cellular responses to a variety of chemical and physical stresses. These latter phenomena apparently involve several mechanisms. Suppression of intracellular putrescine and spermidine levels enhances the cytotoxic activity of alkylating agents, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (3), while diminishing the cytotoxic effectiveness of cis-diaminedichloroplatinum(II) (4). Since polyamines are known to interact with nucleic acids and can contribute to changes in DNA structure (5), it has been suggested that depletion of polyamines alters the action of these DNA-damaging drugs by affecting chromatin structure (6, 7). Polyamine depletion, using DFMO, the enzyme-activated suicide inhibitor of ODC (8), also sensitizes some cell lines to the toxic effects of heat shock (9–11). Since heat shock is not thought to kill cells via a mechanism involving direct DNA damage (12), this effect appears distinct from that involving the two previously mentioned drugs.

The target for ionizing radiation-induced cytotoxicity is thought to include the nuclear DNA of cells, as cell viability measured by colony forming ability is closely correlated with chromosome aberrations produced after irradiation (13). Others have speculated that depletion of endogenous putrescine and spermidine levels would alter the response of cells and tissues to X-irradiation. However, in one cell line, the survival response of log phase cultures to single X-ray doses at high dose rates was the same whether putrescine and spermidine levels were suppressed or not (14). Cell survival responses are known to be affected by a number of parameters. For example, survival increases when radiation dose is split into two or more fractions given over time, and this phenomenon has been termed SLDR (15). Survival also increases after single X-ray doses when certain conditions are used, such as when growth-arrested cells are irradiated and held for periods of time prior to growth stimulation for colony formation (16). This effect has been suggested to be due to the repair of damage that would have been lethal if un repaired, and the phenomenon has been termed PLDR (16, 17).

In this study, we asked two questions relating to the possible role of polyamines in cell survival responses to ionizing radiation. First, we asked whether polyamine content could affect the production of damage in X-irradiated cells, when damage was assessed either by measurement of DNA strand breaks or cell survival after single X-ray doses at high dose rates. Second, we asked whether endogenous polyamines were involved in cellular radiation recovery processes, such as SLDR and PLDR, since the negative report regarding the influence of polyamines in radioresponses cited above (14) did not address this possibility.

MATERIALS AND METHODS

Cell Culture and Irradiation Techniques. All experiments reported here used Chinese hamster cells (line CHO), which were maintained as described elsewhere (18), except that medium contained 10% instead of 20% fetal bovine serum. Cells were irradiated with a 4-Mev linear accelerator (Varian Assoc., Palo Alto, CA) and plated in plastic tissue culture dishes to form colonies for survival determinations as detailed earlier (19). Dose rates used here were ~300 cGy/min. Cells were irradiated at ice bath temperature under different conditions of oxygenation as follows. Cultures were irradiated under what are defined here as oxic conditions either in T-25 flasks while attached, or after removal from the monolayer, at which time they were resuspended at 10^6 cells/ml in full growth medium. One ml of this cell suspension was then drawn up into a 5-ml syringe and irradiated. Colony forming responses of our hamster cells irradiated under these two conditions were indistinguishable. Parallel cell suspensions were irradiated under hypoxic conditions (OER ~2.5–3, see “Results”), produced by metabolic consumption of oxygen, by sealing the syringes with parafilm and incubating the suspensions at 37°C for 30 min prior to irradiation. A similar method of producing hypoxia has been reported by Hall (20). For the experiments reported here, all irradiations were done using cell suspensions and all were incubated in the syringes for 30 min prior to
irradiation, whether they were sealed or not. The colony forming efficiencies of unirradiated control cultures for both of these two conditions were 70–90% and were not different from those of log phase cultures growing as monolayers.

Cell growth rates were determined by plating known numbers of cells into replicate dishes and then harvesting duplicate dishes on subsequent days to determine cell numbers (via a Coulter Particle Counter, Coulter Electronics, Hialeah, FL). For the studies presented here, 0.25 x 10⁶ cells were plated into 5 mL of medium in 60-mm Petri dishes on Day 0. Untreated control cells grew exponentially for 48 h, after an initial lag phase. After 48 h, the cell growth rate declined as cells began to enter plateau phase (18, see also “Results”). Split-dose irradiations were carried out on log phase cultures 24-48 h after seeding. For PLDR studies, cultures were seeded as described above and irradiated 96 h later.

Polyamine Analysis. Polyamines were quantitated using the high-performance liquid chromatography technique described by Seiler and Knodgen (21). Technical details relating to preparation of cell culture samples have been reported elsewhere (22). Briefly, to assess cellular polyamine contents, cultures were harvested, washed, and lysed. Acid-soluble polyamines were separated from proteins and other macromolecules by adjusting cell lysates to 0.2 M HClO₄ and were directly analyzed by high-performance liquid chromatography.

Alkaline and Neutral Elution of DNA. Alkaline elution of DNA from irradiated and unirradiated cells was carried out as described elsewhere for L1210 cells (23), while neutral elution analysis followed the procedure of Bradley and Kohn (24). Cells were grown in medium containing [³²P]thymidine (0.2 μCi/ml, 55.2 mCi/mmol) for 2 days prior to irradiation. After irradiation and repair interval incubations, cells were washed and collected into cold phosphate buffered saline (150 mM NaCl, 85 mM K₂HPO₄, 0.7 mM KH₂PO₄, pH 7.4), and loaded onto 2-μm pore PVC BS filters (Millipore Corp., Bedford, MA). For alkaline elution studies, cells were lysed on the filters at room temperature with a 0.1 % sodium dodecylsulfate, 0.02 M H₄EDTA, solution containing sodium dodecylsulfate, pH 9.6). DNA was eluted with the above elution buffer. Samples were collected and analyzed as previously described (23, 24). Briefly, individual elution profiles were compared by measuring r, the fraction of DNA retained on filters after 200 min of elution (r₀ is for unirradiated controls). The relative frequency of repair of damage is then defined as

\[ 1 - \frac{(\log r₀/r)}{(\log r₀/r₀)} \]

where t is the repair interval and r represents the value immediately after irradiation.

RESULTS

The dependence of endogenous polyamine content on cellular responses to ionizing radiation was investigated by treating cultures with DFMO. As shown in Fig. 1, putrescine content was depleted within 8 h after addition of 5 mM drug to the culture medium. Spermidine levels decreased more slowly, and were found to be suppressed by approximately 50% by 8 h and were minimal by 48 h after addition of DFMO to the culture medium. Spermine contents were unaffected by drug treatment. Cell proliferation was inhibited after 24 h of exposure to the drug. Colony forming ability of these cells was unaffected when treatment times with 5 mM DFMO were 96 h or less (data not shown).

Using these conditions, we next investigated the effect of putrescine and spermidine depletion on the response of Chinese hamster cells to single doses of X-rays. Since polyamines can be oxidized in response to certain cell stresses (1), we measured this survival response of normal and polyamine depleted cells under oxic and hypoxic conditions (Fig. 2). A small decrease in survival of cells irradiated under oxic conditions was observed in cultures that were treated with DFMO for 48 h, an incubation time that optimally suppressed both putrescine and spermidine contents. No modification of the survival response was observed in hypoxic cells, which displayed an oxygen enhancement ratio (ratio of the inverse slopes of the exponential portion of the survival response curves for hypoxic and oxic cells) of approximately 2.5.

To determine whether polyamine depleted cells could express SLDR, cells growing for 48 h in the presence or absence of 5 mM DFMO (see Fig. 1 for polyamine contents and cell densities) were irradiated with 1000 cGy given in two equal fractions separated by various times as shown in Fig. 3. Non-DFMO treated cultures expressed increased survival when plated for colony formation, which was maximal for intervals of about 2 h between doses. However, survival did not increase in polyamine-depleted cells irradiated with fractionated doses. Similar to the results seen in Fig. 2, colony forming ability was slightly lower in DFMO-treated cultures which received 500 cGy in a single dose or 1000 cGy in two equal fractions. To determine whether this inhibition of SLDR was due to depletion of endogenous polyamines and not as result of some unknown effect of DFMO, putrescine was added to parallel DFMO-treated cultures 12 h before irradiation to restore endogenous...
POLYAMINE-DEPENDENT RECOVERY PROCESSES

Fig. 2. Survival response of normal and polyamine-depleted CHO cells, irradiated under oxic or hypoxic conditions, to single, acute doses of X-rays. Cultures were seeded as in Fig. 1 in the presence (open symbols) or absence (closed symbols) of 5 mM DFMO. Forty-eight h later, cultures were harvested, resuspended at 10^6 cells/ml in either open (circles) or sealed (squares) 5-ml syringes and incubated at 37°C for 30 min. Cell suspensions were then irradiated in the syringes with doses as shown. Symbols plotted in this figure, and in Figs. 3 and 4, are the means and SEMs of the survival values from a single representative experiment in a series of three replicates. Survival curves were hand drawn.

polyamine contents (11). This treatment fully restored the ability of DFMO-treated cells to express SLDR.

The possible dependence of PLDR on endogenous polyamines was investigated by seeding cells in the presence or absence of 5 mM DFMO and incubating them without further feeding for 96 h. By this time, cell proliferation was minimal (18) although cell density was different in the DFMO-treated and nontreated cultures due to the cytostatic effect of the drug which was evident after 24 h of incubation. All cultures were irradiated with 1500 cGy. To control for effects of DFMO other than those related to polyamine depletion, putrescine was added after irradiation to a replicate series of DFMO-treated and irradiated cultures. All cells were then returned to the 37°C incubator for varying time intervals. At those times, cells were harvested and stimulated to proliferate by plating for colony formation (Fig. 4). Portions of these samples were also analyzed for polyamine content (see Fig. 5). As shown in Fig. 4, cell survival increased by nearly one log within 2 h after irradiation in nondrug-treated cultures. Survival did not increase when polyamine depleted cells were treated in a similar manner. However, when putrescine was added after irradiation to these DFMO-treated cells, survival increased to the same level, but at a slower rate, as in nondrug-treated cultures. From Fig. 5, one can see that exogenous putrescine addition restored endogenous pools of this amine within 1 h and were maximal and at least four times greater than control values by 2 h. Endogenous spermidine levels accumulated more slowly, reaching control values at a similar time to that when PLDR reached control levels as seen in Fig. 4.

The data in Figs. 3 and 4 showed that radiation recovery mechanisms were dependent on endogenous polyamine contents. Since it has been suggested that DNA strand breaks (DNA single and double strand breaks) are responsible for ionizing radiation-induced cell death and that radiation recovery processes are a result of the repair of these lesions (25, 26), we carried out alkaline and neutral elution studies to study DNA single and double breaks, respectively. The elution assays were carried out either immediately after irradiation or following various repair intervals, to determine whether polyamine depleted cells showed enhanced DNA damage in response to X-irradiation and whether the depleted cells were able to remove X-ray induced DNA strand break damage. Polyamine depletion did not affect the induction of DNA single or double strand breaks. (A quantitative measure of DNA strand break frequencies [-\log (r/r_0)] yielded values of 0.33 ± 0.08, −DFMO, and 0.36 ± 0.06, +DFMO, for alkaline elution, and 0.15 ± 0.06, −DFMO, and 0.12 ± 0.03, +DFMO, for neutral elution.) In these experiments, control or DFMO-treated cells were irradiated with 1000 cGy (alkaline elution) or 5000 cGy (neutral elution) and harvested immediately after irradiation. The rejoining of single and double strand DNA breaks after irradiation was followed in control and DFMO-treated cells. The rejoining of both types of DNA breaks in control cells was nearly complete in 1 h of postirradiation incubation of the cells at 37°C (Fig. 6). The apparent rates of DNA strand break rejoining in DFMO-treated cells were not different from those observed in cells not treated with DFMO.
POLYAMINE-DEPENDENT RECOVERY PROCESSES

Fig. 4. Effect of DFMO treatment on PLDR in growth arrested CHO cells. Cultures were seeded into medium without (O) or containing (A) DFMO as in the previous figures and were incubated at 37°C for 96 h. Cells from all groups were irradiated while attached to substrate with 1500 cGy. To one group (A), 1 mM putrescine was added immediately after the irradiation. All cultures were then returned to the 37°C incubator for the times shown, when they were stimulated to proliferate by harvesting and plating for colony formation.

Fig. 5. Restoration of polyamine contents in growth arrested cultures. Aliquots of the same cells used to measure colony forming ability in the experiment shown in Fig. 4 were processed and analyzed for polyamine contents (PUT, putrescine; SPD, spermidine; SPM, spermine). Symbols correspond to the groups shown in Fig. 4.

DISCUSSION

Polyamine depletion has been shown to alter mammalian cell responses to a variety of chemical (3, 4, 6, 7) and physical (9–11) stresses. We show here that some, but not all, CHO cell survival responses to X-irradiation are also dependent on intracellular polyamine content. Like a previous investigation in which the response of a rat brain tumor cell line was studied (14), we found that the response of our log phase CHO cells to single, acute radiation doses was not affected by DFMO treatment. Radiation recovery processes such as SLDR and PLDR, however, were both suppressed in DFMO-treated cells and both processes could be restored by the reaccumulation of endogenous polyamine contents. Since PLDR recovery closely correlated with the reaccumulation of spermidine but not putrescine levels, we conclude that PLDR is a polyamine-dependent process, and that spermidine is the most likely polyamine involved in this phenomenon. SLDR is also polyamine dependent, but as yet we have not correlated the restitution of putrescine and spermidine contents with SLDR recovery kinetics.

The conclusion that DFMO can inhibit PLDR after X-irradiation has also been reported by Courdi et al. (27). The findings of these authors, who studied the response of a human breast cancer cell line, appear to be different than those reported by us in that they found maximum potentiation of radiation responses after DFMO treatments of 1 h in duration. While no polyamine content information was provided, significant depletion of endogenous putrescine and spermidine levels would not be expected during a 1-h treatment, although synthesis of new polyamines would be inhibited. A second difference in our results and those of Seidenfeld (14), compared to Courdi et al. (27), was that the response of cells to acute radiation exposures was potentiated in the latter study. We have also observed this result in several human cancer cell lines after DFMO treatment times sufficient to deplete intracellular putrescine and spermidine levels (results to be reported elsewhere), as have Tofilon and coworkers (28). This difference probably reflects cell line-dependent differences, although, like the CHO cells, we have also observed inhibition of PLDR in a human adenocarcinoma cell line when we deplete endogenous putrescine and spermidine contents.

The results presented here demonstrate that polyamine depleted cells were quite efficient in rejoining X-ray-induced single and double strand breaks. We interpret these data to mean that neither single nor double strand DNA break rejoining is inhibited in polyamine depleted cells under identical conditions to those which lead to a suppression of SLDR. We have not yet measured strand break rejoining in plateau phase cultures, so we can not comment on the relationship to PLDR. Alkaline elution of DNA detects alkali-labile lesions in DNA in addition to actual scissions of the phosphate backbone. Though both lesions are probably measured, changes in elution rates are indicative of the rejoining of DNA single strand breaks. However, the elution assay does not measure the correctness or fidelity of the strand rejoining. Neutral elution at pH 9.6 of
DNA as carried out here and by others (24) also probably detects these same alkali-labile lesions, and when opposite a radiation-induced single strand break, will be detected as a double strand break (29, 30). Schwartz et al. (31) have observed that fewer DNA breaks are detected when elutions are carried out at pH 7.2, compared to 9.6, but found that the rate of repair was the same at both pHs. It seems reasonable to conclude that the rates of both single and double strand DNA break rejoining are not substantially affected by endogenous polyamine content. Thus, since rejoining was unaffected while SLDR was inhibited by polyamine depletion, these data argue that DNA strand break rejoining is not sufficient for cells to express at least SLDR. Schwartz and coworkers have come to a similar conclusion, finding that CHO mutants which do display differences in the rates of DNA strand break rejoining do not express differences in either SLDR or PLDR (31). Other workers have reported that residual, or unrepaired, damage, rather than the rate of repair, does correlate with the ability of cells to recover from radiation insult (32, 33). The specific mechanism by which polyamine depletion inhibits SLDR and PLDR, apparently independent of effects on the rate of DNA strand break rejoining, is unclear at this time. We are currently testing the hypothesis that DNA topoisomerase II is involved in these phenomena, since we have found that the effects of VP-16, the nonintercalating inhibitor of this enzyme, on both DNA damage and cell survival are dependent on endogenous polyamine content (34). These data may have application in clinical radiotherapy. Human tumor radiosensitivity has been associated with the ability of cells to express PLDR (35, 36). Also, radiotherapy is generally administered in daily fractions over a period of a number of weeks, a condition which should maximize SLDR. Thus, treatment of patients with DFMO prior to and during radiotherapy, to deplete tumor tissue polyanines, could be beneficial in enhancing local control rates of human cancers in response to fractionated radiotherapy. Some dose-limiting normal tissues have either no or a very small proliferative compartment, and thus have low or nondetectable levels of putrescine and spermidine (37). DFMO would have little or no effect on the radioreponse of these tissues and may achieve a therapeutic gain in these cases.

REFERENCES

Inhibition of Ionizing Radiation Recovery Processes in Polyamine-depleted Chinese Hamster Cells

Eugene W. Gerner, Margaret E. Tome, Steven E. Fry, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/48/17/4881

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.