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The Effect of Polyamine Depletion on the Cytotoxic Response to PUVA, Gamma Rays and UVC in V79 Cells In Vitro[1]

Jerry R. Williams,[2] Robert A. Casero and Larry E. Dillehay

The Johns Hopkins Oncology Center, Baltimore, Maryland 21287-5001

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Difluoromethylornithine (DFMO) depletes cells of polyamines, sensitizing cells against the action of antineoplastic drugs and altering ability to repair radiation-induced DNA strand breaks. Others have hypothesized that the mechanism by which polyamine depletion sensitizes cells is through inhibition of DNA strand break repair or through altering the spectrum of initial DNA damage. We have compared the effect of polyamine depletion on cytotoxic effects in V79 cells for three agents that damage DNA: PUVA (8-methoxypsoralen and ultraviolet light, 365 nm), γ-rays and UVC (ultraviolet light, 254 nm) in polyamine depleted V79 cells. DFMO pretreatment sensitizes cells to PUVA and γ-ray toxicity but not to UVC. Unlike UVC photoinduction of DNA lesions, PUVA- and γ-ray-induced DNA damage is modulated by chromatin structure. Our results suggest that polyamine depletion sensitizes cells to the action of PUVA and γ-rays by mechanisms disparate from those for UVC, suggesting that the level or type of initial damage, rather than DNA repair, per se, may be the more important determinant of enhanced cytotoxicity.

The polyamines, spermidine and spermine, and their diamine precursor, putrescine, are naturally occurring polycations whose metabolism and intracellular concentrations are under strict control (1-3); they are essential for growth, differentiation, and in some cases, cell survival (4,5). Their charged nature implicates the polyamines in binding and altering conformation of nucleic acids (6), and particular attention has been focused on the association of the polyamines and DNA (7). The emerging data suggest that the polyamines can have profound and potentially specific effects on chromatin structure (8-14).

Spermidine and spermine have been shown to have a role in x-irradiation damage repair (11,12), and polyamine content also appears to be involved in determining both radiation sensitivity and thermal stability (10,12). Similarly, in Chinese hamster ovary cells, polyamines have been shown to reduce the number of sister chromatid exchanges (SCEs) induced by PUVA (8-methoxypsoralen plus UVA irradiation) (10). Based on these results, we sought to

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[2]Address correspondence to Jerry R. Williams, The Johns Hopkins Oncology Center, Radiobiology Laboratory #2-121, 600 North Wolfe Street, Baltimore, Maryland 21287-5001. FAX:410/955-8780.

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compare the effects of three DNA-damaging regimens [PUVA (8-methoxy-psoralen at 10^{-5}M plus ultraviolet light, 365 nm), γ-rays, and UVC (ultraviolet light, 254 nm)] following polyamine depletion by pretreatment with the ornithine decarboxylase inhibitor, 2-difluoromethylornithine (DFMO) (15). DFMO is a highly specific inhibitor of polyamine biosynthesis and treatment leads to rapid depletion of putrescine and spermidine (16). PUVA is believed to produce its cytotoxic action by psoralen monoadduct production and interstrand DNA crosslinking, as psoralen intercalation and subsequent photoaddition of the intercalated molecule to DNA by UV light produce such lesions (17,18). Gamma-rays interact with intracellular molecules, predominantly water, to produce free radicals that can interact with the DNA and damage accessible sites. Thus, both of these agents may produce elevated levels of DNA damage, if DNA is more accessible, possibly enhancing cytotoxicity. On the other hand, the ability of UVC to produce pyrimidine dimers and other photoproducts does not depend upon the conformation of DNA as measured by accessibility to diffusing species, since photons are absorbed directly by processes driven by the excitation of the nucleic acid moieties (19). All three agents, however, induce enzymatic, excision-based DNA repair processes. We therefore sought to test whether modulation of cytotoxicity by polyamine depletion differs qualitatively or quantitatively between agents whose initial cellular damage is dependent or independent of DNA conformation.

Materials and Methods

Cell pretreatment and polyamine analysis. In all cases, except where noted otherwise, DFMO pretreatment indicates that the cells were grown in normal medium supplemented with 1 mM DFMO, α-difluoromethylornithine, (Merrel Dow Research Institute Cincinnati OH) for 24 hours prior to an assay or subsequent radiation treatment. Intracellular polyamine levels in DFMO-pretreated cells and control cells were determined by high pressure liquid chromatography of the dansylated derivatives using the method of Kabra *et al.* (20).

Cytotoxicity studies. Cytotoxicity was determined by standard clonogenic survival assay (colony formation) as previously described (21). Three hours after plating, cells were exposed to various doses either of PUVA, γ-rays or UVC. Each dish was then rinsed with 10 ml of serum-free medium, covered with 10 ml of normal medium, and placed in the incubator to allow for colony formation. After 6 days of incubation the dishes were stained with crystal violet and the colonies were counted.

PUVA treatment. Following 3 hr incubation to allow for cell attachment, a set of 3 replicate dishes was removed from the incubator, the medium was removed, and each dish was rinsed with 10 ml phosphate-buffered saline (PBS). Each dish received 10 ml of PBS containing 8-methoxypsoralen (8-MOP, Sigma) at a concentration of 10^{-5}M, and were then incubated for 30 min to allow for intercalation. For DFMO-pretreatment PBS was supplemented with 1 mM DFMO, and for DFMO + putrescine-pretreatment PBS was supplemented with 1 mM DFMO plus 10 μM putrescine. Following 30 min incubation, the cells in each dish were exposed to a given dose of ultraviolet light (365 nm, Sylvania FR20T12/UVA) at a rate of 5.0 J/m^2/s at room temperature while still covered by the psoralen solution.

Gamma-ray exposure. Cells were irradiated in a ^{137}Cs irradiator (Atomic Energy of Canada, Gamma Cell 40) at a rate of 1.24 Gy/min at room temperature while still covered by the appropriate medium.

UVC exposure. Following 3 hr incubation, a set of 3 replicate dishes was removed from the incubator, the medium removed, and each dish rinsed with 10 ml PBS. The cells in each dish were exposed to a given dose of ultraviolet light (254 nm) at a rate of 0.29 J/m^2/s at room

temperature. Prior to exposure, lids to the dishes were removed and the dishes then aspirated of covering solution.

Cytotoxicity analysis. The interaction coefficient is the ratio of the cytotoxic effects of both treatments (pretreatment with DFMO followed by PUVA, γ-rays or UVC) divided by the cytotoxic effects if both agents acted independently (22). The mean lethal hit is derived from the survival curves of control cells for each agent, *i.e.*, PUVA, γ-rays and UVC, and represents the D_0, that dose producing one lethal event per cell, if hit theory is applied to the exponential portion of the survival curve (23).

Results

Initial studies identified the optimal dose of DFMO, 1 mM, that would deplete polyamines with minimal perturbation of cell growth as indicated by flow cytometry. After evaluating DFMO doses ranging from 0.5 mM to 5 mM, we selected 1 mM DFMO as our standard dose (data not shown). After 24 hr treatment with 1 mM DFMO, intracellular concentrations of putrescine and spermidine were reduced to nearly undetectable levels, whereas the concentration of spermine was slightly increased compared to untreated controls (Table 1). The V79 cells remained completely viable as determined by trypan blue exclusion and colony formation plating assays.

V79 cells with polyamine depleted by DFMO were treated with PUVA. Figure 1 (panels a and b represent separate experiments) shows the PUVA survival curves for control cells, for cells pretreated with DFMO, and for cells pretreated with DFMO plus putrescine. DFMO-pretreatment at all doses of UVA increases cytotoxicity dramatically compared to controls. Furthermore, the difference in cytotoxicity between DFMO-pretreated and control cells increases progressively as a function of increasing UVA dose. Cells pretreated with putrescine plus DFMO, however, show a survival curve almost identical to that of control cells. This suggests that increased cytotoxicity induced by DFMO pretreatment was due specifically to polyamine depletion, as has been reported for other agents (24,25). Cells pretreated with DFMO were also more sensitive to γ-rays. Figure 2 (panels a and b represent separate experiments) shows γ-ray survival curves for control cells and for DFMO-pretreated cells. DFMO pretreatment, at all γ-ray doses, increases cytotoxicity compared to controls. Again, as with PUVA survival, the difference in cytotoxicity between DFMO-pretreated and control cells increases progressively as a function of increasing γ-ray dose. In contrast to PUVA

Table 1: Polyamine Depletion in V79 Cells

Treatment	Putrescine	Spermidine	Spermine
Control	2.54	9.11	2.98
	2.87	9.19	3.01
Control	1.89	6.70	2.35
	1.93	6.80	2.36
DFMO 1 mM	0.24	0.21	4.47
	0.30	0.24	4.49
DFMO 1 mM	0.14	0.37	4.08
	0.11	0.41	4.12

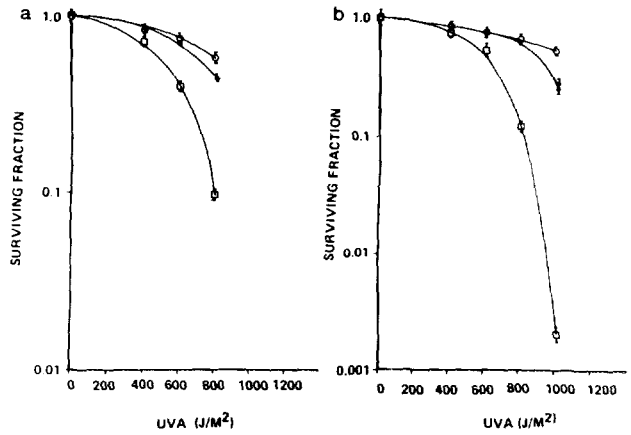

Figure 1. (panels a and b). Cytotoxic response to PUVA: non-pretreated cells (circles), DFMO-pretreated cells (1 mM DFMO for 24 hr) (squares), and putrescine + DFMO-pretreated cells (10 μM putrescine plus 1 mM DFMO for 24 hr) (triangles). The dose of UVA in J/m^2 is plotted on the X axis. The logarithms of cell survival are plotted on the Y axis. Each panel represents a separate experiment.

and γ-ray killing, however, UVC cytotoxicity was not affected by DFMO pretreatment as shown in Figure 3 (panels a and b represent two separate experiments). DFMO pretreatment responses can be compared by reducing the dose of the three toxic agents to "mean lethal hit," the dose for each agent that reduces cell survival to 37% of any value on the exponential part of the control survival curve, and expressing relative survival as the differences between control and treated expressed as logs. These data are shown in Figure 4. The data clearly show a similar pattern of increased killing for DFMO pretreatment before PUVA or γ-rays, but no effect for DFMO pretreatment prior to UVC.

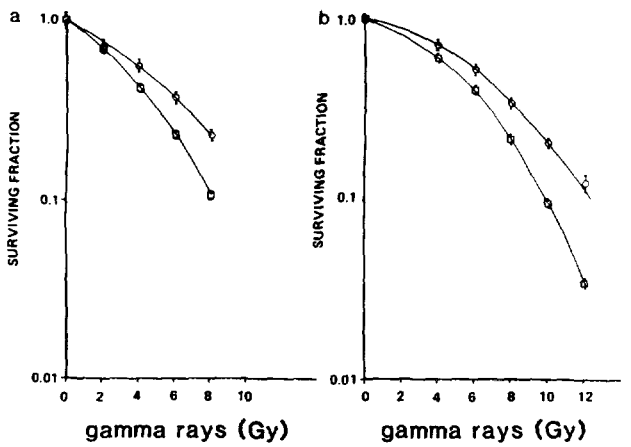

Figure 2. (panels a and b). Cytotoxic response of non-pretreated (circles) and DFMO-pretreated (squares) V79 cells to γ-rays. The dose of γ-rays in Gy is plotted on the X axis. The logarithms of cell survival are plotted on the Y axis. Each panel represents a separate experiment.

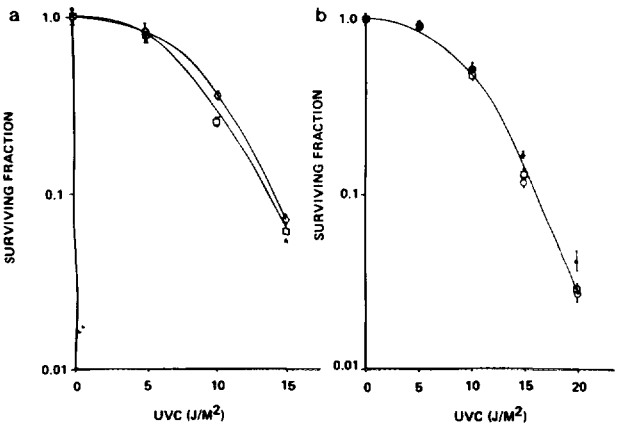

Figure 3. (panels a and b). Cytotoxic response of non-pretreated (circles), DFMO-pretreated (squares), and putrescine + DFMO-pretreated (triangles) V79 cells to UVC. The dose of UVC in J/m^2 is plotted on the X axis. The logarithms of cell survival are plotted on the Y axis. Each panel represents a separate experiment.

Discussion

These studies demonstrate that DFMO depletion of polyamines sensitizes cells to the cytotoxic effects of two DNA-damaging agents, PUVA and γ-rays, but not to a third, UVC. Since adding exogenous putrescine restores normal intracellular polyamine content to DFMO-pretreated cells and also reverses cytotoxic sensitivity to PUVA and to γ-rays, it seems likely that polyamine depletion, rather than any down-stream sequelae, is involved in the increased cytotoxicty. The exact mechanism by which DFMO changes the cytotoxic response of V79 cells to PUVA and γ-rays must still be considered speculative, although the major hypotheses would be modulation of initial damage or modulation of post-damage DNA repair,

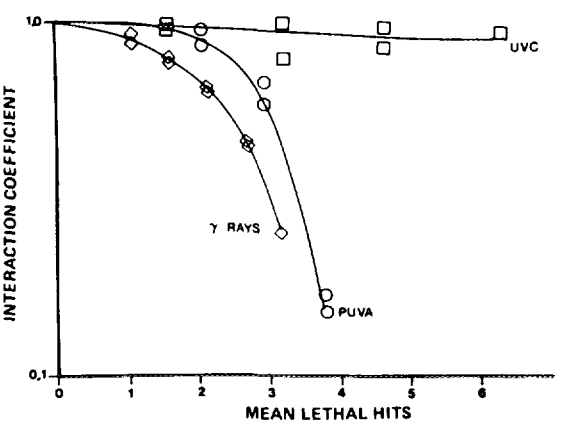

Figure 4. A plot of the degree to which cytotoxicity was potentiated by DFMO pretreatment for PUVA (circles), γ-rays (diamonds) or UVC (squares). The interaction coefficient is plotted as a function of mean lethal hits. See Materials and Methods for calculation procedures.

or both. In any case, the role played by the polyamines putrescine, spermidine and spermine in binding to DNA and stabilizing its structure may be an integral part of the mechanism. Marton and co-workers have shown that polyamine depletion alters DNA conformation in 9L rat brain tumor cells, which may lead to an alteration in chromatin structure; they did not, however, observe altered cytotoxic response to x-irradiation (26,27). This change in DNA conformation or chromatin structure seems to change the accessibility of the DNA to the subsequent action of DNA-damaging agents, increasing the accessibility for some agents and decreasing the accessibility for others. For example, polyamine-depleted 9L rat brain tumor cells subsequently exposed to BCNU show an increase in cytotoxicity and in DNA interstrand crosslinks, compared to untreated 9L cells (13). The same is true for a polyamine-depleted, human lymphoma cell line designated BHM, subsequently exposed to L-phenylalanine mustard (28). Even though polaymine depletion destabilizes chromatin structure and changes the accessibility, DNA accessibility subsequent action of DNA-damaging agents does not always increase. In fact with some agents, accessibility decreases. This is true of polyamine-depleted 9L cells exposed to cis-Platinum. They exhibit a decrease in cytotoxicity and in DNA interstrand crosslinks compared to normal 9L cells. Likewise, a polyamine-depleted human carcinoma exposed to Adriamycin also exhibits a decrease in cytotoxicity compared to controls (29).

On the other hand, Snyder and his colleagues observe a decreased rate of DNA repair as indicated by alterations in nucleoid sedimentation (12). That UVC-induced cytotoxicity (a DNA-repair dependent effect) is not modulated by polyamine depletion seems to suggest that DNA-repair *per se* is not the dominant mechanism.

In summary, we have demonstrated very different patterns of cytotoxic modulation for polyamine-depleted cells treated with agents that induce different types of DNA damage. We hypothesize that the ability of polyamine depletion to modulate cytotoxic effects of many DNA-damaging agents depends upon the topology of DNA and its orientation within the chromatin at the time of exposure. DNA repair, *per se*, cannot be the sole determinant of polyamine depleted modulation of survival. The characterization of the relationship between induced structural changes in chromatin and modification of cytotoxic response to anticancer agents may not only provide insight into the mechanism of the cytotoxic action of some anti-tumor agents, but may also provide a rationale for improved therapy by combinations of cytotoxic agents.

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