The Aminothiol WR-1065 Protects T Lymphocytes from Ionizing Radiation-induced Deletions of the HPRT Gene

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Abstract
Aminothiols, such as WR-2721 and its active free thiol, WR-1065, reduce mutations from ionizing radiation in exponentially growing cells. In this study, human noncycling G0 T lymphocytes were exposed in vitro to γ-irradiation in the presence or absence of WR-1065. The five treatment groups were: (a) control; (b) treatment with 4 mM WR-1065; (c) treatment with 3 Gy of γ-irradiation from a 137Cs source; and (d) and (e) treatment with WR-1065 30 min prior to or 3 h after 3 Gy of γ-irradiation, respectively. A total of 224 cloned HPRT mutants representing 179 independent mutations were analyzed for genetic alterations using multiplex PCR. Ionizing radiation alone significantly increased the percentage of mutations with gross structural alterations compared to controls (P = 0.02). Although the frequency of such large structural mutations was not different from control cells treated with WR-1065 alone, this aminothiol significantly reduced their frequency among irradiated mutants (P = 0.01) when the radioprotector was present during the irradiation. Addition of WR-1065 3 h postirradiation also greatly reduced the percentage of gross structural alterations; however, due to small numbers, this was not statistically significant. This is the first demonstration that the antimutagenicity of WR-1065 in human cells specifically protects against these kinds of large-scale DNA alterations induced by ionizing radiation. WR-1065 and similar aminothiol compounds may afford protection against radiation-induced mutations through polyamine-like processes, e.g., stabilization of chromatin structure, inhibition of cell proliferation, and influences on DNA repair systems.

Introduction
Ionizing radiation, specifically γ-radiation, is an inducer of gross structural alterations in the HPRT (hypoxanthine-guanine phosphoribosyl transferase) gene and other mutation assay systems (1-6). Furthermore, ionizing radiations dramatically increase the percentage of human in vivo HPRT T-cell mutations with gross structural alterations of the gene, as demonstrated on Southern blots. Gross structural alterations are defined as total and partial deletions of the HPRT gene, as well as translocations and molecular weight changes indicating intronic deletions/insertions. Such changes represent 15% of the adult background, with the remaining 85% being base changes or small insertions and deletions that escape detection due to the limited sensitivity of Southern blot analysis (7-11). By contrast, Bradley et al. (9) found that 33% of the T-cell HPRT mutations in patients who had received diagnostic radionuclides showed Southern blot-defined gross structural alterations compared to 1% in pretreatment patients. Similarly, elevated percentages of T-cell mutations with the large-scale DNA changes were observed on Southern blots in radioimmunotherapy patients who had received 131I and/or 90Y-labeled antiferritin antibodies (38.3%) compared to preradioimmunotherapy patients (16.9%) or to normal controls (14.2%; Refs. 12-14). Also, using Southern blot techniques, O'Neill et al. (15) found similar results following in vitro γ-irradiation of human T lymphocytes, in which the percentage of gross structural alterations among the HPRT mutations increased to 75% after 3 Gy of irradiation. Therefore, the mechanisms by which ionizing radiations induce mutations result in large scale DNA changes.

N-(2-Mercaptoethyl)-1,3-diaminopropane (WR-1065) is the in vitro active species of the well-characterized radioprotector WR-2721, which requires in vivo metabolic activation by alkaline phosphatase. There is considerable interest in WR-2721 and WR-1065, because these agents appear to preferentially protect normal rather than neoplastic tissues against the deleterious effects of alkylating agents or ionizing radiations (16, 17). These and other thiol-containing compounds have been shown to exhibit antitumor transforming (18), anticlastogenic (19, 20), anticarcinogenic (21), and antimutagenic (22-24) properties in mammalian cells.

In murine studies, WR-2721 increases the life span and decreases the incidence of lymphocytic lymphomas in animals receiving neutron or γ-irradiations (25, 26). Decreases in Hprt mutant frequencies determined in splenic lymphocytes were also observed (27). Increases in cell survival in vitro in Chinese hamster cell lines have been observed only if the aminothiol, WR-1065, is administered prior to irradiation, but Hprt mutant frequencies decrease regardless of the time of administration (30 min prior to irradiation, 5 min postirradiation, or 3 h postirradiation) of the aminothiol (22, 28). Although the radioprotective effects of these agents have been shown to be antimutagenic and anticlastogenic, few data are available on the molecular nature of these protected mutations.
Because the mutagenicity of γ-irradiation is associated with increases in gross structural alterations, we examined the radioprotective effects of WR-1065 on T-cell mutations in vitro. Human Gp T lymphocytes were incubated with WR-1065 30 min before or 3 h after 3 Gy of γ-irradiation from a 137Cs source. We have reported elsewhere protection by WR-1065 against the in vitro T-cell HPRT mutagenicity of γ-irradiation, as evidenced by markedly reduced frequencies of radiation-induced mutation (29). The resulting mutants from these studies have now been analyzed at the molecular level using multiplex PCR to define the nature of the HPRT changes and to determine the kinds of mutations eliminated from the γ-irradiation mutation spectrum by this radioprotection.

Materials and Methods

Cellular Cloning of HPRT Mutants. In vitro mutagenicity experiments were performed as described previously (30, 31). HPRT mutants were isolated from selection plates inoculated with cells that had been allowed an 8-day expression interval following their irradiation with 3 Gy from a 137Cs source, treatment with WR-1065, or treatment with WR-1065 30 min before or 3 h after 3 Gy of γ-irradiation. Mutant and wild-type HPRT isolates were determined only from experiments that demonstrated a minimum of a 6-fold or greater increase in mutant frequency with irradiation as compared to controls. Isolates were expanded to at least 10⁶ cells using nonselection media (RPMI 1640, 20% HL-1 medium + 5% calf bovine serum (CBS), an interleukin 2 source (supernatant of mononuclear cells cultured with interleukin 2 during lymphotoxine-activated killer therapy), and irradiated HPRT lymphoblastoid feeder cells (a gift of H. Liber and C. Dentaul, Harvard University, Cambridge, MA), as described elsewhere (30). Fifty thousand cells [for multiplex PCR analysis of the HPRT gene or γ-TCR (T-cell receptor) gene analysis] or 5000 cells (for β-TCR gene analysis) were washed in PBS and microcentrifuged for 2 min at 6500 rpm. Supernatants were removed, and the microfuge tube containing the cell pellet was quick frozen in liquid nitrogen and stored at −70°C.

Multiplex PCR Analysis. Dry cell pellets were resuspended in 50 μl of lysis buffer containing 0.45% NP40, 0.45% Tween 20, 100 μg/ml protease K, and T5E1 (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)). Resuspended cell pellets were incubated at 50°C for 1 h, with a final incubation at 96°C for 10 min to inactivate the protease K. Cell lysates were then stored at −20°C until analysis by multiplex PCR. Five μl of cell lysate were added to a 45 μl multiplex HPRT PCR reaction, as described by Gibbs et al. (32). After 33 cycles, one-half of the reaction was analyzed on a 1.4% agarose gel stained with 0.5 μg/ml ethidium bromide. The presence of individual HPRT exons and changes in exon fragment size was visualized under a UV transilluminator. Individual isolates that represented total deletions, partial deletions, or molecular weight changes were obtained from 11 experiments (involving γ-irradiation and WR-1065 treatments) using blood samples from eight different individuals. Multiplex PCR was used to determine the percentage of mutants with gross structural alterations (defined as total deletions, partial deletions, or molecular weight changes of the HPRT gene, as shown by exon bandshifts).

Independence of the mutations was determined by PCR of TCR gene rearrangements. The percentages of gross structural alterations among the HPRT mutations for the different treatment groups are shown in Table 1. After correction for clonality by TCR analysis, it was found that 179 (80%) of the isolated mutants represented independent mutations. In the control mutants, 16.7% demonstrated gross structural alterations in the HPRT gene. The percentage of gross structural alterations among the HPRT mutations in the control group is therefore 14.6% after correction for clonality. The percentage of mutants with large-scale changes was similar for those exposed to WR-1065 alone, e.g., 18.8% of mutants and 18.2% of mutations.

In the cell populations receiving 3 Gy of γ-irradiation and selected after an 8-day expression interval, 38.5% of the mutants and 35.3% of the mutations showed large-scale DNA changes. This elevation was statistically significant (P = 0.02) when compared to the two control populations (control and WR-1065 alone-treated cells). In contrast to this, the presence of WR-1065 during irradiation or added 3 h later dramatically reduced the percentages of radiation-associated gross HPRT alterations. Cells receiving 3 Gy of γ-irradiation in the presence of WR-1065 showed 13.6% of the mutants and 11.9% of the mutations with gross structural alterations of HPRT. Both percentages were significantly reduced from what was observed when irradiated cells were not protected by WR-1065 (P = 0.01). Furthermore, the percentage of radiation-derived mutant and mutations with gross structural alterations when the WR-1065 was not added to the cell population until 3 h after their receiving the 3 Gy of γ-irradiation (8.7% and 12.5%, respec-

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Table I  HPRT multiplex PCR and clonality analyses for HPRT mutant clones from cells treated with and without WR-1065 and 3 Gy of γ radiation in vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of wild-types isolated</th>
<th>No. of mutants isolated</th>
<th>No. of mutations isolated</th>
<th>HPRT multiplex PCR resultsa</th>
<th>TCR pattern resultsb</th>
<th>Frequency of mutants with structural alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>14</td>
<td>60</td>
<td>48</td>
<td>50 no change</td>
<td>1-8-mer, 2-2-mer</td>
<td>10 of 60 mutants (16.7%)</td>
</tr>
<tr>
<td>WR-1065 alone</td>
<td>18</td>
<td>32</td>
<td>22</td>
<td>26 no change</td>
<td>1-3-mer, 1-7-mer</td>
<td>6 of 32 mutants (18.8%)</td>
</tr>
<tr>
<td>3 Gy of radiation alone</td>
<td>3</td>
<td>65</td>
<td>51</td>
<td>40 no change</td>
<td>1-5-mer, 3-2-mer</td>
<td>4 of 22 mutations (18.2%)</td>
</tr>
<tr>
<td>WR-1065 + 3 Gy’</td>
<td>5</td>
<td>44</td>
<td>42</td>
<td>38 no change</td>
<td>1-2-mer</td>
<td>25 of 65 mutants (38.5%)</td>
</tr>
<tr>
<td>3 Gy + WR-1065’</td>
<td>2</td>
<td>23</td>
<td>16</td>
<td>21 no change</td>
<td>1-8-mer</td>
<td>18 of 51 mutations (35.3%)</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>224</td>
<td>179</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a del., deletion.
b mer, identical TCR isolates that have the same HPRT mutation.
c WR-1065 3 hours after 3 Gy of gamma irradiation.
d WR-1065 30 minutes prior to and during 3 Gy of gamma irradiation.

tively) was reduced. However, because of small numbers, this reduction did not achieve statistical significance. There were no statistically significant differences in the percentages of gross structural alterations when control cells, cells receiving WR-1065 alone, or cells receiving 3 Gy of γ-irradiation with WR-1065 added either before or after the irradiation were compared.

The percentages of total deletions, partial deletions, and point mutations were examined to determine to what extent each type of lesion was protected by the WR-1065 (summarized in Table 2). When the treatment of cells in the presence or absence of WR-1065 was examined, total deletions represented 9.09% of the mutations without WR-1065 treatment as compared to 1.25% of the mutations in WR-1065 treatments (P = 0.055). Subdividing treatments by WR-1065 and/or irradiation revealed that 11.8% of the mutations from irradiation treatments were total deletions. In contrast, no total deletions were detected in the combination aminothiol/irradiation treatment.

Discussion

We have reported previously that the γ-radiation-induced increase in HPRT mutations in human G0 T lymphocytes (7–10-fold above control) is reduced to background levels when the aminothiol WR-1065 is present before or even 3 h after irradiation (29). The current study was conducted to determine what types of mutational events are protected by this aminothiol.

Table 2  Analysis of HPRT alterations among HPRT mutations observed in cells from mutants treated or not treated with WR-1065

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total deletionsa</th>
<th>Partial deletions</th>
<th>Point mutationsb</th>
</tr>
</thead>
<tbody>
<tr>
<td>No WR-1065/no irradiation</td>
<td>3 of 48 (6.25%)</td>
<td>4 of 48 (8.33%)</td>
<td>41 of 48 (85.4%)</td>
</tr>
<tr>
<td>WR-1065/no irradiation</td>
<td>1 of 22 (4.55%)</td>
<td>3 of 22 (13.6%)</td>
<td>18 of 22 (81.8%)</td>
</tr>
<tr>
<td>No WR-1065/irradiation</td>
<td>6 of 51 (11.8%)</td>
<td>12 of 51 (23.5%)</td>
<td>33 of 51 (64.7%)</td>
</tr>
<tr>
<td>WR-1065/irradiation</td>
<td>0 of 58 (0.00%)</td>
<td>7 of 58 (12.1%)</td>
<td>51 of 58 (87.9%)</td>
</tr>
<tr>
<td>WR-1065 + 3 Gy’</td>
<td>8 of 99 (9.09%)</td>
<td>16 of 99 (16.2%)</td>
<td>74 of 99 (74.7%)</td>
</tr>
<tr>
<td>WR-1065</td>
<td>1 of 80 (1.25%)</td>
<td>10 of 80 (12.5%)</td>
<td>69 of 80 (86.3%)</td>
</tr>
</tbody>
</table>

a Statistically significant (P = 0.055).
b Mutations that showed no gross structural alterations when analyzed by multiplex PCR were considered point mutations that include single-base changes and small insertions and deletions.

c No irradiation and irradiation-exposed groups from part A combined.
WR-1065 Protects against Radiation-induced Deletions

As has been shown in several studies, an increase in gross structural alterations (deletions, translocations, and inversions) is characteristic of \(\gamma\)-radiation-induced mutations. In the present study, cell populations exposed to 3 Gy of \(\gamma\)-radiation showed that 35.3% mutations had gross structural alterations, as determined by multiplex PCR. In general, multiplex PCR identifies deletions of one or more exons, but bandshifts representing small insertions or deletions can also be detected. Translocations or inversions are only rarely detected (as exon band loss) because of the requirement that the break occur in the small region amplified by an exon primer set (usually breaks occur in the much larger nonamplified intronic regions).

We believe that the signature for ionizing radiation is a high percentage of gross alterations (mutations other than small deletions and point mutations). Although the frequency of gross structural alterations reported here is lower than that reported by O’Neill et al. (Ref. 15; 75% gross structural alterations were detected after \(\gamma\)-irradiations); we used multiplex PCR, whereas the O’Neill study used Southern blots. Southern blots detect deletions over 200–500 bases (but not small deletions), and most translocations and inversions (nearly all if restriction enzymes generating large fragments are used). In the O’Neill et al. (15) study, many structural alterations were detected through Southern blot analysis with both BamHI and HindIII restriction digests, which examines nearly the entire gene. The high percentage of gross rearrangements detected in the O’Neill study indicates that many radiation-induced mutations involve translocations or inversions; this was supported by initial karyotype analysis.\(^3\)

Multiplex PCR alterations, in particular total deletions, are reduced greatly from the postirradiation mutation spectrum when WR-1065 is added before or after irradiation. Therefore, a reduction in mutant frequency observed in our earlier study is due to protection from this class of DNA alteration. This reduction in mutant frequency occurred with no decrease in survival as compared to irradiation alone, indicating that WR-1065 is not eliminating cells containing premutagenic lesions. Our results in regard to total deletions are different from those of Denault and Liber (36), who studied lymphoblastoid cells irradiated in the presence of cysteamine, an endogenous amionothiol similar to WR-1065. They showed a preferential protection against lesions that lead to smaller deletions or rearrangements. Denault and Liber studied a cycling B lymphoblast cell line, whereas we studied noncycling human peripheral T lymphocytes, and it is known that certain stages of the cell cycle are more sensitive to \(\gamma\)-irradiation (37).

The protection against radiation- and chemical-induced mutagenesis afforded by aminothiol compounds has been attributed to free radical scavenging, direct chemical repair via hydrogen donation, and auto-oxidation (38). Although these mechanisms may account for some reduction of \(\gamma\)-radiation-induced HPRT alterations observed when WR-1065 is present during irradiation by reducing the initial damage, these mechanisms cannot account for the reduction of mutations in cells exposed to WR-1065 3 h after irradiation.

In regard to the effects of WR-1065 after irradiation, it is thought that aminothiols act similarly to polyamines. Polymamines can bind to and stabilize DNA, affect DNA replication, and modify DNA repair processes (39). WR-1065 and its disulfide form WR-33278, like polyamines, have been found to bind to DNA (40) and associate with nucleoproteins (41).

WR-33278 has also been reported to effectively compete with polyamines for uptake via a well-characterized polyamine transport system into the lungs of rats (42).

In our previous studies, WR-1065 was shown to transiently delay \(G_2\) lymphocytes after exposure to \(\gamma\)-radiation and mitogen stimulation in the \(G_2\) phase of the cell cycle prior to the first S phase. This result is consistent with those of Murley et al. (43), who found that WR-1065 transiently delayed progression of the cell cycle in late S phase and \(G_2\) phase. As noted, a delay in cell cycle progression could permit a longer time for more accurate repair of DNA damage.

Furthermore, WR-1065 has been shown to directly affect some enzymes involved in DNA function. The disulfide form of WR-1065 increases topoisomerase I activity (44), whereas WR-1065 decreased topoisomerase IIa activity (45). WR-1065 might also affect levels of DNA repair enzymes by a similar mechanism.

In summary, the aminothiol WR-1065 has demonstrated protection against \(\gamma\)-radiation-induced DNA alterations at the HPRT gene in human \(G_2\) T lymphocytes. We have demonstrated that antimutagenicity is detected using this biomarker gene, and we suggest that humans can be monitored for antimutagenicity in vivo using the HPRT T-cell system. The hope is that such studies will demonstrate the efficacy of using aminothiols with traditional, cancer radiation therapies, so that the untoward late effects of treatment-induced secondary cancers can be eliminated or reduced.

Acknowledgments

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