Radioadaptation to the Mutagenic Effect of Ionizing Radiation in Human Lymphoblasts: Molecular Analysis of HPRT Mutants

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Abstract

A 70% reduction of HPRT mutant frequency in radioadapted human lymphoblastoid cells has been reported, as analyzed by the Southern blot method (O. Rigaud et al., Radiat. Res., 133: 94–101, 1993). The data reported here extend the previous molecular analysis to a collection of 118 mutants. Structural rearrangements of the HPRT gene were determined using the multiplex polymerase chain reaction assay. This allows us to define more precisely the deleted exons in mutants and to ensure the absence of small alterations in exons among mutants with no detectable change after Southern analysis. The phenotype of these latter mutants is likely to be due to point mutations. Overall results of both Southern and multiplex polymerase chain reaction analyses confirm that the proportion of deletion-type mutations is decreased in adapted cells (42%) compared to that in mutants treated with the high dose alone (77%). Mutants with no change at the HPRT gene level were further characterized with respect to their HPRT gene expression. The vast majority of adapted mutants (86%) were still expressing mRNA, whereas HPRT transcripts were detected in only 56% of the mutants induced by the high dose alone. From these data and those reported by others, possible mechanisms underlying the adaptive response are proposed.

Introduction

The biological consequences of IR treatments depend upon both the level of damage produced and the ability of cells to deal correctly with this damage. These cellular responses to IR are commonly studied using relatively high doses of radiation since, in most biological systems, significant effects can be reproducibly observed only following treatment with high doses. In reference to the “radiation paradigm” recently presented by Sagan (1), it is stated that low-dose effects can be predicted from those observed after high-dose exposure. As a consequence, it is supposed that all dose levels produce the same harmful effects which are more or less severe depending on the dose. However, the situation is more complex. Indeed, in radiation protection, the existence of a “negligible dose” is evoked because of the background level of spontaneous damage with their subsequent biological effects. This concept of “negligible dose” remains controversial (2).

Moreover, evidence is accumulating concerning low-dose effects, i.e., unpredictable from the high-dose experiments. For instance, effects either at unexpectedly high levels or, on the contrary, at unpredictable low levels have been reported for the low-dose range treatments (3). Among the numerous experimental data, those dealing with radioadaptation are of particular relevance in this context. Olivieri et al. (4) first demonstrated in 1984 that human lymphocytes exposed in vitro to low-level radiation become more resistant to the clastogenic effect of a subsequent high dose. This so-called AR of cells to IR has been confirmed by others (5–8).

The first experimental protocol dealing with AR used continuous exposure to tritiated thymidine as the inducing dose and a high dose of X-rays (1.5 Gy) given in the G2 phase; the amount of chromatid aberrations was reduced compared to that in lymphocytes not exposed to low-level radiation from tritium. This reduction was not due to a delay in the progression of damaged cells or to variations in cell cycle sensitivity (4). The possibility of selective killing by tritiated thymidine against a more radiosensitive cell population was ruled out by using coculture of male and tritiated thymidine-labeled female cells; moreover, since only labeled cells exhibited less chromosomal damage, it was concluded that the cytogenetic adaptation did not involve diffusible factors (9).

In further experiments, a low dose of X-rays replaced the endogenous radiation from tritiated thymidine, since such treatment was restricted to S phase cells. This permitted the demonstration that AR can be induced in either G1, S, or G2 phases of the cell cycle and that it persists for three cell cycles. The protective effect can be reinduced by a low dose reexposure after three cell cycles (10). The possibility of induction of the AR in noncycling cells is still controversial (6, 7, 10). The cytogenetic adaptation reaches a maximum within the 5- to 6-h interval between low- and high-dose exposures (10).

The inducing dose must be delivered at an adequate dose rate depending on the total dose. In other words, the inducing signal for the AR must be a certain number of lesions produced in a certain time (11). This implies that there is a threshold level of radiation dose or dose rate above which the AR can be induced.

The cytogenetic adaptive response is also effective in reducing chromosome-type aberrations, sister chromatid exchanges, and micronuclei (7, 8, 12).

Interindividual variability of the response has been observed among normal donors (5, 13 and references therein). Attempts to determine the reasons for such a variability (cellular metabolic state, individual G2 sensitivity) did not lead to definitive conclusions (14). When the cytokinesis block method was used, which allows the collection of not only G2 cells but also cells exposed at any phase of the cell cycle, the AR induced by a low concentration of H2O2 was constantly evident only for the longest recovery period, indicating that AR variability could be due to the lapse time needed for its appearance (8).

AR is not restricted to human lymphocytes but can occur in human fibroblasts (15); other mammalian cells, such as lymphocytes, bone marrow cells and germ cells from rodents, and plant cells can be adapted after in vitro exposure to the combined treatment with small and high doses (6, 12, 16).

In vivo irradiation with chronic low doses of X-rays was able to induce an AR in rabbit lymphocytes exposed to a subsequent large dose in vitro given in G2 phase cells as well in G0 phase cells. When both inducing and challenging doses were delivered in vivo, the degree of protection of bone marrow cells was related to the low-dose level, i.e., the higher the inducing dose, the weaker the AR (6).

The AR is observed not only with IR but also with bleomycin, H2O2, and mitomycin C. Any of these agents used for the conditioning low-dose treatment in combination with another agent for the challenging high dose leads to protection against the clastogenic effect (17). These results indicate that these different agents lead to common signals which trigger AR.

In conditions leading to clastogenic adaptation, a preexposure of

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2The abbreviations used are: IR, ionizing radiation; AR, adaptive response; PCR, polymerase chain reaction; cDNA, complementary DNA; HPRT, hypoxanthine phosphoribosyltransferase.

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human lymphocytes improved survival of cells challenged with 2 Gy but not always with 4 Gy (18). The increase of cells devoided of chromosomal aberrations cannot solely explain the higher cell survival. In other words, the cellular mechanism induced by the low dose acts on lethal damage that cannot be cytogenetically visible (18). Adaptation in terms of cell survival coupled with adaptation to either the clastogenic or the mutagenic effects was observed in some studies (15, 19) but not in others (20, 21).

The radiation-induced mutant frequency measured at the HPRT locus using 6-thioguanine selection was shown to be decreased in mammalian cells exposed to treatment with small, followed by large, doses. This mutagenic adaptation could be induced after preexposure to either tritiated thymidine (20), low doses of X-rays (22), γ-rays (21), or xanthine-xanthine oxidase (19) but not after protracted exposure to tritiated water (5 cGy/day for 10, 20, or 30 days) (23). Expression of the mutagenic AR occurred within the same interval of time between small and large dose exposures as that described for cytogenetic AR, i.e., 6–24 h.

We recently reported (21) a decreased IR-induced HPRT mutant frequency following adaptive treatment, compared to that observed in cells treated with an acute dose. Southern analysis of mutants indicated that, under adaptive conditions, point mutations or small alterations were predominantly induced in contrast to the high proportion of deletion-type mutations produced by the large dose alone. In the present study, we have attempted to overcome some of the ambiguities left by the Southern blot hybridization methodology by applying the multiplex PCR assay to analyze mutants at the gene level. Data presented here of the molecular analysis of a larger collection of mutants derived from adapted and nonadapted cells confirm our previous results. Moreover, we have extended the molecular characterization of the HPRT mutants by screening those mutants with no structural rearrangements for gene expression. Again, our results indicate differences according to the two treatment conditions; loss of HPRT gene expression was more frequently observed in mutants produced by the high dose alone than in the low plus high dose-induced mutants.

Materials and Methods

Cell Culture, Irradiation Conditions, and Mutant Isolation. Exponentially growing normal human lymphoblastoid cells, AHH-1, were irradiated using a 137Cs source either with a high dose of 4 Gy or with a low dose of 0.02 Gy, followed by a 6-h incubation before the high-dose challenge of 4 Gy. Corresponding controls were cells left unirradiated or exposed to only the low dose (for details, see Ref. 21). In order to ensure the independent origin of mutants, each culture of treated cells was split into several flasks immediately after treatment and cultured separately for 1 week. Cells were seeded into microplates in selective medium containing 6-thioguanine (6 μg/ml). Mutants were isolated after 14—18 days of culture. Large cultures of each mutant were grown for total DNA extraction.

Structural Analysis of Genomic DNA. A detailed description of the genomic DNA preparation, restriction enzyme (PstI and EcoRI) digestion, and Southern blot hybridization method has been reported (21). Multiplex PCR assay was carried out according to the method of Gibbs et al. (24). The amplification of the nine exons was obtained using eight pairs of primers (exons 7 and 8 were amplified as a single fragment). The reaction mixture (final volume, 50 μl) containing mutant DNA (250 ng), the appropriate buffer, and the 16 oligonucleotides at the concentrations as indicated by Fuscoe et al. (25) were overlaid with mineral oil and heated to 80°C for 4 min. Taq DNA polymerase (4 units; Boehringer Mannheim, Germany) was added, and the reactions were heated to 94°C for 4 min. After 30 cycles of denaturation (94°C, 1 min), annealing (59°C, 1 min), and extension (68°C, 2 min), one-fifth of the reaction was loaded on 1.4% agarose-0.5X Tris-borate gel (25 mM Tris, 25 mM boric acid, 25 mM EDTA).

HPRT Gene Expression. Mutants with no detectable alteration of the HPRT gene using the Southern blot hybridization method and/or multiplex PCR assay were tested for their mRNA expression. For this purpose, PCR amplification of cDNA was carried out according to Yang et al. (26) with some modifications (27) in order to detect HPRT transcripts. First-strand cDNA was synthesized directly from cell pellets of 2000—4000 cells by adding 10 μl of a cocktail containing 10 μg/ml of primer and 20 units of reverse transcriptase (Superscript; Gibco BRL). The reaction was incubated at 37°C for 45 min. Five μl of the reaction were then mixed with 0.5 units of Taq polymerase and nested primers in an appropriate buffer in order to synthesize and amplify the double-stranded DNA. After a first round of 30 cycles, 2% of the amplified cDNA served as a template for a second round of PCR (25 cycles). The final product was concentrated and loaded on a 1% agarose-1× Tris-acetate-EDTA gel (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA).

Results

Structural Rearrangements of the HPRT Gene. The molecular analysis by Southern blot hybridization of an HPRT mutant collection including 41 spontaneously arising or 0.02 Gy-induced and 53 γ-rays-induced mutants (27 mutants for the high dose alone and 26 mutants for the adaptive treatment) was recently reported (21). Twenty-four new mutants derived from adapted or nonadapted cells have been tested for structural rearrangements at the HPRT gene level using multiplex PCR assay.

The multiplex PCR assay is a more rapid technique than the Southern blot hybridization method. Moreover, it allows one to detect loss of individual exons (each individual exon is amplified as a single band except for exons 7 and 8) or small deletions in exons. Indeed, in order to specify the deleted exons, the multiplex PCR assay was applied to the mutants which were missing band(s) corresponding to exons 5 and 6 and/or 7—9 associated with a novel band, as previously detected by filter hybridization. For these mutants, exon 5 or exon 6 or exons 7 and 8 failed to be amplified, indicating that the deletion encompassed only exon 5 or 6 and 7, or 8. For 3 other mutants which were missing exons (5 and 6 and/or 7—9) not combined with an additional band, the multiplex PCR confirmed the deletions detected using the Southern blot hybridization method. Furthermore, a wild-type multiplex amplification pattern was observed for the radiation-induced mutants without detectable change in restriction fragment pattern. Nevertheless, it should be kept in mind that structural rearrangements in intron sequences cannot be detected by the multiplex PCR assay.

Results of the analysis by the multiplex PCR assay of the 24 additional mutants are listed in Table 1. Among the 12 mutants induced with the high dose alone, no signal was detected for 5 mutants, indicating a total gene deletion. Four others were missing one or several exons. The 3 other mutants exhibited a wild-type multiplex pattern. A larger proportion of this type of mutant was observed after the combined treatment with low plus high dose (7 of 12). The 5 other mutants were either partially (3 mutants) or totally deleted (2 mutants).

Quantitative distribution of spontaneous mutants and radiation-induced mutants are presented in Table 2. The previously reported

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mutants</th>
<th>Exon(s) deleted</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Gy</td>
<td>3</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>2—9</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>7—8</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>0.02 Gy + 4 Gy</td>
<td>7</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>1—9</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7—8</td>
<td></td>
</tr>
</tbody>
</table>
Table 2 Quantitative distribution of different classes of spontaneous and radiation-induced mutants according to the structural alterations of the HPRT gene

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mutants analyzed</th>
<th>Unrearranged mutants</th>
<th>Total deletion</th>
<th>Partial deletion</th>
<th>Rearranged mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>17 (71)</td>
<td>1 (4)</td>
<td>6 (25)</td>
<td>7 (29)</td>
</tr>
<tr>
<td>0.02 Gy</td>
<td>12</td>
<td>10 (59)</td>
<td>2 (12)</td>
<td>5 (29)</td>
<td>5 (41)</td>
</tr>
<tr>
<td>4 Gy</td>
<td>11</td>
<td>9 (39)</td>
<td>3 (26)</td>
<td>3 (26)</td>
<td>5 (41)</td>
</tr>
<tr>
<td>0.02 Gy plus 4 Gy</td>
<td>9</td>
<td>7 (35)</td>
<td>3 (29)</td>
<td>2 (22)</td>
<td>5 (37)</td>
</tr>
</tbody>
</table>

* Number (%).
* Significantly decreased (0.01 < P < 0.025).

Table 3 HPRT gene expression in radiation-induced mutants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mutants analyzed</th>
<th>Normal size</th>
<th>Abnormal size</th>
<th>&gt;1 transcript</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02 Gy</td>
<td>7</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>9 (100%)</td>
</tr>
<tr>
<td>4 Gy</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>5 (56%)</td>
</tr>
<tr>
<td>0.02 Gy plus 4 Gy</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>4 Gy</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>19 (86%)</td>
</tr>
</tbody>
</table>

* Mutants with no structural alteration of the HPRT gene.

Discussion

Screening of a larger HPRT− mutant collection for structural rearrangements at the HPRT gene level confirms our previous results about the nature of IR-induced mutants following preexposure and nonexposure to a low dose (21). Molecular analysis of additional mutants by multiplex PCR assay showed a molecular distribution similar to that observed for mutants analyzed by the Southern blot hybridization method. Furthermore, the multiplex PCR assay allows one to detect more precisely the deleted HPRT exons. The absence of small alterations of exons in mutants with a wild-type restriction enzyme pattern was confirmed by this assay. The phenotype of these latter mutants is likely to be due to point mutations.

From combined data previously reported and those presented here, it can be concluded that the proportion of radiation-induced mutations of the deletion type is decreased when cells are preexposed to a low dose. The cellular mechanisms triggered by the low dose thus prevented preferentially a certain class of premutagenic lesions, i.e., those leading to deletions. As previously discussed (21), these lesions are likely to be the double-strand breaks. This implies that adapted cells can deal efficiently with this type of damage.

Further characterization of those mutants with no detectable alteration at the HPRT gene level indicated that mRNA expression was more frequently observed in mutants induced under adaptive treatment (19 of 22) compared to mutants produced by the large dose alone (5 of 9). It has been reported that Fanconi anemia cells are characterized by a high frequency of spontaneous or induced deletion mutations and, concomitantly, a large proportion of mutants with no alterations at the HPRT gene level which did not express mRNA (27). It has been suggested that deletion in the promot region of the gene may explain the high frequency of mutants not expressing mRNA (28). This hypothesis could explain the absence of mRNA expression in the 4 Gy-induced mutants, since an acute radiation is very efficient in producing deletions.

Aberant splicing of mRNA as detected by the abnormal size of the HPRT cDNA PCR products was shown to occur in 9 of 19 adapted mutants of the 0.02 plus 4 Gy-induced mutants (Table 3) but in none of the 4 Gy-induced mutants. We are currently carrying out experiments to determine the causative mutations of these aberrant splicings. Our results are in agreement with several reports showing that a large proportion of mutations at the HPRT gene results in aberrant splicings (29 and references therein). Mutations in splice donor or acceptor sites as well as in exons resulting in the splicing out of exons have been described (29).

The mechanism(s) underlying radioadaptation in mammalian cells is still unknown, and different hypotheses can be considered. Some data suggest that the low dose could induce a repair mechanism responsible for the reduction of the chromosomal aberrations. The
cytogenetic adaptation of human lymphocytes was prevented by 3-aminobenzamide, an inhibitor of poly(ADP)ribosyltransferase thought to be involved in the repair of strand breaks (9). Similar observations were reported for human lymphocytes adapted with bleomycin (30) and for radioadapted rodent cells (12). Abolition of the protective effect induced by a low dose of IR was also observed if the protein synthesis inhibitor, cycloheximide, was added within the 4- to 6-h interval after the tickling dose (31). Several new proteins have been detected in cells exposed to 0.01 Gy. Their identification is being determined (31), and their role is still unknown.

An enhanced repair of radiation- or bleomycin-induced double-strand breaks in mouse SR-1 cells preexposed to a low dose of γ-rays or bleomycin was shown to be associated with a resistance to the mutagenetic effect of the second high dose (32). An enhanced excision repair process in α sequences following pretreatment with a first dose of γ-rays in CV1 monkey cells has also been reported (33).

Radioadaptation could be due to another mechanism involving an increased cell ability to remove toxic radicals. This would minimize the indirect damaging effects of IR, i.e., the reaction of free radicals produced by water radiolysis with DNA and the membrane. This could occur via the activation of cellular defense enzymes such as catalase, superoxide dismutase, and peroxidases. As an example of such induction, cells pretreated with xanthine-xanthine oxidase became less susceptible to the mutagenetic and killing effects of a second dose of radiation, and this was associated with a 2-fold increase of superoxide dismutase activity but an unchanged catalase activity (19). Experiments reported by Feinendegen et al. (34) are also of interest in this context. It was shown that radical detoxification is triggered in mice after whole body irradiation with very low doses of γ-rays. A temporary decrease in thymidine kinase (used as an indicator of radical scavenging) reaching a minimum at 4 h postirradiation was observed with a concomitant increase of free glutathione. These results were interpreted as a temporary reduction of DNA synthesis. Indeed, cell growth arrest may allow cells to repair in response to a dose as low as 0.01 Gy (34).

After single-dose treatment with IR, cysteamine protected human lymphoblasts against deletion-type mutations at the HPRT gene (35). Similarly, radioadaptation led to the same effect (data presented here). This argues in favor of a possible role of an increase in scavenging of free radical under adaptive conditions.

It is now well established that, following exposure to IR and other DNA-damaging agents, the expression of a number of genes is modulated. These include genes involved in control of cell cycle, transcription factors, repair enzymes, etc. (36). Modest increases of transcripts encoding nuclear proteins following doses as low as 0.06 Gy have been reported (36). The low dose of IR used in adaptive treatment could trigger such a process, leading to regulation of the expression of genes involved in the genotoxic stress response. This merits further analysis.

It should be kept in mind that the mechanisms evoked here in order to explain the radioadaptive response can be related. Southern blot hybridization and the multiplex PCR methodologies allowed us to characterize the radioadaptive response at the gene level in terms of structural alterations and expression. Sequencing of unarranged mutants is now being carried out in order to establish the molecular spectrum of the mutations. The data generated by these approaches should shed light on the processes triggered by low doses of IR.

Acknowledgments

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