Molecular Markers of Ionizing Radiation-induced Gene Mutations in Mammalian Cells

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We have isolated independent Chinese hamster ovary (CHO) cell mutants at the hypoxanthine guanine phosphoribosyltransferase (hprt) locus from untreated, 60Co γ-ray-exposed, and 220Rn α-exposed cells and identified the molecular changes underlying the mutation determined by multiplex polymerase chain reaction (PCR)-based exon deletion analysis. Both the parental CHO-K1 cells and the X-ray-sensitive mutant xrs-5 cells were studied. The radiosensitive xrs-5 cells are defective in DNA double-strand break rejoining ability and in VDJ recombination, which can be complemented by Ku protein. Of the 71 spontaneous CHO-K1 hprt mutants analyzed, 78% showed no change in exon number or size, 20% showed loss of one to eight exons (partial deletion), and 2% showed loss of all nine hprt exons (total deletion). Exposure of CHO-K1 cells to 6 Gy of γ rays, which reduced survival levels to 10%, produced a high deletion spectrum with 45% of the 20 mutants analyzed showing a loss of one to eight exons and 30% showing total deletion. Exposure to an equitoxic dose of α radiation from 220Rn, a 220Rn daughter, resulted in a spectrum similar to the γ-ray spectrum in that 75% of the 49 mutants analyzed were deletions. The α radiation, however, tended to produce larger intragenic deletions than γ radiation. Of the 92 spontaneous xrs-5 mutants analyzed for deletions, 43% showed a loss of one to eight exons and 14% showed total deletion. This suggests that, in certain regions of the hprt gene, base alterations can be converted into large deletions and alteration in the Ku protein complex can influence this type of mutational process. Exposure to α radiation (10% survival) to xrs-5 cells resulted in a deletion spectrum similar to that seen in CHO-K1 cells. Of the 49 mutants analyzed, 43% showed no change in exon number or size, 16% showed a loss of one to eight exons, and 41% showed total deletion. While the defect in xrs-5 cells has a profound effect on spontaneous mutant spectra, this defect does not appear to affect α-induced mutation spectra. — Environ Health Perspect 104(Suppl 3):675–678 (1996)

Key words: radiation mutagenesis, molecular mutagenesis, gene mutation, γ radiation, α radiation, DNA double-strand breaks

Introduction

The genetic effects of ionizing radiation have been extensively studied in a wide spectrum of biological systems (1,2). Over the past decade, significant progress has been made in our understanding of the molecular basis of ionizing radiation-induced specific-locus mutations (3-8). We have recently undertaken a study to characterize the mutation spectra and identify potential molecular markers of ionizing radiation-induced mutations at the hypoxanthine guanine phosphoribosyltransferase (hprt) locus in Chinese hamster ovary (CHO) cells as a function of radiation dose, radiation dose rate, radiation quality (linear energy transfer [LET]) and DNA repair background of CHO cells.

We report here the deletion spectra of all nine exons of the hprt locus in CHO-K1 cells (9) and a CHO-K1 cell radiosensitive derivative xrs-5 cell (10) exposed separately to 60Co γ rays and to 220Rn α particles using multiplex polymerase chain reaction (PCR)-based exon deletion analysis (11–13). Cells were exposed to acute doses of γ rays or α particles that reduced survival to approximately 10% and increased the frequency of hprt mutants approximately 10-fold over the background.

The greater effectiveness of high-LET radiations (such as α particles) in producing genetic changes is thought to be due to the different spatial distribution of damage produced by various radiation types (14). One might therefore expect different spectra of mutations from high-LET radiations compared to low-LET radiations (such as γ rays). Our results suggest that α radiation produces larger intragenic deletions than that of γ radiation.

Methods

Isolation of Independent Spontaneous hprt Mutants

Both CHO-K1 cells (9,15) and xrs-5 cells (10) were maintained in Ham’s F12 medium containing 5% dialyzed heat-inactivated fetal calf serum (F12FCM5) in a 5% CO2–95% air incubator at 37°C with 100% humidity.

The spontaneous mutant frequency of the hprt gene in CHO cells ranges from 5 to 20 x 10–6 mutants per cell (9,13,15). Independent spontaneous hprt-deficient mutants were isolated according to our established protocol (11,16). Briefly, cells were treated with HAT medium (medium

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Abbreviations: CHO cells, Chinese hamster ovary cells; F12FCM5, F12 medium containing 5% dialyzed heat-inactivated fetal calf serum; HAT medium, medium containing hypoxanthine, aminopterin, and thymidine; hprt gene, hypoxanthine guanine phosphoribosyltransferase gene; LET, linear energy transfer; PCR, polymerase chain reaction; xrs cells, X-ray sensitive cells.
F12FCM5 supplemented with 10^{-4} M hypoxanthine, 10^{-6} M aminopterin, and 10^{-5} M thymidine for 5 days to eliminate preexisting mutants.

Replicate cultures of 1,000 cells/dish were inoculated into 60 mm dishes and allowed to grow to near-confluence in medium F12FCM5. These cultures were then subcultured every 2 days in medium F12FCM5 in 100 mm dishes for 2 to 3 weeks for the expression of the mutant phenotype. hprr-deficient mutants, resistant to 6-thioguanine, were isolated using our standard protocol (9,11,16). To ensure the independent origin of all mutants, only one mutant was randomly isolated from each of the original replicate cultures.

**Radiation Mutagenesis**

Both CHO-K1 cells and xrs-5 cells were pretreated with HAT medium for 5 days followed by recovery in complete medium for 2 days as described above. Cells (5×10^{5}) were kept in suspension for all 212Bi- and γ-ray exposures. The γ-ray source was a γ Beam 650 Irradiator (Atomic Energy of Canada, Ottawa, Ontario). Single-cell suspensions were exposed to 6 Gy 60Co γ rays at a dose rate of 250 cGy/min at room temperature. This dose produces a 10% survival level (16).

The α source used in these studies was 212Bi, a 220Rn daughter that we elute from a 224Th generator (17). Cells were exposed to 212Bi chelated to diethylene triamine pentaacetic acid (212Bi-DTPA) to prevent attachment of 212Bi to the cells and to keep the activity extracellular. Radioactivity was assayed using a calibrated 3×3-in NaI well detector and dose was calculated as described (16,18,19). Exposure time was 4 hr. The dose rate was 0.5 cGy/hr. Exposures were terminated by washing the cells three times with medium.

**Analysis of Cell Survival and Gene Mutations and Isolation of Independent Mutants**

We used a protocol for determination of cellular lethality and mutant frequency at the hprr locus in CHO cells (the CHO/HPRT assay) (9,15) and for the isolation of independent hprr mutants (11,16) developed in our laboratories. To determine radiation-induced cell survival, 200 to 20,000 cells were plated in twenty 100-mm Petri plates immediately after exposure. After 7 to 10 days of incubation, the plates were stained with crystal violet and scored for the presence of surviving colonies. The remaining cells were seeded into 150-cm² flasks and maintained as exponentially growing populations for 8 to 10 days, the previously determined optimum phenotypic expression time for the hprr locus. Mutant frequency was determined by scoring for resistance to 6-thioguanine. Cells were plated in twenty 100-mm plates (10^5 cells/plate) containing 10 μM 6-thioguanine in 10 ml of complete medium. Two hundred cells were also plated at the same time in nonselective medium to determine cloning efficiency. After incubation for 10 to 12 days, single, independent 6-thioguanine-resistant colonies were removed from each plate for expansion and analysis. The remaining colonies were stained in crystal violet and scored to determine mutant frequencies. For each independent experiment, a single 6-thioguanine-resistant clone was expanded, tested for aminopterin sensitivity, and then frozen for molecular analysis. The independent nature of the mutants was assured by isolating only one mutant clone from each independent and separate experiment.

**Multiple PCR-based Deletion Screening**

Multiplex PCR-based exon deletion screening was performed as we have previously described (11–13). Briefly, genomic DNA was isolated from each mutant. All nine hprr exons were simultaneously amplified from 0.1 to 0.5 μg of genomic DNA using eight pairs of oligonucleotide primers that flank eight fragments containing the hprr exons. Exons 7 and 8 were amplified in one fragment. PCR was performed in a Perkin-Elmer DNA Thermal Cycler 480 (Perkin-Elmer, Foster City, CA) for 30 cycles (94°C, 1 min; 55°C, 1 min; 68°C, 1 min). PCR fragments were resolved on a 3% NuSieve 3:1 agarose gel stained with ethidium bromide. 

**Results**

**Analyses of Spontaneous Mutation Spectrum at the hprr Locus in CHO-K1 Cells and xrs-5 Cells**

Mutations develop following radiation exposure from the nonhomologous recombination between two DNA double-strand breaks. Mutant frequency and mutation spectrum should therefore be influenced by the number of breaks per cell, the distribution of breaks within a cell, and the cell's ability to repair.

To study a role of DNA repair in mammalian molecular mutagenesis, we have investigated radiation-induced spectra of mutations at the hprr locus in DNA repair-proficient CHO-K1 cells (9,15) and in a DNA repair-deficient CHO-K1 cell radiosensitive derivative, xrs-5 cells (10). The radiosensitive xrs-5 cell is defective in V(D)J recombination and DNA double-strand break joining (10,20,21). V(D)J recombination is a process that rearranges germ line DNA to assemble exons encoding immunoglobulin and T-cell receptor protein (10,20,21). The defective repair and recombination processes have been shown to be due to an alteration that affects the 80-kDa subunit of the Ku protein (Ku80) (22–24). Ku protein was initially identified in sclerodema-polyangitis syndrome, Ku being derived from the first patient's name (22–24). This protein is necessary for the processing of DNA double-strand breaks induced by genotoxic agents or by endogenous recombination processes. The Ku protein may act by preventing nucleosome digestion of double-strand breaks or by promoting recombination. To determine the role of recombination and DNA double-strand break repair in the production of spontaneous mutations, we analyzed the molecular nature of 92 spontaneous hprr mutants in xrs-5 cells (JL Schwartz, RC Porter, and AW Hsie, unpublished data) and compared this spectrum to that of 64 spontaneous hprr mutants isolated from CHO-K1 cells (25)(Table 1).

In the 64 spontaneous CHO-K1 hprr mutants analyzed, 78% showed no apparent change in exon number or size, 20% showed a change in number of exons, and 2% showed a change in size.

**Table 1. Comparison of spontaneous hprr mutation spectra in CHO-K1 and xrs-5 cells.**

| Type of mutation | CHO-K1 | xrs-5 |
|------------------|--------|-------|
| Base substitutions | 34 (63%) | 11 (12%) |
| Transversions | 12 (19%) | 2 (2%) |
| Transversions | 22 (34%) | 9 (10%) |
| Frameshifts | 1 (2%) | 5 (5%) |
| Deletions | 15 (23%) | 54 (59%) |
| Duplication | 2 (3%) | 0 (0%) |
| Insertions | 3 (5%) | 0 (0%) |
| Splice mutations | 15 (23%) | 3 (3%) |
| Others | 5 (8%) | 19 (21%) |
| CDNA not amplified | 0 (0%) | 15 (16%) |
| Mutations not defined | 5 (8%) | 4 (5%) |
| Total number of mutants | 64 | 92 |

In CHO-K1 cells, total percent exceeds 100 and the total mutant number is less than the sum of specific mutants because some base changes causing aberrant RNA splicing are included in base substitutions. Data from Xu et al. (25) and from unpublished data of JL Schwartz, RC Porter, and AW Hsie.
showed loss of one to eight exons (partial deletion), and 3% showed loss of all nine hprt exons (total deletion) (25).

Of the 92 spontaneous xrs-5 hprt mutants analyzed, more than 50% of the spontaneous xrs-5 mutants had lost one or more exons compared to less than 25% of spontaneous CHO-K1 mutants losing one or more exons. Most of the deletions in xrs-5 cells involved the loss of multiple exons, while single exon deletions predominated in CHO-K1 cells. There was also a nonrandom distribution of breakpoints in both CHO-K1 cells and xrs-5 cells. Most of the deletion breakpoints were 3' to exon 9, around exons 4 to 6, or near exon 1. Although the frequency of base substitutions was lower in xrs-5 cells, the spectrum of base substitutions was qualitatively similar to that of CHO-K1 cells. There was no significant difference in the spontaneous mutation frequency in xrs-5 cells and CHO-K1 cells. The results suggest that, in certain regions of the hprt gene, base alterations can be converted to large deletions and alterations in the Ku protein complex can influence this type of mutational process (JL Schwartz, RC Porter, and AW Hsie, unpublished data).

Deletion Analyses of γ-Ray- and α-Particle-induced hprt Mutants in CHO-K1 Cells and xrs-5 Cells

Previously, we reported on the radiation sensitivity of CHO-K1 cells and mutation induction in this cell line following exposure to X rays and α particles (16). In the present study, we have chosen doses of γ rays (6 Gy) and α particles (2 Gy) that yield approximately 10% survival level; the corresponding hprt mutant frequencies are 147 ± 15 and 138 ± 38 mutants/10⁶ clonable cells, respectively. The mutant frequency in the unirradiated cells was 15 ± 8 mutants/10⁶ clonable cells. Mutants were isolated only from experiments in which survival and mutation levels were observed.

Exposure of CHO-K1 cells to 6 Gy of 60Co γ rays resulted in 75% of the 20 mutants analyzed showing deletions (Table 2). Nine (60%) of the 15 deletion-containing mutants analyzed lost a single exon; the remaining 6 (40%) lost all nine exons (26). By χ² analysis, this pattern of genetic changes was shown to be significantly different from that seen in spontaneous mutants (p < 0.001). It is similar to the spectra published by other investigators (7,8) for X-ray-induced hprt mutations in that these authors also reported deletion frequencies of 60 to 70%. However, for these two studies, total gene deletion frequencies were higher than reported here. Approximately 45% of X-ray-induced hprt mutants were total gene deletions. This difference may well reflect the higher LET of X rays compared to 60Co γ rays.

The majority (72%) of the 49 α-exposed hprt mutants of CHO-K1 cells were also deletions (Table 2). Twenty-six (53%) were total gene deletions in which all nine exons were missing, five (10%) lost a single exon, and seven (14%) lost between two and eight exons. Among the deletion mutants where some but not all of the exons were deleted (partial gene deletions), deletions of exons 1 to 3 and 6 to 9 were found (Table 2); none of these partial gene deletion mutants involved exons 4 and 5. The α-particle deletion spectrum was significantly different from the spontaneous deletion spectrum (p < 0.001) and from the 60Co γ-ray deletion spectrum (p < 0.025).

While most of the spontaneous deletion mutants in xrs-5 cells involve the loss of multiple exons (most of which involve 3' to exon 9), single exon loss predominated in CHO-K1 cells. The difference of α-particle-induced hprt mutants between xrs-5 cells and CHO-K1 cells is not as pronounced as the difference of spontaneous mutation spectrum between these two cell types. There was a slight shift to larger sized deletions in α-particle-induced hprt mutants in xrs-5 cells as in CHO-K1 cells (Table 3) (26).

Discussion

High-LET radiations exhibit greater general relative biological effectiveness than that of low-LET radiations, as might be expected. This has been attributed to the track structure of high-LET radiations, which yield a proportion of high-energy deposition events. These events are thought to give rise to damage that might be qualitatively different from that produced by low-LET radiations. One important form of damage produced by both low- and high-LET radiations is DNA strand breaks. High-LET radiations produce more unrepaired strand breaks than low-LET radiations (2,4,27).

The different energy deposition pattern of high-LET radiations compared to low-LET radiations has led some to suggest that the spectrum of deletion sizes in cells exposed to high-LET radiations might be shifted towards larger sized deletions as compared to the spectrum seen in cells exposed to low-LET radiations. Most studies on the hprt locus in hamster cells, however, suggest that the deletion pattern for low-LET X-ray-induced hprt mutations is similar to that seen for high-LET α-radiation-induced mutations (3,6-8).

Studies on the hprt locus in the human lymphoblastoid cell line TK6 (28,29) and CHO cells (30) have similarly shown that α radiations produce a spectrum indistinguishable from X rays. When mutants are grouped by those showing no change in exon pattern, those showing a loss of one to eight exons (partial gene deletions), and those showing total loss (all nine hprt exons deleted), our results (Tables 2, 3) differ somewhat from similar studies reported by other investigators (28-30). We find no significant difference between low- and high-LET spectra; however, when one considers whether the partial intragenic deletions involve one or multiple exons, then the spectra are significantly different from each other. Alpha radiation produces more partial intragenic deletions.

### Table 2. Analyses of deletion mutations induced by γ rays and by α particles in CHO-K1 cells.

| Treatment | Mutant frequency (x 10⁶) | No. of mutants | Exons deleted |
|-----------|-------------------------|----------------|---------------|
|           |                         | None | Single | Multiple | All |
| None      | 15                      | 71   | 55 (78%) | 11 (16%) | 3 (4%) | 2 (3%) |
| 6 Gy γ    | 154                     | 20   | 5 (25%) | 9 (45%) | 0 (0%) | 6 (30%) |
| 2 Gy α    | 188                     | 49   | 11 (22%) | 5 (10%) | 7 (14%) | 26 (53%) |

Data from Schwartz et al. (26).

### Table 3. Analyses of deletion mutations induced by α particles in xrs-5 cells.

| Treatment | Mutant frequency (x 10⁶) | No. of mutants | Exons deleted |
|-----------|-------------------------|----------------|---------------|
|           |                         | None | Single | Multiple | All |
| None      | 20                      | 92   | 38 (44%) | 20 (23%) | 17 (20%) | 12 (14%) |
| 1 Gy α    | 98                      | 49   | 21 (43%) | 3 (6%) | 5 (10%) | 20 (41%) |

Data from Schwartz et al. (26).
showing multiple exon loss than that of γ radiation (26). Our unique observation is likely due to the sensitivity of our PCR-based multiplex exon deletion assay procedure (11–13) and lack of attention by other groups to analyze intragenic deletion by taking deletion size into consideration (28–30). We suggest the size of intragenic deletions should be considered when comparing spectra from cells exposed to radiations with different LETs.

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