Analysis of spontaneous, gamma ray- and ethyltritosourea-induced hprt mutants in HL-60 cells with multiplex PCR

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MATERIALS AND METHODS

Cell culture

HL-60 is a human acute promyelocytic leukemia cell line described earlier by Collins et al. HL-60 cells were maintained as an asynchronous, exponentially growing population in RPMI 1640 medium (Sigma, St. Louis, USA) supplemented with 10 % fetal bovine serum (SJQ, Hangzhou, China), 100 U/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma), and 2 mM L-glutamine (Gibco, Carlsbad, USA) at 37 °C in an atmosphere of 5 % CO₂. Preexisting hprt mutants that cannot live in ultraviolet (UV) light, ethyl methane sulfonate (EMS), ICR-191 and N-ethyl-N-nitrosourea (ENU) were identified (9-11). Thus, while conventional missense mutants induce predominantly point mutations, ionizing radiation induces both point and deletion mutations.

As a part of our ongoing effort to analyze the nature and spectrum of mutations induced by various types of physical and chemical mutagens, we adopted the multiplex PCR technique for the initial screening of deletion mutants. In this paper, we have characterized the molecular nature of mutations induced by γ-ray and ENU at the hprt locus of human promyelocytic leukemia cells.

CONCLUSION: The spectra of spontaneous mutations differs completely from that induced by EUN or 6Co γ-rays. Although both EUN and γ-ray can cause destruction of genetic structure, mechanism of mutagenesis between them may be different.
one single cell was inoculated in 200 μl medium per well. After incubating for 7 days, colonies per well were counted and the plating efficiency (PE) was calculated with equation:

\[ \text{PE} = \frac{-\ln(\text{Number of negative well/Number of all wells})}{\text{Number of cells per well}} \]

**Mutation experiments**

After expression of gene mutations (8 days) HL-60 cells were added in the 96-well microtiter plates to ensure one cell was inoculated per well. After incubating for 7 days, wells with colony formation were counted as positive wells for cloning efficiency (CE). Meanwhile, cells were added in other 96 microwell plates to ensure that each well received 1x10^5 cells in 200 μl medium containing 1 μg/ml 6-thioguanine (6-TG; Sigma). After incubating for 8 days, positive wells were counted and mutant frequency (MF) was calculated. Three plates were used for CE and MF in each treatment.

\[ \text{MF} = \frac{-\ln(\text{Number of negative wells/Number of all wells})}{\text{Number of cells per well} \times \text{CEF}} \]

**Screening, extension and DNA isolation**

A single positive clone was transferred from the 96-well plate to a 24-microwell plate (Gibco) with 1 ml screening medium containing 2 μg/ml 6-TG in each well and cultured for additional 1-2 days. Then, 10^3 cells was transferred to each well in a new 24-microwell plate in HAT culture medium and cultured for 1-3 days. If the cells in a well were obviously dead, the cloned cells of the well were identified as mutated clones and the remaining cloned cells in the 24-microwell were transferred into culture bottles for extension expression. DNA isolation and purification from wild-type cells and hprt-mutated cells was performed with conventional method.

**Design, synthesis and appraisal of primers**

Eight pairs of oligonucleotide primers were designed by computer software with a minor modification of the literature. The synthesis and appraisal of the 8 pairs of primers were completed by different laboratories of Beckman Company in Beijing, Cybersyn B. J. in American and the Institute of Cellular Biology of Chinese Academy of Science in Shanghai.

Sequence of 8 pairs of oligonucleotide primers was showed in Table 1. Exons 7 and 8 were amplified simultaneously with same primers, because they are only 163 bp apart. All primers except the exon 1 specific ones enabled amplification of the corresponding exons in the multiplex PCR. It was, however, difficult to include exon 1 primers within the remaining set of all primers without having a spurious synthesis of non-specific signal. In our pre-experiments with several primer pairs in one PCR reaction it was difficult to control and optimize the reaction conditions. In addition, insertions and deletions within exons could occur, therefore we restricted the number of primer pairs in a single PCR reaction in order to confirm the distances among of PCR products based on molecular weights. So, false-negative or false-positive rate was reduced. Following some preliminary experiments, 8 pairs of primers of exons were divided into 3 groups, group one was multiplex PCR including exons 2, 5, 6 and 7/8, group two included exons 3, 4, and 9; in group three, exon 1 was amplified separately. Multiplex PCR method was used to analyze 119 mutants.

**PCR analysis**

For amplification of hprt exons, 0.5-2.0 μl of genomic DNA (36-50 ng) was mixed with 50 pmol of each primer in 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH8.8), 0.3-1.05 mM MgCl₂, 0.2 mM dNTPs and 2.5 U of AmpliTaq DNA polymerase (Shenggong, Shanghai, China). After initial denaturation of the template DNA at 98 °C for 7 min, a total of 40 PCR cycles were performed with denaturation at 94 °C for 1.5 min, annealing at 52 °C for 1.5 min and extension at 72 °C for 2.0 min. Exon 1 was synthesized individually with a modified condition: a total of 30 PCR cycles were performed with denaturation at 95 °C for 0.5 min, annealing at 64 °C for 1.0 min and extension at 72 °C for 1 min. The last cycle was extension at 72 °C for 7 min. The PCR product (10 μl) was used for analysis by 3 % agarose gel or by using polyacrylamide gel electrophoresis (Figure 1).

**Table 1 Oligonucleotide primers in multiplex PCR of the human HPRT locus**

| Exons | Primers sequence (5’ - 3’) | Fragment size (bp) |
|-------|---------------------------|-------------------|
| 1     | F TGG GAC GTG TGG TCC AAG GAT TCA | 626 |
|       | R CCG AAC CCG GGA AAC TGG CCC | |
| 2     | F CCT GAT ATG CTC TCA TGG AAA CA | 211 |
|       | R GCT GCT GAT GTT TGA AAT TAA CAC | |
| 3     | F GTT TAA TGA CTA AGA GGT GTT TG | 311 |
|       | R GAA AAC CTA GTG TGG CCA CAT AA | |
| 4     | F GTG TGT GTA CAT AAG GAT ATA CA | 165 |
|       | R TTC TCT TTC TCC TTC AAG ATA CAT AC | |
| 5     | F GGA AAT ACC GGG TTA TTC ATT GT | 125 |
|       | R GTG CAT ACT AAG TTA GAA AGG TC | |
| 6     | F GTG ACT CTC GAT TTA AAG CTA TG | 150 |
|       | R CTG TGT CAA AAT GTC ATA CAT AC | |
| 7/8   | F GTC TCT CTG TAT GTC ATA TTA GTC AC | 379 |
|       | R TGC GTG TTT TGA AAA ATG AGT GAG | |
| 9     | F GTT ACT GCT GTC TCA TCA TCA | 136 |
|       | R CAA ACT CAA CTT GAA CTC TCA | |

**Figure 1 Detection of deletion mutation in human hprt gene by multiplex PCR.** (A)PCR products from normal cells; (B)PCR products from one hprt mutant. (1) PUC Mix Marker; (2)Exons 2,5,6,7/ 8; (3)Exons 3,4,9; (4)Exon 1.
Statistics
All data were analyzed by student's t test and total rate test. The statistical difference P<0.05 was considered as significant and P<0.01 as very significant.

RESULTS
Cytotoxicity and mutagenicity of γ-rays and ENU
The HL-60 cells plating efficiency (PE) gradually decreased with increasing concentrations of ENU. There was a significant difference from the control of PE at concentrations above 100.0 µg/ml of ENU. A linear increase of the mutation frequency (MF) with increasing concentration of ENU was found. At 12.5-200.0 µg/ml ENU, MF was 3.5-20.8 times higher than that in the control cultures. The cytotoxicity and mutagenicity of γ-rays were similar to ones of ENU. The HL-60 cells PE gradually decreased with increasing doses of γ-rays. There was a significant difference of PE at doses above 2.0 Gy of γ-rays in comparison with the control. A linear increase of the MF with increasing doses of γ-rays was found. At 1.0-4.0 Gy γ-rays, MF was 3.6-14.2 times higher than that in the control cultures (Table 2).

Table 2 Cytotoxicity and mutagenicity of γ-rays and ENU in HL-60 cells

| Type          | Dose (µg/ml) | PE(%) | CE(%) | MF(×10^6) |
|---------------|--------------|-------|-------|-----------|
| Control       | 0.0          | 93.01 | 86.64 | 5.13      |
| ENU (µg/ml)   | 12.5         | 75.92 | 85.91 | 17.73*    |
|               | 25.0         | 70.67 | 78.62 | 31.18     |
|               | 50.0         | 61.45 | 82.67 | 64.45*    |
|               | 100.0        | 45.77 | 83.91 | 82.61*    |
|               | 200.0        | 25.63 | 67.02 | 106.70*   |
| γ-ray (Gy)    | 0.5          | 91.63 | 146.0 | 12.30     |
|               | 1.0          | 69.31 | 186.0 | 18.30*    |
|               | 2.0          | 55.34 | 108.2 | 23.20*    |
|               | 4.0          | 39.94 | 69.0  | 73.00*    |

*P<0.05 vs control group, **P<0.01 vs control group.

Multiplex PCR analysis
Analysis of multiplex PCR was done in 13 spontaneous mutants, 59 ENU-induced and 47 γ-rays-induced hprt mutants. Forty-two mutants (35.3 %) of 119 mutants analyzed were found to exhibit no abnormal band in any of the 9 exons, which indicated that these mutants had point mutation without exon deletion or insertion. In 47 of 119 mutants, less than 8 bands were existed for each locus, and showed partial deletions of exons. The remaining 30 mutants had no PCR products, which meant that all exons studied were deleted. Of all mutants analyzed, 64.7 % (77/119) had partial or complete deletions.

Molecular spectrum of HPRT gene
Spontaneously derived, γ-rays- and ENU-induced mutants at the hprt locus were showed in Table 3. The electrophoresis patterns of mutants mainly consisted of three types: “normal pattern” including point mutations, total deletion and partial deletion. γ-rays- (1.0-4.0 Gy) and ENU-induced (12.5-200.0 µg/ml) mutant cells showed mutation spectra that were significantly different from the spectra of spontaneous mutations. Total exon deletion did not existed in any spontaneous mutants but in γ-rays- and ENU-induced mutants.

Analysis of deletion breakpoints
Distribution of the deletions in the 9 exons of the hprt gene found in the 119 mutants analyzed (Table 4). Deletion mutations were found in all 9 exons of the hprt gene, but number of single exon deletion was very small. Most of γ-rays- (59.6 %) and ENU-induced (67.8 %) mutations were chain deletion with multiple exons.

Table 3 Summary of multiplex PCR analysis of THH-induced HPRT mutants in HL-60 cells

| Categories of mutation | Number analyzed | Number showing PCR changes | Percentage deleted | Number showing no change |
|------------------------|----------------|----------------------------|-------------------|--------------------------|
|                        |                | Complete deletion          | Partial deletion  |                          |
| Spontaneous            | 13             | 0                          | 1/ 13 (7.7%)      | 7.7%                     | 12/ 13 (92.3%)          |
| ENU (µg/ml)            |                |                            |                   |                          |
| 12.5                   | 8              | 1/ 8 (12.5%)               | 4/ 8 (50.0%)      | 62.5%                    | 3/ 8 (37.5%)           |
| 25.0                   | 10             | 2/ 10 (20.0%)              | 5/ 10 (50.0%)     | 70.0%                    | 3/ 10 (30.0%)         |
| 50.0                   | 10             | 2/ 10 (20.0%)              | 6/ 10 (60.0%)     | 80.0%                    | 2/ 10 (20.0%)         |
| 100.0                  | 12             | 4/ 12 (33.3%)              | 6/ 12 (50.0%)     | 83.3%                    | 2/ 12 (16.7%)         |
| 200.0                  | 19             | 7/ 19 (36.8%)              | 10/ 19 (52.6%)    | 89.4%                    | 2/ 19 (10.6%)         |
| γ-rays (Gy)            |                |                            |                   |                          |
| 0.5                    | 7              | 1/ 7 (14.3%)               | 1/ 7 (14.3%)      | 28.6%                    | 5/ 7 (71.4%)          |
| 1.0                    | 13             | 3/ 13 (23.1%)              | 5/ 13 (38.5%)     | 61.5%                    | 5/ 13 (38.5%)         |
| 2.0                    | 10             | 3/ 10 (30.0%)              | 3/ 10 (30.0%)     | 60.0%                    | 4/ 10 (40.0%)         |
| 4.0                    | 17             | 7/ 17 (41.2%)              | 6/ 17 (35.3%)     | 76.5%                    | 4/ 17 (23.5%)         |

aP<0.05 vs control group, bP<0.01 vs control group.
DISCUSSION

The X-linked HPRT gene is the most extensively examined mammalian locus for mutagenesis studies\(^ {13-15}\). The enzyme is part of the purine salvage pathway, catalyzing the reaction of 5-phosphoribosyl 1-pyrophosphate with either hypoxanthine or guanine to form precursors that are recycled for use in DNA synthesis. As exploited in mutation analysis, this pathway leads to the killing of wild-type cells exposed to the toxic base analogue 6-TG\(^ {16-18}\). Toxicity and mutagenesis of \(\gamma\)-ray and ENU were characterized in HPRT locus forward mutation test, and found that reverse relationship between them was existed. \(\gamma\)-ray and ENU could serve a dual purpose to clone efficiency of HL-60 cell, which have association with dosages.

DNA hybridization used to be a main method in mutation analysis of HPRT gene, and recently PCR has been used in studies of gene mutation, and improved the precision of mutation analysis\(^ {19-21}\). HPRT gene mutations were studied with multiplex PCR method. As it was difficult to control and optimize the reaction conditions in our preliminary study, insertions and deletions within exons could occur, the number of primer pairs in a single PCR reaction was restricted in order to confirm the distances among PCR products according to their molecular weights, consequently false-negative or false-positive rate were reduced. Therefore, eight pairs of primers were divided into 3 groups: one multiplex PCR included exons 2, 5, 6 and 7/8, second one included exons 3, 4 and 9, and in third one, exon 1 was amplified separately.

ENU is a direct-acting alkylating agent that produces similar ratios of well-characterized ethyl adducts in DNA in solution, in prokaryotes, in cultured mammalian cells, and in various tissues of rats and mice \(\text{in vitro}\)\(^ {22-24}\). Several O-ethyl-adducts, including O-ethylguanine, O-ethylthymine, and O-ethylthymine, have been shown to direct mispairing of bases during DNA replication \(\text{in vitro}\)\(^ {25,26}\), and the results of site-directed mutations are consistent with the types of base substitutions observed in assays of ENU mutation specificity\( ^ {27}\).

### Table 4

Schematic diagram of the distribution of deletion within nine exons of the human HPRT gene

| Types of exons deletion | Mutation clones |
|-------------------------|-----------------|
|                         | Number | Percent (%) |
| Spontaneous mutants     | 12     | 92.3       |
|                         | 1      | 7.7        |
| ENU-induced mutants     | 12     | 20.3       |
|                         | 3      | 5.1        |
|                         | 2      | 3.4        |
|                         | 4      | 6.8        |
|                         | 2      | 3.4        |
|                         | 1      | 1.7        |
|                         | 2      | 3.4        |
|                         | 2      | 3.4        |
|                         | 1      | 1.7        |
|                         | 3      | 5.1        |
|                         | 1      | 1.7        |
|                         | 16     | 27.1       |
|                         | 1      | 1.7        |
|                         | 1      | 1.7        |
|                         | 2      | 3.4        |
|                         | 2      | 3.4        |
|                         | 1      | 1.7        |
| r-rays-induced mutants  | 18     | 38.3       |
|                         | 1      | 2.1        |
|                         | 1      | 2.1        |
|                         | 2      | 4.3        |
|                         | 2      | 4.3        |
|                         | 1      | 2.1        |
|                         | 1      | 2.1        |
|                         | 3      | 6.4        |
|                         | 1      | 2.1        |
|                         | 1      | 2.1        |
|                         | 2      | 4.3        |
|                         | 14     | 29.8       |

\(^{a}\)deletion of a exon.
Furthermore, ENU was used as the model agent for developing the in vivo hprt mutation assays in mouse, rat, and monkey and for defining the age-dependent relationships between chemical exposure, DNA adduction, and phenotypic expression of hprt mutations in T cells of exposed mice.\(^{28,29}\) Ionizing radiation may exert a carcinogenic stimulus, even at low levels of exposure. Such biological effects have been extensively studied. Observed mutation frequencies of γ-ray-induced mutation in HL-60 cells were similar to the results reported previously for X-irradiated human fibroblasts.\(^{30-32}\) Our results support the general observation that the majority of ionizing-radiation-induced mutations at the hprt locus are large deletions, about 60% of mutants of γ-irradiated HL-60 cells exhibited large deletions (1.0-4.0 Gy). These results suggest that the size of genetic alteration appears to be dependent on doses. It is now thought that hprt gene mutations are not well distributed, so the controversy might be due to different methods rather than results. As deletion breakpoints are mapped and sequenced more precisely, it may be helpful in clarifying the mechanisms of induced deletion.

No obvious differences among the absolute numbers of mutants in all 9 exons of hprt gene were found, meaning that there were no clear “hot spots”. However, some reports showed the preferential localization of deletion breakpoints at or toward the 3’ end of the hprt gene.\(^{33,34}\) Our results suggested that 9 exons of hprt gene are not well distributed, so the controversy might be due to different methods rather than results. As deletion breakpoints are mapped and sequenced more precisely, it may be helpful in clarifying the mechanisms of induced deletion.

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