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• BASIC RESEARCH •

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

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Abstract

AIM: To explore the molecular spectra and mechanism of human hypoxanthine guanine phosphoribosyl transferase (hprt) gene mutation induced by ethyluitrosourea (ENU) and ^{60}Co γ-rays.

METHODS: Independent human promyelocytic leukemia cells (HL-60) mutants at the hprt locus were isolated from untreated, ethyluitrosourea (ENU) and ^{60}Co γ-ray-exposed cells, respectively, and verified by two-way screening. The genetic changes underlying the mutation were determined by multiplex polymerase chain reaction (PCR) amplification and electrophoresis technique.

RESULTS: With dosage increased, survival rate of plated cell reduced (in the group with dosage of ENU with 100-200 μg/ml, $P<0.01$; in the group with dosage of ^{60}Co γ-ray with 2-4 Gy, $P<0.05$) and mutational frequency increased (in the group of ENU 12.5-200.0 μg/ml, $P<0.05$; in the group of ^{60}Co γ-ray with 1-4 Gy, $P<0.05$) significantly. In the 13 spontaneous mutants analyzed, 92.3 % of mutant clones did not show any change in number or size of exon, a single exon was lost in 7.7 %, and no evidence indicated total gene deletion occurred in nine hprt exons. However, deletions were found in 79.7 % of ENU-induced mutations (62.5-89.4 %, $P<0.01$) and in 61.7 % of gamma-ray-induced mutations (28.6-76.5 %, $P<0.01$). There were deletion mutations in all 9 exons of hprt gene and the most of induced mutations were chain deletion with multiplex exons (97.9 % in gamma-ray-induced mutants, 88.1 % in ENU-induced mutants).

CONCLUSION: The spectra of spontaneous mutations differs completely from that induced by EUN or ^{60}Co γ-ray. Although both ENU and γ-ray can cause destruction of genetic structure, mechanism of mutagenesis between them may be different.

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INTRODUCTION

Many developments in molecular biology, especially, polymerase chain reaction (PCR) have made procedure of mutation analysis relatively simple[1,2]. Classically, Southern hybridization was the primary method for the molecular analysis of deletion mutations. However, southern analysis not only is time-consuming but also provides incomplete results due to the limited resolution of each exon and possible cross-hybridization with psendogenes. Therefore, using PCR to amplify each individual exon of the hprt gene provides a powerful alternative method to southern analysis[3]. PCR has been used for the analysis of various mutations in human and Chinese hamster cells. These and other studies of the hprt locus in various mammalian cells have shown a wide spectrum of structural aberrations in the hprt gene, which are induced by physical and chemical mutagens[4-7]. Ionizing radiation induces deletion mutations and results in genetic alterations, which can be detected by Southern analysis[8]. Such detectable genetic alterations have been found infrequently after exposure to ultraviolet (UV) light, ethyl methane sulfonate (EMS), ICR-191 and N-ethyl-N-nitrosourea (ENU)[9-11]. Thus, while conventional missense mutagens 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.

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 ℃ in an atmosphere of 5 % CO_2. Preexisting hprt mutants that cannot live in thymidine (Sigma; HAT culture medium) were removed by incubating cells in complete medium supplemented with 10^{-6} M aminopterin (Gibco), 10^{-4} M hypoxanthine (Sigma) and 10^{-5} M HAT culture medium for 24 hours, then the medium was replaced with complete medium containing 10^{-5} M thymidine and 10^{-4} M hypoxanthine and cultured for 48 hours. Following removal this medium, the cells were incubated in normal medium for 7-10 days.

Cytotoxicity

For measuring the cytoxicity of γ-ray and ENU (Tokyo, Japan), exponentially growing HL-60 cells were treated with different doses of γ-ray and ENU. Initial cell number inoculated was 5.0×10^6. Sterile distilled water was used as negative control. After incubation, the cells were harvested and washed twice with D-Hank's medium at 37 ℃, counted and diluted in normal culture medium and transferred to 96 microwell plates (Gibco),

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:

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

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 1×10^4 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.

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

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[12]. 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 $MgCl_2$, 0.2 mM dNTPs and 2.5 U of Amplitaq DNA polymerase (Shenggong, Shanghai, China). After initial denaturation of the template DNA at 98 ℃ for 7 min, a total of 40 PCR cycles were performed with denaturation at 94 ℃ for 1.5 min, annealing at 52 ℃ for 1.5 min and extension at 72 ℃ 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 ℃ for 0.5 min, annealing at 64 ℃ for 1.0 min and extension at 72 ℃ for 1 min. The last cycle was extension at 72 ℃ for 7 min. The PCR product (10 μl) was used for analysis by 3 % agarose gel or by using polyacylamide 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 GTC TGG TCC AAG GAT TCA	626
	R	CCG AAC CCG GGA AAC TGG CCG CCC	
2	F	CCT GAT ATG CTC TCA TTG 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 TTG CCA CAT AA	
4	F	GTG TGT GTA CAT AAG GAT ATA CA	165
	R	TTC TTC CCT TTC AAG ATA CAT AC	
5	F	GGA AAT ACC GTT TTA TTC ATT GT	125
	R	GTG CAT ACT AAG TTA GAA AGG TC	
6	F	GTG ACT CTG AAT TTA AAG CTA TG	150
	R	CTG TGT CAA AAT GTC ATA CAT AC	
7/8	F	GTC TCT CTG TAT GTT ATA TGT CAC	379
	R	TGC GTG TTT TGA AAA ATG AGT GAG	
9	F	GCT ATT CTT GCC TTT CAT TTC AG	136
	R	CAA ACT CAA CTT GAA CTC TCA TC	

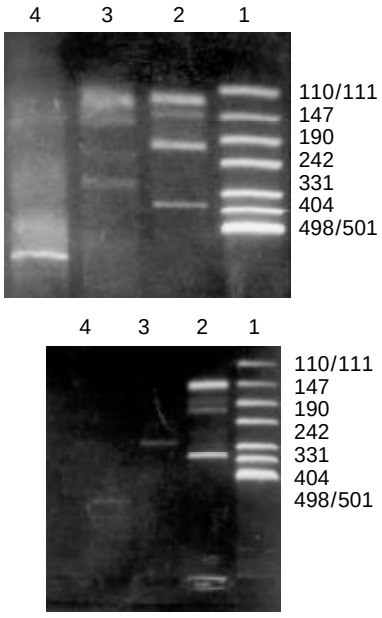

Figure 1 Detection of deletion mutation in human hprt gene by multiplex PCR. (A)PCR products from normal cells; (B)PCR products from one hprt mutant. (1) PUC Mix Marker; (2)Exons 2,5,6,7/8; (3)Exons 3,4,9; (4)Exon 1.

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	PE(%)	CE(%)	MF($\times10^{-6}$)
Control (μg / ml)	0.0	93.01	86.64	5.13
ENU (μg / ml)	12.5	75.92	85.91	17.73[a]
	25.0	70.67	78.62	31.18[b]
	50.0	61.45	82.67	64.45[b]
	100.0	45.77[b]	83.91	82.61[b]
	200.0	25.63[b]	67.02	106.70[b]
γ-ray (Gy)	0.5	91.63	146.0[b]	12.30
	1.0	69.31	186.0[b]	18.30[b]
	2.0	55.34[a]	108.2	23.20[a]
	4.0	39.94[b]	69.0	73.00[b]

[a]$P<0.05$ *vs* control group, [b]$P<0.01$ *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. The proportions of deletion mutations were quite different between spontaneous mutants and induced mutants, and similar change of γ-rays- and ENU-induced mutants occurred at the hprt locus. Over 60 % deletion mutations were found in these two kind of induced mutants while only 7.7 % deletions were found in spontaneous mutants. The proportion of the "normal pattern" in spontaneous mutants and induced mutants were 92.3 % and less than 40 %, respectively. The clearer dose-response relationship was seen in induction of partial- and whole-deletion mutation than in induction of mutants including and kind of mutations.

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%)[a]	62.5%[b]	3 / 8 (37.5%)[a]
25.0	10	2 / 10 (20.0%)	5 / 10 (50.0%)[a]	70.0%[b]	3 / 10 (30.0%)[b]
50.0	10	2 / 10 (20.0%)	6 / 10 (60.0%)[b]	80.0%[b]	2 / 10 (20.0%)[b]
100.0	12	4 / 12 (33.3%)[a]	6 / 12 (50.0%)[a]	83.3%[b]	2 / 12 (16.7%)[b]
200.0	19	7 / 19 (36.8%)[a]	10 / 19 (52.6%)[a]	89.4%[b]	2 / 19 (10.6%)[b]
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%[b]	5 / 13 (38.5%)[b]
2.0	10	3 / 10 (30.0%)[a]	3 / 10 (30.0%)	60.0%[b]	4 / 10 (40.0%)[b]
4.0	17	7 / 17 (41.2%)[b]	6 / 17 (35.3%)	76.5%[b]	4 / 17 (23.5%)[b]

[a]$P<0.05$ *vs* control group, [b]$P<0.01$ *vs* control group.

Table 4 Schematic diagram of the distribution of deletion within nine exons of the human HPRT gene

	Types of exons deletion								Mutation clones	
	1	2	3	4	5	6	7/8	9	Number	Percent (%)
Spontaneous mutants									12	92.3
					– [a]				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
				–	–	–	–		2	3.4
						–	–	–	1	1.7
		–	–				–	–	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.

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 γ-ray and ENU were characterized in HPRT locus forward mutation test, and found that reverse relationship between them was existed. γ-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 of 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 *in vivo*[22-24]. Several O-ethyl-adducts, including O^6-ethylguanine, O^4-ethylthymine, and O^2-ethylthymine, have been shown to direct mispairing of bases during DNA replication *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].

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 aduction, 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 unlikely results from block of transcription and the initial damage leads to irreversible DNA loss.

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[33-35]. 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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