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Evaluation of *in vitro* Genotoxic Potential of Kojic Acid in Human Lymphoblastoid Cells

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Although kojic acid is used as a cosmetic agent for skin whitening, information of its genotoxicity in *in vitro* assay is much complicated. In order to evaluate its genotoxic potentials *in vitro*, we conducted comet assay in regular and acellular versions, chromosome aberration assay, and *TK* mutation assay in human lymphoblastoid cells. Positive results were obtained in all of the comet, chromosome aberration, and *TK* mutation assays at almost identical concentration in both TK6 and WTK1 cells. In the acellular comet assay, kojic acid led to positive responses at pH12 and pH13, suggesting that it induced DNA single strand breaks (SSBs). In the *TK* mutation assay, kojic acid increased the fraction of normal growing but not slowly growing mutants, suggesting that observed gene mutations are due to point mutations within the *TK* locus but not gross structural changes that can form chromosome aberrations observable by a microscopy.

Key words: kojic acid, DNA damage, regular comet assay, acellular comet assay, chromosome aberration, *TK* mutation

Introduction

Kojic acid [5-hydroxy-2-(hydroxymethyl)-4-pyrone; CAS No. 501-30-4] is a secondary metabolic product from various species of *Aspergillus* and *Penicillium* (1, 2). Kojic acid inhibits polyphenol oxidase (tyrosinase) in mushrooms (3), potatoes, apples and crustaceans (4). Since the polyphenol oxidase catalyzes the conversion of tyrosine to melanin *via* 3,4-dihydroxyphenylalanine and dopaquinone (3, 5), kojic acid has been used as an inhibitor of polyphenol oxidase in foods to prevent enzymatic browning of raw crabs and shrimps. Although kojic acid is not used as a food additive now, it is used as a cosmetic agent to lighten the skin because of the excellent skin whitening properties (6, 7) and inhibitory actions on human melanocyte tyrosinase (8).

So far a number of genotoxicity assays *in vitro* has been performed on kojic acid, complicated results have been obtained. In the Ames test, while kojic acid was not mutagenic to *Salmonella typhimurium* TA1537 and TA1538 strains, it led to positive results in TA100, TA1535, and TA98 strains at $\geqq 2000\,\mu g$/plate in the presence of rat S9 mix fraction and at $\geqq 1000\,\mu g$/plate in the absence of rat S9 mix fraction (9-11). It showed positive and negative responses in the *E. coli* rec-assay (12) and *E. coli* SOS spot test (13), respectively, for DNA damage examination. Nohynek *et al*. (14) demonstrated that kojic acid was mutagenic in *S. typhimurium* TA 98, TA 100, TA 1535, TA102 and *E. coli* WP2*uvrA*, but not in TA 1537 by the standard Ames test. Ishikawa *et al*. (15) demonstrated that three different lots of samples of kojic acid were mutagenic in *S. typhimurium* TA100 and their mutagenicities were derived from kojic acid itself, but not from any contaminants. In mammalian cell systems, kojic acid induced chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary CHO cells with and without S9 mix (10). Kojic acid also induced chromosome aberrations in Chinese hamster lung CHL/IU cells at $1000\,\mu g$/mL in the absence of S9 mix (16). However, the clastogenicity in CHL/IU cells was not reproducible at the concentrations up to $5000\,\mu g$/mL (17). The induction of chromosome aberration in V79 cells was shown to be marginal because of cytotoxicity after prolonged (18 h) exposure (14). Furthermore, it did not induce *Hprt* gene mutation in V79 cells (9,14) and mouse lymphoma cells (14).

In this study, we queried the fate of the DNA damages induced by kojic acid in *in vitro* systems. To examine whether the kojic acid induced-DNA damages can be fixed as chromosome aberrations and/or gene mutations, we compared the induction of DNA damage (detected by the comet assay in regular and acellular versions), chromosome aberration, and *TK* gene mutation

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in human lymphoblastoid cells, TK6$^{TK+/-}$, having wild type *p53* and WTK1$^{TK+/-}$, having a mutant *p53*, after treatment with kojic acid. To detect whether the DNA damages induced by kojic acid include DNA strand breaks, not only comet assay in regular version but also comet assay in acellular version was conducted. Since WTK1 cells are more resistant to clastogenic effects than TK6 cells (18), both WTK1 and TK6 cells were used to know whether the clastogenic effect of kojic acid play an important role in its genotoxicity and cytotoxicity.

Materials and Methods

Chemicals: Kojic acid kindly provided by Alps Pharmachemical Industry Co., Ltd. (Gifu, Japan) was dissolved in DMSO (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Either 4-nitroquinoline 1-oxide (4NQO; Wako Pure Chemical Industries) dissolved in DMSO or bleomycin hydrochloride (BLM; Wako Pure Chemical Industries) dissolved in saline was used as a positive control chemical.

Cell: The $TK^{+/-}$ heterozygote of the TK6 and WTK1 human lymphoblastoid cell lines (kindly provided by Dr. Honma, National Institute of Health Sciences) were used (TK6 and WTK cells have wild type *p53* and a mutant *p53*, respectively). Cells were maintained in culture using RPMI1640 medium (Nissui Pharmaceutical Co., Ktd.) supplemented with 10% horse serum (SAFC Biosciences), 200 μg/mL sodium pyruvate, and 200 μg/mL streptomycin at 37°C under a 5% CO_2 atmosphere. Cells were maintained in logarithmic growth. For treatment, cells were centrifuged and re-suspended at a concentration of 5×10^5 cells/mL in culture medium. Cells were placed in 10 cm dishes at a concentration of 5×10^5 cells/mL in 20 mL of culture medium, containing kojic acid, and the cell culture dishes were incubated for 4 or 20 h. After the incubation, the cell cultures were centrifuged, washed twice with the fresh medium and re-suspended in the fresh medium. The cell cultures were used for the comet assay, chromosome aberration assay, and *TK*gene mutation assay.

Comet assay: We performed regular and acellular comet assays. The regular comet assay was conducted immediately after the 4- and 20-h treatment with kojic acid. Cell viability for each dose was determined by Trypan blue exclusion method at slide preparation. Cells were embedded in LGT agarose (Nakalai Tesque, Kyoto, Japan) dissolved in saline at 1% following kojic acid treatment. Then, slides were placed in a chilled lysing solution (2.5 M NaCl, 100 mM Na_4EDTA, 10 mM Tris-HCl, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, pH 10) and kept at 0°C in the dark for $\geqq$60 min, then the slides were placed on a horizontal gel electrophoresis platform, and covered with pH > 13 alkaline solution made up of 300 mM NaOH and 1 mM Na_2EDTA for 20 min in the dark at 0°C. Electrophoresis was conducted at 0°C in the dark for 20 min at 25 V (0.96 V/cm, approximately 300 mA).

In the acellular comet assay, comet slides from untreated WTK1 cells were prepared as outlined above and the slides were lysed immediately in a solution at 0°C for 60 min as described above. Lysed slides were neutralized in 400 mM Tris HCl buffer (pH 7.5) for 15 min and immersed in that buffer containing different concentrations of the kojic acid for 2, 4, and 24 h at room temperature in the dark. After the treatment period, the slides were rinsed three times for 5 min by immersion in cold distilled water, the slides were placed on a horizontal gel electrophoresis platform, and covered with pH > 13 alkaline solution made up of 300 mM NaOH and 1 mM Na_2EDTA or pH12 alkaline solution made up of 10 mM NaOH, 300 mM NaCl and 1 mM Na_2EDTA for 20 min in the dark at 0°C, then electrophoresis was conducted as above.

The slides were then neutralized and stained with 50 μL of 20 μg/mL ethidium bromide. Photographs of Comet images were taken using Fuji Neopan Presto 400 Black & White film and the length of the whole comet (head to tail) was measured manually using a scale for 50 nuclei for each dose and differences between the means in treated and control plates were compared with the Dunnett test after one-way ANOVA. A *p*-value less than 0.05 was considered statistically significant.

Chromosome aberration assay: The mitotic preparations were prepared immediately after kojic acid treatment for 20 h and after the cultivation for 16 h in fresh medium following to kojic acid treatment for 4 h. The mitotic preparations were stained with 2% Giemsa solution in 1/15 M phosphate buffer (pH 6.8) for 10 min and 100 well spread metaphases per culture were analyzed for chromosome aberrations. Breakage-type chromatid aberrations (ctb, chromatid breaks and chromatid fragments), exchange-type chromatid aberrations (cte, interchange and intrachange between chromatids), breakage-type chromosome aberrations (csb, chromosome breaks and chromosome fragments) and exchange-type chromosome aberrations (cse, interchange and intrachange between chromosomes) were scored. The number of aberrant cells was statistically analyzed using the chi-square test.

***TK* mutation assay:** In the mutation test, cells were cultured in fresh medium for 72 h after the kojic acid treatment for 4 and 20 h. The cells were seeded onto 96-well cluster dishes at 1.6 cells/well to evaluate cytotoxicity (RS0) immediately after the treatment with the kojic acid and the cultivation for 72 h with fresh medium. After 12 days, numbers of wells without colony were recorded. To detect *TK* mutation, TK6 and WTK1 cells were seeded onto 96-well cluster dishes at 10^4 and 2000 cells/well, respectively, using selection medium containing 3.0 μg/mL of trifluorothymidine. After 12 and 30

days, numbers of wells with normally growing and slowly growing colonies were recorded to evaluate trifluorothymidine resistant mutation frequency, respectively. A chemical is here considered to induce significant mutation if the mutant frequencies (NMF and SMF) of treated culture are more than 3-fold higher dose-dependently than those of control culture.

Results

In the regular comet assay, significant increase in the mean tail length was observed in TK6 and WTK1 cells after 4- and 20-h treatment with $\geqq 1250\,\mu$g/mL kojic acid (Table 1). At that time, cell viability ranged from 71.2 to 108% except for 20-h treatment at 5000 μg/mL, showing that observed DNA migration was not due to cell death. In the acellular comet assay at both pH12 and pH13, the treatment with kojic acid for 24 h at $\geqq 625\,\mu$g/mL increased the tail length significantly (Table 2). BLM used as a positive control chemical in the acellular comet assay led to positive responses at both pH13 and pH12.

In the chromosome aberration experiment, significant increases in the frequency of aberrant cells was also observed in both cell lines after 4- and 20-h treatment with kojic acid (Table 3). Induced aberrations were not exchange-type chromatid aberrations, but breakage-type. Significant increase in polyploidy was not observed.

The *TK* mutation assay in TK6 and WTK1 cells showed that the frequencies of normal growing mutant colony in cultures after 4- and 20-h treatment with kojic acid were $\geqq$3-fold higher dose-dependently than those in control cultures (Table 4). Kojic acid did not lead to positive responses in the frequency of slowly growing mutant colony in either cells. 4NQO used as a positive control chemical led to positive responses in the regular comet, chromosome aberration, and *TK* mutation assays.

Discussion

Considering the carcinogenic process, it is important to inspect whether DNA primary lesions are repaired, un-repaired DNA lesions result in cytotoxicity, or unrepaired DNA lesions form chromosome aberrations and/or gene mutations. In the present study *in vitro*, we compared the induction of DNA primary lesions, chromosome aberrations, and *TK* gene mutations. Kojic acid induced DNA primary lesions, chromosome aberrations, and *TK* gene mutations at almost identical con-

Table 1. DNA damage detected by the regular comet assay in human lymphoblastoid cells treated with kojic acid

Cell line	Chemical	(μg/mL)	DNA migration (μm, mean ± SEM of 50 cells) 4 h*	20 h*
TK6	Kojic acid	0	23.1 ± 0.51 (100)†	22.9 ± 0.44 (100)
		313	22.9 ± 0.66 (108)	23.2 ± 0.41 (95.3)
		625	24.4 ± 1.04 (84.1)	26.4 ± 0.53 (81.3)
		1250	29.3 ± 1.51 (88.1)	33.1 ± 1.47§ (77.2)
		2500	34.3 ± 1.81§ (71.2)	34.1 ± 1.57§ (74.5)
		5000	33.2 ± 1.53§ (73.7)	—‡ (22.3)
	4NQO	0.4	63.4 ± 3.16§ (75.2)	— (10.4)
WTK1	Kojic acid	0	22.7 ± 0.61 (100)	22.1 ± 0.52 (100)
		313	24.9 ± 1.19 (95.8)	21.4 ± 0.49 (85.3)
		625	23.6 ± 0.95 (89.1)	24.3 ± 1.25 (95.0)
		1250	29.9 ± 1.86 (105)	35.7 ± 1.67§ (88.1)
		2500	32.1 ± 1.81§ (91.2)	35.9 ± 1.59§ (73.1)
		5000	34.3 ± 1.72§ (81.3)	— (39.1)
	4NQO	0.4	57.7 ± 1.99§ (78.3)	— (11.2)

Reproducibility was ascertained by three independent experiments and representative data are shown in this Table.
*, Exposure period
†, Numbers in parenthesis show relative viability (%) measured using Trypan blue dye exclusion.
‡, Not evaluated because of excessive toxicity.
§, Significant difference from untreated control: $p < 0.05$.

Table 2. DNA damage detected by the acellular comet assay in human lymphoblastoid WTK1 cells treated with kojic acid

Cell line	Chemical	(μg/mL)	DNA Migration (μm, Mean ± SEM of 50 cells) 2 h* pH13	2 h* pH12	4 h* pH13	4 h* pH12	24 h* pH13	24 h* pH12
WTK1	Kojic acid	0	27.5 ± 0.32	31.1 ± 1.61	27.6 ± 1.43	29.8 ± 1.37	30.4 ± 1.94	30.4 ± 1.47
		313	27.3 ± 0.11	29.9 ± 1.21	ND	ND	ND	ND
		625	28.9 ± 1.20	30.3 ± 1.81	28.6 ± 1.68	30.4 ± 2.06	65.8 ± 2.01†	57.8 ± 4.20†
		1250	27.9 ± 0.44	29.2 ± 0.80	28.8 ± 1.58	30.7 ± 1.82	64.7 ± 1.73†	53.8 ± 4.49†
		2500	27.6 ± 0.34	30.9 ± 1.42	29.1 ± 0.65	29.7 ± 1.65	72.9 ± 1.31†	55.4 ± 4.82†
		5000	32.6 ± 1.39	33.3 ± 2.30	29.2 ± 1.58	30.5 ± 1.94	79.7 ± 1.98†	75.7 ± 5.92†
	BLM	5	38.3 ± 2.83†	35.0 ± 2.92†	36.2 ± 4.44†	36.5 ± 3.13†	48.2 ± 3.46†	53.5 ± 6.21†

Reproducibility was ascertained by three independent experiments and representative data are shown in this Table.
*, Exposure period
ND, not done
†, Significant difference from untreated control: $p < 0.05$.

Table 3. Chromosome aberrations in human lymphoblastoid cells treated with kojic acid

Cell line	Kojic acid		Treatment period (h)	Aberrant cells(%)		No. of chromosome aberrations/100 cells							Polyploidy (%)	Mitotic index (%)
						Chromatid type			Chromosome type			Others		
		(μg/mL)		+gap	−gap	ctg	ctb	cte	csg	csb	cse			
TK6		0	4–16*	2	2	2	1	1	0	0	0	0	2	3.5
		156	4–16	3	3	1	3	0	0	0	0	0	2	3.1
		313	4–16	7	6	5	13	2	0	0	0	0	4	3.2
		625	4–16	6	5	4	12	1	0	0	0	0	3	2.3
		1250	4–16	9	7	2	13	0	0	0	0	1	5	2.1
		2500	4–16	13‡	12‡	1	18	1	0	0	0	0	1	1.2
		5000	4–16	12‡	11‡	2	31	1	0	0	0	0	2	0.4
	4NQO	0.4	4–16	22‡	19‡	4	10	29	1	0	1	3	2	2.1
		0	20–0	2	2	1	2	0	0	0	0	0	2	3.6
		156	20–0	2	2	0	3	0	0	0	0	0	2	3.9
		313	20–0	9	7	2	11	1	1	0	0	1	5	3.1
		625	20–0	8	8	0	13	2	0	0	0	1	5	1.9
		1250	20–0	16‡	16‡	5	27	3	0	1	0	0	4	1.8
		2500	20–0	29‡	29‡	6	65	2	0	0	1	0	3	0.3
		5000	20–0	—†	—	—	—	—	—	—	—	—	—	0
	4NQO	0.1	20–0	43‡	38‡	12	28	63	0	5	1	1	1	1.1
WTK1		0	4–16	3	2	3	2	0	0	0	0	0	2	3.8
		156	4–16	4	4	0	10	0	0	0	0	0	5	4.1
		313	4–16	6	5	5	9	1	0	0	0	1	3	4.2
		625	4–16	5	5	0	5	3	0	0	0	0	3	3.3
		1250	4–16	7	4	7	4	1	0	0	0	0	4	3.1
		2500	4–16	11‡	11‡	1	14	2	0	0	0	0	3	2.2
		5000	4–16	4	3	1	3	0	0	0	0	0	4	0.8
	4NQO	0.4	4–16	29‡	25‡	7	13	28	4	2	2	2	4	1.9
		0	20–0	2	2	1	1	1	0	0	0	0	3	3.1
		156	20–0	5	5	1	5	1	0	0	0	0	5	2.9
		313	20–0	6	6	1	5	2	0	0	0	0	6	3.4
		625	20–0	7	6	3	7	1	0	0	0	0	3	2.3
		1250	20–0	15‡	14‡	6	19	2	0	0	1	0	7	1.9
		2500	20–0	33‡	30‡	6	55	1	0	0	0	0	6	1.2
		5000	20–0	—	—	—	—	—	—	—	—	—	—	0
	4NQO	0.1	20–0	51‡	46‡	10	33	71	2	4	5	0	12	0.9

*, Exposure period–recovery period

†, not evaluated due to lack of mitoses.

‡, Significant difference from untreated control: $p < 0.05$.

ctg, chromatid gaps; ctb, chromatid breaks and fragments; cte, interchange and intrachange between chromatids; csg, chromosome gaps; csb, chomosme breaks and fragments; cse, interchange and intrachange between chromosomes.

centration range. In the comet assay, both alkali-labile sites and DNA single strand breaks (SSBs) can be detected at pH > 13, but only SSBs can be detected at pH12 (19). In the regular comet assay, DNA initial lesions can be repaired and/or can form alkali-labile sites such as AP sites by cell functions before comet slide preparation. On the other hand, in the acellular comet assay where any cell functions cannot act, initial lesions such as SSBs persist and are detected. Therefore, our present finding that kojic acid led to positive responses at pH12 in the acellular comet assay suggests it can induce SSBs. At 625 μg/mL, the positive responses were observed in the acellular assay only, may suggest that DNA lesions other than SSBs induced by kojic acid at 625 μg/mL are repaired and cannot form alkali-labile sites. In the acellular assay, the positive responses were not observed after 2 and 4 h treatment, may suggest that SSB-induction by kojic acid treatment for 2 and 4 h is too low to be detected by this assay. Since SSBs cannot be repaired in the acellular assay, SSBs would accumulate up to detectable level during long treatment period (24 h). The negative responses in acellular assay at 4 h seem to be contradictory to the positive responses in regular assay at 4 h. Considering that acellular assay can detect SSBs and that regular assay can detect not only SSBs but also alkali-labile sites, the present results could be explained by followings; initial lesions induced by kojic acid would be both SSBs and lesions that can form alkali-labile sites, the induction level of SSBs at 4 h is too low to be detected by the acellular assay, the induction level

Table 4. Toxicity and *TK* mutant frequency in human lymphoblastoid cells treated with kojic acid

Cell line	Chemical	Concentration (μg/mL)	Treatment period (h)	RS0* (%)	RTG+ (%)	NMF‡ /10^6 cells	RNMF§ (%)	SMF‖ /10^6 cells	RSMF** (%)
TK6	Kojic acid	0	4	100	100	4.40	100	2.61	100
		625	4	92.0	99.3	3.66	81.6	1.71	65.3
		1250	4	81.1	46.5	13.4#	299	4.60	176
		2500	4	40.6	26.7	23.5#	523	5.04	193
		5000	4	23.1	14.2	27.5#	614	4.85	185
	4NQO	0.2	4	34.6	21.4	51.8#	1156	35.0	1341
	Kojic acid	0	20	100	100	4.18	100	2.37	100
		313	20	97.0	85.1	7.90	189	1.84	77.3
		625	20	76.7	37.2	16.7#	399	3.11	131
		1250	20	79.0	26.7	28.3#	677	4.75	200
		2500	20	24.4	12.1	32.8#	784	4.12	173
		5000	20	0	0	Toxic	Toxic	Toxic	Toxic
WTK1	Kojic acid	0	4	100	100	82.8	100	50.8	100
		625	4	89.4	93.1	73.2	77.5	53.8	106
		1250	4	76.6	45.7	289#	349	74.8	147
		2500	4	39.3	29.3	427#	516	82.0	161
		5000	4	26.9	16.0	477#	576	75.1	148
	4NQO	0.2	4	36.5	16.2	503#	453	314	450
	Kojic acid	0	20	100	100	111	100	69.8	100
		313	20	139	134	193	174	126	180
		625	20	72.9	66.6	300	270	106	152
		1250	20	84.8	28.6	536#	482	98.3	141
		2500	20	22.6	11.4	567#	510	126	179
		5000	20	0	0	Toxic	Toxic	Toxic	Toxic

Reproducibility was ascertained by two independent experiments and representative data are shown in this Table.
*, Relative survival at day 0; +, Relative total growth during the 72 h expression period; ‡, Frequency of normally growing mutant colonies; §, Relative normally growing colony mutant frequency; ‖, Frequency of slowly growing mutant colonies; **, Relative slowly growing colony mutant frequency; Toxic, not assayed due to toxicity.
#, more than 3-fold higher than control.

of lesions that can form alkali-labile sites at 4 h is high enough to be detected by the regular assay, and only alkali-labile sites were detected by the regular assay at 4h. It is, however, remained for further studies whether kojic acid can induce base damages. Chromosome aberrations are induced by kojic acid at $\geqq 2500\ \mu$g/mL where it led to positive responses in the comet assay, may suggest the possibility that its induced DNA lesions that can form alkali-labile sites result in chromosome aberrations.

Since both WTK1 cells and TK6 were derived from WIL-2 cells and WTK1 cells are *p53* mutant cells, WTK1 cells are more resistant to clastogenic effects than TK6 cells (18) and are more sensitive to genotoxic effects than TK6 cells (20). In TK6 and WTK-1 cells, normally growing *TK* mutants that grow at the same rate as the wild type (doubling time, 13 to 17 h) are produced mainly due to the mutations of the *TK* locus, while slowly growing mutants (doubling time, of >21 h) are induced by gross structural changes involving the growth-regulating gene outside the *TK* locus (18). In this study, great difference in the mutation induction and cell survival was not observed between TK6 and WTK1 cells. Kojic acid increased the frequency of normal growing but not slowly growing mutants, suggesting that observed gene mutations are due to point mutations of the *TK* locus but not gross structural changes that can result in chromosome aberrations observable by a microscopy. However, kojic acid has been reported not to induce *Hprt* gene mutation in V79 (9,14), which contradict with our results. Gross structural changes of *Hprt* locus involving closely linked essential genes on monosomic X chromosome lead to poor recovery of viable mutants (21). Therefore, if kojic acid yields gross structural changes at the targeted *Hprt* locus, it would be reasonable that its mutability could not be detected as *Hprt* mutant colonies. However, since our results suggest the possibility that its mutability is not due to its clastogenic effect, the contradiction may be due to the difference of sensitivity of used cell lines and/or target locus. PE0 and RTG decreased remarkably at $\geqq 2500\ \mu$g/mL after 4 h treatment and $\geqq 625\ \mu$g/mL after 20 h treatment. These concentration ranges coincide with those where induction of normally growing *TK* mutants and chromosome aberrations were observed. Considering that slowly growing mutants that reflect gross struc-

tural changes at *TK* locus that can result in chromosome aberrations observable by a microscopy did not increased, cells having chromosome aberrations might die rather than survive and form normally growing *TK* mutants.

As a summary, our *in vitro* results would suggest the possibility that kojic acid-induced DNA damage detected by the comet assay results in chromosome aberrations, gene mutations, and cell death and that a contribution of chromosome aberrations to cell death is larger than that to gene mutations.

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