Suppressing Effect of Tannic Acid on UV and Chemically Induced Chromosome Aberrations in Cultured Mammalian Cells

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UV-induced structural chromosome aberrations were suppressed by post-treatment with tannic acid (*m*-galoyl gallic acid) in cultured Chinese hamster ovary cells. A significant decrease in the frequencies of chromosome aberrations in the presence of tannic acid was observed in cells treated with mitomycin C or methylmethanesulfonate but not with X-rays or bleomycin. The effects of tannic acid were dependent on the DNA pre-replicative G1 phase of the cell cycle. Furthermore, tannic acid enhanced the liquid-holding effect on UV-irradiated cells at G1 phase. On the other hand, the number of surviving UV-irradiated cells increased in the presence of tannic acid. These results suggest the possibility of promotion by tannic acid of an excision repair system of mammalian cells.

Recently a number of antimutagenic compounds which suppress the process of mutagenesis in bacteria have been identified. These compounds include caffeine,¹⁾ cinnamaldehyde,^{2~4)} tannic acid,⁵⁾ vanillin,⁶⁾ gallic acid,⁷⁾ and benzaldehyde⁸⁾ which exist in our daily foods as plant components or food additives. It is of interest to elucidate the effects of these compounds on genetic damage in mammalian cells. We have recently reported the modifying effects of vanillin on chromosome aberrations and sister-chromatid exchanges induced by mitomycin C in mammalian cells in culture.^{9,10)} In this study we investigated the effects of tannic acid on structural chromosome aberrations induced by UV, X-rays, or chemical mutagens in cultured Chinese hamster ovary cells. Tannic acid is a hydrolytic product of tannins which are widely present in plants, including green tea.

MATERIALS AND METHODS

Chemicals. Tannic acid, bleomycin, dimethylsulfoxide,

colchicine, and TN16 were obtained from Wako Pure Chemical Industries, Osaka. Mitomycin C and methylmethanesulfonate were purchased, respectively, from Kyowa Hakko Ltd., Tokyo and Eastman Kodak, New York, U.S.A. Tannic acid and methylmethanesulfonate were dissolved in dimethylsulfoxide. The final concentration of solvent in the medium was 0.5%. Mitomycin C, bleomycin, and colchicine were dissolved in Hank's balanced salt solution (pH 7.0).

Cells and media. The CHO K-1 Chinese hamster ovary cell line was originally obtained from the American Type Culture Collection and cloned in our laboratory. Mycoplasma-free cells were grown in Ham's F12 medium (Nissui Pharmaceutical Co., Ltd., Tokyo) supplemented with 10% foetal bovine serum (HyClone Laboratories, Inc., U.S.A.), 50 IU/ml penicillin G, 50 µg/ml streptomycin sulfate, and 2.5 µg/ml fungizone, in a humidified atmosphere with 5% CO₂ at 37°C. All antibiotics were purchased from Flow Laboratories, Inc., U.S.A. Doubling time was about 22 hr.

Assay of chromosome aberration. Cells were seeded at a density of 1.0×10^6 cells/100-mm dish. Cells were treated with mitomycin C, methylmethanesulfonate, or bleomycin for one cell cycle of 22 hr in culture medium. At the end of the treatment period, the cells were washed to remove the mutagen. The cells were irradiated with X-ray or UV in a

dish without medium using an X-ray radiation apparatus (Hitachi, Tokyo) with a radiation radiometer (Victoreen, U.S.A.), or a 10-W germicidal lamp with a UV radiometer (Topcon, Tokyo), respectively. Treated cells were then cultured in the presence or absence of tannic acid for 24 hr. Mitotic cells were collected by treatment with colchicine for 2 hr at a final concentration of 50 µg/ml. Air-dried chromosome preparations were stained with 2% Giemsa solution in phosphate buffer (pH 6.8) for 10 min and 100 well-spread metaphase cells per culture were analyzed for chromosome aberrations. The numbers of metaphase cells with any types of chromosome aberrations, breakage-type chromosome aberrations (chromatid gaps and breaks, acentric fragments, and fragmentations), and exchange-type chromosome aberrations (chromatid exchanges and rings) were analyzed using the Chi-square test.

Experiments by pulse-treatment with tannic acid for 3 hr were conducted on synchronized cells. For arresting mitotic cells, cells were incubated in the presence of 10 µM TN16 which has colchicine-like effects.¹¹⁾ Mitotic cells collected by shake-off were seeded in dishes, immediately irradiated by UV at 20 J/m², and then cultured in fresh medium. Pulse-treatments with tannic acid at 3.3 µg/ml were made every 3 hr after the UV-irradiation. Three hours after the addition of tannic acid, cells were washed to remove tannic acid and cultured in fresh medium. Since the cell cycle after the UV-irradiation at 20 J/m² was prolonged to about 30 hr, chromosome preparations were prepared 30 hr after UV-irradiation. Mitotic cells were collected by treatment with colchicine at 50 µg/ml for 30 min. The periods of cell stages (G1, S, and G2 phase) were identified by the method described previously.¹²⁾

Liquid holding effect. To hold cells in G1 phase,¹³⁾ they were incubated in an isoleucine-free F12 medium (prepared in our laboratory) containing 10% dialyzed foetal bovine serum (GIBCO Laboratories, U.S.A.) for 36 hr.

Cells irradiated by UV at 20 J/m² were incubated in the presence or absence of tannic acid at 3.3 µg/ml for 12 or 24 hr. After the treatment with tannic acid, cells were washed and cultured in complete F12 medium. Eighteen hours later, colchicine was added at 50 µg/ml to arrest the first mitotic cells after G1 holding. Chromosome preparations were prepared 6 hr after the addition of colchicine.

Effects of tannic acid on cell cycle. To find the effects of tannic acid on the cell cycle, the mitotic index and doubling time of mutagen-treated cells were measured in the presence and absence of tannic acid. The mitotic index was scored as the number of metaphase cells per 1000 cells in chromosome preparations. To measure the doubling time, numbers of cells were scored just before and after (N1 and N2, respectively) the incubation in the presence and absence of tannic acid for 48 hr. Doubling time (DT) was calculated by the following formula:

$$DT = \frac{48 \times \log 2}{\log N2 - \log N1}$$

Effects of tannic acid on survival. UV-irradiated cells were incubated in the presence and absence of tannic acid for 24 hr. An appropriate number of cells were then seeded in 60-mm dish and cultured for 10 days. Cells were fixed with ethanol, and stained with Giemsa solution, and the number of colonies was counted.

RESULTS

The effects of post-treatment with tannic acid on the induction of chromosome aberrations by UV-irradiation are shown in Table I. Assays were done at UV doses of 15, 20, and 25 J/m² giving about 20~60% aberrant cells.

TABLE I. SUPPRESSING EFFECTS OF POST-TREATMENT WITH TANNIC ACID ON THE FREQUENCY OF CELLS WITH CHROMOSOME ABERRATIONS INDUCED BY UV-IRRADIATION

UV (J/m ²)	Tannic acid (3.3 µg/ml)	Metaphase cells with chromosome aberrations (%)			Mitotic index (%)	Doubling time (hr)
		Total	Breakage-type	Exchange-type		
0	—	1	1	0	5.0	22.1
	+	0	0	0	5.2	22.0
15	—	21	18	18	5.1	22.0
	+	7*	6**	5**	5.2	21.8
20	—	40	26	33	1.8	29.9
	+	12***	6***	10***	1.7	29.9
25	—	64	42	54	0.9	35.8
	+	36***	20***	25***	0.9	35.9

UV-irradiated cells were cultured with or without tannic acid at 3.3 µg/ml for 24 hr. A significant decrease (*: 0.01 < p < 0.05, **: 0.001 < p < 0.01, and ***: p < 0.001) in the number of metaphase cells with chromosome aberrations was observed in tannic acid-containing culture compared with tannic acid-free culture.

The number of metaphase cells with chromosome aberrations per 100 cells was apparently decreased (67, 70, and 44%, respectively) in the

FIG. 1. Suppressing Effect of Tannic Acid on UV-Induced Chromosome Aberrations.

Cells were irradiated by UV at 20 J/m². ●, total number of cells with chromosome aberrations; ▲, cells with breakage-type chromosome aberrations; ■, cells with exchange-type chromosome aberrations. See Table I for the level of significance.

presence of 3.3 µg/ml tannic acid. Figure 1 shows a dose-dependent suppressing effect of tannic acid on UV-induced chromosome aberrations. Both the frequency of metaphase cells with breakage-type and exchange-type aberrations were suppressed. Table II shows the effects of tannic acid on the induction of chromosome aberrations by mitomycin C or methylmethanesulfonate. Post-treatment with tannic acid caused significant decreases in the frequency of metaphase cells with both breakage-type and exchange-type chromosome aberrations. Mitotic index and doubling time of mutagen-treated cells in the presence and absence of tannic acid were also shown in Tables I and II. Neither a decrease nor an increase in the mitotic index or doubling time was observed by the post-treatment with tannic acid. The results suggest that tannic acid did not delay the cell-cycle or kill cells. On the other hand, structural chromosome aberrations induced by X-rays or bleomycin were not suppressed by tannic acid at all (Fig. 2).

To analyze the suppressing effect of tannic acid on UV-induced chromosome aberrations, further investigation was carried out on syn-

TABLE II. SUPPRESSING EFFECTS OF POST-TREATMENT WITH TANNIC ACID ON THE FREQUENCY OF CELLS WITH CHROMOSOME ABERRATIONS INDUCED BY MITOMYCIN C OR METHYLMETHANESULFONATE

Mutagen (μM)		Tannic acid (μg/ml)	Metaphase cells with chromosome aberrations (%)			Mitotic index (%)	Doubling time (hr)
			Total	Breakage-type	Exchange-type		
Mitomycin C	0.23	0	41	39	37	2.0	24.5
		0.33	34	30	29	2.2	24.4
		1.0	27*	20**	17**	2.5	24.6
		3.3	21**	12***	17**	2.1	24.6
	0.30	0	68	48	48	1.8	28.0
		0.33	54*	38	41	1.4	28.4
		1.0	45**	28**	31*	1.4	28.2
		3.3	36***	23***	25***	1.8	28.2
Methylmethane-sulfonate	100	0	25	18	20	3.2	22.6
		0.33	15	10	15	3.5	22.7
		1.0	13*	4***	10*	3.4	22.5
		3.3	8**	3***	6**	3.3	22.4
	200	0	65	43	45	2.5	24.7
		0.33	56	35	37	2.5	24.7
		1.0	31***	18***	22***	2.7	24.6
		3.3	24***	12***	14***	2.8	24.6

Cells were treated with mitomycin C or methylmethanesulfonate for 22 hr. Then cells were post-treated with tannic acid at the indicated concentrations for 24 hr. See Table I for the level of significance.

FIG. 2. Absence of Suppressing Effect of Tannic Acid on Chromosome Aberrations Induced by X-Rays (Left) or Bleomycin (Right).

Cells treated with X-rays or bleomycin at the indicated doses were cultured with (●) or without (○) 3.3 µg/ml tannic acid for 24 hr.

FIG. 3. Effects of Tannic Acid at Different Cell Stages on Chromosome Aberrations Induced by UV.

Synchronized cells were irradiated by UV at 20 J/m², then pulse-treated with tannic acid for 3 hr. See Table I for the level of significance.

chronized cells. Cells were pulse-treated with tannic acid for 3 hr at every cell stage. As shown in Fig. 3, the frequencies of metaphases with chromosome aberrations were decreased when cells at G1 phase were treated with tannic acid. In contrast, no effect of tannic acid was observed on cells in S and G2 phase. To know the relationship between the effects of tannic acid and DNA pre-replicative G1

FIG. 4. Effects of Tannic Acid on Liquid-holding Cells in G1 Phase.

Cells held in G1 phase were irradiated by UV at 20 J/m². Cells were incubated in the presence (●) and absence (○) of tannic acid. A significant decrease (*: 0.01 < *p* < 0.05, and **: 0.001 < *p* < 0.01) was observed in tannic acid-containing culture compared with tannic acid-free culture at the same holding time. A significant decrease (a: 0.01 < *p* < 0.05, and b: *p* < 0.001) was observed in culture with liquid holding compared with culture without liquid holding.

phase, a liquid-holding study was done. Cultured cells from rodents are held in G1 phase when they are incubated in isoleucine-

FIG. 5. Survival of CHO K-1 Cells as a Function of UV-Dose.

UV-Irradiated cells were cultured in medium containing tannic acid at 0 (○), 1.0 (●), or 3.3 $\mu\text{g}/\text{ml}$ (■) for 24 hr.

free medium containing dialyzed foetal bovine serum.¹³⁾ Figure 4 shows the effects of tannic acid on UV-irradiated G1 cells during liquid holding. The frequency of UV-induced chromosome aberrations was gradually decreased by liquid holding in the absence of tannic acid for 12 and 24 hr. However, a greater decrease was observed with the addition of tannic acid. These results showed that tannic acid acted in G1 phase and suppressed the formation of chromosome aberrations.

Since a possible promoting effect of tannic acid on a repair system in G1 phase was suggested, the effects of tannic acid on survival of UV-treated cells was investigated. An apparent increase in the number of surviving colonies was observed in the presence of 3.3 and 1.0 $\mu\text{g}/\text{ml}$ tannic acid in the medium (Fig. 5).

DISCUSSION

In this study, we showed suppression by

tannic acid of chromosome aberrations induced by treatment with UV, mitomycin C or methylmethanesulfonate. As explanations for the decrease in the number of induced chromosome aberrations in the medium containing tannic acid, the following possibilities are considered: 1) inhibition of the formation of chromosome aberrations during DNA synthesis at S phase, 2) enhancement of post-replicative repair activity at G2 phase, and 3) a decrease in the number of DNA lesions responsible for chromosome aberrations by enhancing DNA excision repair activity at G1 phase. Chromosome aberrations reflect DNA double-strand breaks and recombinations between their broken ends. According to Bender,¹⁴⁾ DNA double-strand breaks are generated secondarily as a consequence of normal DNA synthesis from DNA lesions. It is considered that DNA double-strand breaks which are generated in S phase are repaired by G2 repair activity and that unrepaired DNA strand breaks result in chromosome aberrations.^{15,16)} If G2 repair activity is enhanced, suppression is expected to be observed in DNA post-replicative G2 phase. We previously demonstrated that the frequency of metaphases with chromosome aberrations was decreased by post-treatment with vanillin, an example of this case.⁹⁾ As shown in this study, however, DNA prereplicative G1 phase but not S phase or post-replicative G2 phase is sensitive to tannic acid. Since a suppressing effect of tannic acid was observed in G1 holding cells without DNA replicating activity, it is considered that tannic acid may enhance a DNA repair system acting in cells held in G1 phase. UV irradiation forms pyrimidine dimers¹⁷⁾ and mitomycin C induces bulky adducts in DNA of mammalian cells.¹⁸⁾ Alkylated bases in DNA are formed by methylmethanesulfonate.¹⁹⁾ It has been shown that alkylated lesions in DNA are repaired in cells held in G1 phase by a nucleotide excision repair system²⁰⁻²²⁾ or a similar base-excision repair process involving DNA glycosylase.²³⁾ X-rays and bleomycin cause DNA strand breaks.²⁴⁾ The repair process for DNA strand

breaks seems to be different from that for UV damage, because ataxia telangiectasia cells, which are sensitive to X-rays and bleomycin, do not show any increased sensitivity to UV.²⁵⁾ In our observation, X-ray- or bleomycin-induced chromosome aberrations were not suppressed by tannic acid. Then, we assume that some DNA pre-replicating repair process in G1 phase, probably an excision repair, is modified by tannic acid. This supposition may be supported by the fact that UV-irradiated cells showed higher colony-forming ability in the presence of tannic acid. In the study using *Escherichia coli*, tannic acid was reported to have an antimutagenic effect on UV-induced mutagenesis in an excision repair-proficient strain, but not in the excision repair-deficient (*uvrA*⁻ or *uvrB*⁻) strain.⁵⁾ Their proposed mechanism is also that tannic acid enhances the excision repair activity. To further investigate the effects of tannic acid on excision repair in mammalian cells, studies using excision repair-deficient human cells such as those from patient with xeroderma pigmentosum (XP) and Fanconi's anemia are now in progress.

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