3.2. Transformation assays of initiators

The results are summarized in Table 1. Fig. 1A and B shows the results of Bhas initiation and promotion assays of MNNG, respectively. Significant increase of focus formation was observed in the initiation assay at the concentrations of 0.5 μg/mL and above. As mentioned above, MNNG did not induce transformed foci in the promotion assay up to 0.1 μg/mL. With concentrations over 0.2 μg/mL in the promotion assay, cell flattening with enlarged cell size and without increased cell number was observed after triple dosing with concentrations over 0.2 μg/mL in the promotion assay.

Results of the initiation and promotion assays for MCA are shown in Fig. 1C and D. MCA showed a response similar to that with MNNG. MCA induced transformed foci in the initiation assay at concentrations of 0.5 μg/mL and higher. In the case of the promotion assay, no focus formation was observed at concentrations up to 0.1 μg/mL and severe cytotoxicity was observed at concentrations over 0.2 μg/mL.

AFB_1 showed negative results in both initiation and promotion assays (Fig. 1E and F). In the case of promotion assay, 1 μg/mL of AFB_1 was the maximum concentration tested because of its cytotoxicity. Metabolic activation of AFB_1 by cytochrome P450 (CYP) 2A6, 3A4 and 1A2 is necessary to exert mutagenic and carcinogenic activities [24,25]. Fig. 1G shows results of the initiation assay of AFB_1 metabolically activated by S9 mix, and there was significant foci induction at 1 and 2 μg/mL.

3.3. Transformation assays of tumor promoters

Initiation and promotion activities of three tumor promoters were also examined and the results are summarized in Table 1. Fig. 2A and B shows results on TPA. No increase of focus number was observed in the initiation assay up to 100 ng/mL (Fig. 2A). In contrast, significant increase of focus formation was observed in the promotion assay at all concentrations tested up to 100 ng/mL (Fig. 2B).

Fig. 2C and D shows results on LCA. As in the case of TPA, transformed foci were not induced in the initiation assay at all concentrations tested (Fig. 2C), whereas transformed foci were observed in the promotion assay at all concentrations tested (Fig. 2D).

Okadaic acid, up to 10 ng/mL, did not induce transformed foci (Fig. 2E) in the initiation assay. A significant increase of focus formation was observed in the promotion assay at 8 and 10 ng/mL at which high cytotoxicity was shown (Fig. 2F).

3.4. Transformation assays with various schedules of treatment

Fig. 3 shows results of treatment with MCA (100 ng/mL) and/or TPA (50 ng/mL) under various treat-

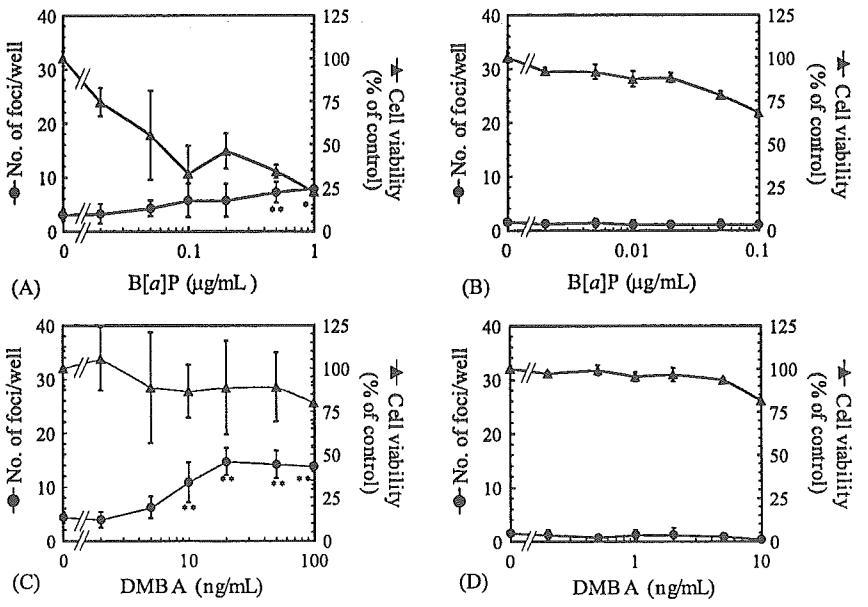

Fig. 4. Results of initiation and promotion assays of B[*a*]P and DMBA (Group I). A and C show results of initiation assay and B and D show results of promotion assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

ment schedules. When Bhas 42 cells were seeded at 2×10^3 cells/mL, cells were in growth phase almost until Day 7. In order to treat cells repeatedly during the period of growth phase, change of medium containing MCA or TPA was performed every 2 days (periods 1–3 in Fig. 3). After Day 7, when cells became confluent, the change of MCA- or TPA-containing medium was carried out twice a week during the subsequent period (periods 4–6 in Fig. 3).

Schedules B–E were performed as confirmation test of initiation and promotion assays for MCA and TPA. The number of transformed foci in Schedule F did not increase from that in Schedule B, indicating that the additional treatment with MCA during stationary phase had little effect. In contrast, MCA-treatment during cell-growth phase followed by TPA-treatment during stationary phase clearly enhanced the formation of transformed foci (Schedule G). Repeated treatment with MCA during

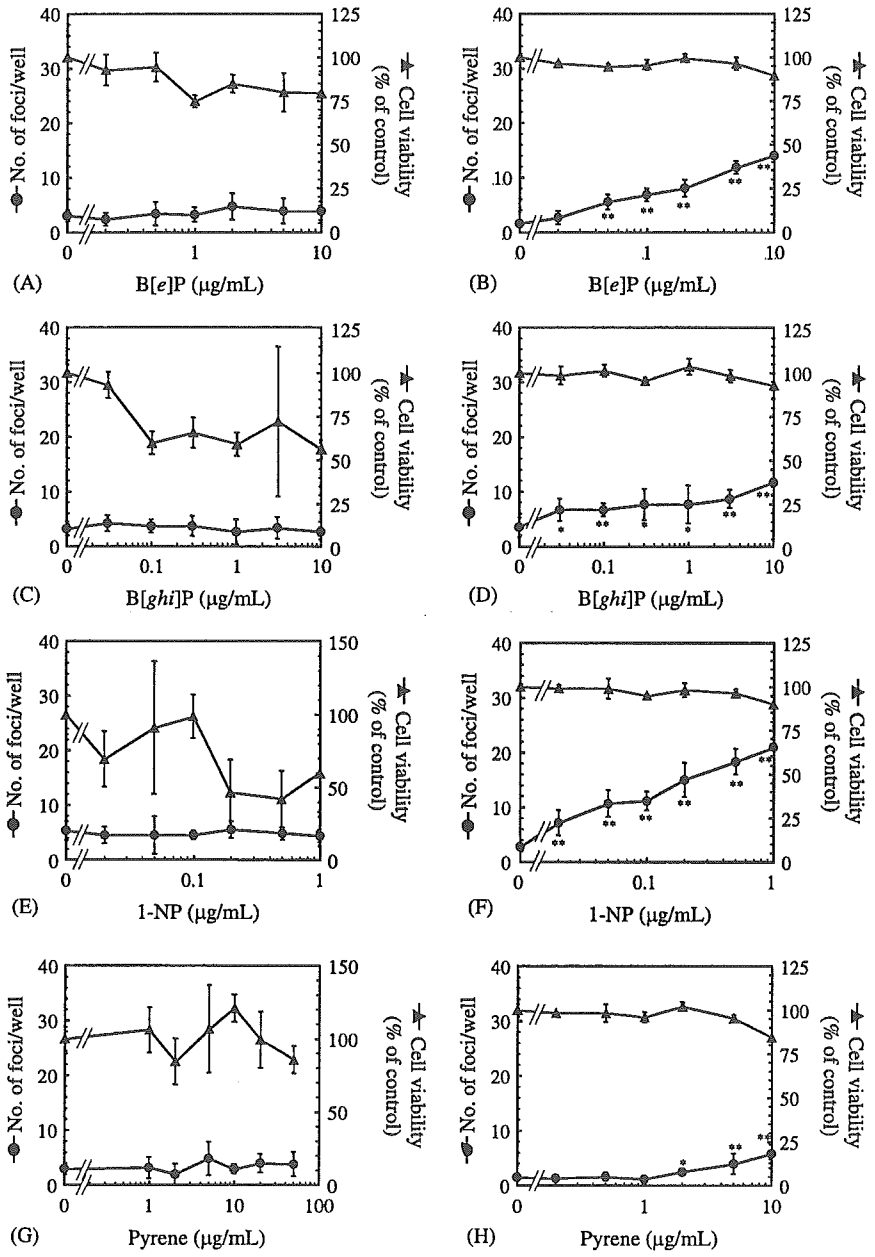

Fig. 5. Results of initiation and promotion assays of B[*e*]P, B[*ghi*]P, 1-NP and pyrene (Group II). A, C, E and G show results of initiation assay and B, D, F and H show results of promotion assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

the cell-growth phase (Schedule H) and the whole period (Schedule J) increased the number of transformed foci. It is remarkable that cell growth was retarded and cells did not reach subconfluence on Day 7 because of the cytotoxicity of MCA. Repeated TPA treatment during growth phase (Schedule I) was ineffective for inducing transformed foci. The number of transformed foci in Schedule K (TPA treatment during cell-growth phase and stationary phase) did not increase from that in Schedule E.

3.5. Initiation and promotion assays of PAHs

Results described above showed that MCA induced foci in the initiation assay without metabolic activation by S9 mix. This means that Bhas 42 cells have the capacity to metabolize PAHs, like the parental cell line BALB/c 3T3. Sixteen PAHs were evaluated for their initiating and promoting activities using the present methods. Response to PAHs in both methods could be categorized into four groups: showing only initiation

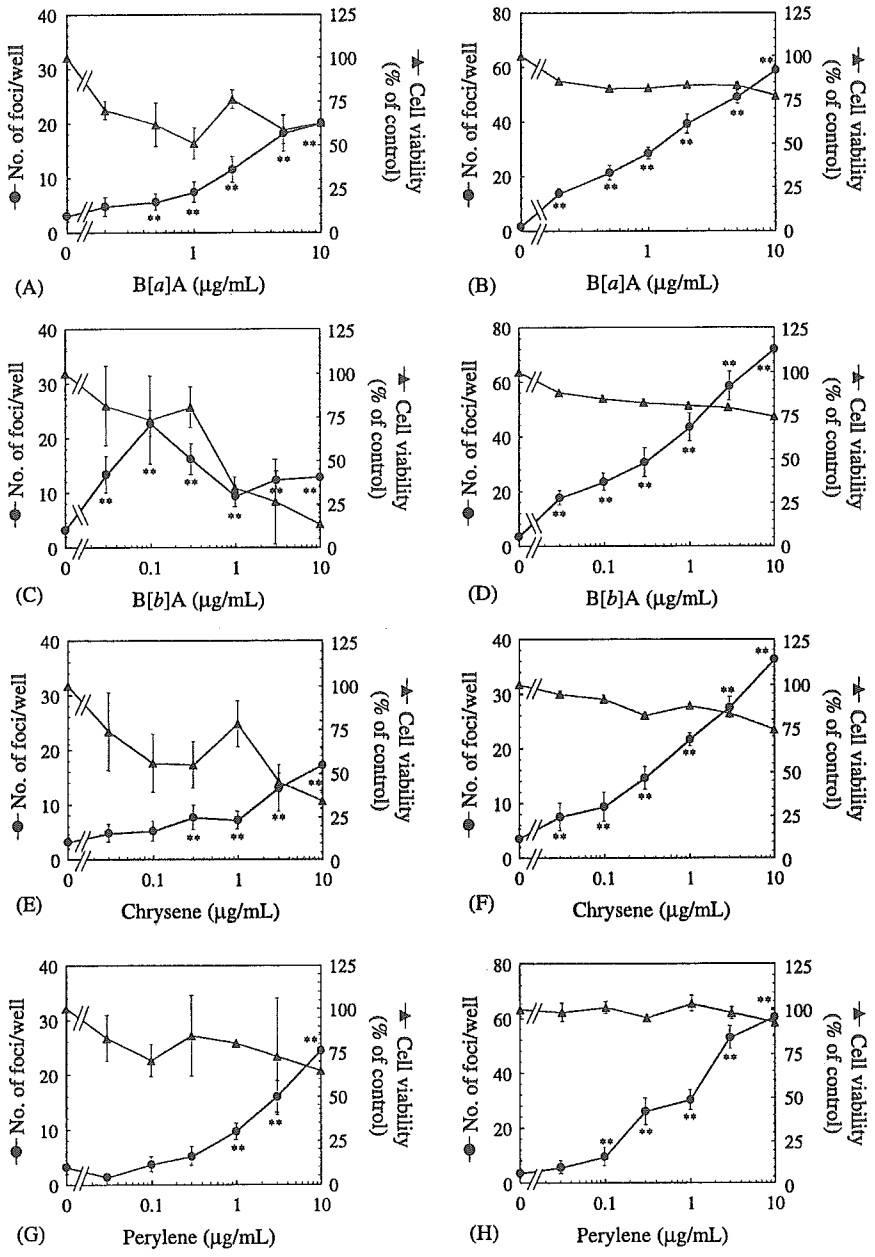

Fig. 6. Results of initiation and promotion assays of B[*a*]A, B[*b*]A, chrysene and perylene (Group III). A, C, E and G show results of initiation assay and B, D, F and H show results of promotion assay. $^{*}p<0.05$, compared with solvent control. $^{**}p<0.01$, compared with solvent control.

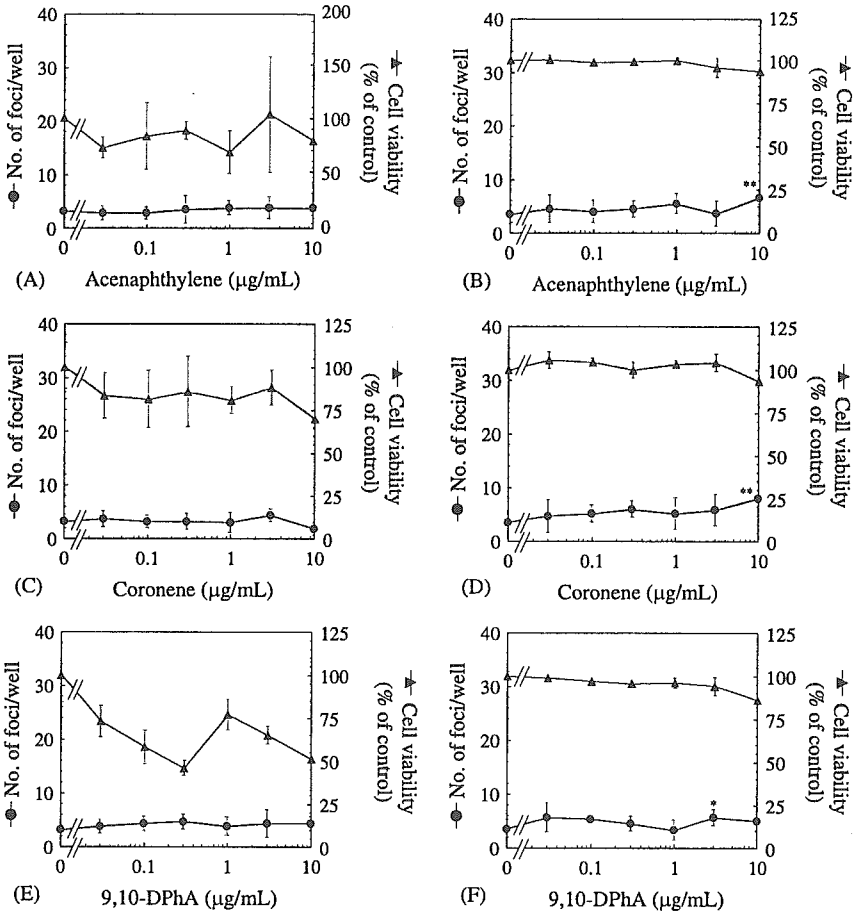

Fig. 7. Results of initiation and promotion assays of acenaphthylene, coronene and 9,10-DPhA (Group IV). A, C and E show results of initiation assay and B, D and F show results of promotion assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

activity (Group I), only promotion activity (Group II), both initiation and promotion activities (Group III), and equivocal or negative response in both methods (Group IV).

Fig. 4 shows results of two chemicals showing positive responses only in the initiation assay (Group I). B[*a*]P at concentrations of 0.5 and 1 μg/mL (Fig. 4A) and DMBA at concentrations of 10 ng/mL and above (Fig. 4C) induced transformed foci in the initiation assay only. In the case of the promotion assay, no transformed foci were induced at any concentrations tested for B[*a*]P (Fig. 4B) or DMBA (Fig. 4D).

Chemicals classified in Group II consisted of B[*e*]P, B[*ghi*]P, 1-NP, and pyrene, which showed positive responses only in the promotion assay (Fig. 5). No increase of foci was observed in the initiation assay with these chemicals (Fig. 5A, C, E and G). In contrast, they induced significant increase of foci in the promotion assay (Fig. 5B, D, F and H).

Results for Group III are shown in Fig. 6. B[*a*]A at concentrations of 0.5 μg/mL and above (Fig. 6A), chrysene at concentrations of 0.2 μg/mL and above (Fig. 6E) and perylene at concentrations of 1 μg/mL and above (Fig. 6G) induced foci in the initiation assay. In the initiation assay of B[*b*]A, the number of foci increased on increasing the dose up to 0.1 μg/mL, but it decreased at concentrations over 0.2 μg/mL (Fig. 6C). In the promotion assay, a marked increase in focus formation was observed with these chemicals at a non-cytotoxic concentration range from 0.1 to 10 μg/mL (Fig. 6B, D, F and H).

Fig. 7 shows results of initiation and promotion assays of acenaphthylene, coronene, and 9,10-DPhA. No increase of foci was observed in the initiation assay at all concentrations tested for acenaphthylene (Fig. 7A), coronene (Fig. 7C) and 9,10-DPhA (Fig. 7E). These chemicals weakly induced foci in the promotion assay at the highest concentration (Fig. 7B, D and F). Results of initiation and promotion assays for anthracene, naph-

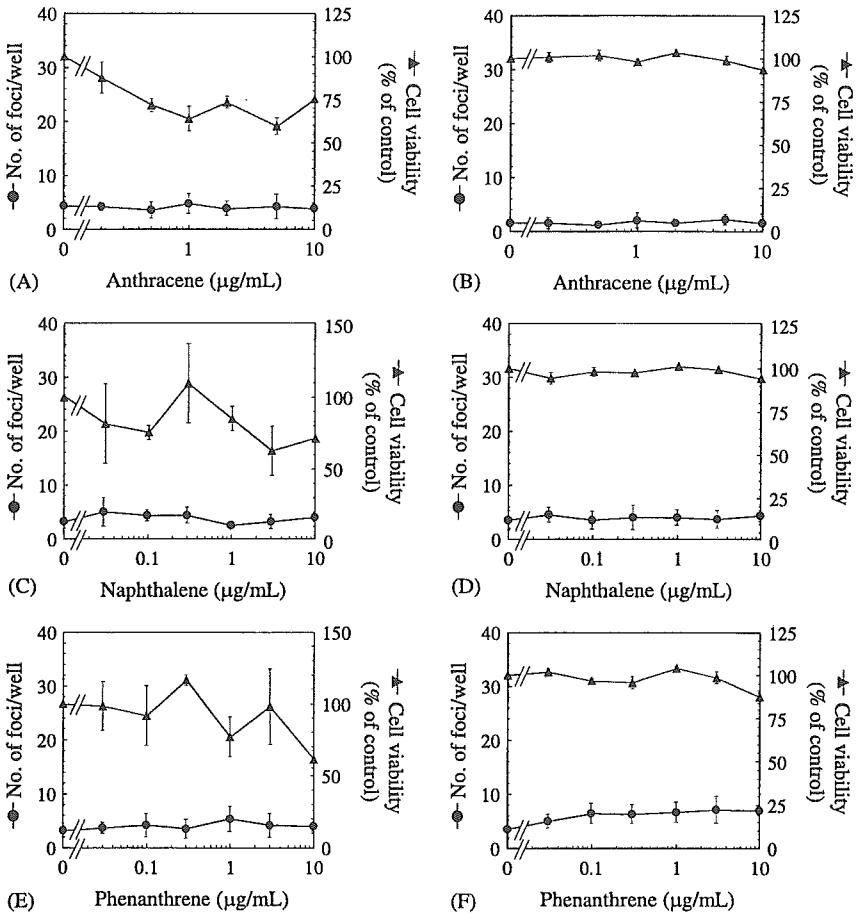

Fig. 8. Results of initiation and promotion assays of anthracene, naphthalene and phenanthrene (Group IV). A, C and E show results of initiation assay and B, D and F show results of promotion assay. $^{*}p < 0.05$, compared with solvent control. $^{**}p < 0.01$, compared with solvent control.

thalene, and phenanthrene are shown in Fig. 8. These chemicals were entirely negative in both initiation and promotion assays at concentrations up to 10 μg/mL.

4. Discussion

In addition to the Bhas promotion assay, we have developed a method for evaluation of initiating activity of chemicals using Bhas 42 cells. In the promotion assay, cells are treated with test chemicals at subconfluent condition, in which further cell division is limited, whereas the initiation assay was designed for cells at a lower cell density to be able to divide several times after treatment with test chemicals. The fact that tumor initiators could transform Bhas 42 cells only under cell proliferating conditions was important in aiding understanding of the two-stage model of chemical carcinogenesis. MNNG and MCA, both tumor initiators, were positive in the initiation assay but negative in the promotion assay. In contrast, TPA, LCA, and okadaic acid, tumor promoters [26–31], were positive in the promotion assay but were negative in the initiation assay.

Some tumor initiators are considered to have tumor-promoting activity. This recognition came from the experimental observation that repeated application at subtumorigenic doses can induce tumors in mouse skin without any post-treatment with a promoter [1]. In order to probe why repeated treatment with MNNG or MCA could not induce transformed foci in the Bhas promotion assay, an experiment with various schedules of treatment was conducted (Fig. 3). This demonstrated that MCA was effective only when cells were in a dividing phase, consistent to the current notion for the mechanism of initiator action, i.e., the fixation of genetic damage resulting in genetic alteration, namely mutation, after several cell divisions. On the contrary, the promoter, TPA, induced transformed foci when cell division was limited but it was not effective during the cell-dividing phase. It is well known that tumor promoters inhibit the interaction with neighboring cells (metabolic cooperation) [32–35].

Table 2
Summary of Bhas initiation and promotion assays for 22 tested chemicals, and published carcinogenicity and mutagenicity assay summaries

Chemical	CAS no.	IARC	Bhas 42 assay		In vivo carcinogenicity[a]		Ames' test				*E. coli*		Metabolic cooperation		MLA		References
			Initiation assay	Promotion assay	Carcinogenicity	Promotion	Frameshift mutation[b]		Missense mutation[c]		S9 (−)	S9 (+)	S9 (−)	S9 (+)	S9 (−)	S9 (+)	
							S9 (−)	S9 (+)	S9 (−)	S9 (+)							
Group I																	
MNNG	70-25-7	2A	+	−	+		+	+	+	+	+	+	+		+	+	[42,44–46,48,50,55]
MCA	56-49-5		+	−	+			+		+	−	−		+			[42,44,45,48,55]
B[a]P	50-32-8	2A	+	−	+		−	+		+			−	+		+	[38,41,42,55,57,61]
DMBA	57-97-6		+	−	+		+	+	+	I	−	−	−	+	−	+	[41,42,44,48,55,60]
AFB1	1162-65-8	1	+[d]	−	+[e]		I			+			−	+			[39,42,55]
Group II																	
TPA	16561-29-8		−	+		+		−		−			+				[1,42,62]
LCA	434-13-9		−	+	−		−	−	−	−	−	−	+		−	−	[41,44,47,60,62]
Okadaic acid	78111-17-8		−	+		+		−		−				I			[30,54,56]
B[e]P	192-97-2	3	−	+	−	+	−	+	−	−	−	−	−				[38,41–44]
B[ghi]P	191-24-2	3	−	+	−	−	−	+									[38,41,49,50]
1-NP	5522-43-0	2B	−	+	+		I	I									[39,49,50,58]
Pyrene	129-00-0	3	−	+	−	I	−	+	−	I	−	−					[38,41,42,44]
Group III																	
B[a]A	56-55-3	2A	+	+	+	+		+					−	+			[38,42,53,55]
B[b]A	92-24-0		+	+			−	+	−	−							[51]
Chrysene	218-01-9	3	+	+	+	−	−	+									[38,41,42,59]
Perylene	198-55-0	3	+	+	−	−	−	+		+					−	+	[38,41,50,52,60]
Group IV																	
Acenaphthylene	208-96-8		−	±													
Anthracene	120-12-7	3	−	−	−		−	+	−	−	−	−			−	+	[38,41,42,44,60]
Coronene	191-07-1	3	−	±	+	−	−	+									[38,41,43]
9,10-DPhA			−	±													
Naphthalene	91-20-3	2B	−	−	−		−	−	−	−	−	−			−	+	[40–42]
Phenanthrane	85-01-8	3	−	−	−	−	−	−	−	−	−	−					[38,41,42,44]

+, positive result; −, negative result; ±, equivocal result; I, inconclusive result.

[a] Carcinogenicity was tested by skin application to mice and promoting activity was assessed by mouse-skin initiation-promotion assay.

[b] Frameshift mutation was detected with TA97, TA98, TA1537 and TA1538.

[c] Missense mutation was detected with TA100 and TA1535.

[d] Initiating activity of AFB1 required metabolic activation with S9.

[e] Carcinogenicity of AFB1 was shown in liver.

Tsuchiya and Umeda [36] have demonstrated that a subconfluent phase of MNNG-initiated BALB/c 3T3 cells was the most sensitive stage for the induction of transformed foci by TPA.

Bhas 42 cells were considered as initiated cells by virtue of transfection of the v-Ha-*ras* gene. Here, however, we demonstrated that the additional treatment with initiating chemicals is effective for inducing transformed foci. Carcinogenesis is now considered to be a multi-step phenomenon. Sugimura stated in his review that several mutations could lead to tumor formation [37]. Thus, the results are interpreted as showing that initiator-induced mutations of oncogene(s) other than *ras* oncogene in Bhas 42 cells can result in the induction of cell transformation.

These discussions may pertain to a specific in vitro experimental assay, but the phenomena support the hypothetical mechanism for tumor formation in the mouse skin, where repeated treatment with tumor initiator causes genetic damage of several oncogenes in the skin cells. The basement cell layer of mouse skin comprises actively dividing stem cells, wherein genetic damage can be fixed and result in mutations. Therefore, we hypothesize that tumor formation from tumor initiator exposure does not involve promoting activity, such as disturbed interaction between neighboring cells, i.e., loss of metabolic cooperation, but involves fixation and accumulation of genetic damage after cell divisions.

In order to investigate the applicability of Bhas initiation and promotion assays to an in vitro screening for carcinogenesis, various PAHs and other initiators and promoters were evaluated by the assays; these could be classified into four groups (Table 2). Group I consists of chemicals showing positive results in the initiation assay but negative in the promotion assay. MNNG, MCA, AFB_1, and carcinogenic PAHs were classified in this group. Group II chemicals are negative in the initiation assay but positive in the promotion assay. Tumor promoters, TPA, LCA, and okadaic acid, are Group II chemicals. These promoters are also negative in bacterial mutation assays. Group III consists of chemicals showing positive response in both initiation and promotion assays (B[*a*]A, B[*b*]A, chrysene and perylene). Group IV consists of chemicals showing equivocal or negative response in both assays. These results showing that there are various kinds of PAHs with initiating and/or promoting activities suggest carcinogenic risk in mixtures of PAHs such as soot from exhaust gas, and particulate matter in polluted air.

In the present study, a metabolic activation system was not used in the examination of PAHs. Some chemicals negative in the Bhas initiation assay (Groups II and IV) are positive in the bacterial reverse mutation assay in the presence of an exogenous metabolic activation system (Table 2). It is, therefore, necessary to include a metabolic activation system in the Bhas initiation assay. To incorporate the metabolic activation system into the Bhas assay, we tried to modify the present Bhas initiation assay. AFB_1, as a model initiator that requires metabolic activation by CYP enzymes such as 2A6, 3A4 and 1A2 [24,25], was used to validate the incorporation of the metabolic activation system. AFB_1 did not induce transformed foci in the initiation assay in the absence of the metabolic activation system but it induced transformed foci in its presence.

In summary, Bhas assays for the detection of both initiating and promoting activities of chemicals are more sensitive and economical than other cell transformation assays, and deserve consideration as a promising screening tool. However, the results obtained are still limited, and further studies are necessary to confirm the applicability of the Bhas assays especially in the presence of an exogenous metabolic activation system. In addition, the Bhas assays may be able to play an important role for understanding the mechanism of chemical carcinogenesis.

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Practical threshold for micronucleated reticulocyte induction observed for low doses of mitomycin C, Ara-C and colchicine

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Micronucleus induction was studied for the DNA target clastogens mitomycin C (MMC) and 1-β-D-arabinofuranosylcytosine (Ara-C), and also the non-DNA target aneugen colchicine (COL) in order to evaluate the dose–response relationship at very low dose levels. The acridine orange (AO) supravital staining method was used for microscopy and the anti-CD71-FITC based method was used for flow cytometric analysis. In the AO method, 2000 reticulocytes were analysed as commonly advised, but in the flow cytometric method, 2000, 20 000, 200 000 and 1 000 000 reticulocytes were analysed for each sample to increase the detecting power (i.e. sensitivity) of the assay. The present data show that increasing the number of cells scored increases the statistical power of the assay when the cell was considered as a statistical unit. Even so, statistically significant differences from respective vehicle controls were not observed at the lowest dose level for MMC and Ara-C, or the lower four dose levels for COL, even after one million cells were analysed. When the animal was considered as a statistical unit, only the top dose group for each chemical showed significant increase of micronucleated reticulocytes frequency. As non-linear dose–response curves were obtained for each of the three chemicals studied, these observations provide evidence for the existence of a practical threshold for the DNA target clastogens as well as the non-DNA target aneugen studied.

Introduction

As chemical safety evaluations are performed, the existence of a threshold is an important issue when considering genotoxic carcinogens. There are two important threshold concepts, i.e. 'absolute' threshold and 'practical or biological' threshold in genotoxicity. The 'absolute' threshold is defined as a concentration below which a cell would not 'notice' the presence of the chemical, and the 'practical or biological' one is considered as a concentration below which any effect is biologically unimportant (1–3). Some chemicals clearly exhibit a threshold, and non-DNA target mechanisms of action (e.g. spindle apparatus disturbance, topoisomerase II inhibition, DNA synthesis inhibition, overloading homeostatic defence, and physiological perturbation) provide rationale for the non-linear responses that are observed (4). For instance, the spindle poison, colchicine (COL), damages spindle fibres, but the effect on chromosome movement should be detected only at the concentration that damages enough microtubules to impair the anchorage of the chromosome. This mechanism is thought to explain the threshold that is observed for this particular non-DNA target chemical (5). Up to the present, a widely held view is that genotoxic carcinogens do not have a threshold, and thus it has been difficult to determine the acceptable daily intake (ADI) safety exposure level. For this reason, such chemicals have been banned for use in daily life, most notably in food and food-related chemicals. Recently, however, discussion on the strategy for evaluating genotoxicity for risk assessment has been initiated (6–8). Moreover, in many cases and especially in the European Union, the principle of reducing exposure to unavoidable toxic compounds to levels that are as-low-as-reasonably-achievable (ALARA) has been advocated (9–11).

Adding to this complex discussion are reports by Fukushima and his group (12–14) who have demonstrated the existence of practical thresholds for the genotoxic hepatocarcinogens 2-amino-3, 8-dimethylimidazo *[4,5-f]* quinoxaline (MeIQx) and *N*-nitrosodiethylamine, and even hormesis for phenobarbital. These investigators studied carcinogenicity, glutathione *S*-transferase placental form (GSTP) positive focus, gene mutation, DNA oxidative damage and DNA adduct formation at very low dose levels. They showed GSTP positive focus induction at the dose level at which carcinogenicity could not be detected; gene mutation could be observed at the dose level at which GSTP positive foci could not be detected, and so on. Therefore, they concluded that at least practically, a threshold for carcinogenicity existed. It has been claimed that one of the shortcomings of their proposal is the lack of discussion of the sensitivity of the assays they performed, because, from a statistical view point, the power of the assay (i.e. sensitivity) largely depends on the number of cells analysed.

The micronucleus assay has been widely used for evaluating chemical genotoxicity *in vivo*. One of the characteristics of the rodent peripheral blood micronucleus assay is its simple endpoint, i.e. a small DNA containing cell inclusion in the cytoplasm of enucleated erythrocytes. Because of this simplicity, automation of analysis has been achieved by image analysis (15–17) and flow cytometry (18–23). We have developed a high performance manual method using acridine orange (AO) supravital staining (24,25). We have also developed a flow cytometric method utilizing an erythrocyte surface antigen for CD71 to identify young erythrocytes and the use of malaria infected erythrocytes as an instrument calibration standard for accurate measurement (21–23,26). In the present study, we aimed to show the dose–effect relationship of micronucleus inducers with different modes of action at extremely low dose levels. We applied two methods, i.e. the manual AO supravital staining method and flow cytometry, on three model

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chemicals: mitomycin C (MMC), which is a cross-linking agent and typical micronucleus inducer (27) frequently used as a positive control in the micronucleus assay; 1-β-D-arabinofuranosylcytosine (Ara-C), which is a long-patch repair inhibitor and known inducer of small micronuclei (28); and COL, a spindle poison that induces large micronuclei (29). Another aim was to evaluate the degree to which the statistical power of the assay depends on the number of target cells interrogated.

Further impetus to study low dose effects came from reports by Grawé *et al.* (30) and Abramsson-Zetterberg (31), who measured genotoxicant-induced micronuclei using an alternate, dual laser flow cytometric technique. We discuss the interpretation of very weak micronucleus induction, if any, at low dose levels and also the existence of a threshold effect.

Materials and methods

Chemical substances

Three micronucleus inducers with different modes of action, MMC (CAS number: 50-07-7), Ara-C (CAS number: 147-94-4), and COL (CAS number: 64-86-8) were obtained from Kyowa Hakko (Tokyo, Japan), Merck (PA, USA) and Sigma (Mo, USA), respectively. MMC was dissolved with sterile distilled water and COL and Ara-C were dissolved with physiological saline. Solvents were used as negative controls.

AO pre-coated slides for supravital staining were obtained from Toyobo Co., Osaka. Fixative, anticoagulant and other all materials necessary for diluting, fixing and shipping specimens for flow cytometric analysis were from Mouse MicroFlow® Basic Kits (transported from Litron Laboratories, NY).

Animals

Healthy seven-week-old male CD-1 mice (ICR, Charles River Japan Inc., Hino, Japan) with a mean body weight of 34.6 g were used after a week's acclimation. The mice were housed at 21 ± 1°C and 55 ± 10% relative humidity and exposed to 12-h light-dark cycle. Five mice per group were assigned randomly and were given commercial food pellets (CE-2, CLEA Japan Inc., Tokyo Japan) and tap water *ad libitum* throughout the acclimation and the experimental period.

Micronucleus assay

The highest dose level of each chemical was determined experimentally or by referring to the published data (27,32–34). The objective was to choose a high dose level that induced micronuclei slightly, but statistically significantly. We selected 0.3 mg/kg for MMC, 6 mg/kg for Ara-C, and 0.8 mg/kg for COL as the high dose levels. The six dose levels for MMC and five dose levels for Ara-C and COL were spaced by the square root of 10. A solvent control was assigned to each experiment as reference. Each chemical was delivered intraperitoneally (10 ml/kg) once and blood was collected 48 h after treatment based on data from a previous paper (25). Treatment, sample preparation and AO scoring were performed at Nitto Denko, Osaka, Japan and fixed blood samples were sent by air on dry ice to Litron Laboratories, NY for flow cytometric analysis.

All AO supravital staining slides were coded and analysed without knowledge of treatment information. All tubes containing fixed blood cells for flow cytometric analysis were also coded. All codes were broken only after analysis was completed.

AO supravital staining micronucleus assay

The AO supravital staining micronucleus assay was performed according to the method of Hayashi *et al.* (24,25). Aliquots of 5 μl of peripheral blood, obtained from the tail blood vessel of each mouse, was put on an AO coated glass slide, and immediately covered with a glass coverslip. Two thousand reticulocytes were analysed by fluorescence microscopy (Model:AHBT3-RFC, Olympus, Tokyo Japan) with blue-excitation filter set, and the number of micronucleated reticulocytes (MNRET) was scored.

Flow cytometric analysis

At the same time of AO supravital sampling, another 100 μl blood was collected via orbital sinus into the Mouse MicroFlow® basic kit-supplied anticoagulant solution using a cleaned glass capillary. Each sample was fixed in duplicate with ultracold (−80°C) methanol, agitated vigorously and immediately placed at −80°C until shipment on dry ice from Nitto Denko to Litron Laboratories for flow cytometric analysis.

On the day of analysis, samples were washed out of fixative with ~12 ml Hank's Balanced Salt Solution (HBSS). A high-density/CD71-associated fluorescence thresholding technique was used (35,36). Briefly, with this method, 80 μl of each washed cell pellet was added to polypropylene tubes

Table I. Frequencies of micronucleated reticulocytes (%) assay by manual and flow analysis

Chemical	Dose (mg/kg body wt)	Number of cells analysed				
		Manual	Flow cytometry			
		2 K	2 K	20 K	200 K	1 M
MMC	0	0.22 ± 0.06	0.23 ± 0.16	0.25 ± 0.09	0.23 ± 0.06	0.23 ± 0.05
	0.00095	0.33 ± 0.06	0.17 ± 0.04	0.21 ± 0.05	0.24 ± 0.06	0.24 ± 0.05[a]
	0.00300	0.24 ± 0.08	0.25 ± 0.11	0.27 ± 0.05	0.25 ± 0.05	0.25 ± 0.05[b]
	0.00948	0.24 ± 0.17	0.22 ± 0.09	0.28 ± 0.04	0.27 ± 0.03[b]	0.27 ± 0.04[b]
	0.03000	0.20 ± 0.06	0.22 ± 0.08	0.24 ± 0.08	0.19 ± 0.08	0.23 ± 0.08
	0.09480	0.35 ± 0.14	0.27 ± 0.10	0.25 ± 0.08	0.28 ± 0.07[b]	0.27 ± 0.06[b]
	0.30000	0.64 ± 0.15[d,b]	0.36 ± 0.10	0.40 ± 0.11[e,b]	0.41 ± 0.06[d,b]	0.39 ± 0.05[d,b]
Ara-C	0.000	0.32 ± 0.10	0.21 ± 0.05	0.21 ± 0.05	0.23 ± 0.04	0.22 ± 0.02
	0.060	0.26 ± 0.15	0.19 ± 0.07	0.24 ± 0.07	0.24 ± 0.07	0.24 ± 0.07
	0.190	0.28 ± 0.18	0.30 ± 0.06	0.23 ± 0.06	0.25 ± 0.04[c]	0.25 ± 0.03[c]
	0.600	0.32 ± 0.03	0.21 ± 0.13	0.25 ± 0.05	0.27 ± 0.05[b]	0.27 ± 0.05[b]
	1.890	0.42 ± 0.21	0.24 ± 0.11	0.29 ± 0.07[b]	0.29 ± 0.05[b]	0.28 ± 0.04[b]
	6.000	0.81 ± 0.38[d,b]	0.34 ± 0.10[d]	0.41 ± 0.11[e,b]	0.39 ± 0.09[e,b]	0.39 ± 0.09[e,b]
COL	0.00000	0.32 ± 0.08	0.22 ± 0.10	0.25 ± 0.05	0.27 ± 0.05	0.28 ± 0.06
	0.00800	0.18 ± 0.11	0.21 ± 0.09	0.22 ± 0.06	0.22 ± 0.04	0.22 ± 0.03
	0.02520	0.31 ± 0.13	0.16 ± 0.09	0.20 ± 0.04	0.22 ± 0.04	0.21 ± 0.04
	0.08000	0.13 ± 0.08	0.22 ± 0.11	0.23 ± 0.06	0.21 ± 0.05	0.21 ± 0.03
	0.25200	0.34 ± 0.19	0.21 ± 0.08	0.28 ± 0.05	0.26 ± 0.04	0.27 ± 0.05
	0.80000	0.56 ± 0.19 [b,d]	0.41 ± 0.14	0.41 ± 0.13[b]	0.44 ± 0.13[b]	0.44 ± 0.13[b]

Data are shown as mean ± SD (%) of five mice.
[a]Only 881 389 instead of 1 M cells were analysed in one of five mice.
[b]Fisher's exact test (all groups) significant difference at 1%.
[c]Fisher's exact test (all groups) significant difference at 5%; no mark, not significant.
[d]Student's *t*-test (20 and 200 K, and 1 M groups) significant difference at 1%.
[e]Student's *t*-test (20 and 200 K, and 1 M groups) significant difference at 5%.

containing 80 μl RNase/antibodies (1.0 ml HBSS, 10 μl anti-CD71-FITC, 5 μl anti-CD61-PE and RNase A at 5 mg/ml). Antibodies, and all other flow cytometry reagents, including fixed malaria-infected rodent blood (malaria biostandard), were from Mouse MicroFlowPLUS® Kits (available from Litron Laboratories, Rochester, NY, and BD Biosciences-Pharmingen).

Following successive 30-min incubations at 4°C and ~37°C, the cells were placed at 4°C until analysis (same day). For analysis, each sample was resuspended in approximately 1.5 ml propidium iodide (PI) staining solution. Of the stained blood sample 100 μl was transferred to a separate tube containing 400 μl PI. This diluted sample was used to determine the percentages of reticulocytes and micronucleated normochromatic erythrocyte (MN-NCE) of each blood sample by the analysis of 1 000 000 (1 M) total erythrocytes.

The corresponding undiluted sample was then analysed using the CD71-thresholding technique whereby CD71-negative erythrocytes (the majority of the cells) were omitted from acquisition (35,36). The frequency of micronuclei was then measured for each sample using each of the following stop modes: 2000 (2 K), 18 000 (18 K), 180 000 (180 K) and 800 000 (800 K) reticulocytes. By adding the successive values, percentage of MNRET frequencies could be calculated based on the analysis of 2 K, 20 000 (20 K), 200 000 (200 K) and 1 M reticulocytes.

Statistical analysis

P-values for each comparison with respective controls were calculated by Fisher's exact method. For the flow cytometry data based on 20 K or more cells analysed, a Student's *t*-test was used after normality of the data was confirmed. When determining statistical significance, a Bonferroni correction was used to adjust for the multiple (i.e. 5) comparisons made.

Results

The group means of five mice/group are summarized in Table I for the AO supravital staining method based on the observation of 2 K reticulocytes and for the flow cytometric method based on the observation of 2, 20 and 200 K, and 1 M reticulocytes. The *P*-values for all three chemicals were <0.01 at the highest dose group when the Fisher's exact test was applied. However, when considering individual differences, this was not the case for all COL high dose datasets when evaluated using the Student's *t*-test. Dose-response relationship curves of MMC, Ara-C and COL are shown in Figure 1. Dose-response curves of each chemical were similar between AO supravital analysis and flow cytometric analysis, although there was a tendency for the absolute values of induced MNRET to be higher by the AO supravital method than by the flow cytometric method, especially at higher dose levels.

It is evident that the variation among mice in each dose group decreased depending on the number of cells analysed. Even so, individual differences among animals in each group were observed even when 1 M reticulocytes were scored per sample. Likewise, the smoothness of the dose–response curves tended to increase as the number of cells analysed increased. As a representative example, Figure 2 shows individual scattergrams of MMC at 2, 20 and 200 K, and 1 M reticulocytes analysed. There are not, however, essential differences among results based on the number of cells analysed.

Discussion

According to the present data, MNRET frequencies obtained using the AO supravital staining method tended to be higher than those by flow cytometric analysis. This phenomenon may be explained by modest differences in the age cohort of reticulocytes analysed by each method, i.e. AO supravital staining, where the analysis is restricted to Types I, II and III reticulocytes, and the flow cytometric method, where the analysis was restricted to reticulocytes with the CD71-positive phenotype. In both analysis procedures, the method of defining reticulocytes was kept consistent for all samples.

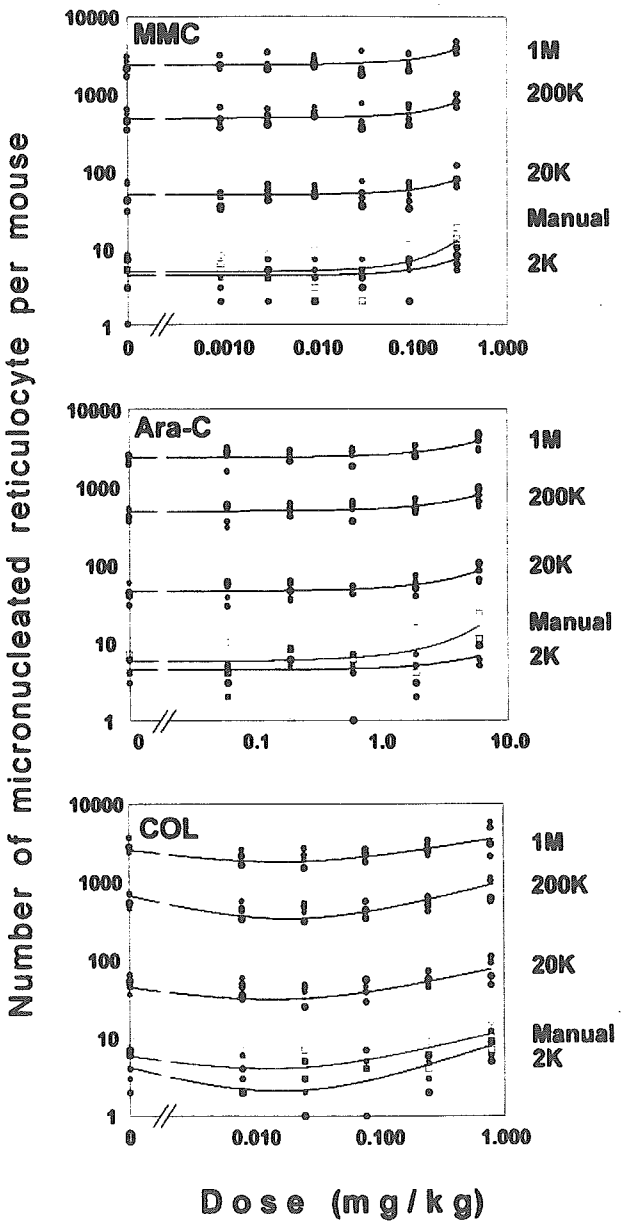

Fig. 1. Dose–response curves for MMC, Ara-C and COL. Each symbol shows individual number of micronucleated reticulocytes. In each dose group, a colour shows identical mouse for each experimental size for flow cytometry (closed symbol) and manual analysis (open symbols).

For flow cytometry, our data show that 20 and 200 K were sufficient to obtain reliable data for the evaluation of micronucleus induction. While flow cytometric data associated with analysis of 2 K reticulocytes were slightly more variable than corresponding microscopy-based measurements, automated acquisition of 20 K reticulocytes yielded essentially equivalent power of detection compared to the AO method. When 200 K and 1 M reticulocytes were analysed per specimen, assay sensitivity was observed to improve significantly, as evidenced by the lower doses at which statistical significance was noted when evaluated by Fisher's exact test. This was true when cells were the statistical unit evaluated, but not when individual mice were considered to be the unit. This issue is discussed

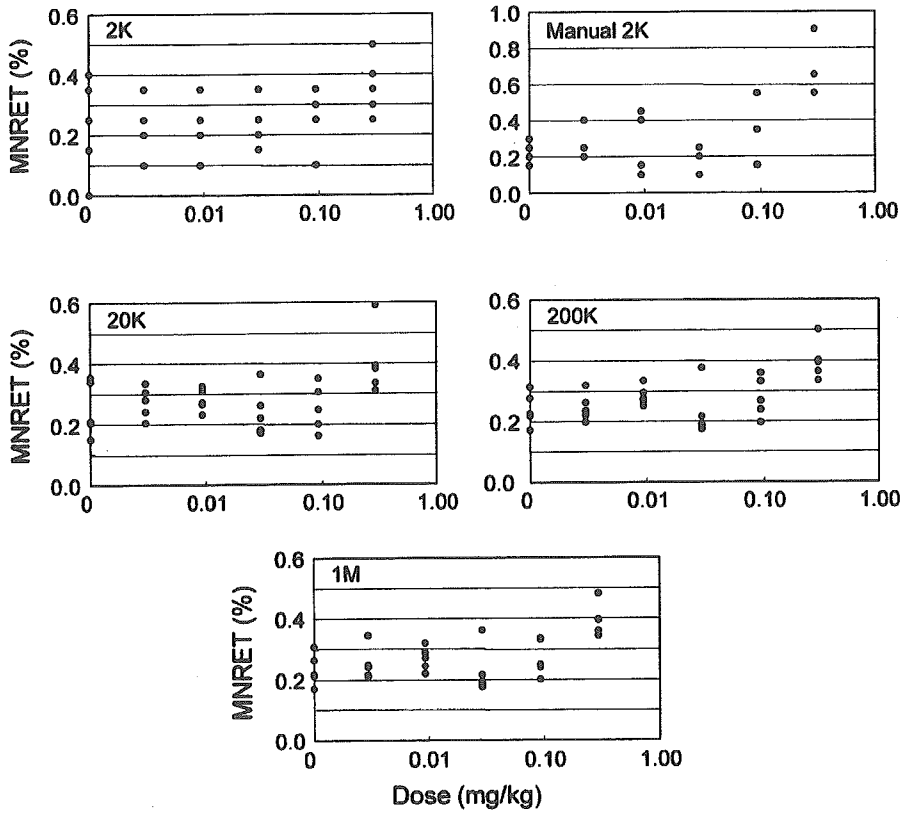

Fig. 2. Frequencies of micronucleated reticulocytes (%) for MMC based on 2, 20 and 200 K, and 1 M for flow cytometry and 2 K for manual microscopy. Each symbol shows the frequency of one mouse with exceptions because of overlapping (five animals per group).

in a companion paper wherein data from a reconstruction experiment are presented (37). In that study it was observed that when 200 K or 1 M cells per sample were analysed, the scoring error decreased and converged to a respective value. However, in the present study, the differences among the individual animals became apparent and there was more data variability within each dose group.

Grawé *et al.* (30) reported low dose effects on MMC, diepoxybutane, cyclophosphamide and COL using flow cytometry (stained with Hoechst 33342 for DNA and thiazole orange for RNA). Generally, one animal per dose level was used and 200 K polychromatic erythrocytes were analysed. Our present data agree with the COL data showing a non-linear dose–response, but in contrast to our present results, they showed linear dose–response relationships for extremely low dose levels of MMC, 0.007 mg/kg; diepoxybutane, 0.44 mg/kg; and cyclophosphamide, 0.3 mg/kg. Abramsson-Zetterberg *et al.* (31) also showed linear dose–response curves for acrylamide down to very low dose levels (1 mg/kg body wt) and observed no threshold. Although we did not evaluate acrylamide as a model chemical in this study, we did not observe linear dose–response curves, even for MMC and Ara-C, which are DNA-reactive clastogens. We could not find any rationale to explain such differences at the present time, and believe it is necessary to continue studying chemicals that interact directly with DNA to better understand their effect at extremely low dose levels.

To confirm biological and statistical relevance of the present study data, we performed a reconstruction model experiment using serial dilutions of malaria-infected blood with non-treated mouse blood (37). The samples were analysed by flow cytometry based on 2, 20 and 200 K (Experiment 1) and up to 1 M (Experiment 2) cells. These data show extremely high performance of the flow cytometric assay in terms of accuracy, especially when 200 K or more reticulocytes are evaluated per specimen. This result shows that the statistical power of the assay depended on the number of analysed cells. This dilution experiment supports our conclusion that thresholds were present for micronucleus induction in reticulocytes for the three model chemicals analysed at very low dose levels.

It might be difficult to prove the existence of thresholds in toxicology or biology in general using statistical methods (38), and it is not easy to discuss the threshold concept from the biological viewpoint. However, when we only consider mean values, for example in the case of Ara-C, the MNRET frequencies appeared to increase linearly, but when the individual values (Table II) were evaluated, the differences among

Table II. Individual data in the Ara-C study by flow cytometry analysed 1 M cells

Mouse ID	Dose (mg/kg body wt)					
	0.00	0.06	0.19	0.60	1.89	6.00
1	0.24	0.26	0.25	0.28	0.34	0.39
2	0.21	0.32	0.30	0.31	0.24	0.31
3	0.19	0.16	0.23	0.31	0.27	0.50
4	0.25	0.28	0.21	0.18	0.24	0.46
5	0.22	0.16	0.24	0.25	0.29	0.29
Mean	0.22	0.24	0.25	0.27	0.28	0.39
SD	0.02	0.07	0.03	0.05	0.04	0.09

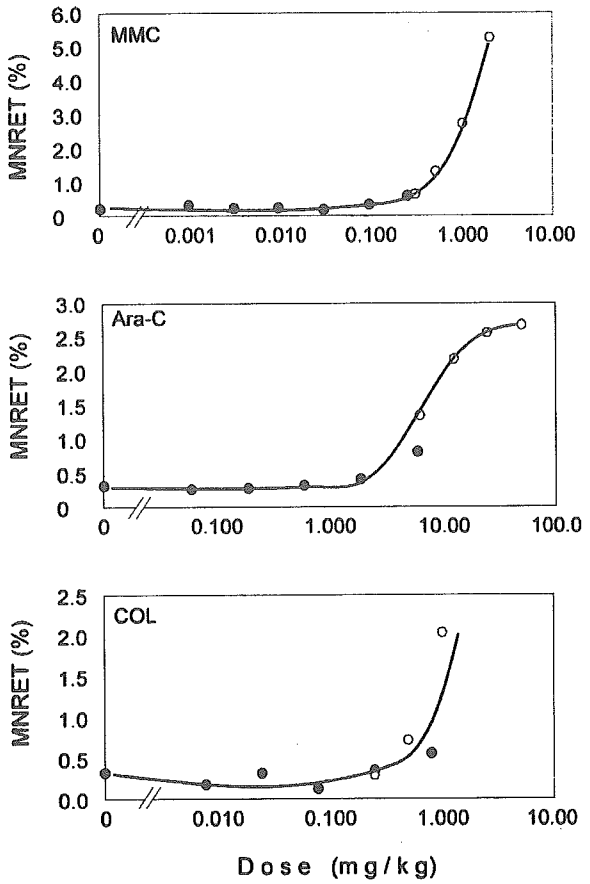

Fig. 3. Dose–response curves of MMC, Ara-C and COL in wider range of dose levels superimposed to the present data with the results of our published data. MMC: Hara *et al.*, 1992 (Table I) Study 1, 48 h; Ara-C: Iwakura *et al.*, 1992 (Table I) Laboratory B, 48 h; COL: Kondo *et al.*, 1992 (Table II) Laboratory 4, 48 h.

animals became clearer. Two individuals at the lowest dose group (mouse ID 3 and 5) demonstrated the lowest MNRET frequency (0.16%) even when including the vehicle control group. The control data variation in the Ara-C experiment was less than that in the MMC or COL experiments. When the Student's *t*-test was applied to the data obtained by flow cytometry based on the analysis of ≥20 K cells, only the highest dose groups were significantly different from the concurrent control for MMC and Ara-C. Therefore, we should consider the individual animal differences to determine the micronucleus induction ability of the chemical being studied. It is likely that at very low doses of genotoxicant, as were studied here, individual differences in DNA repair activity, metabolism related cytochrome P450 activity, or anti-oxidant concentration etc. play a larger role in dictating the micronucleus incidence of each individual of an exposure group.

In addition, Figure 3 shows the dose–response curves of these three model chemicals in a wider range of dose levels superimposed on the results of published data (27,32,34) using the same strain of mouse and same manner of experiments by AO supravital staining. Closed circles represent the data from the present study and open circles are published data for higher dose levels. The dose–response curves became clearer by adding the data from higher dose levels and the practical threshold or the threshold were shown. Moreover, COL even shows the tendency of inhibition in induction of micronuclei at extremely low dose levels (U- or J-shape response). Hormesis usually implies increased repair capability or some other protective, adaptive response in the field of radiobiology (1). The COL data presented herein is suggestive of a hormesis-like effect, and further work aimed at elucidating the mechanisms and significance of this observation is warranted.

Considering the data detailed above, an important conclusion is the existence of a biological or practical threshold in the genotoxicity assay on DNA target chemicals as well as non-target chemicals. Although we used only three model chemicals in the present study, we could draw the following conclusions: (i) the flow cytometric micronucleus assay method is a powerful tool when ≥20 K cells were analysed; (ii) the AO supravital staining micronucleus assay method and the flow cytometric method gave qualitatively similar results; (iii) when the cell is considered the statistical unit and more cells are analysed, both power and assay sensitivity at lower dose levels is significantly enhanced as evidenced by the significant differences observed when compared to vehicle control; and (iv) non-linear dose–response curves were obtained for the model chemicals studied here when evaluating the individual animal as a unit, suggesting the existence of a practical threshold for the DNA target micronucleus inducers (MMC and Ara-C) as well as the non-DNA target chemical (COL).

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Short Communication

4-Oxo-2-hexenal, a Mutagen Formed by ω-3 Fat Peroxidation, Causes DNA Adduct Formation in Mouse Organs

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Abstract: To identify mutagens formed in a model reaction of lipid peroxidation, linolenic acid methyl ester and hemin were reacted with dG. As a result, a 4-oxo-2-hexenal-dG adduct (dG*) was identified in the model reaction mixture. The 4-oxo-2-hexenal (4-OHE) showed mutagenic activity in the Salmonella typhimurium strains TA100 and TA104. After 4-OHE was orally administered to mice, dG*, 4-OHE-dC- and 4-OHE-5-methyl-dC adducts were detected in esophageal, stomach and intestinal DNA. In the vapor phase released from the methyl linolenate-hemin model system, and in the smoke released during the broiling of fish, 4-OHE was detected by GCMS. The 4-OHE seems to be produced by the auto-oxidation of ω-3 polyunsaturated fatty acids. These results provide a warning to workers dealing with ω-3 fats, who may be exposed to this volatile mutagen.

Key words: 4-oxo-2-hexenal, ω-3 polyunsaturated fatty acids, DNA adduct

It is important to identify environmental mutagens and to remove them to prevent cancer in industry workers. We have been interested in the mutagens produced by lipid peroxidation[1,2], because they may be formed during the extraction and purification of edible oils, or by frying foods with vegetable-oil. Many low molecular-weight mutagens are released into the atmosphere by oil factories, the food industry, and restaurant kitchens. Various lipid peroxide compounds are produced by the oxidative fragmentation of fats catalyzed by iron and heat or light. Our study focused on the identification of mutagens in the lipid peroxide products formed from ω-3 polyunsaturated fatty acids, such as linolenic acid.

It is well known that many mutagens react with DNA components, particularly with deoxyguanosine (dG)[3]. To identify the mutagens formed in a model reaction of lipid peroxidation, linolenic acid methyl ester and hemin were reacted with dG in phosphate buffer (pH 7.4), as an emulsion for 72 h at 20°C. The dG-mutagen adducts were isolated by HPLC, and their structures were determined by mass-, UV-, ^{1}H- and ^{13}C-NMR spectra. As a result, the 4-oxo-2-hexenal-dG adduct (dG*) was identified in the model reaction mixture. M+Na, m/e 384.1256 (384.1284 calcd. for $C_{16}H_{19}N_5NaO_5$); UV λmax in H_2O, 228, 280 nm. The structure was confirmed by comparison with an authentic dG* sample prepared from synthetic 4-oxo-2-hexenal (4-

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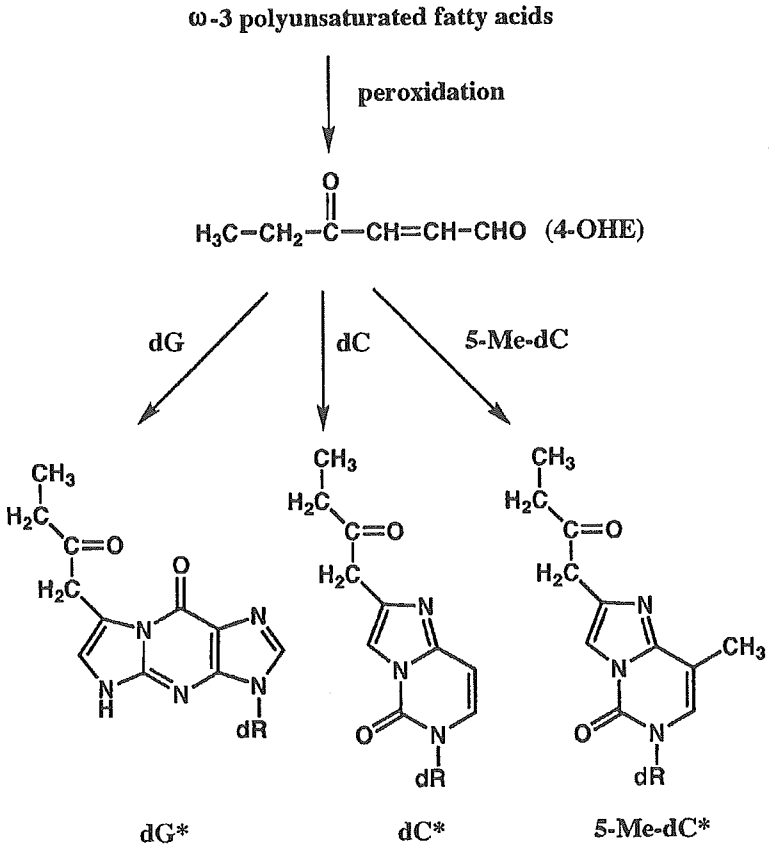

Fig. 1. Structures of DNA adducts formed by 4-oxo-2-hexenal.

OHE) and dG, and by X-ray crystallography of the 4-OHE-9-ethylguanine-adduct. The 4-OHE was mutagenic in the Salmonella strains, TA100 and TA104, without S9 mix. Its specific mutagenic activities against TA100 and TA104 were 78 and 67 revertants/μg, respectively. The mutagenicity of 4-OHE has not been reported thus far. It also induced apoptosis in human HL-60 cells. This is reasonable because DNA damage is a common trigger for apoptosis induction in mammalian cells[4].

The 4-OHE reacts not only with dG but also with dC and 5-methyl-dC to yield adducts (dG*, dC*, 5-methyl-dC*). The structures of dC*and 5-methyl-dC* were determined mainly by mass-, ^{1}H- and ^{13}C-NMR- spectra. dC*: M+Na, m/e 344.1192 (344.1222 calcd. for $C_{15}H_{19}N_3NaO_5$); UV λmax in H_2O, 227 nm. 5-methyl-dC*: M+Na, m/e 358.1372 (358.1379 calcd. for $C_{16}H_{21}N_3NaO_5$); UV λmax in H_2O, 278 nm. The structure of dC* was confirmed by X-ray crystallography. After the reaction of 4-OHE with double stranded calf thymus DNA, the yield of these DNA modifications were dC* > 5-methyl-dC* > dG*. The more efficient formation of dC* than dG* in DNA may be due to the fact that the amino group of dC in DNA is located in the major groove, while that of dG is in the minor groove and is more sterically hindered. As a prelude for testing its genotoxicity *in vivo*, 4-OHE (3 mg) was orally administered to mice, and after 24 h, the DNA was isolated from the esophagus, stomach, intestine, liver and kidney. The DNA was enzymatically digested to deoxynucleosides, and was subjected to LC/MS/MS analyses. Particularly, in the esophagus, stomach and intestine DNA, dC* was detected in the range of 2.0, 5.5 and 1.1 adducts / 10^7 dC, respectively. Lower amounts of 5-methyl-dC* and dG* were also detected in these organ DNAs.

It is important to confirm the formation of 4-OHE in a model system of lipid peroxidation, as well as during the heat processing of edible vegetable oil, and during cooking. From ethylacetate traps (extracts) of the vapor phase released from the heated methyl linolenate-hemin model system, and the smoke released during the broiling of fish, 4-OHE was detected by GCMS. Commercial perilla oil, which is rich in linolenic acid (40–50 μg/g), the edible part of broiled fish (5–170 μg/g), and various fried foods (5–50 μg/g) also

contained high levels of 4-OHE. It was present at an especially high concentration (70–170 μg/g) in broiled saury. However, 4-oxo-2-nonenal[5], a cognate longer carbon chain derivative, was only detected in an one order of magnitude lower range in these food products. This may be due to the fact that the major natural ω-3 polyunsaturated fatty acids, such as linolenic acid, DHA and EPA, the precurser of 4-OHE, are more easily oxidized than ω-6 fatty acids, such as linoleic acid.

Workers involved in the manufacturing of vegetable oils, such as colza oil and soybean oil, which contain linolenic acid, will be exposed to 4-OHE during the crude oil extraction and purification. The same is true for workers manufacturing instant cup noodles and snack foods, and producing EPA and DHA from fish oil. Kitchen workers involved in frying foods or broiling fish in restaurants may also be exposed to 4-OHE. Since 4-OHE induces DNA adduct formation in experimental animal organs, it is possible that 4-OHE is an important human carcinogen. Further studies on the carcinogenicity of 4-OHE and its detection in working environments will be required.

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Regular Article

Characterization of Genotoxicity of Kojic Acid by Mutagenicity in Salmonella and Micronucleus Induction in Rodent Liver

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Three lots of kojic acid (KA) which were produced for use as a reagent, food additive and in cosmetics were shown to be mutagenic in *S. typhimurium* TA100 with or without S9 mix, with a specific activity of around 100 revertants per mg of KA. Since there are contradictory reports on genotoxicity of KA, we examined, using HPLC, whether the mutagenicity to *S. typhimurium* is due to KA itself, or due to contaminants present in the KA samples. Although two UV absorbing fractions were separated by HPLC, mutagenicity was detected only in the major fraction and the specific mutagenic activity of KA did not change before and after HPLC separation. The material in the major peak fractions on HPLC was confirmed to be KA by NMR. Thus it was demonstrated that KA itself is mutagenic and no mutagenic contaminants were detected in the three lots of samples. Since KA is known to produce liver tumors in mice, we further examined the genotoxicity of KA in the liver of rodents. KA induced micronuclei (MN) in the regenerating liver of adult mice by its gastric intubation at 1 g per kg body weight. However, no MN were induced in young mice (3 weeks old) without partial hepatectomy. Since it was recently found that KA had no tumor-initiating activity in the liver of mice in a two-step carcinogenicity study, there is no evidence that the genotoxicity detected in the mouse liver is involved in liver carcinogenesis.

Key words: mutagenicity, liver micronuclei, genotoxicity, *S. typhimurium*, kojic acid.

Introduction

Kojic acid (KA) [5-hydroxy-2-hydroxymethyl]-4*H*-pyran-4-one; CAS No. 501-30-4; (Fig. 1)] is a natural substance produced by various fungi, *Penicillium* spp., *Aspergillus orizae, flavus and lamarii*, and also by certain bacteria (1). KA which had been used as a food additive for the prevention of enzymatic browning of shell fish, raw crabs and shrimp, owing to its inhibitory activity on tyrosinase, was found to be genotoxic *in vitro*, inducing *his*$^+$ reverse mutations in *S. typhimurium* (2-5). It was also found to be genotoxic *in vivo*, inducing micronuclei (MN) in peripheral blood of rats (6).

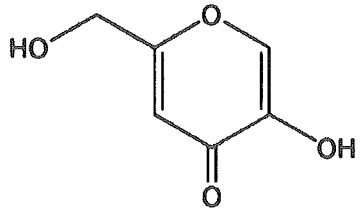

Fig. 1. Structure of kojic acid (KA).

KA had been reported to induce hepatomas and thyroid adenomas in mice (7). Thyroid adenomas or hyperplasia production in the mouse or rat was suggested to be due to promoting activity of KA (7-9). In contrast, the possibility of involvement of genotoxicity of KA in mouse hepatoma development could not be excluded (10). In 2003, the Ministry of Health, Labour and Welfare, Japan noticed that KA was no longer used for prevention of browning of shell fish in market due to the development of modern technology, and KA was withdrawn from the list of existing food additives in the same year. Since KA is a fungal product, soy sauce, miso and sake were suspected to contain KA. Out of 32 samples of fermented foods examined for the presence of KA, three samples were found to be positive, but the levels were not so high, being at the maximum, 1 ppm (personal communication by Dr. Tamio Maitani, National Health Sciences, Tokyo, Japan, Dec. 7, 2005). Further, KA is still used in cosmetics as a skin lightening agent (quasi-drug) at concentrations of up to 1.5% due

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to its inhibiting action on human melanocyte tyrosinase (11). Thus, the safety of KA needs to be confirmed from various points.

Recently, results of an extensive study on genotoxicity of KA have been reported, in which KA showed only weak mutagenicity without a dose-dependent response in *S. typhimurium* (12). Many contradictory or inconsistent results had been reported for the genotoxicity of KA. KA induced MN in peripheral blood, but not in the liver of young rats by a single application through gastric intubation (6). However, it did not induce MN in bone marrow of mice (12,13) by a single application through intraperitoneal injection. Furthermore, MN were not induced in human keratinocytes SVK14 nor in human hepatocellular carcinoma cells, HepG2, *in vitro* (12) but were induced in human lymphoblastoid cells, TK6 and WTK-1 (personal communication by Dr. Masamitsu Honma, National Institute of Health Sciences, Tokyo Japan, December 12, 2005). KA did not induce $HPRT^-$ mutations in Chinese hamster V79 cells or mouse L5178Y cells (12), but induced TK^- mutation in TK6 ($TK^{+/-}$) and WTK-1 ($TK^{+/-}$, $P53^{-/-}$) cells (unpublished observations, Yu F Sasaki). As for chromosome aberrations and sister chromatid exchanges *in vitro*, positive results were reported in Chinese hamster cells (5).

Since there had been no standards for composition of KA in food hygiene law, while the manufacturers' specifications for cosmetic use was at least 97% pure, there was a possibility that the discordance reported for genotoxicity was due to differences in composition of KA samples used. Thus, it is important to clarify whether the mutagenicities of some KA samples were due to KA itself or contaminant included. In the present study, samples of various lots of KA produced by different companies that are used as a food additive, reagent and cosmetics ingredient were purified by HPLC. The resulting fractions were tested in order to determine whether the reported mutagenicity in *S. typhimurium* was due to KA itself or to another component present in the KA samples. This testing showed that the mutagenicity is due to KA itself and not to contaminants. As KA has been reported to be hepatocarcinogenic in mice (7), we further examined whether KA shows *in vivo* genotoxicity in rodent liver, by MN assay.

Materials and Methods

Chemicals: Kojic acid, reagent grade, lot no. 052K2516 was purchased from Sigma (St. Louis), 5312 used for food additive (content is 100.6%) was supplied from Alps Pharmachemical Industry (Gifu, Japan) and 2Y181 [at least 97% pure, but more typically (by HPLC) was >99% pure] used for cosmetics was supplied from Sansho Seiyaku Co., Ltd (Fukuoka, Japan). For *in vivo* genotoxicity studies, KA for food additive provided by Alps Pharmachemical Industry was used. Cyclophospamide, diethylnitrosamine (DEN), dimethyl sulfoxide (DMSO), 4-nitroquinoline 1-oxide and trifluoroacetic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan), and 1,2-dimethylhydrazine (1,2-DMH) and benzo[*a*]pyrene were from Tokyo Kasei Kogyo (Tokyo, Japan). Solutions of KA were prepared immediately before use.

HPLC separation: Analytical HPLC was performed under three systems. System 1; an LC-20A series from Shimadzu Co. (Kyoto, Japan) equipped with Shimadzu SPD-M20A photodiode detector. A Mightysil RP-18 GP column (5 μm particle, 3 mm i.d. $\times$50 mm; Kanto Chemical Co., Tokyo, Japan) was used at 40°C, with an isocratic eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) and a flow rate of 0.15 mL/min: System 2; a Shimadzu LC-10A series was used with a Mightysil RP-18 GP column (5 μm particle, 4.6 mm i.d. $\times$250 mm), an eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) and a flow rate of 0.7 mL/min: System 3; the same equipment and column with system 2, but the eluent used was 0.1% propionic acid-0.05N perchloric acid and a flow rate of 0.7 mL/min. Preparative HPLC was performed on an LC-8A series from Shimadzu Co., using a large volumetric flow cell in the UV detector. A Mightysil RP-18 GP column (5 μm particle, 10 mm i.d. $\times$250 mm; Kanto Chemical Co., Tokyo, Japan) and an eluent of methanol-0.05 % v/v trifluoroacetic acid (3:97) were used with a flow rate of 4.0 mL/min. After separation, each fraction was lyophilized and weighed. All fractions were subjected to mutagenicity testing.

NMR analysis: ^1H and ^{13}C spectra were recorded on an ECP-600 spectrometer (JEOL Ltd., Akishima, Tokyo) using DMSO-d_6 as a solvent.

Mutagenicity assay: The mutation assays were performed using *S. typhimurium* TA100 with and without S9 mix (14). The S9 purchased from Oriental Yeast Co. Ltd (Tokyo, Japan) was prepared from Crj:CD (SD), male rat treated with phenobarbital and 6-naphthoflavone. KA was dissolved in 0.1 mL of distilled water and the assay was performed by the method of preincubation (15). For the mutagenicity test, after separation of samples by HPLC, 1 mg of dried residues was applied to a plate for each of fractions 6, 8 and 9. For fraction 7, a dose-dependent response with 0.5, 1.0 and 1.5 mg/plate was examined and the specific activity was calculated based on the linear regression of the least square method. For other fractions, the residues were dissolved in 500 μL water, and 10 or 100 μL were applied to each plates. Statistic analysis was performed by multiple regression analysis.

Animal and KA administration: Male ddY mice of 3 and 8 weeks old, and male Fischer 344 rats of 8 weeks