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Genotoxic and Nongenotoxic Effects of Glycidyl Methacrylate on Human Lung Fibroblast Cells[1]

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Objective To evaluate the genotoxic and nongenotoxic effects of short-term exposure to glycidyl mathacrylate (GMA) on human lung fibroblast cells (2BS cells) *in vitro*. **Methods** DNA strand breakage was determined by single cell gel electrophoresis, and DNA ladder formation assay and flow cytometric analysis were carried out to detect apoptic responses of cells to GMA exposure. The HPRT gene mutation assay was used to evaluate the mutagenicity, and the effect of GMA on gap junctional intercellular communication (GJIC) in the exposed cells was examined with the scrape loading/dye transfer technique. The ability of GMA to transform 2BS cells was also tested by an *in vitro* cell transformation assay. **Results** Exposure to GMA resulted in a dose-dependent increase in DNA strand breaks but not apoptic responses. GMA was also shown to significantly induce HPRT gene mutations and morphological transformation in 2BS cells *in vitro*. In contrast, GMA produced a concentration-dependent inhibition of GJIC. **Conclusions** GMA elicits both genotoxic and nongenotoxic effects on 2BS cells *in vitro*. The induction of DNA damage and gene mutations and inhibition of GJIC by GMA may casually contribute to GMA-induced cell transformation.

Key words: Glycidyl methacrylate; DNA damage; Comet assay; HPRT gene mutation; Gap junctional intercellular communication; Cell transformation

INTRODUCTION

Glycidyl Methacrylate (GMA) is unique among the family of acrylic monomers, offering dual functionality through acrylic and epoxy groups. The dual functionality of GMA brings together the chemical resistance of an epoxy with the weatherability of an acrylic, making GMA a wide application as a comonomer in resin, coating, adhesive and plastic industries[1-2]. Exposure to GMA can occur at the workplaces where it is produced or used. As GMA is one among other epoxides which are generally direct-acting mutagens in *Salmonella typhimurium*, and some of which have been identified as rodent carcinogens or potential human carcinogens[3-4], exposure to GMA is thus of an occupational health concern. Studies from our laboratory as well as that from others have demonstrated that GMA

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displayed direct-acting mutagenicity in the *Salmonella*/microsome test system and increased micronucleus frequency in bone marrow of mice or cultured V79 cells[4,5-7]. Premutagenic lesions or gene mutations were also observed in plasmid DNA or mammalian cells exposed to GMA *in vitro*[6,8]. It has been shown that covalent binding of GMA to mammalian DNA, increased DNA repair in human and rat lymphocytes and elevated abnormality rate of sperm cells in mice occurred after exposed to GMA[9]. Formation of GMA-DNA adducts in blood and various organs of rats orally administrated with GMA was also determined[10]. Our recent *in vitro* studies further showed that GMA could induce morphological transformation of several types of mammalian or human cells with neoplastic properties, demonstrating its carcinogenic potential[11-14]. However, the mechanism by which GMA induces cell transformation remains to be fully elucidated. Although mutation and/or hyperexpression of p53, c-myc and GST-*p* are suggested to be of importance in GMA-induced cell transformation[11,13,15], the genotoxicity of GMA especially effects of acute GMA exposure do not seem to have been extensively investigated *in vitro*. On the other hand, it has become clear that nongenotoxic mechanisms are also involved in cell transformation, while the accumulation of genetic changes in a somatic cell is considered essential for this process[15]. In this regard, however, little is known about the nongenotoxic effects of GMA on cell transformation so far.

In this study, the ability of GMA to induce DNA strand breaks in the 2BS human lung fibroblast cells (2BS cells) was determined by the single cell gel electrophoresis or comet assay. To verify whether apoptosis was associated with the GMA-induced DNA damage, DNA ladder formation assay and flow cytometrc analysis were performed under the same conditions. GMA was also tested for its mutagenic and transforming activity in 2BS cells by the hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene mutation assay and *in vitro transformation* assay, respectively. To explore whether GMA elicits potential nongenotoxicity implicated in transforming processes, effect of GMA on gap junctional intercellular communication (GJIC) was examined by the scrape loading/dye transfer (SL/DT) technique. All these experiments were performed using the same cell type and GMA from a single source. These studies are of value in assessing the early genotoxic and nongenotoxic effects of GMA and should provide more insight into the mechanisms by which GMA induces cell transformation.

MATERIAL AND METHODS

Chemicals

GMA (CAS No. 106-91-2, >99.9% pure) was obtained from the No. 601 Research Institute (Beijing, China). *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG), 12-tetradecanoylphorbol-13-acetate (TPA), 6-thioguanine (6-TG), proteinase K, propidium iodide, ethidium bromide, Lucifer yellow CH and dimethyl sulphoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of reagent grade and purchased from Sino-America Biotechnology Company (Beijing, China) unless mentioned otherwise.

Cells and Cell Culture

The 2BS human lung fibroblast cell line was obtained from Beijing Research Institute of Biological Products (Beijing, China). The cells were grown exponentially in Eagle's minimum essential medium (Gibco BRL, Gaitherburg, MD, USA) supplemented with 4 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% heat- inactivated (56℃ for 30 min) fetal bovine serum (FBS) and maintained at 37℃ in a humidified atmosphere

containing 5% CO_2. They were subcultured twice a week using 0.25% trypsin solution (Gibco BRL) in phosphate buffered saline (PBS).

Comet Assay

Exponentially growing 2BS cells were plated at a density of 2.5×10^5 cells/ 25-cm^2 flask containing 5 mL complete culture medium, and incubated at 37℃ with 5% CO_2. Twenty-four hours later, the cells were treated with 0.5-5 μg/mL GMA for 2 h, or with 5 μg/mL GMA for up to 24 h. DMSO (0.1%) and potassium dichromate (PD, 300 μg/mL) were used as solvent and positive controls, respectively. After treatment, the cells were washed with PBS, detached by trypsinization, centrifuged and resuspended to an approximate density of 5×10^6 cells/mL in PBS on ice. Cell viability was examined by trypan blue exclusion technique and expressed as percentage of that in DMSO controls.

The comet assay was performed under alkaline conditions as described by Singh *et al.*[17], with some modifications. Briefly, fully frosted microscope slides were pre-coated with 0.5% normal melting point agarose (Sigma) in water. Two gels of 75 μL of mixture of cells and 0.5% low melting point agarose (LMPA, Sigma) in Ca^{2+}- and Mg^{2+}-free PBS were set on each pre-coated slide as duplicates. LMPA (110 μL) was then applied to cover the cells. The slides containing agarose-embedded cells were immersed in cold, freshly prepared lysing solution (2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris-HCl, 1% sodium sarcosinate, pH 10; 1% Triton X-100 and 10% DMSO added just before use) and kept at 4℃ for 1 h. The slides were then placed on a horizontal gel-electrophoresis tank and covered with cold alkaline electrophoresis buffer (0.3 mol/L NaOH, 1 mmol/L Na_2EDTA, pH 13) for 30 min prior to electrophoresis at 25 V and 300 mA for 15 min. After electrophoresis, the slides were neutralized in 0.4 mol/L Tris buffer (pH 7.5) and stained with ethidium bromide. All of the steps described above were conducted under very dim light or in the dark to prevent additional DNA damage. Comets were visualized using a fluorescence microscope (IX 50, Olympus) with an image analysis system (Image-Pro Plus, Media Cybernetics, Inc., Silver Spring, MD, USA). One hundred cells (50 per replicate) were scored at 400 × magnification, and the length of comet tail from digitized images was determined by measuring the distance between edge of head and end of tail, and expressed as DNA migration in microns. All experiments were repeated in an independent test.

Measurement of Apoptosis

To verify whether apoptosis was associated with the DNA-damaging effect of GMA, assay for genomic DNA fragmentation or DNA ladder formation, one of characteristic features for apoptotic cells, and flow cytometric analysis, which allows for quantitatively detecting cells undergoing apoptosis were performed with 2BS cells under the same conditions as in the Comet assay. For DNA ladder formation assay, 2BS cells were treated with 0.5-5 μg/mL GMA or DMSO (0.1%) for 12 h. Thymocytes freshly isolated from a male Kunming mouse (4-6 weeks of age, Institute of Experimental Animals, Chinese Academy of Medical Sciences, Beijing, China) were treated with dexamethasone (1 μmol/mL, 4 h) or PBS and used as positive and vehicle controls in the meantime. After treatment, cells (5×10^6) were lysed with 2 mL lysing buffer (10 mmol/L Tris-HCl, pH 8.0; 0.1 mol/L Na_2EDTA, pH 8.0; 50 μg/mL RNAase, and 0.5% sodium dodecyl sulfate) and incubated at 37℃ for 1 h. Proteinase K was then added to a final concentration of 100 μg/mL, and incubated at 50℃ for 3 h. After phenol extraction and ethanol precipitation, DNA samples were subjected to electrophoresis on a 1.5% agarose gel at 80 V for 3 h. The gels were then stained with

ethidium bromide and the DNA was visualized and photographed under UV illumination. For flow cytometric analysis, 2BS cells were treated with 0.5-5 μg/mL GMA for 2 h, or 5 μg/mL GMA for 12 or 24 h. DMSO (0.1%) was used as solvent control. After treatment, the cells were trypsinized, washed once with PBS, and suspended in 70% ethanol for fixation overnight at 4℃. Cells were then resuspended and incubated in PBS containing 50 μg/mL RNAase at 37℃ for 30 min, and stained with propidium iodide (65 μg/mL) for 30 min at 4℃ in the dark. After filtered through a 70 μm mesh the cells were analysed for cell cycle on a Coulter ELITE flow cytometer (Coulter, USA).

HPRT Gene Mutation Assay

The HPRT gene mutation assay was carried out following standard test procedures[6]. A total of 2.5×10^5 2BS cells were seeded into a 25-cm^2 flask for each point. Twenty-four hours later, the cells were treated with GMA (1-8 μg/mL) for 6 h. MNNG (10 μg/mL) and DMSO (0.1%) were used as positive and vehicle controls, respectively. After treatment, cells were washed twice with PBS and detached by trypsinization. For the cytotoxicity assay, 200 cells were seeded into each of four 25-cm^2 flasks. The cells were fixed with methanol, stained with Giemsa and counted on the 7th day of cultivation. For the determination of mutation frequency, cells were seeded and subcultured every 2-3 days at a 1 to 4 split ratio to maintain them in a growth state during the expression period of 8 days. Then 2.5×10^5 cells were reseeded into each of four flasks with selective medium containing 6-TG (5 μg/mL). At the same time, plating efficiency was determined by seeding 200 cells into each of four flasks containing non-selective medium. After 7 days, the colonies were fixed, stained and counted. Mutant frequencies were determined by correcting mutant counts for plating efficiency and expressed as the number of mutant colonies per 10^6 survivors. To ensure reproducibility of the effects observed, all mutation tests were repeated in independent experiments.

GJIC Assay

The GJIC was assessed by scrape loading/dye transfer (SL/DT) method described by El-Fouly *et al.*[18]. Briefly, the confluent cultures of 2BS cells growing in 35-mm dishes (3 dishes for each treatment) were treated with GMA (0.5-5 μg/mL) for 12 h. TPA (0.1 μg/mL) and DMSO (0.1%) were used as positive and vehicle controls, respectively. After treatment, the cells were rinsed three times with PBS. An aliquot of 1.5 mL of PBS containing 0.05% Lucifer yellow CH was then added and three scrapes (cuts) were made on the monolayer of each culture dish using a surgical blade. After 5 minutes of incubation at room temperature, the dye was discarded, and cells were washed three times with PBS. GJIC was visualized using a fluorescence microscope (IX 50, Olympus) with an image analysis system (Image-Pro Plus, Media Cybernetics), and analyzed by measuring the intensity of fluorescence of ten randomly selected sites along with each scrape. Cell viability was examined by trypan blue exclusion technique.

Cell Transformation Assay

Cell transformation assays previously performed in our laboratory have demonstrated the transforming activity of GMA in several assay systems, and positive dose-related responses were repeatedly observed with the exposure doses ranging from 1-16 μg/mL[11-14]. In this study, the transformation assay was performed to ascertain if GMA could induce morphological transformation in the current human cell strain, according to the procedure

described previously[14]. In brief, 2BS cells were seeded onto 25-cm^2 flasks at a concentration of 4×10^4 cells per flask for the transformation assay (ten flasks used for each group) and 200 cells per flask for the colony formation assay (three flasks used for each group). Twenty-four hours later, the cells were treated with GMA at a single dose of 8 μg/mL or DMSO (0.1%) as control for 72 h. The cells were then washed three times with PBS and cultured with fresh medium for 24 h. These processes were again repeated twice and 3 times of GMA treatment in total were given. The cells were subsequently fed with fresh medium twice weekly and cultured for an additional 10 days for the colony formation assay and 10 weeks for the transformation assay. The colonies were then fixed with methanol, stained with Giemsa and counted.

Statistical Analysis

Comparisons of DNA migration and intensity of fluorescence between treatment and control groups were performed with one-way analysis of variance (ANOVA), followed by Student's *t*-test. Gene mutation frequency and transformation efficiency were analyzed by chi-square test. The level of significance was set at $P<0.05$.

RESULTS

Induction of DNA Strand Breaks

Exposure of 2BS cells to GMA was found to induce significantly elevated levels of DNA damage as revealed by the increased DNA migration in Comet assay, when compared to DMSO controls. The induction of DNA damage by GMA was dose-dependent with significantly increased DNA migration occurred at the dose as low as 0.5 μg/mL (Fig. 1A). At the highest dose (5 μg/mL) of GMA tested, a time-dependent increase in DNA migration was found as compared with that in DMSO controls. The DNA migration was first observed

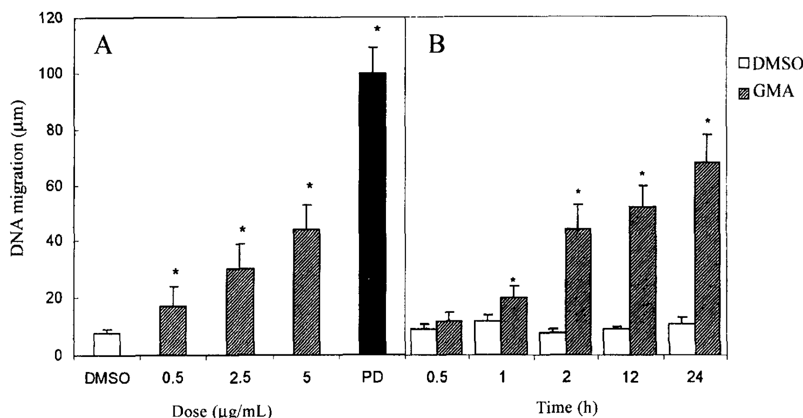

FIG. 1. Dose-response and kinetics of GMA-induced DNA migration in 2BS cells. 2BS cells were treated with (A) 0.5-5 μg/mL GMA for 2 h or (B) 5 μg/mL GMA for 0.5-24 h. DMSO (0.1%) and passium dichroum (PD, 300 μg/mL) were used as negative and positive controls, respectively. Data represent $\bar{x}\pm s$ of two independent experiments. $^*P < 0.05$ compared with negative control.

to be significantly elevated at 1 h after exposure to GMA at 5 μg/mL, and persistently increased up to 24 h (Fig. 1B). Under the present experimental conditions, no significant cytotoxicity of GMA was observed in 2BS cells. Viability of these cells, as determined by trypan blue exclusion technique, was above 90% relative to that in DMSO controls.

To determine whether GMA, in addition to its effect on DNA damage, induced apoptosis that may consequently contribute to the strand breaks observed above, 2BS cells were treated with GMA under the same conditions and assessed for apoptotic responses. Dexamethasone, a known apoptosis inducer, caused a typical apoptotic change, *i.e.*, DNA ladder formation in mouse thymocytes after treatment (Fig. 2). However, neither the GMA-treated 2BS cells, nor the DMSO-treated 2BS and PBS-treated thymocytes were observed to exhibit this apoptotic feature, showing GMA did not induce apoptosis in 2BS cells under the current exposure conditions. This was further confirmed by the results of flow cytometric analysis as shown in Fig. 3. The levels of apoptotic cells were very low (1%-3%) and similar with or without GMA treatment, in contrast to the marked difference in the Comet assay under the identical circumstances. No typical "sub-G_1" peak, which was recognized as a pattern of apoptotic DNA, was observed in the DNA fluorescence histogram analysis (Fig. 3). These results indicate that the short-term exposure to GMA results in DNA strand breaks in the cultured 2BS cells, and that this DNA-damaging effect of GMA is not associated with apoptosis.

Induction of HPRT Gene Mutations

A striking difference was found in the induction of HPRT gene mutations, determined as the number of 6-TG resistant-mutants per 10^6 survivors, between GMA-exposed 2BS cells and controls after a 6-h treatment (Fig. 4). The effect of GMA was seen to be dose-dependent with 5 to 10-fold of mutation frequency in GMA-treated cells over control cultures. These results show that GMA, in the absence of extraneous metabolic activation system, induces gene mutations in 2BS cells, indicating its direct-acting mutagenic activity. Under the given test conditions, the cell survival in GMA-treated groups was slightly reduced, with the lowest level of 80% at the highest dose (8 μg/mL) tested relative to that in controls.

FIG. 2. Detection of DNA ladder formation in 2BS cells. 2BS cells were treated with (A) 0.5, (B) 2.5, (C) 5 μg/mL GMA for 12 h. The 2BS treated with (D) DMSO (0.1%, 12 h) and the primary mouse thymus cells treated with (E) PBS or (F) dexamethasone (1 μmol/L) for 4 h were used as negative and positive controls, respectively. The DNA was extracted and subjected to electrophoresis on a 1.5% agarose gel at 80 V for 3 h. (M) 200 bp DNA ladder marker.

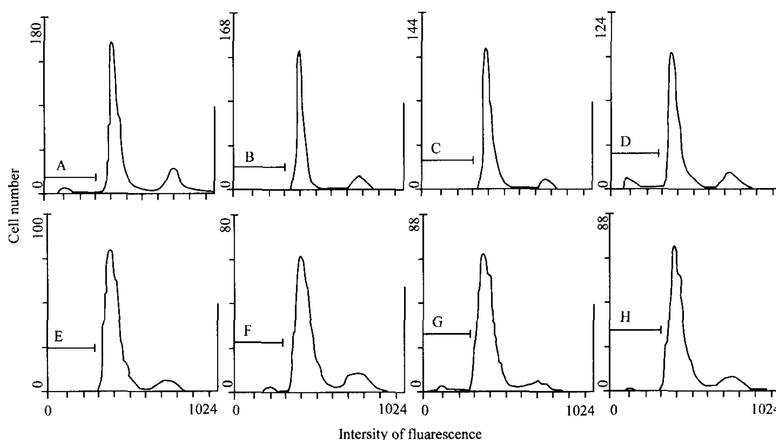

FIG. 3. Flow cytometric analysis of 2BS cells. 2BS cells were treated with various concentrations of GMA or DMSO (0.1%) for up to 24 h. The cells were stained with propidium iodide (65 μg/mL) and analysed for cell cycle on a Coulter ELITE flow cytometer. (A). DMSO, 2h; (B). 0.5 μg/mL GMA, 2 h; (C). 2.5 μg/mL GMA, 2 h; (D). 5 μg/mL GMA, 2 h; (E). DMSO, 12 h; (F). 5 μg/mL GMA, 12 h; (G). DMSO, 24 h; (H). 5 μg/mL GMA, 24 h.

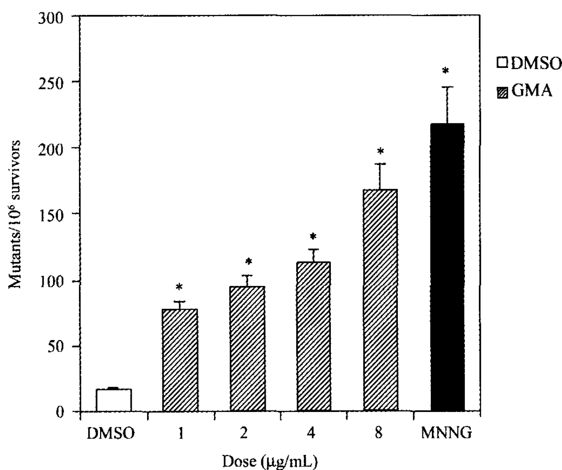

FIG. 4. HPRT gene mutation induced by GMA in 2BS cells. 2BS cells were treated with various concentrations of GMA or DMSO (0.1%) for 6 h. DMSO (0.1%) and MNNG (10 μg/mL) were used as negative and positive controls, respectively. Data represent $\bar{x}\pm s$ of two independent experiments. *$P < 0.05$ compared with negative control.

Inhibition of GJIC

In order to probe into the potential nongenotoxic effects of GMA, the ability of the

GMA-treated 2BS cells to communicate through gap junctions was assessed with the SL/DT technique. In contrast to its inductive effects on DNA strand breaks and gene mutations, GMA inhibited GJIC in 2BS cells in a dose-dependent fashion (Fig.5). Significant inhibition of GJIC was observed at the two higher doses (2.5 and 5 μg/mL), with the maximal inhibition (approximately 50% of the control level) occurred at 5 μg/mL.

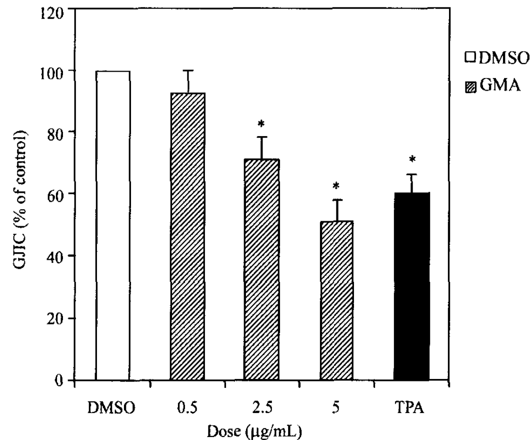

FIG. 5. Inhibition of GJIC by GMA in 2BS cells. Cells were treated with 0.5-5 μg/mL GMA for 12 h. DMSO (0.1%) and TPA (0.1 μg/mL) were used as negative and positive controls, respectively. The GJIC was assessed using the SL/DT technique. Data represent $\bar{x}\pm s$ of 90 measurements from triplicate dishes. $^{*}P < 0.05$ compared with negative control.

Cell Transforming Activity

The transformation efficiency in GMA-treated 2BS cells was significantly increased and above 15-fold higher than that in controls (Table 1). Morphological examinations showed that the GMA-transformed cells obtained in the present experiment were densely multilayered, randomly oriented at focus edge and invaded into monolayer. The transformed cells also showed various properties of malignantly transformed cells, such as evident ultrastructural alterations, anchorage-independent growth, increased agglutinating ability toward agglutinin, karyotypic alterations and chromosomal aberrations (data not shown).

TABLE 1

Morphological Transformation of 2BS Cells Induced by GMA

Treatment (μg/mL)		CFE (%)[a]	RCE (%)[b]	Number of TF[c]	TE (%)[d]
GMA	8	0.84	83	128	3.81*
DMSO	0.1%	1.01	100	10	0.25

Note. [a]Colony-forming efficiency (%) = (Number of colonies per flask/ 200 cells per flask) × 100; [b]Relative colony-forming efficiency (%) = (CFE of GMA/CFE of DMSO) × 100; [c]Total transformed foci from 10 flasks at the end of experiments; [d]Transformation efficiency (%) = [Total transformed foci/ (4 × 10^4 × number of flasks × CFE)] × 100; *Significantly different from DMSO control at $P < 0.05$.

DISCUSSION

The past database of GMA-induced genotoxicity, mutagenicity, and cell transformation was generated largely on various rodent cell models or microbial systems. In this study, the effects of short-term GMA exposure on induction of two genetic endpoints which are commonly used in genotoxicity testing and population monitoring, *i.e.*, DNA damage and HPRT gene mutations, and inhibition of GJIC, a typical nongenotoxic endpoint involved in tumor-promotion process, were investigated using the 2BS human lung fibroblast cells. GMA was also tested for the ability to transform the same human cells *in vitro*. The results showed that GMA induced DNA strand breaks, HRPT gene mutations and morphological transformation, but impaired GJIC in the cultured 2BS cells.

In accordance with our previous results[9], we demonstrated by the Comet assay that exposure to GMA led to a dose- and time-dependent increase in DNA damage in 2BS cells. The results showed that short-term exposure to GMA at relative low concentrations significantly increased DNA strand breaks in the cultured human cells. Compared with the data from our previous genotoxicity tests[9], the DNA damage detected by the Comet assay is a relatively sensitive indicator for GMA exposure, suggesting it may serve as a useful biomarker for monitoring studies. Though it was considered that the majority of the DNA lesions observed in the Comet assay may be repaired before being fixed as mutation[19], the DNA-damaging effect of GMA on 2BS cells reported here is of genotoxic importance. This is supported by the time course studies showing that GMA-induced DNA migration increased with the duration of exposure and no evidence of recovery was observed up to 24 h. The idea is further validated by the results of gene mutation assay, in which GMA clearly induced HPRT gene mutations in 2BS cells after a 6-h treatment. Moreover, we have reported previously that GMA, at the concentrations similar to those used in the Comet assay, displayed a positive transforming activity in various *in vitro* transformation assays[11-14]. These results suggest that the DNA strand breaks may be related to the measured gene mutations and most likely one of the early events in the initiation stage of cell transformation induced by GMA.

The mechanisms by which GMA induces DNA strand breaks are not clear. Particularly, in the event that a positive response in the Comet assay is obtained, it is critical to assess the possibility that the increased DNA migration is not associated with genotoxicity, because increase in DNA migration can also occur in company with DNA fragmentation associated with cytotoxicity arising through necrosis or apoptosis. It has been shown that apoptosis results in the extensive formation of DNA double-strand breaks (DSB)[20]. Similarly, the DNA of necrotic cells also undergoes extensive degradation due to the induction of DSB[21]. Several investigators have concluded that based on the characteristic appearance of the comets, apoptotic cells can be readily distinguished from necrotic cells in the alkaline assay[22]. However, this characterization may not be completely accurate[23]. In response to this concern, we further investigated whether GMA could induce apoptosis in 2BS cells, though it was observed that GMA was only slightly cytotoxic under the same experimental conditions. The fact that GMA did not show any detectable apoptotic effect under the current experimental conditions suggests that DNA damage observed in the Comet assay is not likely associated with apoptosis, providing further evidence that DNA-damaging effect of GMA on the exposed cells may elicit genotoxic outcomes. We propose that DNA damage observed may be the consequence of strand breaks and alkaline-labile sites induced by the covalent banding of GMA to DNA, or appears during the excision repair process following GMA exposure. In deed, we have previously demonstrated that covalent binding of GMA to

mammalian DNA, and increased DNA repair in human and rat lymphocytes occurred after *in vitro* exposure to GMA[9]. Formation of several GMA-DNA adducts was also recently determined in blood and various organs of exposed rats[10]. In addition, GMA-induced lipid peroxidation and potential oxidized DNA damage may also result in these strand breaks as already suggested earlier[24,25].

While the accumulation of genetic changes in a somatic cell is considered essential for transformation or genesis of a cancer, more and more evidences suggest that nongenotoxic mechanisms related to changes in cytoplasmic membrane, signal transduction systems, cell cycle control factors as well as many other factors are also involved in this process. One conceptually well-accepted hypothesis of mechanisms of nongenotoxic carcinogenesis is the block of GJIC that directly links the interiors of neighboring cells[16]. The role of GJIC in the regulation of cell behavior, while still not known in detail, seems to involve in the transfer of ions and small molecular weight regulatory molecules through the gap junction channels[26]. Most tumors display a reduction in GJIC activity, either between themselves or with other cell types[27]. Characterization of the relationship between morphological transformation and GJIC involving compounds such as phorbol esters, cAMP modulators, retinoids, and inhibitors of protein kinase C, indicated a role for decreased communication in morphological transformation, since enhancement of transformation is companied by a decrease in GJIC[16]. Studies also showed that the extent of neoplastic transformation *in vitro* is dependent on GJIC activity, as evidenced by an inverse correlation between the ability of cells to communicate and their ability to form colonies in soft agar[28]. In the present study, we clearly demonstrated that GMA inhibited GJIC in 2BS cells in a dose-dependent fashion. To our knowledge, this is the first report showing that GMA elicits an adverse effect on GJIC, a nongenotoxic event involved in tumor promotion process. It is interesting to note that GMA may play dual roles in the transformation process. Presumably, GMA could not only initiate a cell by directly reacting with DNA that may ultimately result in molecular changes of critical genes, but also induce a down-regulation of GJIC activity that would lead to the removal of growth inhibitory signaling from surrounding normal cells, thereby providing a selective advantage for the initiated cell. Since the inhibition of GJIC in 2BS cells occurred shortly after GMA exposure (12 h), this early effect may favor the birth of the new initiated cell and subsequently the clonal expansion. While the mechanism of the inhibition of GJIC is not clear, our results strongly suggest that GMA might have tumor-promoting potential.

Morphological transformation is considered as a useful *in vitro* model for *in vivo* carcinogenesis. Although the assay does not directly detect genetic and/or nongenetic alterations or damage, it detects at least the ability of a test compound to transform mammalian cells into cells with neoplastic potential. Human cell transformation, in particular, is widely viewed as the system with the greatest feasibility to model and predict human neoplasia[29]. In this study, we demonstrated that GMA induced morphological transformation in 2BS cells, in agreement with our previous transformation assays with Syrian hamster embryo cells, BALB/c 3T3 cells and human KMB-13 cells[14-17]. We have shown that a single exposure to GMA could readily transform cultured rodent cells, but not human cells[13]. In order to transform human cells into the type III transformed foci, repeated treatments with GMA are required. We assume that the primary DNA damage such as DNA strand breaks or gene mutations induced by a single treatment with GMA can be eventually repaired in the exposed human cells. In the case of repeated treatments, however, repair systems in the exposed cells may be overloaded or even overwhelmed, which lead to accumulation of DNA damage and gene mutations, and consequently result in transformation. Although no epidemiological evidence or animal studies showing carcinogenic effect of GMA are available so far, the results of the present study together with that obtained from

previous transformation assays suggest that exposure to GMA may contribute the occupational cancer risk which remains to be further investigated in the future.

In summary, the results of the present study demonstrate that GMA elicits both genotoxic and nongenotoxic effects on human 2BS cells. These findings add valuable in vitro data to complete the overall elucidation of potential carcinogenic activity of GMA and may also provide a clue regarding the possible role of GMA in transformation. Although the importance of DNA damage, gene mutations and block of GJIC in transformation and carcinogenesis is obvious, more information is needed with respect to the causal relationship. On the other hand, it would be of particularly interest to characterize changes in structure/expression of GMA responsible genes within the target cells during the transformation process. Knowledge of relationship between these alterations and genotoxicity and epigenotoxicity of GMA as well as the development of neoplastic phenotype should facilitate our understanding of the mechanism of GMA-induced cell transformation. This work is currently underway in our laboratory.

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