Inorganic Arsenic Compounds and Methylated Metabolites Induce Morphological Transformation in Two-Stage BALB/c 3T3 Cell Assay and Inhibit Metabolic Cooperation in V79 Cell Assay

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We have performed two-stage transformation assay using BALB/c 3T3 cells to determine initiating and promoting activities of disodium arsenate, sodium arsenite, monomethylarsonic acid (MMAA) and dimethylarsinic acid (DMAA). Treatment with these arsenic compounds at the initiating stage induced significant numbers of transformed foci when cells were post-treated with 12-O-tetradecanoylphorbol-13-acetate (TPA). Disodium arsenate was active at the concentrations of 15–30 μM, sodium arsenite 5–20 μM, and DMAA 1–2 mM. MMAA required 10 mM to induce cell transformation. The concentrations of these compounds (except DMAA) that induced transformation were highly growth-inhibitory (more than 50%). DMAA induced transformation foci at growth inhibition levels of 66 to 84%. In experiments on promoting activity, cells pretreated with a sub-threshold dose of 20-methylcholanthrene (MCA, 0.2 μg/ml) or sodium arsenite (10 μM) were used. Transformation was enhanced by post-treatment with disodium arsenate (1–10 μM), sodium arsenite (0.5–2 μM), and MMAA (200–1000 μM), but not with DMAA. Studies of gap junctional intercellular communication using the V79 cell metabolic cooperation assay showed that the arsenic compounds (except DMAA) exhibited inhibitory activity. Thus, most arsenicals were shown to have not only initiating activity, but also promoting activity. In addition, inorganic arsenicals, especially trivalent sodium arsenite, were more active than organic ones and exhibited promoting activity at one-order of magnitude lower than initiating activity. These results suggest that from the viewpoint of human hazard, more attention should be paid to the tumor promoting activity of inorganic arsenic compounds.

Key Words: arsenic compounds; BALB/c 3T3 cells; initiating activity; metabolic cooperation; tumor-promoting activity; two-stage cell transformation.

On the basis of epidemiological studies, arsenic compounds are considered to be highly hazardous and carcinogenic to man (IARC, 1987), but their carcinogenicity in rodents has been poorly defined; results with inorganic arsenic compounds in carcinogenicity bioassays have been equivocal (IARC, 1987). Since the 1980’s, however, carcinogenic activities of arsenic compounds have been reported. Several studies have shown carcinogenicity of inorganic arsenic in experimental animals using relatively high doses (Germolec et al., 1997; Ishinishi et al., 1983; Pershagen et al., 1984; Pershagen and Bjorklund, 1985; Yamamoto et al., 1987). Studies with dimethylarsinic acid (DMAA), a methylated arsenate metabolite, have shown that it acts as a tumor promoter (Wanibuchi et al., 1996; Yamamoto et al., 1995; Yamanaka et al., 1996), although negative results in DMAA were also reported by Seike et al. (2002). Long-term exposure to DMAA caused tumors (Hayashi et al., 1998; Wei et al., 1999).

Various in vitro examinations have been performed: arsenic compounds were negative or weakly positive in bacterial mutation assays (Rossman, 1995), positive in chromosomal aberration and DNA repair inhibition tests (Rossman, 1995), and were co-mutagenic and induced sister chromatid exchanges (SCEs) (Lee et al., 1985). In an in vitro short-term assay for tumor promoters, pentavalent arsenic acid was positive in metabolic cooperation assay using V79 cells (Loch-Caruso et al., 1991).

Regarding in vitro cell transformation assays, inorganic arsenic compounds were reported to induce transformation in colony assay using Syrian hamster embryo cells (Lee et al., 1985), in focus formation assays using BALB/c 3T3 cells (Bertolero et al., 1987) and C3H10T1/2 cells (Landolph, 1994), and in an assay using a rat liver epithelial (TRL 1215) cell line (Zhao et al., 1997). Inorganic arsenic compounds enhanced transformation of bovine papillomavirus DNA-transfected C3H10T1/2 mouse embryo cells (Kowalski, 1996).

Although many reports on arsenic compounds have been published, including reviews of their toxicity, carcinogenicity,
and potential mechanisms of action (Rossman, 2003; Simeonova and Luster, 2000), the true nature of carcinogenic action of arsenic compounds still remains to be elucidated.

Transformation assay using BALB/c 3T3 cells can simulate the process of two-stage carcinogenesis, initiation, and promotion by treating the cells at different growth state, and therefore potentially detect not only initiating activity, but also promoting activity of chemicals (Tsuchiya and Umeda, 1995). However, the promoting activity of arsenic compounds has not been reported in the two-stage transformation assay using BALB/c 3T3 cells. Recently, Ohmori et al. (2004) have reported that arsenic trioxide has promoting activity by the use of promoter-sensitive Bhas 42 cell assay.

In the present study, inorganic arsenic compounds as well as the methylated metabolites monomethylarsonic acid (MMAA) and dimethylarsinic acid (DMAA), were examined using initiation and promotion testing regimes of the BALB/c 3T3 cell transformation assay. The effect of these compounds on gap-junctional intercellular communication in the V79 metabolic cooperation assay was also examined as this endpoint has been used for prediction of tumor promoting activity of chemicals (Sakai et al., 2002; Tong and Williams, 1987; Trosko et al., 1983; Trosko and Ruch, 1998). The results showed that most arsenicals tested had both initiating and promoting activities. Inorganic arsenicals, especially trivalent sodium arsenite, were more active than organic ones, and exhibited promoting activity at less concentration range than initiating activity.

MATERIALS AND METHODS

Cells, media, and culture conditions. Cells used in morphological transformation were BALB/c 3T3 A31-1-1 clonal cells originally supplied by Dr. T. Kakunaga. They were grown in a medium consisting of Eagle’s minimum essential medium (MEM; Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FBS (Moregate; Brisbane, Australia, lot No. 174014) in a humidified incubator under 95% air and 5% CO₂. Cells were subcultured before confluence, usually twice a week. In order to obtain constant transformation results, many frozen stock amoules of the cells were prepared at passage 10. One amoule was thawed and used at each individual transformation experiment.

Wild-type V79 cell line (hypoxanthine/guanine phosphoribosyl transferase-positive; HPRT⁺) and a V79 line mutated at HPRT locus (HPRT⁻) were provided by the Japanese Cancer Research Resources Bank (presently Health Science Research Resources Bank, Tokyo, Japan). They were grown in MEM supplemented with a mixture of Eagle’s non-essential amino acids, 10 µM sodium pyruvate and 3% FBS.

Other media used were obtained as follows: DMEM/F-12 from GibCO Laboratories (Grand Island, NY), and ITES (200 µg/ml bovine pancreas insulin, 200 µg/ml human transferrin, 12.2 µg/ml ethanolamine and 0.034 µg/ml sodium selenite) from Wako Pure Chemical Industries. MMAA and DMAA were obtained from Tori Chemical Institute (Yamanashi, Japan), and 6-thioguanine (6-TG) from Sigma (St. Louis, MO). MCA and TPA were dissolved in dimethyl sulfoxide and diluted in the medium to obtain desired concentration. Other chemicals were dissolved in sterile distilled water (D. Water).

Cytotoxicity assay. A preliminary cytotoxicity assay was carried out for determination of test concentrations. For the evaluation of growth-inhibitory and cytotoxic effect of initiating chemicals, cells were seeded at a density of 1 × 10⁴ cells per 60-mm dish (four dishes per group). Twenty-four h later, the cultures were exposed to test chemicals and kept for three days. Cells were then fixed with 10% formalin and stained with 1% crystal-violet (CV) solution. After extraction of the stained CV with 0.02N HCl-50% ethanol, OD₅₇₀ values were measured. Concentrations of a chemical to be used for the transformation assay were determined according to the cytotoxicity results.

For the determination of test concentrations in promotion experiments, cultures were prepared as in the transformation assay and cultivated for seven days. Test chemicals were then added and cells treated for another four days. The cultures were fixed and stained, and the stained CV values were determined as mentioned above.

In order to ensure the integrity of every transformation assay, a cytotoxicity assay was carried out in parallel.

Transformation assay. The assay procedure for transformation experiments was described in detail in a previous report (Tsuchiya et al., 1999). Exponentially growing BALB/c 3T3 A31-1-1 cells were plated at a density of 1 × 10⁵ cells per 60-mm dish (Sumilon MS-10600, Sumitomo Bakelite, Japan) in 10 plates per condition. The medium was MEM + 10% FBS. After a 24-h incubation, the first chemical was added as the initiation treatment. On Day 4, the dishes were replenished with a fresh DMEM/F-12 + 1% ITES + 2% FCS (D12F) medium. Afterwards, D12F medium was changed twice per week. The second chemical was added in the medium on Day 7, 11, and 14 as the promotion treatment. On Day 25 the cells were fixed with methanol and stained with Giemsa solution.

In order to examine initiating activity, cells were exposed to test chemicals at the initiation stage, with or without subsequent 0.1 µg/ml TPA treatment at the promotion stage. When a promotional effect was examined, non-treated cells or cells treated with MCA (0.2 µg/ml) or sodium arsenite (10 µM) at the initiation stage were exposed to test chemicals at the promotion stage.

Scoring of transformed foci was performed according to the criteria, which discriminate transformed foci by four morphological characteristics: (1) foci of more than 2 mm in diameter, (2) deep basophilic staining, (3) piling up of cells forming a dense multi-layer, and (4) random orientation of cells at the edge of foci.

Data were analyzed statistically using the composite maximum contrast method (Nishiyama et al., 2002).

Metabolic cooperation assay. The metabolic cooperation assay using V79 cells was performed according to the method described by Trosko’s group (Tsuchimoto et al., 1982) with minor modifications. Four hundred thousand V79/HPRT⁻ cells and 200 V79/HPRT⁺ cells were together seeded per 60 mm dish. After a 4-h incubation the cultures received various concentrations of test chemicals, and after another 15 min 6-TG at a final concentration of 10 µg/ml. After a three-day treatment, the cells were cultivated in a fresh 6-TG medium for another four days, and then fixed with methanol. Formed colonies were counted after staining with 0.1% CV solution.

For the parallel cytotoxicity experiment, 200 V79/HPRT⁺ cells were seeded per 60 mm dish, and the ensuing procedures were the same as in the metabolic cooperation assay dishes. In both metabolic cooperation and cytotoxicity assays, six dishes per concentration were used.

Data were statistically analyzed by the Bonferroni method.
RESULTS

Initiating Activity of Arsenic Compounds in BALB/c 3T3

Cell Transformation

Disodium arsenate (pentavalent), sodium arsenite (trivalent), MMAA, and DMAA were examined for their initiating activity in the two-stage transformation assay using BALB/c 3T3 cells and for cytotoxicity (Table 1). High cell growth inhibition was observed at more than 15 μM of disodium arsenate and 5 μM of sodium arsenite, respectively. MMAA showed growth inhibition at a dose as high as 10 mM, and DMAA at more than 2 mM. The organic compounds thus induced growth inhibition at concentrations about three orders of magnitude greater than inhibitory doses of the inorganic compounds.

The results of transformation assays were that arsenic compounds tested at the initiation stage without the subsequent promoting treatment with TPA generally induced very few transformed foci. Slight increase of transformed foci was obtained with sodium arsenite and DMAA. When TPA was added at the promotion stage, significant and dose-dependent increase of cell transformation was observed for the initiating treatment with disodium arsenate (15–30 μM), sodium arsenite (5–20 μM), and DMAA (1–2 mM). The transformation rates were similar to the rate obtained from 0.2 μg/ml MCA.
treatment as a positive control. In the case of MMAA, a few transformed foci were induced only at the highest concentration tested (10 mM). It is noteworthy to mention that the transformation was induced with arsenic compounds at concentrations that were highly growth-inhibitory (less than 50%), except for DMAA, which induced transformation at growth inhibition level of 66 to 84%.

**Promoting Activity of Arsenic Compounds in BALB/c 3T3 Cell Transformation**

Table 2 shows data of transformation promoting activity experiments with arsenic compounds, without and with MCA treatment at the initiation stage, respectively. Cultures treated with distilled water at the initiation stage and subsequently treated with arsenic compounds rarely developed transformed foci (0 to 0.1 foci/dish). Concentrations used in this experiment were those with little or no growth inhibitory effect, being an order of magnitude lower than concentrations examined for initiating activity. In cultures pre-treated with MCA and post-treated with disodium arsenate (1–10 μM), sodium arsenite (0.5–2 μM), or MMAA (200–1000 μM), transformed foci were induced. The induction was statistically significant, but the rates were less marked (at most 1.2 to 2.3 foci/dish) than the positive MCA/TPA control (5.0 foci/dish). DMAA was negative for promoting activity.
Sodium arsenite could induce transformed foci. Sodium arsenite was used for the initiating activity because it was most potent in the initiating activity (Table 3). Cultures treated with sodium arsenite at both initiation and promotion stages could induce transformed foci. Sodium arsenite was used for the initiating activity because it was most potent in the initiating activity (Table 3). Cultures treated with sodium arsenite (10 μM) at the initiation stage only showed 0.4 foci/dish, whereas subsequent treatment with disodium arsenate (5 μM), sodium arsenite (1 μM), or MMAA (500 μM) significantly enhanced the formation of transformed foci (1.3, 1.6, or 2.9 foci/dish, respectively). DMAA (50 μM), however, had no significant promotional effect. These results suggested that arsenic compounds would act as complete carcinogens in BALB/c 3T3 cell transformation.

**Complete Transforming Activity of Arsenic Compounds in BALB/c 3T3 Cell Transformation**

Since the tested arsenic compounds were shown to have both initiating and promoting activities in two-stage cell transformation, we next examined whether only treatment with arsenic compounds at both initiation and promotion stages could induce transformed foci. Sodium arsenite was used for the initiating activity because it was most potent in the initiating activity (Table 3). Cultures treated with sodium arsenite (10 μM) at the initiation stage only showed 0.4 foci/dish, whereas subsequent treatment with disodium arsenate (5 μM), sodium arsenite (1 μM), or MMAA (500 μM) significantly enhanced the formation of transformed foci (1.3, 1.6, or 2.9 foci/dish, respectively). DMAA (50 μM), however, had no significant promotional effect. These results suggested that arsenic compounds would act as complete carcinogens in BALB/c 3T3 cell transformation.

**Inhibition of Metabolic Cooperation by Arsenic Compounds**

The metabolic cooperation assay is based on the use of hypoxanthine-guanine phosphoribosyltransferase (HPRT)-deficient variants (V79/HPRT− cells) in the presence of HPRT-proficient cells (V79/HPRT+ cells) and 6-thioguanine (Loch-Caruso et al., 1991; Sakai et al., 2002). Chemicals that inhibit the transfer of the lethal metabolite of 6-thioguanine from HPRT-proficient to HPRT-deficient cells will allow for recovery of the 6-thioguanine-resistant (HPRT-deficient) cells.

Figure 1 shows the results of testing the arsenic compounds in the intercellular communication assay. In this experiment recovery level of V79/HPRT− cells in the non-treated control was 9.3%. Under the assay conditions, recovery of colonies was clearly observed by treatment with disodium arsenate, sodium arsenite, and MMAA (up to 22–27%). The recovery was dose-dependent and statistically significant. DMAA, however, did not inhibit metabolic cooperation under these conditions.

**DISCUSSION**

Results of in vitro cell transformation assays have shown a relatively high correlation to carcinogenicity bioassays (Barrett et al., 1986). This applies not only to initiators but also to tumor promoters (Sakai et al., 2002), and as such we examined the activity of arsenic compounds in the BALB/c 3T3 cell transformation assay.

The present results revealed that treatment with arsenic compounds at the initiation stage only, with no ensuing promoter exposure, resulted in very few foci. However, with subsequent TPA treatment at the promotion stage, a considerable number of foci were induced with the tested arsenic compounds. It was evident that the induction was at concentrations exhibiting high growth inhibition, except with DMAA. Thus, most arsenic compounds have initiating activity at cytotoxic concentrations. This may explain difficulties in obtaining positive results in previous standard animal experiments. In fact, tumor induction in hamsters by inorganic arsenic required repeated administration of relatively high doses (Ishinishi et al., 1983; Pershagen et al., 1984; Pershagen and Bjorklund, 1985; Yamamoto et al., 1987). Similarly, use of tumor-sensitive Ha-ras transgenic mice has revealed arsenic’s carcinogenicity (Germolec et al., 1997). In the case of transformation assay on DMAA, less growth-inhibitory...
concentrations induced transformed foci. This less cytotoxic nature of DMAA may facilitate long-term exposure and tumor formation in animals (Hayashi et al., 1998; Wei et al., 1999).

When BALB/c 3T3 cells were treated with arsenic compounds at the promotion stage, foci were induced in cultures treated with MCA at the initiation stage. DMAA though did not appear active as a promoter. The transformation rates were relatively low, but the effective concentrations were non-toxic and about one order of magnitude lower than those exhibiting initiation activity. This then appears to mirror long-term in vivo administration of arsenicals, shown to result in tumors in animals previously administered with initiator(s) (Wanibuchi et al., 1996; Yamamoto et al., 1995). Thus, weak but evident promotion activity of arsenic compounds is demonstrated in vitro and in vivo.

Metabolic cooperation assay on the inorganic compounds using V79 cells also showed positive results at test concentrations similar to those of the transformation assay (except DMAA). This may suggest that inhibited gap-junctional intercellular communication—a mechanism linked to many tumor promoters (Sakai et al., 2002; Tong and Williams, 1987; Trosko et al., 1983; Trosko and Ruch, 1998)—plays a role in cell transformation by inorganic arsenic compounds. In the case of MMAA metabolic cooperation was inhibited above 5 mM, but cell transformation assay using BALB/c 3T3 cells induced transformed foci at 100 μM or above. This concentration difference cannot be explained at this moment, although susceptibility difference of respective cells may be suggested. On the other hand, metabolic cooperation assay on DMAA was negative as in the results of cell transformation assay for promoting activity.

The finding that arsenic compounds have both initiation and promotion activities was further confirmed by using sodium arsenite-initiated cells. Here again, the concentration of sodium arsenite used in the initiation stage was one order of magnitude higher compared with those used in the promotion stage. Thus, the use of high dose at the initiation stage and of non-toxic lower dose at the promotion stage is suggested in order to induce cell transformation with arsenic compounds.

Regarding the metabolism of arsenic compounds in animals, many but not all mammalian species methylate inorganic arsenic (Vahter, 1994). Pentavalent inorganic arsenic is metabolized to the trivalent form, which is then methylated. Methylated forms of arsenic compounds, MMAA and DMAA, are excreted in the urine of men chronically exposed to inorganic arsenic in the drinking water (Aposhian et al., 2000; Del Razo et al., 2001), and in the bile of rats receiving arsenite intravenously (Gregus et al., 2000). When DMAA was administered, neither inorganic arsenic compounds nor MMAA were detected in the urine of mice, hamsters, and rabbits (Marafante et al., 1987; Vahter and Marafante, 1983), or in tissues of mice (Hughes et al., 2000). This means that methylated arsenic compounds are not demethylated to inorganic forms. The present results together with the above-quoted animal data suggest that the most biologically active

FIG. 1. Effects of disodium arsenate, sodium arsenite, MMAA, and DMAA on the recovery of 6-TG-resistant cells under the condition of metabolic cooperation. A significant increase of mutant recovery is indicated in comparison with controls, (*p < 0.05, Bonferroni method). Points, results of six dishes; bars, SD; #, small colonies.
arsenic compound is trivalent inorganic arsenite, followed by pentavalent inorganic arsenate. The methylated organic compounds used here were pentavalent, and showed less activity.

Although the present study showed that DMAA seemed very weak or negative in promoting activity, there are some reports from animal experiments supporting a promotion activity of DMAA. It is difficult to explain this discrepancy at this moment. Further investigations on organic arsenicals including trivalent forms are required.

Upham et al. (1998) emphasized that both mutagenic and epigenetic events are involved in a multi-stage and multi-mechanism process in carcinogenesis. Our results demonstrated that treatment with most arsenic compounds induced transformed foci at both initiation and promotion stages. However, concentrations of arsenicals used for the initiation assay were at cytotoxic level and one-order magnitude higher than those exhibiting promoting activity. This means that epigenetic event rather than mutagenic event plays a major role in animal and probably human carcinogenesis with arsenicals. Attention should be paid therefore to the potential promoting activity of arsenicals, especially inorganic ones, in human populations with prolonged exposure.

Recently, Food and Drug Administration (FDA) has approved arsenic trioxide for the treatment of patient with acute promyelocytic leukemia (APL) (FDA, 2000). As the approval is for patients who have APL that does not respond to first line therapy, its treatment must be a final selection to respective patients. Cancers develop after a long latency period, and therefore from the viewpoint of benefit versus risk the treatment can be acceptable. Our results confirming transformation-inducing effects of arsenicals, however, strongly inspire the necessity of advance explanation of its use and consent of patients.

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REFERENCES

