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Cytotoxicological Aspects of Organic Arsenic Compounds Contained in Marine Products Using the Mammalian Cell Culture Technique

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Arsenobetaine, arsenocholine, trimethylarsine oxide and tetramethylarsonium iodide, which are contained in marine fishery products, were examined for their potencies on cell growth inhibition, chromosomal aberration and sister chromatid exchange (SCE). Arsenobetaine, the major water-soluble organic arsenic compound in marine animals, exhibited very low cytotoxicity towards mammalian cells. This compound showed no cell growth inhibition at a concentration of 10 mg cm^{-3} and the cytotoxicity was lower than 1/14 000th of that of sodium arsenite and 1/1600th of that of sodium arsenate towards BALB/c 3T3 cells. Thechromosomalaberrationscausedbyarsenobetaine at a concentration of 10 mg cm^{-3} consisted mainly of chromatid gaps and chromatid breaks, but in this concentration chromosomal breakage owing to its osmotic pressure is likely to be considerable. No SCE was observed at a concentration of 1 mg cm^{-3}. Arsenocholine and trimethylarsine oxide also showed no cell growth inhibited at a concentration of 10 mg cm^{-3}. However, tetramethylarsonium iodide inhibition the growth of BALB/c 3T3 at a concentration of 8 mg cm^{-3}. These compounds exhibited a low ability to induce chromosomal aberrations at a concentration range of 2–10 mg cm^{-3} and no SCE was observed at a concentration of 1.0 mg cm^{-3}. These results suggested that the major and minor organic arsenic compounds contained in marine fishery products are much less cytotoxic inorganic arsenic, methylarsonic acid and dimethylarsinic acid. © 1997 John Wiley & Sons, Ltd.

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INTRODUCTION

Marine products often contain higher levels of arsenic compared with terrestrial products. Most of the arsenic in marine organisms is in the form of water-soluble organic arsenic compounds.[1–4] Arsenobetaine was isolated from the tail muscle of the western rock lobster by Edmonds *et al*. in 1977.[5] Subsequently, it was recognized that arsenobetaine was widely distributed in a variety of marine animals, such as crab,[6, 7] shrimp,[6, 8, 9] lobster,[10] fish[6, 8, 11–16] and shellfish.[17, 18] Arsenobetaine was frequently present as the major water-soluble arsenic compound at a level of more than 90% of the total arsenic in the edible part of marine fishery products.[19] It was found in seafood at concentrations from several micrograms to several hundred micrograms of arsenic per gram.[19] This compound is, at present, considered the main metabolite of inorganic arsenic in the arsenic cycle of marine ecosystems.[20–23]

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The other methylated arsenic compounds, such as arsenocholine,[24] trimethylarsine oxide[25] and tetramethylarsonium iodide,[26] were found in several marine organisms. Arsenocholine, which was detected at low levels in marine animals, is the precursor of arsenobetaine in marine ecosystems.[25] Marine algae were also arsenic-rich[19] and contained dimethylarsenic compounds instead of arsenobetaine. Arsenic derivatives of riboses, which have a dimethylarsenic moiety in their molecule, were designated as arsenosugars[27–29] and observed in marine edible brown algae and some red algae. It was thought that these arsenic compounds were derived from inorganic arsenic by the uptake of arsenic in marine organisms from seawater or through the food chain in marine ecosystems.[21–23]

Since many marine organisms are among the most important foodstuffs, it is certain that a large number of people consuming a variety of marine food products are exposed every day to many arsenicals. Generally, inorganic arsenicals have potential toxicity; the toxicity of organic arsenic compounds differs, and depends on their chemical species. Therefore, it is necessary to confirm the toxicological properties of organic arsenic compounds in marine foods for mammals from the viewpoint of food safety hygiene. We have reported that arsenobetaine was shown to have no substantial acute toxicity and was excreted in the urine without being metabolized in mice.[30] Other arsenic compounds, such as arsenocholine and trimethylarsine oxide, also have a low toxicity towards experimental animals.[31–33] However, there are a few reports on the cytotoxicity of marine organic arsenic compounds using the cell culture technique. The purpose of the present study was to investigate the toxicological properties of several water-soluble organic arsenic compounds contained in marine animal products.

MATERIALS AND METHODS

Chemicals

Sodium arsenite was purchased from the Kanto Chemical Co. (Tokyo, Japan), and disodium arsenate and dimethylarsinic acid were from the Wako Pure Chemical Co. (Osaka, Japan). Dimethylarsinic acid was recrystallized from methanol before use. Methylarsonic acid was obtained from the Tri Chemical Co. (Yamanashi, Japan). Arsenobetaine, arsenocholine, trimethylarsine oxide and tetramethylarsonium iodide were synthesized as described in our previous papers[30–33] and recrystallized three times. Colcemid, trypsin, 5-bromodeoxyuridine (5-BrdU) and Hoechst 33258 were of commercial biochemical grade.

Cells

The A31-1-1 clone of mouse BALB/c 3T3 cells for the cytotoxicity test was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Human umbilical cord fibroblasts for the assay of chromosomal aberrations and the sister chromatid exchange (SCE) test were established in primary culture and were cryo-preserved in liquid nitrogen before use. V79 Chinese hamster cells for the SCE test were obtained from the Institute of Medical Science, University of Tokyo. The cells were grown in Eagle's MEM (Nissui Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and kanamycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

Cell growth inhibition

BALB/c 3T3 cells were suspended in MEM media and seeded at a density of 5×10^4 cells cm^{-3} in 12-well plates. Cells were pre-incubated for 1 h and incubated for 48 h in media that contained each of the arsenic compounds at various concentrations. The 50% growth inhibitory concentration (IC_{50}) of the arsenic compounds was measured by counting the viable cells, as compared with growth of untreated control cells, under a microscope with a hemocytometer after the cells were washed and trypsinized.

Assay of chromosomal aberrations

Human umbilical cord fibroblast cells (2×10^5) were seeded with MEM media in a 60 mm dish and cultured for 48 h. The cells were then incubated with each arsenic compound for 20 h. Colcemid (0.2 $\mu g\ cm^{-3}$) was added to the culture media for the harvest of cells at metaphase and the culture was terminated after 4 h. The cells were trypsinized and gathered by centrifugation. Harvested cells were exposed to a hypotonic solution of 0.075 M KCl for 15 min

and fixed in methanol–acetic acid (3:1, v/v). Slides were prepared by air drying and stained with 5% Giemsa solution for 10 min. Three hundred well-spread metaphases were analyzed for structural aberrations. Chromosomal aberrations were listed as a percentage of the aberrant metaphase.

Induction of sister chromatic exchange (SCE)

Human umbilical cord fibroblast cells and V79 cells were seeded at a density of 2×10^5 cells/dish in a 60 mm dish with MEM media and cultured for 24 h. Arsenic compounds were added in a medium with 5-BrdU (4 μM) under dark conditions and incubated for 52 h. Cells were treated with colcemid (0.2 μg cm^{-3}) and the incubation was carried out for an additional 4 h (human fibroblast cells) and for further 2 h (V79 cells). Cells were trypsinized, harvested by centrifugation, treated with 0.075 M KCl hypotonic solution for 15 min at room temperature and fixed in methanol–acetic acid (3:1, v/v). After preparation of the cells on a glass slide using the air-drying technique, they were immersed in Hoechst 33258 solution (10 μg cm^{-3}) for 20 min and irradiated with a mercury lamp for 2 h. The slides were heated for 25 min at 60 °C and stained with 5% Giemza solution for 10 min. For each concentration level, the frequency of SCE was scored for at least 20 cells.

RESULTS

Cell growth inhibition

Arsenite(III) and arsenate (V) showed potent growth inhibition for BALB/c 3T3 cells at concentrations of 0.001–0.02 mg cm^{-3} as shown in Fig. 1. The IC_{50} values calculated from the response curves of each were 0.0007 mg cm^{-3} and 0.006 mg cm^{-3}, respectively (Table 1). Arsenite was eight times more toxic than arsenate. Methylarsonic acid and dimethylarsinic acid, which were metabolites of inorganic arsenic in mammals, exhibited inhibition of cells at concentrations of 0.2–2 mg cm^{-3}, the IC_{50} values being 1.2 mg cm^{-3} and 0.32 mg cm^{-3}, respectively. The cell growth inhibition of methylarsonic acid was three figures lower than that of arsenite and that of dimethylarsinic acid was two figures lower than that of arsenite. The growth inhibition of dimethylarsinic acid was more potent for mammalian cells than methylarsonic acid. Arsenobetaine, arsenocholine and trimethylarsine oxide showed no inhibition of cell growth at a concentration of 10 mg cm^{-3}. This concentration of the arsenic compounds was probably an upper limit for examination without

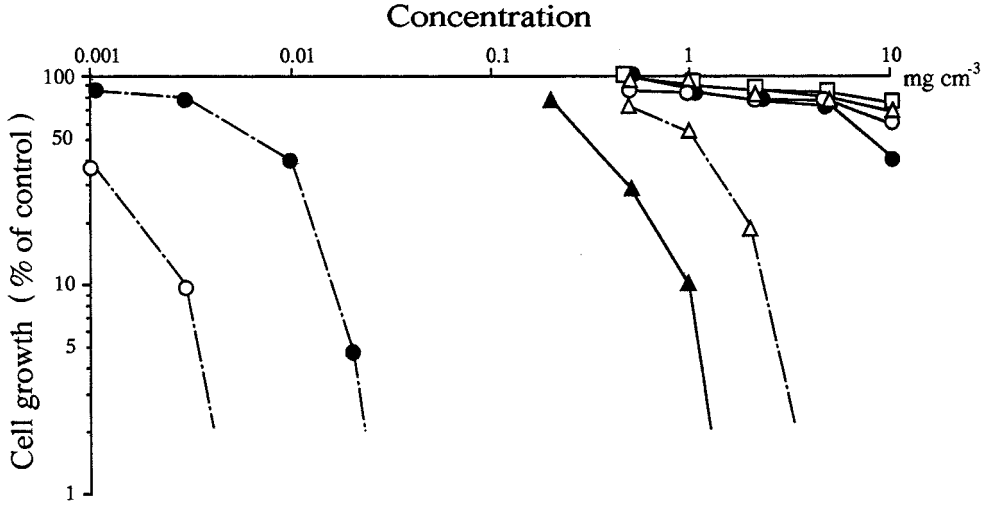

Figure 1 Dose–response curves of arsenic compounds in the growth inhibition test: ○—-—○, arsenite; ●—-—●, arsenate; △—-—△, methylarsonic acid; ▲——▲, dimethylarsinic acid; ○——○, trimethylarsine oxide; □——□, arsenobetaine; △——△, arsenocholine; ●——●, tetramethylarsonium iodide.

Table 1 Toxicity of arsenic compounds to experimental animals and cultured cells

Arsenical	LD_{50}[a] (g kg^{-1})	IC_{50}[b] (mg cm^{-3})	Chromosomal aberrations (%)[c]	Chromosomal aberrations Concentration (mg cm^{-3})	SCE[d] (mg cm^{-3} cell^{-1})
Arsenite	0.0345	0.0007	20	0.001	0.001[e]
Arsenate		0.006	33	0.02	0.02[e]
Methylarsonic acid	1.8	1.2	37	1	0.5[e]
Dimethylarsinic acid	1.2	0.32	90	0.5	0.1[e]
Trimethylarsine oxide	10.6	>10	42	2	1.0[e]
Arsenobetaine	>10	>10	18	10	1.0[e]
Arsenocholine	6.5	>10	15	10	1.0[e]
Tetramethylarsonium	0.9	8	24	10	1.0[e]
Arsenosugar[f]		2	15	5	1.0[e]

[a] 50% lethal dose. (Refs 30, 31, 34, 35.) [b] 50% growth inhibition. [c] Percentage of aberrant. [d] Sister chromatid exchange (SCE) metaphase. [e] Not induced. [f] Ref 47.

influence of the osmotic pressure. These organic arsenic compounds in marine animals did not reduce the rate of growth of the cells by more than 50% of the growth of untreated controls at each concentration. Therefore, their IC_{50} values were estimated simply to be over 10 mg cm^{-3}, as summarized in Table 1. Tetramethylarsonium iodide showed a value of 8 mg cm^{-3} for the IC_{50}, but it was also less toxic than inorganic arsenic, methylarsonic acid and dimethylarsinic acid in this experiment. The order of cytoxicity evaluated from the IC_{50} in the BALB 3T3 cells was as follows: arsenite > arsenate > dimethylarsinic acid > methylarsonic acid > tetramethylarsonium iodide > trimethylarsine oxide = arsenobetaine = arsenocholine (Table 1).

Chromosomal aberrations

We observed the inducibility of organic arsenic compounds contained in marine animals and the other arsenicals for the structural chromosomal aberrations on cultured human fibroblasts. Arsenite induced potential chromosomal aberrations in human fibroblast cells after treatment for 24 h at concentrations of 0.0001–0.001 mg cm^{-3}, as shown in Fig. 2. Chromatid aberrrations were observed in 10–20% of the metaphase at these concentrations. Arsenite, arsenate, methylarsonic acid and trimethylarsine oxide induced mostly chromatid gaps and chromatid breaks, and rarely disruption of centromeres.

The untreated control induced 0.33% of aberrant metaphases. Arsenate induced 16–33% of aberrant metaphases at concentrations of 0.005–0.02 mg cm^{-3}. Methylarsonic acid induced 19–37% of the aberrant metaphases at concentrations of 0.2–1 mg cm^{-3}. However, dimethylarsinic acid induced 28–90% of the aberrant metaphases at concentrations of 0.1–0.5 mg cm^{-3} with high frequency. The behavior of dimethylarsinic acid regarding chromosomal aberrations differed from that of the other arsenic compounds examined in this work. Dimethylarsinic acid induced mainly chromosome pulverizations while the other arsenic compounds induced chromatid gaps and chromatic breaks. Arsenocholine, arsenobetaine and tetramethylarsonium iodide were less toxic than inorganic arsenic, methylarsonic acid and dimethylarsinic acid in the experiment to induce structural chromosomal aberrations (Fig. 2). On the other hand, arsenobetaine, arsenocholine and tetramethylarsonium iodide induced 15–24% aberrations at a concentration of 10 mg cm^{-3}. The ranking order of these arsenic compounds in terms of clastogenic potency was arsenite > arsenate > dimethylarsinic acid > methylarsonic acid > trimethylarsine oxide > tetramethylarsonium iodide > arsenobetaine = arsenocholine, as summarized in Table 1. These results were similar to those on the cytotoxicity of arsenic compounds in the growth inhibition test using BALB/c 3T3 cells. Therefore, these arsenicals in marine products had a low propensity to cause chromosomal aberrations.

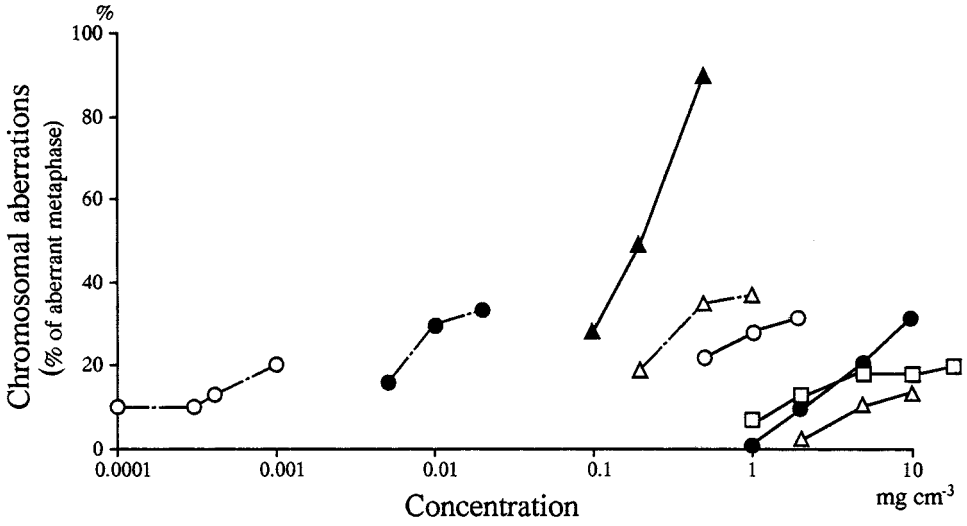

Figure 2 Chromosomal aberrations by arsenic compounds: ○—-—○, arsenite; ●—-—●, arsenate; △—-—△, methylarsonic acid; ▲——▲, dimethylarsinic acid; ○——○, trimethylarsine oxide; □——□, arsenobetaine; △——△, arsenocholine; ●——●, tetramethylarsonium iodide.

SCE

No SCEs for human fibroblasts cells and V79 Chinese hamster cells were observed after exposure to the eight kinds of arsenic compounds at concentrations of 1–10 mg cm^{-3}. V79 cells were more sensitive than human fibroblast cells to chemicals. No effects of organic arsenic compounds contained in marine animals were observed in mammalian cells. These results are summarized in Table 1.

DISCUSSION

We have already reported the acute toxicity of some arsenic compounds. The 50% lethal doses (LD_{50}) value of arsenic trioxide was found to be 34.5 mg kg^{-1} in mice; hence this arsenic form was extremely toxic.[30] In this experiment, inorganic arsenic similarly showed potent growth inhibition for BALB/c 3T3 cells. However, the LD_{50} values of arsenobetaine, arsenocholine, trimethylarsine oxide and tetramethylarsonium iodide was more than 10 g kg^{-1},[30] 10.6 g kg^{-1},[31] 6.5 g kg^{-1},[34] and 0.9 g kg^{-1},[35] respectively, in mice. The order of the acute toxicity evaluated from the LD_{50} in mice was as follows: arsenite > dimethylarsinic acid > methylarsonic acid > tetramethylarsonium iodide > arsenocholine > trimethylarsine oxide > arsenobetaine. The LD_{50} values of arsenic compounds are shown in Table 1. These results clearly showed that the tendency towards the cytotoxicological potency of arsenic compounds for BALB/c 3T3 cells was quite similar to that found in the acute toxicity test. We reported that the depletion of glutathione in cells enhanced the cytoxic effects of arsenite, arsenate, methylarsonic acid and trimethylarsine oxide, whereas the cytotoxicity of dimethylarsinic acid was markedly reduced by the depletion of glutathione. Therefore, glutathione played a role in protecting the cells against the toxic effects of arsenite, arsenate, methylarsonic acid and trimethylarsine oxide, whereas it was involved in the induction of the cytotoxic effects of dimethylarsinic acid.[36] Dimethylarsinic acid is one of the main metabolites of inorganic arsenic in mammals and is the most potent in terms of the ability to cause morphological changes in the characteristics of apoptosis.[37] Yamanaka *et al.* reported that dimethylarsinic acid caused potent induction of gene damage.[38–40] We recently reported that the exhausion of cellular glutathione with L-buthionine sulfoximine increased the incidence of chromosomal aberrations by arsenite, arsenate and methylarsonic acid. Dimethylarsinic acid markedly suppressed the induction of chromosomal aberrations with the depletion of glutathione by L-buthionine sulfoximine. Dimethylarsinic acid produced highly clastogenic effects in the presence of glutathione.[41]

Arsenobetaine is widely distributed in marine animals, especially carnivorous fish, carnivorous conch and crustaceans. In this experiment, we demonstrated that arsenobetaine showed no cytotoxicity in BALB/c 3T3 cells, no inducibility of chomosomal aberrations in human fibroblasts and no SCE in human fibroblasts and V79 cells. The other water-soluble organic arsenic compounds in marine animals—arsenocholine, trimethylarsine oxide and tetramethylarsonium iodide—were less toxic than inorganic arsenic, methylarsonic acid and dimethylarsinic acid in the growth inhibition test, exhibited a low propensity to induce chromosomal aberrations, and produced no SCE.

In an earlier study, Cannon *et al.* reported the mutagenic effects of arsenobetaine in strains of *Salmonella typhimurium*.[42] Arsenobetaine gave consistently negative results. Jongen *et al.*[43] observed no mutagenicity of arsenobetaine in *S. typhimurium*, TA97, TA98 or TA100 without activation or after the addition of a liver enzyme fraction on gut–flora extract. Also, arsenobetaine was negative in the mutation assay of the HGPRT gene and in the test for SCE in V79 Chinese hamster cells at concentrations up to 10 mg cm^{-3}. No inhibition of metabolic cooperation of arsenobetaine between V79 cells was observed at concentrations up to 10 mg cm^{-3}. Meanwhile, Sabbioni *et al.* reported that cytotoxicity and morphological neoplastic transformation assays gave obvious negative results for arsenobetaine as high as 500 μM in BALB/c 3T3 cells.[44] Irvin and Irgolic reported that arsenobetaine lacks subacute and acute prenatal toxicity at a concentration of 20 μg cm^{-3} used rat embryos with intact yolk sacs.[45] They mentioned that these results suggested that prenatal toxic effects were unlikely to occur in humans after the consumption of seafood that naturally contains arsenobetaine at concentrations comparable with those experimental amounts in which the rat embryos grew. In our recent study, arsenite and arsenate showed potent cytoxicity towards macrophages and lymphocytes. The cytotoxicity of arsenate for macrophages and lymphocytes was about 30–200-fold lower than that of arsenite. On the other hand, arsenobetaine and arsenocholine had no immunotoxicity to either macrophages or lymphocytes.[46]

We reported that an arsenosugar,[47] contained in edible marine algae, had a cytoxicity of 1/2800th that of arsenite and 1/300th that of arsenate. The chromosomal aberrations caused by the arsenosugar at a concentration of 5 mg cm^{-3} mainly consisted of chromatid gaps and breaks, and no SCE was observed at a concentration of 1 mg cm^{-3}, as shown in Table 1.

These results indicate that the organic arsenic compounds in marine products are much less toxic than inorganic arsenic compounds, and consumption of foodstuffs from a marine organism body is confirmed to be safer with regard to the toxicological aspects. Therefore, it is suggested that an examination using a cell culture technique of mammalian cells is of great use for the evaluation of the cytotoxicity and genotoxicity of organic arsenic compounds in marine foodstuffs.

CONCLUSIONS

We demonstrated that inorganic arsenic showed potent cytotoxicity for mammalian cells. Arsenobetaine, arsenocholine and trimethylarsine oxide, which are water-soluble organic arsenic compounds contained in marine animals, showed very low cytotoxicity towards BALB/c 3T3 cells, and exhibited a low ability to induce chromosomal aberrations and no SCE. Arsenobetaine was much less cytotoxic than inorganic arsenic compounds.

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