**Letter**

**Arsenite but not arsenate inhibits general proteoglycan synthesis in cultured arterial smooth muscle cells**

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(Received May 21, 2008; Accepted May 30, 2008)

**ABSTRACT** — Epidemiological and experimental studies have suggested that exposure to metalloid arsenic constitutes a risk factor for vascular disease associated with atherosclerosis. Since in atherosclerosis, types of proteoglycans (PGs) present change depending on the stage, we investigated the effect of 2 chemical forms of inorganic arsenic—a trivalent sodium arsenite and a pentavalent sodium arsenate—on the synthesis of PGs in cultured arterial smooth muscle cells. The results indicate that arsenite but not arsenate, at a noncytotoxic level, inhibits general PG synthesis independent of cell density. Arsenite may be one of the chemical forms of inorganic arsenic that influences the PG composition in blood vessel walls during the progression of vascular disorders such as atherosclerosis.

**Key words:** Arsenite, Glycosaminoglycan, Proteoglycan, Vascular smooth muscle cell, Atherosclerosis

**INTRODUCTION**

Exposure to environmentally derived inorganic arsenic is associated with an increased risk of vascular diseases such as atherosclerosis (Wu et al., 1989; Wang et al., 2002). Experimental studies have also shown that arsenic accelerates atherosclerosis in apolipoprotein E-deficient mice—a model of human atherosclerosis (Simeonova et al., 2003). However, the mechanisms underlying this process are not fully understood.

Proteoglycans (PGs) are macromolecules composed of a core protein and one or more glycosaminoglycan (GAG) side chains (heparan sulfate, dermatan sulfate, or chondroitin sulfate chains); the type of core proteins in PGs differs depending on the stage of atherosclerosis (Evanko et al., 1998). The predominant types of PGs found in atherosclerotic vascular walls are as follows: a large heparan sulfate PG (HSPG), perlecan; small dermatan sulfate PGs (DSPGs), biglycan and decorin; and a large chondroitin sulfate PG (CSPG), versican, all of which are mainly derived from vascular smooth muscle cells (Wight, 1985).

We have proposed that the mechanisms underlying the vascular toxicity of heavy metals involve abnormal PG metabolism in vascular cells (Fujiwara, 2004). Recently, we showed that trivalent arsenite inhibits both HSPGs and CSPGs/DSPGs synthesis in vascular endothelial cells (Fujiwara et al., 2005). However, the effect of inorganic arsenic on the PG synthesis in vascular smooth muscle cells is unknown.

In the present study, we demonstrate that trivalent arsenite but not pentavalent arsenate inhibits general PG synthesis in cultured arterial smooth muscle cells.

**MATERIALS AND METHODS**

**Materials**

Arterial smooth muscle cells derived from bovine aorta were provided as a gift by Dr. Yasuo Suda (Kagoshima University Graduate School of Sciences and Engineering, Kagoshima, Japan). Dulbecco’s modified Eagle’s medium (DMEM) and tissue culture dishes and plates were obtained form Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan) and Iwaki (Chiba, Japan), respectively.

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Fetal bovine serum (FBS) and $[^{35}S]$Na$_2$SO$_4$ (carrier-free) were obtained from MP Biomedicals (Irvine, CA, USA). Sodium arsenite (NaAsO$_3$), sodium arsenate (Na$_3$HAsO$_4$), and diethylaminomethyl (DEAE)-Sephaloc were purchased from Sigma (St. Louis, MO, USA). PD-10 columns (disposable Sephadex G-25M) and the lactate dehydrogenase (LDH) assay kit were purchased from Amersham Biosciences (Piscataway, NJ, USA) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Cetylpyridinium chloride (CPC), and other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

Morphological examination and nonspecific cell damage

Arterial smooth muscle cells were cultured in DMEM supplemented with 10% FBS in 100-mm dishes in a humid atmosphere of 5% CO$_2$ in air until confluence. The cells (density, $5 \times 10^4$ cells/cm$^2$) were transferred into 24-well culture plates and cultured for 24 hr (“sparse culture”) or until confluence (“dense cultures”). The medium was discarded and the cell layer washed twice with DMEM supplemented with 10% FBS. The cell layer was then incubated at 37°C for 24 hr in fresh DMEM supplemented with 10% FBS containing sodium arsenite (5 or 10 μM) or sodium arsenate (5 or 10 μM). After incubation, the conditioned medium was harvested and an aliquot of the medium was used for assay of LDH activity—an indicator of cell death. The cell layer was washed with Ca$^{2+}$- and Mg$^{2+}$-free phosphate-buffered saline (CMF-PBS), fixed with methanol and stained with Giemsa.

Incorporation of $[^{35}S]$sulfate into GAGs

Dense and sparse cultures of arterial smooth muscle cells were incubated at 37°C for 24 hr with sodium arsenite (0.5, 1, 2, 5, or 10 μM) or sodium arsenate (5 or 10 μM) in the presence of $[^{35}S]$sulfate (1 MBq/ml). After incubation, the conditioned medium was harvested, and solid urea was the added to bring the concentration to 8 M. The cell layer was washed with CMF-PBS and extracted with an 8 M urea solution containing 0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 10 mM $N$-ethylmaleimide, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M phenylmethanesulfonyl fluoride, 0.1 M NaCl, 50 mM Tris base, and 2% Triton X-100 (pH 7.5) at 4°C for 15 min; the cell extraction solution was then harvested. The conditioned medium and cell extracts were used to determine the incorporation of $[^{35}S]$sulfate into the GAGs by the CPC precipitation method (Wasteson et al., 1973) as follows: portions of the cell extracts were spotted on filter papers and washed 5 times for 1 hr in 1% CPC containing 0.05 M NaCl. The radioactivity of the PGs precipitated on the dried filter paper was measured by liquid scintillation counting.

DEAE-Sephaloc ion-exchange chromatography

Dense cultures of arterial smooth muscle cells were treated with sodium arsenite (10 μM) for 24 hr in the presence of $[^{35}S]$sulfate (2 MBq/ml). After treatment, the conditioned medium was harvested, and solid urea was added to bring the concentration to 8 M. The cell layer was extracted with an 8 M urea cell extraction solution and then harvested by scraping with a rubber policeman. The conditioned medium and cell extract were chromatographed on PD-10 columns equilibrated with 8 M urea buffer (pH 7.5) containing 2 mM EDTA, 0.25 M NaCl, 0.5% Triton X-100, and 50 mM Tris base to obtain macromolecules with high molecular mass (> 3 kDa). To separate PGs into HSPGs, CSPGs, and DS PGs on the basis of the difference in their charge density, the macromolecules were chromatographed on a DEAE-Sephaloc (5 ml resin) column equilibrated with 8 M urea buffer (pH 7.5) containing 2 mM EDTA, 0.25 M NaCl, 0.5% Triton X-100, and 50 mM Tris base. Any unbound radioactivity was removed from the column by washing with 30 ml of the buffer. The bound radioactivity was eluted from the column with a linear gradient of 0.25-0.7 M NaCl in urea buffer (total volume, 50 ml).

Statistical analysis

The data were analyzed for statistical significance by analysis of variance and Bonferroni’s multiple t-test, where possible. P values of less than 0.05 indicated statistically significant differences.

RESULTS AND DISCUSSION

First, nonspecific cell damage to arterial smooth muscle cells after exposure to arsenite and arsenate was evaluated by morphological observation and LDH leakage assay (Fig. 1). It was observed that neither arsenite nor arsenate caused nonspecific damage to the cells at both high and low cell densities. However, in sparse cultures, arsenite caused a decrease in the cell number. Under this nontoxic condition, arsenite (≤ 10 μM) significantly decreased the accumulation of $[^{35}S]$sulfate-labeled PGs in the cell layer and conditioned medium of both dense and sparse cultures (Fig. 2). However, in sparse cultures, the decrease in PG accumulation is regarded as a reflection of a decrease in the cell number. Thus, it is suggested that arsenite inhibits PGs synthesis in arterial smooth muscle cells when cell density is high. In contrast, pentavalent arsenate did not alter the accumu-
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Fig. 1. Morphological appearance of dense and sparse vascular smooth muscle cells after exposure to sodium arsenite and sodium arsenate (original magnification, ×40) and LDH leakage from the cells into the medium. Dense (A1-A3 and B1-B3) and sparse (A4-A6 and B4-B6) cultures of bovine aortic smooth muscle cells were incubated at 37°C for 24 hr in the presence of sodium arsenite (A1-A7) or sodium arsenate (B1-B7) at 5 μM (A2, A5, B2, and B5) or 10 μM (A3, A6, B3, and B6). Values of A7 and B7 are means ± S.E. of 4 samples.

In order to determine the types of PGs whose synthesis is inhibited by arsenite, [35S]sulfate-labeled PGs derived from arterial smooth muscle cells at a high cell densi-
Fig. 2. Incorporation of [35S]sulfate into GAGs accumulated in the cell layer and the conditioned medium of dense and sparse vascular smooth muscle cells after exposure to sodium arsenite and sodium arsenate. Dense and sparse cultures of bovine aortic smooth muscle cells were incubated at 37°C for 24 hr with sodium arsenite (0.5, 1, 2, 5, or 10 μM) (upper panels) or sodium arsenate (5 or 10 μM) (lower panels) in the presence of [35S]sulfate. Values are means ± S.E. of 4 samples. The scores were significantly different from those of the corresponding control, *p < 0.05; **p < 0.01.

ty after exposure to arsenite were applied to a DEAE-Sephacel column (Fig. 3). In the cell layer, with or without arsenite treatment, the [35S]sulfate radioactivity eluted from the column by the NaCl gradient showed 3 peaks—at approximately 0.40, 0.50, and 0.55 M; these peaks have been previously confirmed to contain HSPGs,
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DSPGs and CSPGs, respectively (Fujiwara et al., 2000 and 2002; Yamamoto et al., 2005b). Arsenite obviously decreased the radioactivity of these 3 peaks to a similar degree. In the conditioned medium, PGs were eluted by NaCl at a concentration of approximately 0.50 and 0.55 M; arsenite decreased the bound radioactivity in both peaks to a similar degree. The DEAE-Sephacel profiles clearly indicate that arsenite inhibits general PG synthesis in arterial smooth muscle cells at high cell density. We have demonstrated that lead selectively inhibits the synthesis of versican in arterial smooth muscle cells when cell density is high (Fujiwara et al., 2000), whereas cadmium specifically increases that of DSPGs, biglycan and decorin in the cell layer when the cell density is low (Fujiwara et al., 2002). The effects of toxic metal(loid)s on PG synthesis in arterial smooth muscle cells may distinctly depend upon the type of metal(loid).

The predominant types of PGs are different between vascular endothelial and smooth muscle cells; the former particularly synthesize perlecan and biglycan (Yamamoto et al., 2005a), and the latter synthesize versican, biglycan and decorin (Wight, 1985). However, the present and previous (Fujiwara et al., 2005) studies have suggested that arsenite inhibits all types of PGs expressed in each cell type, whereas arsenate does not exhibit any such activity. Therefore, arsenite but not arsenate may be one of the
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chemical forms of inorganic arsenic that influences the PG composition in blood vessel walls during the progression of vascular disorders such as atherosclerosis.

ACKNOWLEDGMENTS

This work was supported by the “Academic Frontier” Project for Private Universities, Ministry of Education, Culture, Sports, Science and Technology of Japan, 2005-2009; Specific Research Fund of Hokuriku University (to T.K.); a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science (to T.K.); and a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y.F.).

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