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Suppression of Fas ligand expression on endothelial cells by arsenite through reactive oxygen species

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Abstract

Chronic exposure to arsenite is associated with vascular disease, such as arteriosclerosis. However, the cellular mechanisms for vascular disease in response to arsenic are not well known. The present study has demonstrated that arsenite not arsenate decreased the Fas ligand (FasL) expression on ECV304 cells through reactive oxygen species. Incubation of ECV304 cells with arsenite decreased the FasL expression and increased the intracellular peroxide levels. In addition, hydrogen peroxide was found to suppress FasL expression in a dose-dependent manner. The antioxidant, *N*-acetyl-cysteine, blocked the suppression of FasL expression in response to arsenite. These data suggested that arsenite initiates endothelium dysfunction, at least partly, by suppressing the FasL expression through activating reactive oxygen species sensitive endothelial cell signaling.

Keywords: Arsenite; Fas ligand; Reactive oxygen species (ROS); ECV304

1. Introduction

The monolayer of endothelial cells that coat the luminal surface of the vessel wall has numerous physiological functions including prevention of coagulation, control of vascular permeability and regulation of leukocyte extravasation (Ross, 1993). The recruitment of leukocytes at sites of inflammation is a multi-step process that involves tethering, rolling, firm adhesion and the migration of these cells to the subendothelial space (Springer, 1995). Though much is known about the chemoattractant and adhesion molecules that regulate leukocyte recruitment in response to bacterial infection, relatively little is known about

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mechanisms that may actively control the transendothelial cell migration (Picker and Butcher, 1992). Recently, Sata and Walsh found that the downregulation of Fas ligand (FasL) by the endothelium is essential for cytokine-induced leukocyte extravasation (Sata and Walsh, 1998). The Fas–FasL system has been implicated in the regulation of physiological cell turnover, particularly in the immune system (Nagata and Golstein, 1995). Activated T cells express both Fas and FasL, whereas most other tissues express only Fas (French et al., 1996). Immune privileged tissues also express FasL, where it is thought to inhibit the immune response by inducing apoptosis in inflammatory cells (Bellgrau et al., 1995; Griffith et al., 1995; Hahne et al., 1996; Lau et al., 1996; Strand et al., 1996). It has been, recently, demonstrated that vascular endothelium express functional FasL, and its expression is downregulated upon exposure to the inflammatory cytokine TNFα. These studies suggested that FasL serves an atheroprotective function on the endothelium through its ability to induce apoptosis in mononuclear cells attempting to invade the vessel wall in the absence of normal inflammatory stimuli (Sata and Walsh, 1998). The activity of FasL to function as a potent inhibitor of neointima formation has also been reported (Sata et al., 1998). Therefore, these findings suggest that FasL expression on the vascular endothelium functions to inhibit inflammatory responses that are often associated with vascular disorders (Walsh and Sata, 1999).

Arsenic exists ubiquitously in our environment, and various forms of arsenic circulate in water, air, and living organism. Adverse health effects caused by arsenic compounds have long been recognized, including neurotoxicity, liver injury, peripheral vascular disease and increased risk of cancer (Chen et al., 1992; Lai et al., 1994; Chen et al., 1995, 1998a). Mechanism for the effects of arsenic on vascular disease is unclear. We, therefore, investigated the expression of FasL on primary cultured endothelial cells and ECV304 cells in response to arsenic. We found that arsenite suppressed FasL expression on endothelial cells, at least partly, through reactive oxygen species.

2. Materials and methods

2.1. Reagents

Sodium arsenite, sodium arsenate, *N*-acetyl-L-cysteine (NAC), 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were obtained from Sigma Chemical Co. (St Louis, MO). Recombinant TNFα was purchased from R&D systems (Minneapolis, MN).

2.2. Cell culture

HUVEC cells were cultured in EGM (endothelial growth medium) medium and were used for this study at less than six passages. HUVEC cells were isolated as described and grown in medium 199 with 20% endotoxin-free heat-inactivated fetal calf serum (FCS). The immortalized human endothelial cell line ECV304 cells was obtained from the Culture Collection and Research Center of Food Industry Research and Development Institute (Hsinchu, Taiwan). ECV304 cells were cultured in medium 199 (HEPES modification) containing 10% endotoxin-free heat-inactivated fetal calf serum (GIBCO, Grand Island, NY), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine, and cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. When the cells reached a confluent monolayer, they were activated by incubation in medium containing TNFα (25 ng/ml). Various concentrations of the tested compounds dissolved in DMSO were added together with TNFα. The human promyelomonocytic HL-60 cells was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Cell viability assay

ECV304 cells were seeded in 96-well tissue culture plates and allowed to grow to confluence; the cells were treated with different concentrations of arsenite for 24 h. The cell viability was assayed with CellTiter 96 Non-Radioactive Cell Proliferation Assay Kit as suggested by the manufacturer

(Promega, Madison, WI). Briefly, 20 μl of combined solution of a tetrazolium compound MTS (3-(4,5,-dimethyl thiazol-2-yl)-5-(3-carboxymethhoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) and an electron-coupling reagent, phenazine methosulfate, were added to each well. After incubation for 1 h at 37°C in a humidified 5% CO_2 atmosphere. $A_{490\ nm}$ was recorded using an ELISA plate reader (Dynatech MR-7000, Dynatech Labs, Chantilly, VA).

2.4. Western blots

Total cellular extract was prepared using Gold lysis buffer (10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM sodium pyrophosphate, 20 mM Tris–HCl pH 7.9, 100 μM β-glycerophosphate, 137 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin). Total proteins (for FasL, Hsp90 and α-tubulin) containing 10–30 μg of proteins were separated on 10% sodium dodecyl sulfate-polyacrylamide minigels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membrane was incubated overnight at 37°C with 10% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) to block non-specific immunoglobulins and then incubated with anti-human FasL, anti-Hsp90 (Transduction Laboratories, Lexington, KY) or anti-α-tubulin mouse monoclonal antibody (Oncogene Science, Cambridge, UK). FasL, Hsp90 and α-tubulin proteins were detected by chemiluminescence (ECL, Amersham), or by incubation with coloregenic substrates nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as suggested by the manufacturer (Sigma Chemical Co.).

2.5. Flow cytometric analysis of FasL on ECV304 cell surfaces

ECV304 cells, at approximately 90% confluence, were incubated with either basal medium or sodium arsenite (30 μM) or hydrogen peroxide (50 μM) at 37°C, 5% CO_2 for 16 h. ECV304 cells were detached from culture plates using 0.5% trypsin/EDTA and incubated with anti-FasL monoclonal antibody or with mouse IgG. Cells were then washed and stained with FITC-conjugated anti-mouse IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoflorescence staining was analyzed by flow cytometry analysis system (Becton Dickinson, San Jose, CA).

2.6. ROS determination

ECV304 cells were treated with arsenite (30 μM) or hydrogen peroxide (50 μM) or TNFα (25 ng/ml), and DCFH-DA (50 μM) was added into the medium for 1 h, then the ROS production was monitored by flow cytometry. DCFH-DA is a stable nonpolar compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within the cells. Hydrogen peroxide or low molecular weight peroxides produced by the cells oxidize DCFH to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus, the florescence intensity is proportional to the amount of peroxide produced by the cells.

2.7. Cytotoxicity assay

The ability of FasL on ECV304 cells to induce apoptosis in Fas-positive target cells was assessed by co-incubating the Jurkat cells with ECV304 cells using a technique described earlier (Sata and Walsh, 1998). Briefly, ECV304 cells were seeded in 6-well tissue culture plates and allowed to grow to confluence, and then cells were cotreated with NAC and hydrogen peroxide or arsenite or TNFα for 12 h. The cells were then washed once with sterile PBS and fixed with 2% paraformaldehyde at 4°C for 1 h. After the cells were washed two times with serum free RPMI 1640 medium, 2 ml Jurkat cell suspensions (10×10^5 cells) was added to 6-well. After 48 h of coculture, floating Jurkat cells were collected and fixed in 70% ethanol at −20°C at least 1 h. After fixation, cells were washed twice, incubated in 0.5 ml of 0.5 triton X-100/PBS at 37°C for 30 min with 1 mg/ml of RNAase A, and stained with 0.5 ml of 50 μg/ml propidium iodide. The propidium iodide–DNA complex was quantitated by FACScan flow cy-

tometry analysis system (Becton Dickinson, San Jose, CA).

2.8. Statistical analysis

All values in the figures are expressed as the mean ± S.E. of n observations, n represents the number of evaluated. Data sets were examined and individual group means were then compared with Student's unpaired t-test. A P value < 0.05 were considered significant.

3. Results

High concentrations of arsenite increase phosphorylation of endothelial heat shock proteins, deplete nicontinamide adenine dinucleotide levels, promote DNA strand breaks, and cause apoptosis (Robaye et al., 1989; Lynn et al., 1998). Otherwise, epidemiological data suggest that low concentrations of arsenic, which do not cause cell death, promote vascular disease (Engel and Smith, 1994; Engel et al., 1994). Therefore, the effect of sodium arsenite was examined to define the threshold for toxicity and overt stress to the transformed endothelial ECV304 cells. As shown in Fig. 1, human transformed endothelial cells (ECV304) were treated with various concentrations (0–70 μM) of arsenite for 24 h and the viability of ECV304 cells was determined. Higher concentrations of arsenite (As^{3+}) (> 30 μM) significantly decreased the viability ($P < 0.05$). In contrast, the viability of endothelial cell was just minimally affected by treatment with lower than 30 μM concentrations of arsenite. However, treatment with 5–70 μM arsenate (As^{5+}) had no effect on viability of ECV304 cells (data not shown).

After exposure to non-lethal concentrations of 10, 20, 30 μM arsenite or arsenate, the expression of FasL was evaluated in ECV304 cells. Immunoblot analyses revealed that treatment of ECV304 cells with arsenite but not arsenate suppressed the FasL expression in a dose-dependent manner (Fig. 2A). Flow cytometric analysis was performed to examine FasL expression on the cell surface of ECV304 cells. As shown in Fig. 2B, the dotted line is for mouse IgG used as an isotype control. The shaded histogram represents nontreated ECV304 cells, and the solid line indicates arsenite-treated ECV304 cells. The FasL expression on arsenite-treated cells was present at lower levels than that on nontreated cells (control cells). Consistent with data from the western blot analyses, incubation with arsenite decreased the expression of FasL on ECV304 cells. In agreement with earlier findings, TNFα decreased the expression of FasL on ECV304 cells (data not shown). These data indicated that arsenite is able to decrease the FasL expression in a concentration-dependent manner, however, arsenate cannot affect the FasL expression even at 30 μM. Arsenite also decreased the expression of FasL on primary human umbilical vein endothelial cells (HUVEC) in a dose dependent manner; likewise a lower dose is required for suppression of FasL.

As shown in Fig. 3, ECV304 cells treated with 30 μM sodium arsenite or 25 ng/ml TNFα both significantly increased intracellular peroxide levels ($P < 0.05$). Cells treated with 50 μM hydrogen peroxide was used as a positive control for an increase of intracellular peroxide levels. These

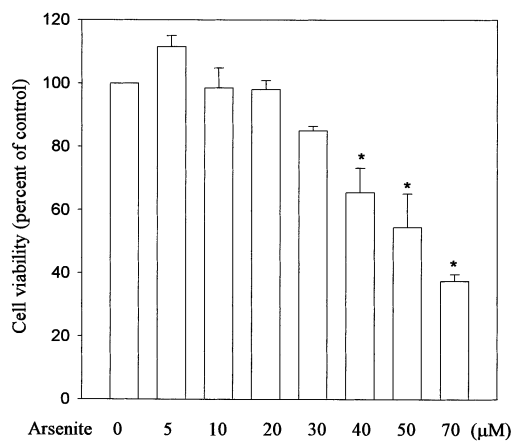

Fig. 1. Effects of arsenite on endothelial cell viability. Confluent monolayer ECV304 cell cultures were treated in complete medium with increasing concentrations of sodium arsenite for 24 h. After treatment 20 μM MTS mixture, (3-(4,5,-dimethyl thiazol-2-yl)-5-(3-carboxymethhoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) and phenazine methosulfate, was added to the cultures for an additional 1 h and analyzed as indicated in Section 2. Data represent mean ± S.D. ($n = 3$). *, Statistically significant decrease in cell viability compared with control samples ($P < 0.05$).

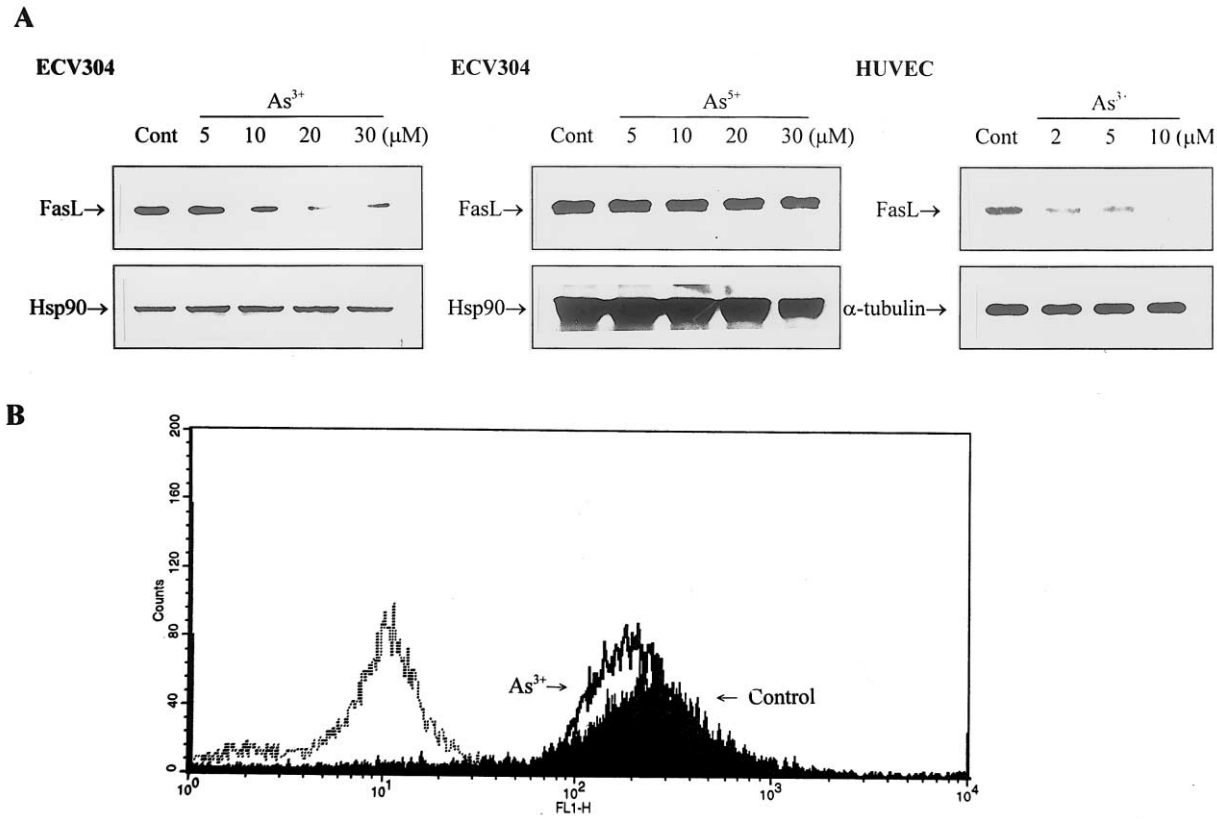

Fig. 2. Effects of arsenite and arsenate on the expression of FasL in ECV304 cells and HUVEC. (A) Western blot analysis of total protein extracts from ECV304 cells or HUVEC treated with the indicated concentrations of arsenite or arsenate for 15 h. Extracts were separated on 10% SDS-PAGE and analyzed as indicated in Section 2. Similar results were obtained in three independent experiments. (B) Flow cytometric analysis of FasL expression on ECV304 cells. ECV304 cells were incubated in either basal medium (shaded histogram) or 30 μM arsenite (solid line) for 15 h. ECV304 cells were detached and incubated with anti-FasL monoclonal antibody (middle panel and right panel) or with mouse IgG in PBS with 10% FBS (left panel), followed by incubation with FITC-conjugated anti-mouse IgG. The left panel is used as an isotype control for mouse IgG. This is representative of three separate experiments.

data indicated that the increment of ROS might play a role as a mediator in arsenite-suppressed FasL expression. To further demonstrate the suppression of FasL expression is mediated by ROS, ECV304 cells were incubated with various concentrations of hydrogen peroxide. Immunoblot analyses demonstrated that hydrogen peroxide was able to decrease FasL expression in a dose-dependent manner (Fig. 4A), and flow cytometric analysis also revealed that hydrogen peroxide downregulated FasL expression on ECV304 cells (Fig. 4B).

In the next serial of experiments, we investigated whether NAC prevents the suppression of FasL expression by arsenite, hydrogen peroxide, or TNFα. ECV304 cells were treated with or without 5 mM NAC for 12 h prior to 30 μM arsenite, 30 μM hydrogen peroxide, or 25 ng/ml TNFα. In Fig. 5A, western blot analyses show that NAC is able to protect ECV304 cells from arsenite, hydrogen peroxide, or TNFα-mediated downregulation of FasL expression. The interference of NAC with FasL expression on ECV304 cells was further confirmed in cytotoxicity analyses (Fig. 5B). Cytotoxicity analyses were applied to determine whether FasL expression on

ECV304 cells is functional. The ECV304 cells were cotreated with NAC and arsenite or treated with NAC or arsenite only for 12 h. Then the ECV304 cells were fixed and co-cultured with Jurkat cells to assess the ability of FasL on ECV304 cells to induce apoptosis in Fas-positive target cells (Jurkat cells). Prior treatment of ECV304 cells with arsenite significantly decreased the sub-G1 ratio of Jurkat cells. In contrast, cotreatment with arsenite and NAC could prevent decrease in the sub-G1 ratio of Jurkat cells. These results suggest that antioxidant-NAC could prevent the arsenite, hydrogen peroxide, TNFα-mediated downregulation of FasL.

4. Discussion

Arsenite is the most toxic form of inorganic arsenic found in the environment. Well waters in areas of Taiwan, where Blackfoot's disease is an endemic peripheral vascular disease, contain between 4.8 and 15.6 μM arsenite (Tseng et al., 1997). Pi et al. (2000) reported that the mean blood concentrations of arsenite in subjects exposed to it was three to nine times lower than the mean environmentally relevant of arsenic in drinking water (Pi et al., 2000). In addition, epidemiological studies in the US have indicated that chronic exposure to >0.25 μM arsenite by drinking water is associated with increased mortality from cardiovascular disease (Engel et al., 1994). However, the underlying cellular mechanisms that initiate vascular disease in response to arsenic are

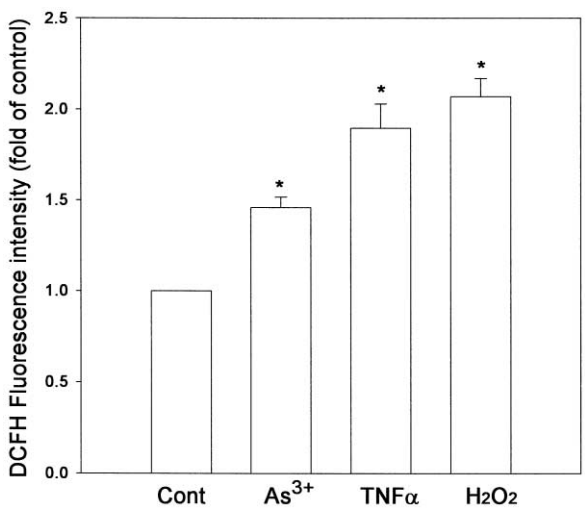

Fig. 3. Effect of arsenite on the production of reactive oxygen species (ROS) in ECV304 cells. Cells were treated with arsenite (30 μM) or TNFα (25 ng/ml) and incubated with 50 μM DCFH-DA for 1 h, and the fluorescence in the cells was immediately assayed using cytometry. Cells were treated with 50 μM hydrogen peroxide and incubated with 50 μM DCFH-DA for 1 h was used as a positive control. Data are presented as fold of green fluorescence intensity, which is compared with control (control is as 1). Results are the means of two independent experiments. *, Statistically significant increase in fluorescence intensity compared with control samples ($P<0.05$).

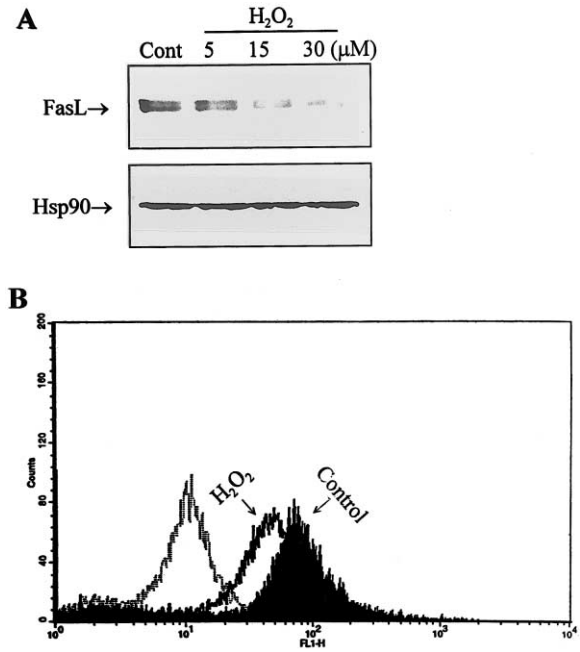

Fig. 4. Effects of hydrogen peroxide on the expression of FasL in ECV304 cells. (A) Western blot analysis of the total protein extracts from ECV304 cells treated with the indicated concentration of hydrogen peroxide for 15 h. Extracts were separated on 10% SDS-PAGE and analyzed as indicated in Section 2. Similar results were obtained in three independent experiments. (B) Flow cytometric analysis of FasL expression on ECV304 cells. ECV304 cells were incubated in either basal medium (shared histogram) or 30 μM hydrogen peroxide (solid line) for 15 h. ECV304 cells were detached and incubated with anti-FasL monoclonal antibody (middle panel and right panel) or with mouse IgG in PBS with 10% FBS (left panel), followed by incubation with FITC-conjugated anti-mouse IgG. The left panel is for mouse IgG used as an isotype control. This is representative of three separate experiments.

not known. Endothelial cell functions are essential to both normal and abnormal vascular biology. Activation of dysfunction of vascular endothelial cells in response to low levels of chemicals or inflammatory agents induces an endothelial cell phenotype that is proinflammatory and retains monocytes in the vessel wall (Bencko, 1987; Guy et al., 1993). However, our group has reported that high levels of arsenite not arsenate could induce apoptosis through release of cytochrome C to cytosol, activation of CPP32 protease, and PARP degradation in NIH3T3 cells (Chen et al., 1998b). In this study, we focus on the effects of low levels of arsenite on ECV304 cells and endothelial cells. A recent report suggests that the deregulated expression of FasL, Fas, or signaling pathway components of this system may be a feature of endothelial cell dysfunction in response to injurious agents leading to inflammatory-fibroproliferative disorders of the vessel wall (Walsh and Sata, 1999). The data presented in the current study are the first observation that non-lethal concentrations of arsenite can suppress the expression of FasL on endothelial and ECV304 cells, and this suppression of FasL was mediated through ROS.

In this study, we have investigated downregulated FasL expression in both primary and transformed human endothelial cells. We first investigated the particular role played by ROS as a mediator for downregulation of FasL. Intracellular ROS are generated in different cellular compartments and electron transfer reactions (Halliwell and Gutteridge, 1990). We found that

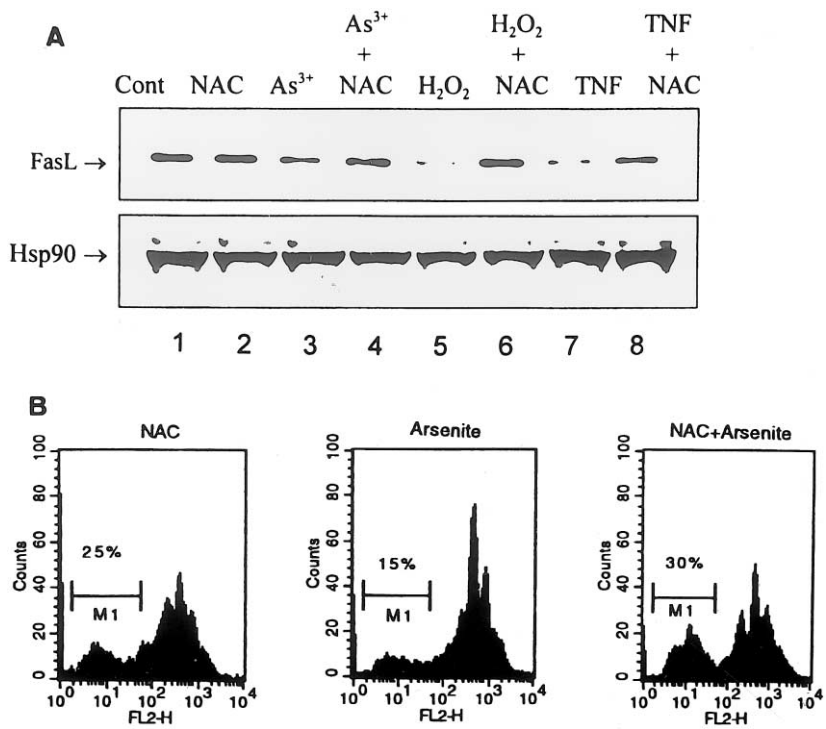

Fig. 5. Effects of NAC on FasL expression in response to arsenite, hydrogen peroxide, or TNFα. (A) Western blot analysis of total protein extracts from ECV304 cells treated with or without the 5 mM NAC for 12 h prior to arsenite (30 μM), hydrogen peroxide (30 μM), TNFα (25 ng/ml) treatment. Extracts were separated on 10% SDS-PAGE and analyzed as indicated in Section 2. Similar results were obtained in three independent experiments. (B) FACScan analyses of propidium iodide staining of Jurkat cells co-cultured with ECV304 cells. The cells were treated as indicated in Section 2. After co-culture 48 h, Jurkat cells were isolated and analyzed by flow cytometry. The ratios of sub-G1 cells were gated (M1). Similar results were obtained from three separated experiments.

peroxide level in ECV304 was increased upon stimulation with arsenite or TNFα, as measured at concentrations of exposure to stimulate that gave strong suppression of FasL expression in these cells. This is consistent with study by Barchowsky et al. (1996) in pocine aortic endothelial cells, where they showed that non-lethal levels of arsenite could elevate intracellular oxidant formation including superoxide and hydrogen peroxide. However, low concentrations of arsenite did not increase NO levels (Barchowsky et al., 1996). TNFα has also been found to generate ROS in various cell types (Schulze-Osthoff et al., 1992, 1993). Adding hydrogen peroxide to ECV304 cells, however, suppressed FasL expression. In addition, antioxidant-NAC could prevent the downregulation of FasL by arsenite, hydrogen peroxide, or TNFα. These findings strongly indicate that ROS are indeed able to interfere FasL expression. Based on the results of a reverse-transcription PCR assay, Bauer et al. (1998), Vogt et al. (1998) both reported that 500 μM hydrogen peroxide significantly increased FasL transcripts in Jurkat cells and microglial cells (Bauer et al., 1998; Vogt et al., 1998). However, the results of the present study clearly show that even lower concentration of hydrogen peroxide (15 μM) suppressed the FasL expression (Fig. 4). This phenomena may due to the effects of hydrogen peroxide on FasL expression are cell types specific. In addition, one possible explanation for these apparently contradictory results is the difference in assays used to measure FasL expression or bi-phasic reaction of hydrogen peroxide to FasL expression. However, this problem deserves further investigation.

The data in the present study indicate that environmentally relevant concentrations of arsenite initiate endothelial cell signaling. Arsenite treatment of endothelial cells results in induction of ROS and suppression of FasL expression, which is operative during the course of vascular dysfunction in response to arsenic. Thus, this study suggests that non-lethal concentrations of arsenite promote vascular injury, at least partly by suppressing FasL expression on endothelium through ROS signaling.

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