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Low Concentrations of Arsenic Induce Vascular Endothelial Growth Factor and Nitric Oxide Release and Stimulate Angiogenesis In Vitro

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Arsenic (As) is widely distributed in nature, and its contamination in drinking water remains a major public health problem. Exposure to As may lead to degenerative peripheral vascular diseases. The purpose of this study is to clarify the role of As in modulating cell proliferation and in vitro angiogenesis in human umbilical vein endothelial cells (HUVECs) and to scrutinize the contributing factors of these events. The results revealed that lower concentrations (up to 1 μM) of sodium arsenite stimulated HUVEC cell growth. An in vitro angiogenesis assay indicated that low concentrations of As increased vascular tubular formation, which was abrogated by hemoglobin, a potent nitric oxide scavenger. In contrast, higher concentrations of As (>5 μM) revealed cytotoxicity and inhibition to angiogenesis. We also demonstrated that lower concentrations of As upregulated the expression of constitutive nitric oxide synthase (NOS3) at both transcriptional and translational levels and that lower concentrations of As implicated a modulatory role in vascular endothelial growth factor (VEGF) expression. In addition, low concentrations of As (<1 μM) increased von Willebrand Factor (vWF) antigen expression, whose elevation paralleled the onset of angiogenesis and was considered an early indicator of endothelial activation in tumor metastasis. VEGF and basic fibroblast growth factor can synergistically upregulate the vWF gene expression. Therefore, we conclude that the treatment of HUVECs with As leads to cell proliferation and activation, which preferentially enhances angiogenesis in vitro, possibly via the VEGF−NOS signaling pathway. The molecular mechanism(s) by which As facilitates angiogenesis remains to be elucidated.

Introduction

Human beings are exposed to As[1] through environmental, medical, and occupational sources (*1*). Drinking water contamination by As remains a major public health problem. Acute and chronic As exposure via drinking water has been reported in many countries of the world. There are sufficient epidemiological evidences revealing a causal association between As exposure and human disease. General health effects that are closely associated with As exposure include significantly higher standardized mortality ratios and cumulative mortality rates for cancers of the bladder, kidney, skin, liver, and colon in many areas of As pollution (*2*−*4*). In addition, As exposure leads to a higher mortality ratio with cardiovascular diseases, such as atherosclerosis (*5*−*7*) and degenerative peripheral vascular diseases such as black-foot disease, which is endemic in a limited area on the southwestern coast of Taiwan (*8*, *9*). These epidemiological studies strengthened the association of As exposure with the occurrence of peripheral vascular diseases. Barchowsky et al. reported that low levels of As trioxide stimulate proliferative signals in primary vascular cells without activating stress effector pathways (*10*). In vitro evidence revealed that As trioxide induces apoptosis of endothelial cells (ECs) and prevents capillary tubule and branch formation (*11*).

Angiogenesis, the sprouting of new capillaries from a preexisting network, is a complex process that encompasses activation, migration, and proliferation of ECs. This process is essential for reproduction, development, repair, and angiogenic and neoplastic diseases (*12*). VEGF is a secreted endothelial specific growth factor that is strongly angiogenic in vivo (*13*−*15*). Besides its involvement in vascular development, VEGF has been demonstrated to play a key role in both physiologic and tumor angiogenesis in adult mammals (*14*, *16*). NO was initially identified as endothelium-derived relaxing factor, a molecule with profound vasomotor regulatory effects (*17*). Subsequent investigations established a number of diverse regulatory activities for NO, including inhibition of platelet aggregation, leukocyte adherence, and smooth muscle proliferation (*18*−*20*). A previous investigation provided direct evidence that NO may induce angio-

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[1] Abbreviations: arsenic, As; bromodeoxyuridine, BrdU; extracellular matrix, ECM; human umbilical vein endothelial cells, HUVECs; nitric oxide, NO; endothelial constitutive nitric oxide synthase, NOS3; vascular endothelial cell growth factor, VEGF; von Willebrand factor, vWF.

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genesis in vitro (*21*). Furthermore, Ziche et al. established the first line of evidence that NO can induce angiogenesis in vivo (*22*, *23*). The objective of this study is to investigate whether As participates in the regulation of cell proliferation and other functions in vascular ECs, particularly focusing on angiogenesis and its related regulators. In addition, we studied the effect of As on EC tubular formation and its possible signaling mechanism.

Experimental Procedures

Cell Culture and Identification. HUVECs were isolated from fresh umbilical cords stored in Hank's balanced salt solution (HBSS; Life Technology/Gibco BRL, Grand Island, NY) at 4 °C, using the modified method of Jaffe et al. (*24*). Briefly, the cord was washed with ice-cold PBS and the sterile three way stopcocks were tightened with nylon string at both ends of the umbilical vein. The lumen was flushed with HBSS three times, and 0.1% collagenase type IV (Sigma, St. Louis, MO) was injected into the vein and incubated at 37 °C for 20 min. The reaction was stopped by addition of minimal essential medium with 10% fetal bovine serum (FBS). The cell pellet was collected by centrifugation at 1000 rpm at 4 °C for 7 min. Then, the cells were fed with Isobecove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-treated FBS (Life Technology/Gibco BRL), 100 μg/mL penicillin (Life Technology/Gibco BRL), 100 units/mL streptomycin (Life Technology/Gibco BRL), 15 μg/mL endothelial cell growth supplement (ECGS; Upstate Biotechnology, Lake Placid, NY), 100 μg/mL heparin (Sigma), and 10 μg/mL insulin (Life Technology/Gibco BRL). The media were renewed every 3 days until confluency. HUVECs from the second to the third passage were applied to the following experiments. HUVECs from the second passage were used to identify the characteristics of ECs by immunodetection of vWF. The cell viability counting was assisted by adding trypan blue, to determine the ability of dye exclusion of live cells. Sodium arsenite at concentrations of 0.1−20 μM were added to HUVECs for the following experiments.

MTS Cell Proliferation Assay. A commercially available kit CellTiter96 AQueous proliferation assay kit, Promega, Madison, WI, was used to detect the cell proliferation according to the manufacturer's instruction. HUVECs were seeded in a 96 well plate at the cell density of 2500 cells/well. After they were incubated overnight, the sodium arsenite at indicated concentrations was added to the culture media and incubated for 24 h. The MTS reagent contained tetrazolium salt, [3-4,5-dimethylthiazol-2-yl-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium], premixed with the electron coupling reagent (phenazine ethosulfate), which was added into each well at 20 μL. The plate was then incubated for 1−2 h at 37 °C. The optical density value was detected by a microplate reader (MRX-II, Dynex Technology, Chantilly, VA), whose detecting and reference wavelengths were set at 490 and 690 nm, respectively (*25*).

Flow Cytometric Detection. Flow cytometer was used to measure cell cycle profile, BrdU incorporation, and vWF antigen expression in HUVECs. For cell cycle analysis, HUVECs treated with or without As for 24 h were harvested by trypsinization and centrifugation. After they were washed with PBS, the cells were fixed with ice-cold 70% ethanol for 30 min, washed with PBS, and then treated with 1 mL of 1 mg/mL of RNase A solution (containing 0.112 mg/mL of trisodium citrate) at 37 °C for 30 min. Cells were harvested by centrifugation at 400*g* for 5 min and further stained with 250 μL of DNA staining solution (10 mg of propidium iodide [PI], 0.1 mg of trisodium citrate, and 0.03 mL of Triton X-100 were dissolved in 100 mL of H_2O) at room temperature for 30 min in the dark. After 750 μL of PBS was loaded, the DNA contents of 10 000 events were measured by FACScan (Elite ESP, Beckman Coulter, Brea, CA) and the cell cycle profile was analyzed from the DNA content histograms with WinCycle software.

The second method allowed an assessment for the proportion of cells undergoing replicative DNA synthesis. HUVECs treated with or without As for 20 h were further pulse-labeled with 10 μM BrdU (Sigma) for 4 h before harvest. Cells were then fixed with 70% ethanol, treated with 0.1 N HCl, and heated for 10 min at 97 °C to expose the labeled double strand DNA. Cells were neutralized with 0.1 N NaOH, stained with anti-BrdU-conjugated FITC (Immunotech, France), and then counterstained with PI. The percentage in S phase was analyzed by gating on BrdU/PI dot plots using Windows Multiple Document Interface software (WinMDI).

For the detection of vWF antigen expression, HUVECs treated with or without As were harvested by scraping and centrifugation. After they were washed with PBS, the cells were fixed with a mixture of acetone and methanol (1:1) at −20 °C for at least 30 min. Following washes with PBS, the cells were then treated with 50 μL of rabbit antihuman vWF antibody (DAKO, Carpinteria, CA) with 1:50 dilution at room temperature for 1 h. After washes with PBS, the immunoreactive signal was linked to secondary FITC-conjugated goat antirabbit IgG antibody (Chemicon, Temecula, CA) at 1:1000 dilution and incubated at room temperature for 1 h. The cells were finally washed five times with PBS, passed through a 30 μm mesh, and detected by FACScan. The mean fluorescent intensity (MFI) of each group was analyzed using WinMDI software.

In Vitro Angiogenesis Assay. The commercially available Matrigel, purchased from Becton Dickinson Labware (Bedford, MA), was used for the extracellular substrate in the in vitro angiogenesis assay. The Matrigel substrate (10 mg/mL) was diluted 2-fold with culture medium containing no FBS and kept on ice to avoid gelling. An appropriate volume of diluted Matrigel (50 μL/cm^2 growth area) was added into each well of a 24 well plate and placed at 37 °C for at least 30 min until complete gelling. Then, the trypsinized HUVECs pretreated with different concentrations of sodium arsenite for 24 h were seeded onto the gel at 4×10^4 cells per well. The cells were cultivated at 37 °C in 5% CO_2 and a humidified incubator. Hemoglobin (Hb; Sigma), a potent NO scavenger, was used at 10 μg/mL to abrogate the possible angiogenic effect of As (*26*). The tubing morphogenesis was documented microscopically at indicated time points, and the tube length was measured at five distinct fields under an inverted phase contrast microscope.

Total RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from 1×10^6 HUVECs using the TRIzol method (Life Technology/Gibco BRL) and processed as recommended by the manufacturer's specification. We prepared the first strand cDNA from the total RNA by avian myeloblastosis virus (AMV) reverse transcriptase. The reverse transcription reaction was performed in a final volume of 20 μL of reaction buffer containing 50 mM Tris-HCl, at pH 8.3, 10 mM magnesium chloride, 50 mM potassium chloride, 10 mM DTT, 0.5 mM spermidine, 0.5 μg oligo-d$(T)_{15-18}$ primer (Promega, Madison, WI), 1 μM each deoxyrionucleotides (Pharmarcia, Sweden), 20 units of rRNasin ribonuclease inhibitor, 2.5 U AMV reverse transcriptase (Promega), and 2 μg of total RNA. The reaction was allowed to proceed at 42 °C for 60 min. The sample was then heated at 99 °C for 5 min followed by incubation at 4 °C for 5 min.

For amplification of the mRNAs of β-actin, NOS3, basic fibroblast growth factor (bFGF), and VEGF, the primer pairs were synthesized as follows: β-actin, (sense) 5′-TCC TGT GGC ATC CAC GAA ACT-3′ and (antisense) 5′-GAA GCA TTT GCG GTG GAC GAT-3′; NOS3, (sense) 5′-GTG ATG GCG AAG CGA GTG AAG-3′ and (antisense) 5′-CCG AGC CCG AAC ACA CAG AAC-3′; bFGF, (sense) 5′-AGA GAG AGG AGT TGT GTC-3′ and (antisense) 5′-GGT CCT GTT TTG GAT CCA-3′; and VEGF, (sense) 5′-CGA AGTGGT GAA GTT CAT GGA TG-3′ and (antisense) 5′-ATA TCC ATC ACA CTG GCG GCC GC-3′. The annealing temperatures for the above primer pairs were 60, 63, 50, and 58 °C, respectively. The profile of the amplification cycle was as follows: 30 s melting at 94 °C, 45 s at annealing temperature, and 60 s extending at 72 °C. Thirty PCR cycles

were performed in a DNA thermal cycler (Hybaid, Middlesex, U.K.). The sizes of the amplified fragments were 314 base pair (bp) for β-actin, 422 bp for NOS3, 222 bp for bFGF, with the exception that four alternative spicing isoforms of the VEGF gene were simultaneously detected by the gene specific primer designed by Iijima et al. (*27*). The amplified fragments were 403, 535, and 603 bp for $VEGF_{121}$, $VEGF_{165}$, and both $VEGF_{189}$ and $VEGF_{206}$, respectively. The PCR products were loaded on a 1.8% agarose gel for electrophoresis, stained with ethidium bromide, and further visualized by UV illumination. The visualized gels were recorded and analyzed on a digital imaging system (Alpha Imager 2000, Alpha Innotech Corp., San Leandro, CA). PCR products were titrated to establish standard curves for documenting linearity and permitting semiquantitative analysis. Levels of gene expression were expressed as the ratios of densities between PCR products and β-actin in the same sample.

Protein Extraction and Western Blotting Analysis. Total cell extracts from cultured HUVECs were obtained by lysing the cells in ice-cold RIPA buffer (50 mM Tris-HCL, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.4% sodium cacodylate, and 150 mM NaCl) in the presence of a cocktail of protease inhibitors (Roche, Molecular Biochemicals, Mannheim, Germany). After they were centrifuged, supernatants were quantified using the Bio-Rad protein assay (Hercules, CA) and an equal amount of total protein for each lane was subjected to SDS−PAGE, using 12 or 15% acrylamide gels under reducing conditions. Proteins were subsequently electrotransferred onto a nitrocellulose membrane (Sartorius AG, Göttingen, Germany) following conventional protocols. Blots were blocked in 5% skimmed milk in PBS-T (PBS, pH 7.4, with 0.1% Tween-20) overnight at 4 °C, followed by 1 h of incubation with primary antibodies at room temperature. The working concentrations of primary antibodies diluted in PBS-T were 0.5 μg/mL for NOS3 (clone N-20, Santa Cruz, CA) and 2 μg/mL for GAPDH (MAB374, Chemicon), respectively. After five washes with PBS-T, the blots were incubated with secondary antibodies (horseradish peroxidase coupled with antimouse or antirabbit immunoglobulin G) at 1:5000 dilution. The enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Piscataway, NJ) detection system and BioMax film (Kodak, Rochester, NY) were used to visualize the presence of immunoreactive proteins on the blots according to the manufacturer's instructions. The exposure time for each protein (ranging from 1 min to 1 h) was calibrated by documenting linearity and permitting semiquantitative analysis. The visualized films were recorded on a digital imaging system (Alpha Imager 2000) and analyzed in a densitometrical analysis system (Apha Ease version 3.23, Alpha Innotech Corp.). Relative protein levels were expressed as the ratios of densities between proteins and GAPDH in the same sample.

NO Assay. Nitrite/nitrate concentrations were measured by the modified Griess reaction to estimate total amounts of NO release from the HUVECs (*28*). Cultured HUVECs were treated with different concentrations of sodium arsenite for 24 h or incubated with 1 μM sodium arsenite at indicated time points. The supernatants were collected and centrifuged to remove cell debris. A total of 50 μL of samples and sodium nitrate standards were loaded into microtiter plates and replenished with reaction buffer (50 mM MOPS, 1 mM EDTA, pH 7.0) to a final volume of 85 μL. Then, 5 μL of nitrate reductase (0.01 units/well; Sigma) and 10 μL of 2 mM β-NADH (reduced form, Sigma) were added into each well. The plate was placed on an orbital shaker and incubated at room temperature for 20 min. Subsequently, 50 μL of color reagent A (sulfanilamide dissolved in 3 N HCl) and an equal volume of color reagent B [*N*-(1-naphthyl)ethylenediamine dihydrochloride dissolved in H_2O] were added into each well and incubated at room temperature for 5 min. The optical density was detected with a microtiter plate reader (MRX-II, Dynex Technology) at 540 nm, and then, the value of the blank controls (medium without cells) was subtracted. The nitrite/nitrate concentrations were calculated with the standard curve.

VEGF and bFGF Protein Quantification. Cultured HUVECs were treated with different dosages of sodium arsenite for 24 h or 1 μM sodium arsenite at indicated time points. Angiogenic factors release from HUVECs, i.e., VEGF and bFGF were assayed by commercially available ELISA kits (Assay Design Inc., Ann Arbor, MI) according to manufacturer's specifications.

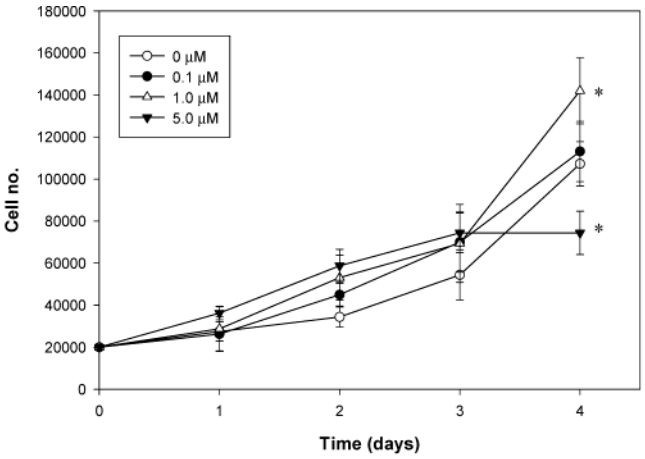

Figure 1. Lower concentrations of sodium arsenite stimulate HUVEC cell growth. HUVECs were seeded in a 24 well plate at 2×10^4 cells per well and cultivated overnight until cell attachment. Arsenite at the indicated concentrations was added into the culture media in triplicate. The number of viable cells was counted by trypsinization and assisted with trypan blue dye exclusion. * indicates that $p < 0.05$ as compared to the control.

Statistical Analysis. All results were expressed as means ± SD and analyzed by using the statistical analysis system (SPSS, SPSS Inc., Chicago, IL). Differences among groups were analyzed by Student's *t*-test. Values of $p < 0.05$ were considered significant for all statistical tests.

Results

Low Concentrations of As Stimulate HUVEC Proliferation. The biostimulatory effect of sodium arsenite on HUVECs was determined by the growth curve of HUVECs measured by direct cell counting (Figure 1). The cell number of HUVECs treated with 1 μM As for 4 days was significantly higher than that of the control group ($p < 0.05$). On the other hand, the concentrations of As higher than 5 μM exhibited an inhibitory effect on cell growth ($p < 0.05$), while As at 20 μM completely inhibited cell proliferation (not shown). The morphological observations revealed that As at 10 μM caused significant cytotoxicity, whereas low concentrations of As (<1 μM) showed no influence on cell morphology (not shown). The activity of mitochodrial dehydrogenase enzymes, detectable by catalyzing MTS reagent, correlated with the cell viability. The cell viability of HUVECs treated with sodium arsenite at different dosages is shown in Figure 2. An increase in cell viability was observed in the groups treated with As at concentrations lower than 3 μM, while an inhibitory effect was observed at concentrations higher than 5 μM. The cell cycle distribution and BrdU incorporation of HUVECs treated with As for 24 h is shown in Table 1. There was no significant alteration in the cell cycle profile observed in the group treated with 0.1 μM As as compared to no treated control. When cells were treated with 1 μM As, the percentage of S phase subpopulation (% S) was significantly reduced whereas % G_2/M increased ($p < 0.05$). Higher concentrations of As (>10 μM) exhibited a remarkable S phase arrest reflected by an increase in % S and a decrease in % G_2/M, as revealed by a simultaneous decrease in BrdU incorporation.

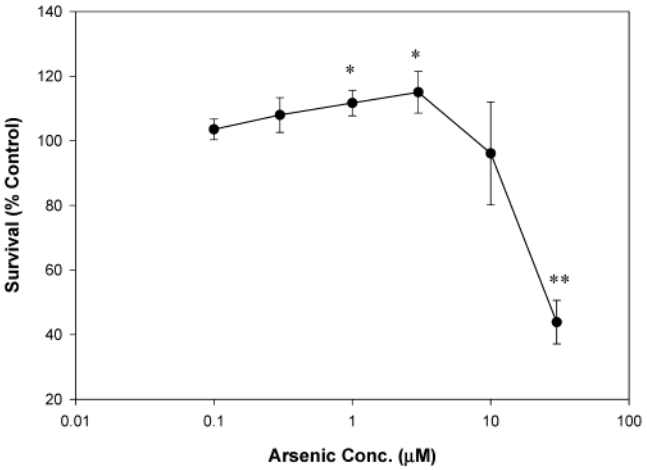

Figure 2. Lower concentrations of sodium arsenite stimulate cell viability in HUVECs. Cells were seeded in a 96 well plate at 3000 cells per well and cultivated overnight until cell attachment. Arsenite at the indicated concentrations was added into the culture media in triplicate and incubated for 24 h. Cell viability was detected using MTS reagent in the proliferation assay kit with an incubation of 1 h. * indicates that $p < 0.05$, and ** indicates that $p < 0.01$ as compared to the control.

Table 1. Cell Cycle Distribution and BrdU Incorporation of HUVECs in Response to As Treatments[a]

As concn (μM)	cell cycle distribution			
	% G_1	% S	% G_2/M	% BrdU (+)
0	79.1 ± 3.5	4.8 ± 0.6	16.1 ± 2.1	16.55 ± 5.1
0.1	79.4 ± 2.8	5.0 ± 0.8	15.6 ± 3.2	17.21 ± 3.6
1.0	78.4 ± 1.6	3.0 ± 0.5*	18.7 ± 1.6*	15.35 ± 3.3
10	75.5 ± 3.3	10.4 ± 1.2*	14.1 ± 1.2*	8.66 ± 2.1*
20	58.3 ± 4.2**	31.7 ± 2.3**	10.0 ± 1.6**	2.41 ± 1.3**

[a] The data are expressed as mean ± SD. *: $p < 0.05$; **: $p < 0.01$, $n = 3$.

Low Concentrations of As Promote Angiogenesis In Vitro. An in vitro experimental model for angiogenesis was adopted in this study. Tubular morphogenesis spontaneously occurs within 4 h, when cultured HUVECs are seeded onto the commercially available Matrigel, in which ECM substrates and a trace of growth factors provide the signals for angiogenesis induction. This tubular formation network can be enhanced by exogenous angiogenic factors such as VEGF and TNF-α, but the integrity of tubings only remains for 48 h in our experimental conditions. Figure 3A shows microphotographs of the tubing morphogenesis of HUVECs on Matrigel 24 h after seeding. The integrity of the tubing network in the group pretreated with 0.1 μM As remained as intact as that in the control group. Concentrations higher than 5 μM seemed to hamper the induction of tubing morphogenesis. Furthermore, this enhanced angiogenic activity was also reflected by the observation that an earlier initiation of tubing morphogenesis was noticed in 0.1 μM As-treated groups (with an incubation shorter than 3 h) as compared to the control groups (emerging after 4 h of incubation). However, this enhancing effect was profoundly abrogated by the addition of Hb at 10 μg/mL, when the pretreated cells were seeding onto Matrigel. The quantitative analysis of angiogenic activity was done by measuring the length of tubing networks in microphotographs. The result revealed that pretreatment with low concentrations of As (at 0.1 μM) significantly increased the tube length ($p < 0.05$), whereas concentrations higher than 5 μM disrupted the tubing integrity ($p < 0.05$) (Figure 3B). The tube length in the group pretreated with 0.1 μM As can be elevated up to 1.54-fold of the control level ($p < 0.05$). Addition of Hb at 10 μg/mL can significantly reduce this elevation back to the control level ($p < 0.05$) (Figure 3C).

Low Concentrations of As Upregulate NO and VEGF Release from HUVECs. Semiquantitative RT-PCR was performed to determine the effects of As on the gene expression of angiogenic factors, i.e., bFGF, VEGF, and NOS3 (Figure 4A). The results revealed that the transcription of NOS3 was significantly upregulated by As treatment. The relative intensity of gene expression normalized to the level of housekeeping β-actin revealed that the concentrations equal to and lower than 5 μM significantly induced NOS3 gene expression (Figure 4B), while this induction peaked at 0.5 μM (about 3.5-fold). A pair of primers designed to simultaneously detect three distinct isoform transcripts of VEGF was used in PCR amplification. The expression of $VEGF_{121}$ transcript in HUVECs was increased by As treatment, while the maximal induction was noticed by 2.8-fold when exposed to 0.1 μM. A mild induction of gene expression of bFGF, $VEGF_{165}$, and $VEGF_{189/206}$ peptides at the concentrations tested was also noticed but not significant. It is worth mentioning that due to the limitation of RT-PCR detection, it is not distinguishable whether the up- and downregulation of gene expression by As treatment is mediated by affecting transcriptional machinery or by altering the stability of transcripts. The direct effect of As on the machinery and the regulatory elements for gene expression needs to be further elucidated.

The result of Western blotting revealed that low concentrations of As exhibited a maximal stimulation of NOS3 protein expression at 0.5 μM and a marked inhibition at concentrations higher than 5 μM (Figure 5A). Densitometrical analyses revealed that an elevation of NOS3 protein expression was increasingly noticed at lower concentrations up to 1 μM As, where the maximal induction was induced by 1.5-fold (Figure 5B). Figure 6 showed the effects of As on NO and VEGF release into supernatants of HUVECs. The nitrite/nitrate concentrations in HUVECs treated with 0.1 (13.6 ± 0.9 μM/10^5 cells) and 1 μM (14.1 ± 0.8 μM/10^5 cells) of As for 24 h were significantly higher than that of the negative control (10.7 ± 0.2 μM/10^5 cells) ($p < 0.05$) (Figure 6A). The time course experiments confirmed the results of the stimulating effect on NO production by 1 μM As (data not shown). The concentrations of VEGF in culture supernatants at 24 h after treatment in the groups of 1 (38.0 ± 7.1 pg/mL) and 5 μM (48.2 ± 9.9 pg/mL) As were significantly higher than that of negative control (23.4 ± 5.8 pg/mL) ($p < 0.05$) (Figure 6B). A time course experiment confirmed the upregulation of VEGF release by 1 μM As (data not shown). In addition, no significant alteration of bFGF release from HUVECs was observed by ELISA detection (data not shown).

All results taken together, the pattern of NOS3 protein expression differed from that of gene expression in that the concentrations higher than 5 μM still retained the upregulation of NOS3 transcription but markedly downregulated the NOS3 translation. The discrepancy may reflect the possibility that As may regulate transcription and translation of genes separately. In addition, we observed different profiles between nitrite/nitrate release and VEGF release in response to As treatment. When exposed to As concentrations higher than 5 μM, the

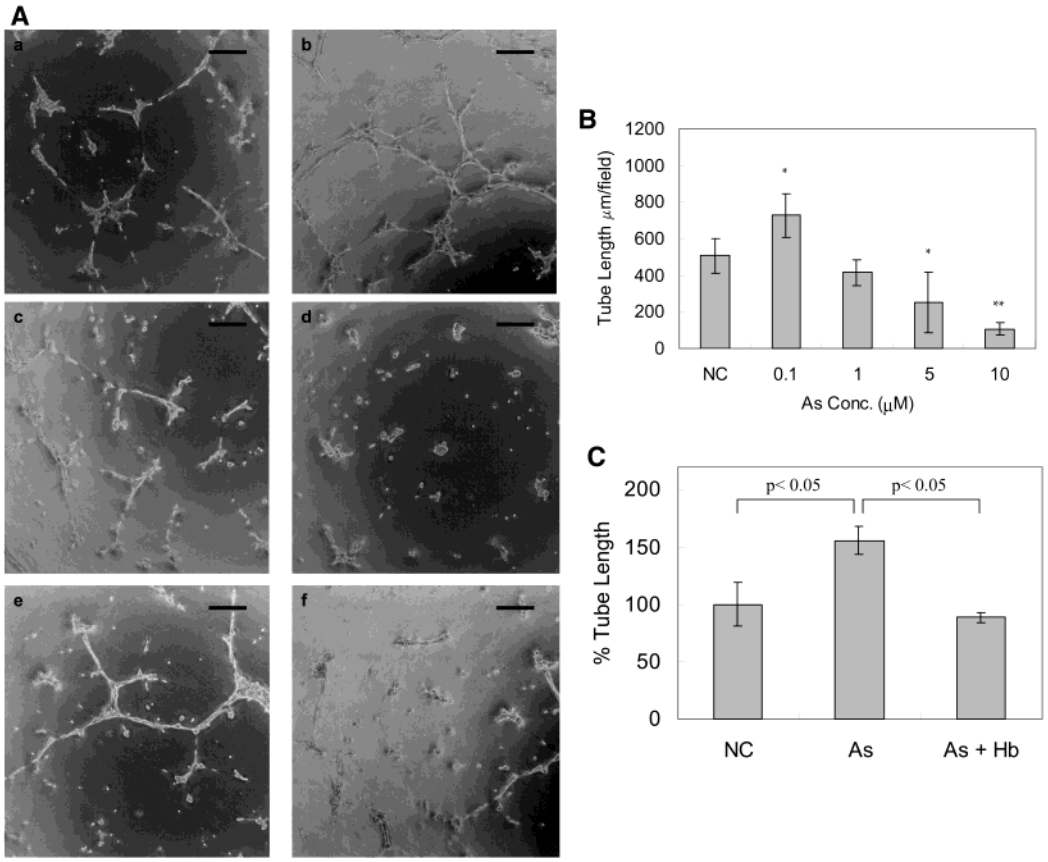

Figure 3. Lower concentrations of sodium arsenite enhance angiogenesis in vitro. A total of 4×10^4 HUVECs pretreated with As for 24 h were seeded onto Matrigel and incubated with or without 10 μg/mL Hb. (A) The tubing morphogenesis 24 h after seeding was documented by microphotography. (a) Negative control; (b) 0.1 μM As pretreatment; (c) 1 μM As pretreatment; (d) 5 μM As pretreatment; (e) 0.1 μM As pretreatment and subsequent Hb addition; (f) 1 μM As pretreatment and subsequent Hb addition. Bar = 120 μm. (B) The absolute value of tube length was measured at five distinct low power fields. The averaged tube length of each group was expressed as micrometer per field. (C) The relative tube lengths of HUVECs pretreated with 0.1 μM As and those simultaneously with 10 μg/mL Hb addition (As + Hb) were normalized by comparison to negative control. * indicates that $p < 0.05$, and ** indicates that $p < 0.01$ as compared to either the control or between groups.

soluble VEGF release was still higher than the control level, whereas the nitrite/nitrate content in supernatants became lower. A possible explanation is that the chemical insult may affect not only peptide synthesis of these two proteins but also the enzymatic activity of NOS3, thus leading to a lower amount of nitrite/nitrate release. Furthermore, this may partially explain why the proangiogenic activity was abrogated by 5 μM As pretreatment, at which concentration the maximal induction of VEGF release was found and its proangiogenic effect was supposed to be the maximum. The role of NO as an important contributor to angiogenesis is, thus, deduced from our observations again.

Low Concentrations of As Increase vWF Antigen Expression in HUVECs. vWF antigen, a biomarker specific to ECs, was recently demonstrated to be closely associated with the occurrence of angiogenic events in metastatic tumor tissue (*29*). In this study, a flow cytometry detection method was conducted to monitor the alterations in terms of this antigen expression in HUVECs treated with As. The results revealed that As at concentrations ranging from 0.1 to 1.0 μM stimulated the expression of vWF on HUVECs (Figure 7). The values of MFI in As-treated groups were significantly higher as compared to the control ($p < 0.05$). A time course experiment confirmed the upregulation of vWF expression by 1 μM As (data not shown).

Discussion

As is an atypical carcinogen. To date, no animal model of As-induced cancer has been developed (*30*, *31*). Recent in vitro evidence indicates that the mechanism underlying the unique properties of As involves changes in mitogenic signaling (*32*). In this study, we demonstrated that the lower concentrations of sodium arsenite (<1 μM) could stimulate the HUVEC growth by direct cell counting (Figure 1), indirect mitochondrial enzymatically colorimetric assay (Figure 2), and DNA synthesis (unpublished data). In contrast, high concentrations of sodium arsenite (>10 μM) resulted in an S phase arrest, as evidenced by the reduction of BrdU incorporation (Table 1). This finding suggested that high concentrations of sodium arsenite elicited a prominent cytotoxicity, which may be associated with the aberration of DNA repairing enzymes, thereby interrupting the cell cycle progression (*31*, *33*, *34*). On the contrary, As at submicromolar levels may disrupt the normal regulation of proliferative events, thereby priming cells to display an amplified mitogenic response upon growth factor stimulation (*32*). Supportive evidence was provided by the observation that the basal MAP kinase activity in cells under long-term exposure to sodium arsenite was elevated and this perturbation contributed to an exaggerated mitogenic response.

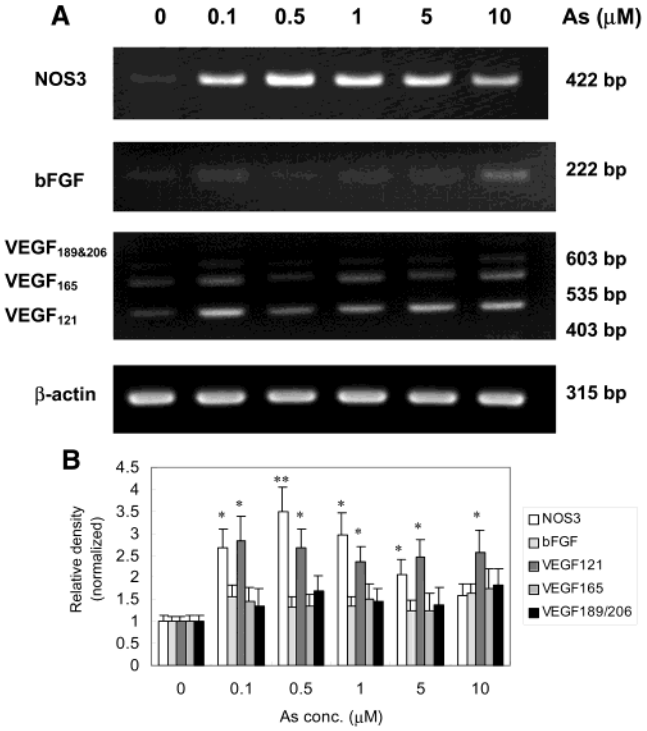

Figure 4. Sodium arsenite upregulates NOS3, bFGF, and VEGF gene expression in HUVEC. HUVECs were seeded in a six well plate at 2×10^5 cells per well and cultivated overnight until cell attachment. Arsenite at the indicated concentrations was added into the culture media in triplicate and incubated for 4 h. (A) The total cellular RNA was extracted, and the semiquantitative RT-PCR was performed. (B) The expression levels of genes in each group were normalized as the ratio of density values of genes to their respective β-actin densities. The ratio for untreated control was considered as 1.0. * indicates that $p < 0.05$, and ** indicates that $p < 0.01$ as compared to the respective control groups.

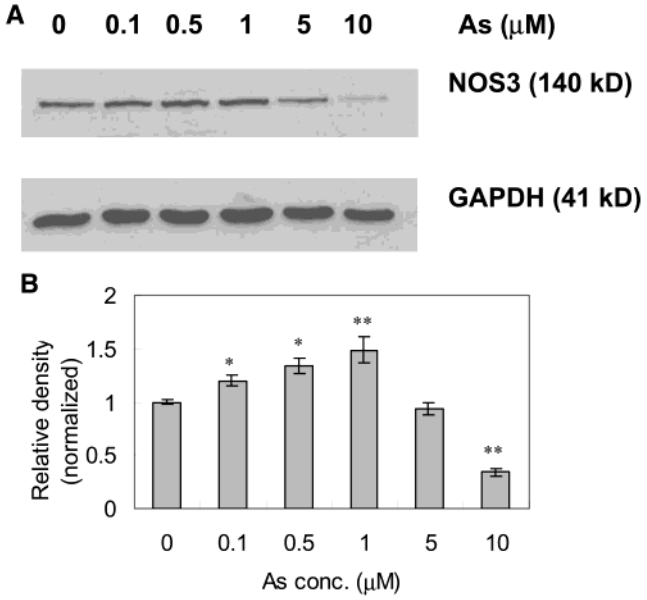

Figure 5. Sodium arsenite at lower concentrations stimulates NOS3 protein expression. HUVECs were seeded in a six well plate at 2×10^5 cells per well and cultivated overnight until cell attachment. Arsenite at the indicated concentrations was added into the culture media in triplicate and incubated for 24 h. (A) The total cellular proteins were collected, resolved by SDS−PAGE, and detected by specific antibody. (B) The protein expression level of NOS3 in each group was normalized as the ratio of density value of NOS3 to its respective GAPDH density. The ratio for untreated control was considered as 1.0. * indicates that $p < 0.05$, and ** indicates that $p < 0.01$ as compared to the control groups.

Although the impact of acute As exposure on various cellular processes has been studied extensively, the relationship between As and angiogenesis is unclear. Herein, we report that at lower concentrations, sodium arsenite elicited an angiogenic effect in vitro. Angiogenesis is thought to play an important role in the pathophysiological functions of ECs. The ECM substrates in Matrigel used for this experiment contain a trace of growth factors for ECs, such as bFGF, EGF, etc. The HUVECs seeded on Matrigel can easily result in tubular morphology within 4 h without addition of exogenous proangiogenic factors. The earlier formation of a tubular network noticed in the 0.1 μM As-treated group (sooner than 4 h) indicated that pretreatment with lower concentrations of As accelerated the initiation of the angiogenic processes. Furthermore, As pretreatment at this level was able to maintain the integrity of tubing network on Matrigel for a longer time than did both the control and the higher As concentration groups. These findings suggest that As may stimulate some proangiogenic factors, which promote in vitro tubular formation in an autocrine fashion. On the other hand, higher concentrations of sodium arsenite (>5 μM) resulted in a significant inhibition in angiogenic morphogenesis, suggesting that the cytotoxic effect induced by high concentrations of As plays an inhibitory role in tubular formation.

Current studies revealed that angiogenesis is a complex process involving factors including cell−cell interactions, various intracellular signaling pathways, and the appropriate extracellular microenvironment (*35*). The best-studied angiogenic factor is VEGF and its two receptors, flt-1 and KDR. In vitro mitogenic responses of human ECs are primarily mediated by KDR (*36*−*40*). Endothelial NOS appears to play an essential role in VEGF-induced angiogenesis via NO activity (*21*). In vivo study revealed that VEGF augments NO release from quiescent rabbit and human vascular endothelium (*41*). In addition, VEGF induces NO-dependent hyperpermeability in coronary venules (*42*, *43*). Recent investigations demonstrated that VEGF upregulates NOS3 mRNA, protein, and NO production in human ECs (*39*, *40*, *44*−*46*). These effects are possibly mediated through the action of VEGF receptor-2 (KDR). Experiments performed in mice lacking NOS3 suggested that NO may act as an important downstream mediator for VEGF (*46*). In contrast, NO can upregulate the VEGF gene expression via the hypoxia response element in its promoter site (*47*−*49*). This evidence implicates a reciprocal regulation between VEGF and NO. These two factors may contribute not only to the mitogenesis but also to the motogenesis in ECs (*50*, *51*). In this study, we demonstrated that lower concentrations of As significantly upregulate the VEGF gene expression and its peptide release from HUVECs, as well as, gene expression of NOS3 and subsequent NO production. First, there are three isoforms of VEGF peptides, i.e., $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, containing intramolecular heparin-binding sites, thus leading to their confinement to the cell surface and ECM. In contrast, $VEGF_{121}$ is secreted in a soluble form (*52*). The limitation that only soluble peptide is detectable by ELISA may underestimate the responsiveness of VEGF expression induced by As treatment in our study. Second, the addition of a NO scavenger, i.e.,

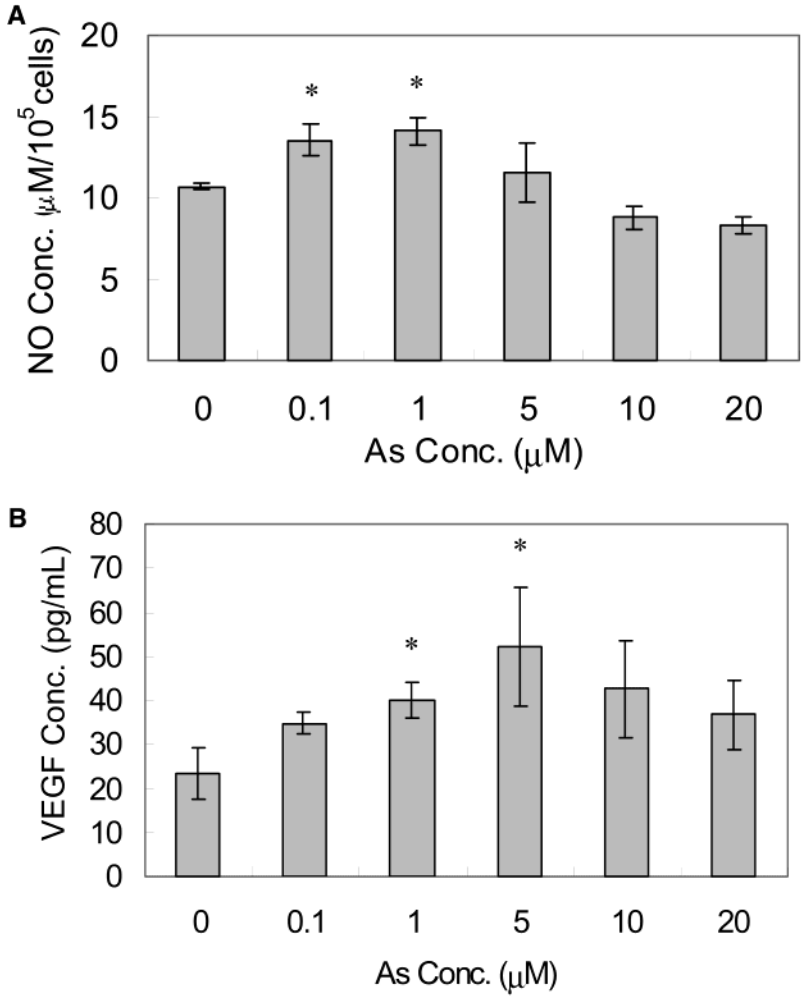

Figure 6. Sodium arsenite stimulates NO production and VEGF release from HUVECs. HUVECs seeded in a six well plate at 2 $\times$ 10^5 cells per well were cultivated overnight until cell attachment. Sodium arsenite at the indicated concentrations was added into the culture media in triplicate. The supernatants were collected after 24 h for subsequent analyses. (A) NO concentration was measured by Griess reagent. (B) VEGF concentration was measured using an ELISA detection kit designed for soluble $VEGF_{165}$ peptide. * indicates that $p < 0.05$ as compared to the negative control.

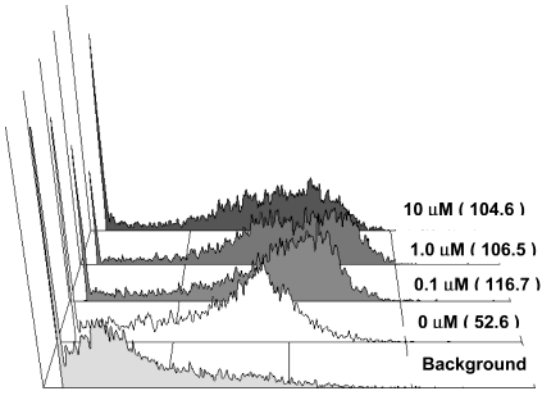

Figure 7. Sodium arsenite stimulates vWF antigen expression in HUVEC. HUVECs were seeded in a six well plate at 2 $\times$ 10^5 cells per well and cultivated overnight until cell attachment. Arsenite at the indicated concentrations was added into the culture media in triplicate. After 24 h, the cells were collected for immunostaining. The MFI of each group was detected by counting 10 000 cells using FACScan and expressed in parentheses.

hemoglobin, abrogated the tubular formation on Matrigel. These findings strongly indicate that at low concentrations, As enhances angiogenesis via the VEGF−NOS−NO signaling pathway.

The gene expression of NOS3, but not inducible NOS (NOS2), was significantly upregulated in HUVECs treated with lower concentrations of sodium arsenite. Endothelial constitutive NOS is a calmodulin-requiring enzyme, and its activation is mediated in a calcium-dependent way (*53*, *54*). In contrast, the activity of NOS2 is primarily mediated at the transcriptional level. The cloning and characterization of NOS2 indicate that NOS2 peptide contains a calmodulin-binding consensus sequence, which can bind tightly to a calmodulin subunit (*55*). Therefore, only a trace of calcium ions is essential for NOS2 activation, its activation is not affected by intracellular calcium concentrations. On the other hand, NOS3 can be activated in a calcium-dependent and reversible mode (*56*). The increase in NO production in HUVECs may be mediated through a calcium-dependent signaling pathway. We found NOS3 upregulation, but not NOS2, induced by As treatment implicates a much longer lasting influence on the physiological function in ECs. On the contrary, Barchowsky et al. reported that low levels of As trioxide stimulate vascular cell proliferation without affecting NO release (*57*). The discrepancy between the report of Barchowsky et al. and our result may be, at least in part, due to the different chemical compounds, phenotypes of cultured cells, and their responsiveness to As treatment.

There are several reports indicating that As is closely associated with vascular malignancies, e.g., primary hepatic angiosarcoma and hemangioendothelial sarcoma (*58*−*61*). In vitro studies demonstrate that two angiogenic factors, bFGF and VEGF, can synergistically upregulate the vWF gene expression (*29*). In addition, the vWF mRNA levels in colon carcinoma are higher than in normal controls and the mRNA level of vWF is related to the blood vessel counts of colon carcinoma. These results suggest that the vWF mRNA expression may be a biomarker for activation of the endothelium. Increased vWF expression levels reflect the elevated levels of VEGF and bFGF, or possibly other angiogenic factors, in angiogenesis. More recently, it was demonstrated that low concentrations of arsenite at micromolar levels decreased the expression of Fas ligand on HUVECs in a dose-dependent manner (*62*). The Fas−Fas ligand apoptosis pathway has been implicated in the regulation of physiological cell turnover, T cell homeostasis, maintenance of local immune privilege, and cytolytic activity of T and NK cells. In this study, we demonstrated that low concentrations of As increased the expression of vWF and VEGF production in HUVECs and stimulated in vitro angiogenesis. These results provide supportive evidence for the reports that As plausibly induces vascular malignancies.

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