Effects of glutamine on adhesion molecule expression and leukocyte transmigration in endothelial cells exposed to arsenic

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Running title: Glutamine modulates CAM expression induced by arsenic

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Abstract

This study analyzed plasma glutamine (GLN) concentrations in mice exposed to arsenic. Also, we evaluated whether GLN concentration was related to endothelial surface molecule expression and the migration of polymorphonuclear neutrophils (PMNs) through endothelial cells (ECs) stimulated by arsenic. Experiment 1: Mice were assigned to either a control (drank deionized water) or an arsenic group (drank water with arsenic). Each control and arsenic group was divided into subgroups with or without GLN supplementation. Five weeks later, mice were sacrificed for plasma GLN analysis. Experiment 2: Human umbilical vein endothelial cells (HUVECs) and PMNs were treated with different GLN concentrations (0, 300, 600, and 1000 μM) for 24 h. After that, we stimulated HUVECs for 3 h with 0.5 uM arsenic and PMNs were allowed to transmigrated to through ECs for 2 h. HUVEC surface expressions of cell adhesion molecules and integrin (CD11b) and interleukin (IL)-8 receptor expressions on PMNs were measured. The transendothelial migration of PMNs was also analyzed. The results showed that plasma GLN levels of mice exposed to arsenic were lower than those in the control group, and that GLN supplementation reversed the depletion of plasma GLN levels. The in vitro study revealed that cell adhesion molecule and integrin expressions in arsenic groups were higher than those without arsenic. Among the arsenic groups, the expression of vascular cell adhesion molecule-1 on ECs and CD11b on PMNs were lower with 600 and 1000 μM than with 300 μM GLN. IL-8 secretions from ECs and PMNs were higher with 300 uM than with 600 and 1000 uM GLN, and this was consistent with the expression of the IL-8 receptor on PMNs. PMN transmigration was significantly higher with 300 μM GLN than with other GLN concentrations. These results suggest that ECs and PMNs were activated after arsenic stimulation. A low GLN concentration resulted in higher

adhesion molecule expression and greater transendothelial migration of neutrophils. GLN administration at levels similar to or higher than physiologic concentrations reduced IL-8 and adhesion molecule expression, PMN transmigration was also decreased after stimulation with arsenic.
Keywords: Arsenic exposure, Glutamine, Adhesion molecule expression, Interleukin-8, Polymorphonuclear neutrophils transmigration

1. Introduction

Arsenic is a notorious environmental toxicant known as both a carcinogen and atherogen in human beings. The pathogenic mechanisms are not completely understood. Previous reports have shown that arsenic results in the generation of reactive oxygen species in a variety of mammalian cells [1,2]. In a study by Wu et al. [3], they showed that ingestion of arsenic-contaminated well water increased the levels of reactive oxidants and decreased the levels of antioxidant capacity in plasma of individuals. Oxidative stress may be associated with the occurrence of arsenic-related diseases [2]. An in vitro study showed that oxidative stress has an impact on the atherogenic process by modulating intracellular signaling pathways in vascular tissues affecting inflammatory cell adhesion, migration, and proliferation [4].

Adhesion molecules play a key role in cell-cell interactions and cell-extracellular matrix interactions. Intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are members of the immunoglobin superfamily of cell adhesion molecules (CAMs) [5,6]. CAMs are important in the adhesion of monocytes, lymphocytes, and neutrophils to activated endothelium [5,6]. Blood leukocytes are mediators of host defense localized in the earliest lesions of inflammation. Activated leukocytes express surface glycoproteins known as integrins, of which the β_2 integrins (CD18) are particularly important [7]. CD11b/CD18 is abundant in neutrophils and contributes to neutrophil migration into sites of inflammation [8]. Activation of integrins is required for a strong attachment to the endothelium and subsequent transmigration. Migration of leukocytes into a tissue is part of the host response for protecting an organ against tissue damage; however, excessive expression of integrin is harmful and may have deleterious effects including tissue destruction, ischemia-reperfusion injury, and autoimmune disease [9].

Glutamine (GLN) is the most-abundant amino acid in blood and tissue fluid. GLN provides a substrate for protein synthesis and precursors for nucleic acid biosynthesis [10]. GLN is an important carrier of nitrogen and carbon, is a precursor for gluconeogenesis in the liver, and takes part in the acid-base homeostasis in the kidney and liver [10,11]. GLN was formerly classified as a nonessential amino acid, because it can be synthesized in the body. However, it is considered to be essential during certain catabolic conditions [12,13]. Previous reports showed that GLN requirements are increased in catabolic conditions such as burn injury, major surgery, and sepsis [12-14]. A lack of GLN promotes mucosal atrophy, increases intestinal permeability and bacterial translocation, and reduces synthesis of glutathione (GSH), a major antioxidant and a vital component of a host's defense [15-17]. Several publications have described the beneficial effects of GLN supplementation on enhancing immune function, improving the nitrogen balance, and better protecting the morphology of the intestinal mucosa in metabolically stressful conditions [12-16,18]. A study by Fukatsu et al. [19] showed that compared with conventional total parenteral nutrition, GLN-supplemented parenteral nutrition reduced ICAM-1 expression in intestinal homogenates. Also, Arndt et al. [20] demonstrated that GLN administration reduced leukocyte adhesion and transmigration in indomethacin-induced intestinal inflammation in rats. As we know, there is no study investigating the effect of GLN on the expression of CAMs and leukocyte transmigration under arsenic exposure. We hypothesized that chronic inflammation induced by arsenic results in depletion of plasma GLN, and GLN administration comparable to physiologic concentrations should decrease CAM expression in HUVECs stimulated by arsenic and thus reduce the immigration of PMNs.

2. Materials and Methods

We conducted 2 experiments in this study.

2.1. Experiment 1: Animal study

2.1.1. Animals

Male BALB/c mice weighing 10-15 g (4 weeks of age) were used in this study. All mice were housed in temperature- and humidity-controlled rooms and were allowed free access to standard chow for 1 wk prior to the experiment. Care of the animals followed the guidelines for the care and use of laboratory animals established by the Animal Care Committee of Taipei Medical University, and protocols were approved by that committee.

2.1.2. Study protocol

Thirty-two mice were randomly assigned to the control or experimental groups, 16 mice to a group. Mice in the control group drank deionized water, whereas those in the experimental group drank water containing 20 ppm sodium arsenite ($NaAsO_2$), which is equivalent to 3-4 mg/kg/d. Each control and experimental group was divided into 2 subgroups with 8 mice to each subgroup. One subgroup was fed a common semipurified diet, while the diet of the other group was supplemented with GLN, replacing 25% of the total amino acid nitrogen (Table 1). This amount of GLN was proven to have an immunomodulating effect in rodents [21]. The groups were isonitrogenous and identical in energy and nutrients distribution. There were 4 groups in this study: the CC group (n = 8), to which no arsenic or GLN was administered; the CG group (n = 8), to which no arsenic was given but GLN was supplemented; the AC group (n = 8), to which arsenic but no GLN was given; and AG group (n = 8), with both arsenic and GLN supplementation. Food and water intake was recorded every day during the experimental period. After 5 wk, all mice were anesthetized and sacrificed by heart puncture.

2.1.3. Plasma GLN level analysis

Blood samples were collected in tubes containing heparin and immediately centrifuged. Plasma amino acid was analyzed by standard ninhydrin technology (Beckman Instrument, model 6300, Palo Alto, CA, USA), after deprotienization of the plasma with 5% salicylic acid [22].

2.2. Experiment 2: In vitro study

2.2.1. HUVEC isolation and culture

HUVECs were isolated from the umbilical cord vein according to the method of Jaffe et al. [23]. The umbilical vein was cannulated, washed with PBS, and perfused with PBS containing 0.1% collagenase for 10 min at 37 °C in 5% CO_2. HUVECs were collected and established as a primary culture in medium-199 (M-199) containing 20% fetal bovine serum (FBS), 20 mM $NaHCO_3$, 25 mM HEPES, antibiotics (100 U/ml penicillin and 100 ug/ml streptomycin), 10 IU/ml heparin sodium, and 15 mg/L endothelial cell growth factor at 37 °C in 5% CO_2 and 95% humidity. Cells were serially passaged 2~3 times for the experimental assay.

2.2.2. Polymorphonuclear neutrophils (PMNs)

Venous blood from a 25-year-old healthy woman was drawn into heparinized tubes. A buffy coat, diluted 3-fold with PBS, was applied on a Ficoll-Hypaque gradient and centrifuged for 30 min at 300 *g* at 4 °C. PMNs were then isolated by Ficoll-Hypaque gradient centrifugation, with a density of 1.077 for 30 min at 300 *g*. Neutrophils (95% ± 2% by May-Grunwald-Giemsa panoptic staining) obtained from the pellet of the Ficoll gradient centrifugation were washed twice with PBS and resuspended in M-199 supplemented with 1% heat-inactivated FCS for 24 h to stabilize the PMNs, after which the PMNs were incubated with different concentrations of GLN (0, 300, 600, and 1000 μM) for 24 h. The viability of PMNs after incubation was > 95% after confirmation by May-Grunwald-Giemsa panoptic staining.

2.2.3. PMN migration across the endothelial monolayer

HUVECs (1×10^5 cells/well) from second subcultures were grown on fibronectin-coated inserts (3-μm pore size, 6.4 mm, Becton Dickson) which were then placed in a 24-well plate until the monolayer was confluent. They were then incubated in M-199 (without FBS) with different concentrations of GLN (0, 300, 600, and 1000 μM) for 24 h. The viability of HUVECs after incubation was > 95% after confirmation by trypan blue staining. Subsequently, cells were washed twice with PBS and cultured with various concentrations of GLN (without FBS), and 0.5 uM sodium arsenite dissolved in medium-199 was added in a final volume of 1 ml for 3 h in the arsenic group. Control groups were also cultured with various GLN concentrations but only medium-199 was added. After that, PMNs (1×10^5 cells/100 μl per well) were added to the wells and allowed to migrate across the ECs for 2 h. The time schedule was determined according to a preliminary study that at this time point the expression of CAMs was the highest during arsenic stimulation for 1-6 hrs. PMNs migrating and falling into the lower chamber were quantified by a microscopic counter in a hemocytometer.

2.2.4. Measurements of CAM and interleukin-8 receptor expressions on PMNs and HUVECs

After the PMN-HUVEC interaction had proceeded for 2 h, HUVEC surface expressions of ICAM-1 and VCAM-1, and PMN expressions of CD11b and the interleukin (IL)-8 receptor were measured. Solutions in the upper chambers of the transwells were collected and centrifuged at 1200 rpm for 10 min, and the pellets were suspended in 100 µl PBS for PMN analysis. After removing the supernatant, HUVECs were washed twice with PBS containing 2 mM iced EDTA to detach adherent PMNs, then the pellets were incubated with 100 µl M-199 (FBS free, containing 2 mM iced EDTA) for a further 30 min at 4 ℃ with the addition of fluorescein-conjugated mouse anti-human VCAM-1 (CD 106) and phycoerythrin-conjugated mouse anti-human ICAM-1 (CD 54). The suspension was collected into a tube and resuspended in 500 µl PBS (containing 0.3 ml of 350 mM formaldehyde). The fluorescence intensity of a 5000-cell population was counted and analyzed by flow cytometry (Coulter, Miami, FL, USA). To determine the integrin and IL-8 receptor expressions on PMNs, fluorescein-conjugated mouse anti-human CD11b (Serotec, Oxford, UK) and phycoerythrin-conjugated mouse anti-human CDw128a (Serotec) were added to 100 µl of the PMN suspension. Fluorescence data were collected on 1 x 10^5 viable cells and analyzed by flow cytometry (Coulter).

2.2.5. Measurements of interleukin-8 concentrations

Solutions in the upper chambers of the transwells were collected to determine IL-8 secretions by ECs and neutrophils. IL-8 was measured using commercial ELISA microtiter plates, with antibodies specific for human IL-8 having been coated onto the wells of the microtiter strips provided (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.3. Statistical analysis

Data are expressed as the mean ± SD in the animal study. Values in the in vitro study are also given as mean ± SD of triplicate measurements. Two-way ANOVA using Duncan's test were performed to determine whether GLN and arsenic affect the outcome. A p value of < 0.05 was considered statistically significant.

3. Results

3.1. Body weight and plasma GLN levels

There were no differences in initial body weights among the groups. No differences in food and water intake were observed across the groups during the experimental period (data not shown). After feeding for 5 weeks, the body weights of mice in the arsenic groups (AC and AG) were significantly lower than those of the control groups (CC and CG). There were no differences in body weights between the arsenic groups with and without GLN supplementation. Plasma GLN levels in the AC group were significantly lower than those in the control groups, however, plasma GLN levels in the AG group did not differ from those of the control groups (Table 1).

3.2. CAM expressions on HUVECs

ICAM-1 and VCAM-1 expressions on HUVECs were higher when stimulated with arsenic than those without arsenic. Among the groups stimulated with arsenic, VCAM-1 expressions on HUVECs were higher when incubated with 300 uM GLN compared to those with 0, 600, and 1000 μM GLN. There were no differences in ICAM-1 expressions among samples incubated with 300, 600, and 1000 μM GLN (Table 2).

3.3. CD11b and IL-8 receptor expressions on PMNs

CD11b and IL-8 receptor expression on PMNs were higher when stimulated with arsenic than those without arsenic. Among the groups stimulated with arsenic, the expressions of CD11b and the IL-8 receptor with 600 and 1000 μM were lower than those with 300 μM GLN. CD11b expression was even lower with 1000 uM than with 600 uM GLN, whereas there were no differences in IL-8 receptor expressions between 600 and 1000 μM GLN (Table 2).

3.4. IL-8 production by ECs and neutrophils

IL-8 production by ECs and neutrophils were higher when stimulated with arsenic than those without arsenic. IL-8 levels was significantly lower with 600 and 1000 uM than with 300 μM GLN when stimulated with arsenic. There were no differences in IL-8 levels between 600 and 1000 μM GLN in arsenic groups (Fig. 1).

3.5. PMN migration across the endothelial monolayer

There were no differences in PMN migration across the endothelial monolayer among various GLN concentrations in the groups without arsenic. Among the groups stimulated with arsenic, PMN transmigration was the highest with 300 μM GLN compared to those with other GLN concentrations (Fig. 2).

4. Discussion

This study showed for the first time that arsenic exposure results in depletion of plasma GLN, and GLN supplementation can normalize plasma GLN levels in mice. In this study, 20 ppm $NaAsO_2$ was used. This dosage of arsenic was in the range used by other studies which showed that arsenic accelerates atherosclerosis formation and promotes tumor initiation [24,25]. The results of this study showed that all mice exposed to arsenic survived, but their body weights were significantly lower than those of the control groups, although the amounts of food and water consumed were comparable among the groups. This finding indicates that the dosage and duration of arsenic administered in this study were not fatal but resulted in metabolic stress in the mice. A previous study showed that arsenite causes oxidative damage to the protein, pyruvate dehydrogenase, thus inhibiting its enzyme activity [26]. Since pyruvate dehydrogenase facilitates entry of pyruvate into the mitochondria, inactivation of this enzyme may interfere with energy metabolism and consequently result in weight loss.

We observed that plasma GLN levels in mice exposed to arsenic were significantly lower than those in the control group. This finding is compatible with previous reports that plasma GLN is reduced during catabolic conditions such as inflammation, infection, and injury [11,12,14]. In order to understand whether GLN concentrations may have an effect on CAMs expression in arsenic exposure, we treated ECs and PMNs with different GLN concentrations including 300, 600, 1000 uM GLN in an in vitro study under the stimulation of arsenic. Three hundred uM GLN is considered low level and may be observed in patients with catabolic conditions, whereas 600 uM is approximate to physiological levels in human plasma. Previous studies reported that plasma total arsenic levels were less than 1 uM in subjects with chronic arsenic exposure [27]. We used 0.5 uM sodium arsenite as a stimulant, because this amount of arsenic has proven to generate reactive species and induce the release of inflammatory cytokines in in vitro studies [27,28]. In this model we observed an increase of CAM and IL-8 expressions in arsenic groups as compared to groups without arsenic, indicating that ECs and PMNs were activated after stimulation by arsenic.

In this study, we observed that adhesion molecules (CD11b, ICAM-1, and VCAM-1) and IL-8 receptor expressions on HUVECs and neutrophils were lowest in the arsenic groups without GLN (0 uM) than with GLN administration. Arsenic group with 300 uM GLN had higher VCAM-1 and CD11b expressions than did the corresponding groups with 0, 600, 1000 uM GLN. Although glucose is the primary fuel for leukocytes and rapidly proliferating cells, GLN is the preferred energy source for the cells [29,30]. It is possible that in the condition without GLN, glucose is used as an energy source for maintaining the viability and basal function of the cells. Whenever GLN is available for use, adhesion molecules are highly expressed in response to the stimulation of arsenic and may consequently result in inflammatory response. VCAM-1 and CD11b are adhesion molecules that play critical roles in neutrophil adhesion and migration [31]. Lower VCAM-1 and CD11b expression on ECs and PMNs observed in arsenic group with GLN at levels similar to or higher than physiological concentrations may reduce leukocyte adhesion and transmigration. Previous studies have shown that nuclear factor (NF)-kB is involved in the regulation of many cytokines and adhesion molecules [32,33]. Whether NF-kB is responsible for decreasing GLN-mediated CAM expression is under investigation in our laboratory.

IL-8 is a potent neutrophil chemoattractant and activator. It is an early marker of the inflammatory process because IL-8 initiates the acute inflammatory cascade [34]. An in vitro study by Huang et al. [35] showed that GLN decreases LPS-induced IL-8 production in Caco-2 cells. In this study, we found that IL-8 secretion with 600 and 1000 μM was significantly lower than that with 300 μM GLN stimulated by arsenic. This finding parallels that of the effects of GLN on PMNs IL-8 receptor expression. These results suggest that a lower GLN concentration resulted in higher IL-8 expression, while nearly normal or higher than physiological GLN reduces IL-8 production stimulated by arsenic. Coeffier et al. [36] reported that a high GLN concentration had inhibitory effects on IL-8 mRNA and protein expression. Huang et al. [35] revealed that GLN-mediated decrease in LPS-stimulated IL-8 production is not associated with NF-kB nuclear binding. Determining the mechanisms by which GLN decreases arsenic-induced IL-8 production in HUVECs requires further investigations.

In this study, we used monolayers of cultured endothelial cells as a barrier to investigate the effects of GLN on leukocyte migration through the endothelium under stimulation by arsenic. We only measured the transmigration of neutrophils because PMNs are the largest population of leukocytes. A study by Galdiero et al. [37] found that among leukocyte populations, transmigration of neutrophils was most obvious when bacterial products were used as stimulants. In this study, we found that transmigration of PMNs was significantly lower with physiologic and higher levels of GLN than with low GLN levels. We speculated that GLN administration at levels similar to physiological conditions reduced IL-8 and CAM expressions, which may consequently result in a lower extent of PMN-HUVEC interactions and PMN migration. Hong et al. [17] found that GLN-supplemented nutrition protects the liver during hepatic injury by preserving GSH stores. An in vitro study by Babu et al. [38] also found that GLN can prevent the liver from damage possibly mediated via GSH synthesis. GLN was found to be rate limiting for GSH synthesis, and the availability of GLN is critical in generating GSH stores [39]. Studies have shown that organic arsenicals inhibit glutathione reductase activity resulting in depletion of cellular GSH concentrations and a decreased ability of cells to protect against oxidants [2,40]. It is possible that the antioxidant property of GLN may be implicated in reducing arsenic-induced oxidative stress and has favourable effect on decreasing inflammatory-related CAM expression.

In summary, this study showed that arsenic exposure results in depletion of plasma GLN, and GLN supplementation can normalize plasma GLN levels in mice.

The in vitro study showed that ECs and PMNs were activated after stimulation with arsenic. A low GLN concentration resulted in higher CAM expressions and greater transendothelial migration of neutrophils. GLN administration at levels similar to or higher than physiological concentrations reduced IL-8 and CAM expression on ECs and neutrophils; PMN transmigration also decreased under arsenic stimulation.

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Figure legends

Fig. 1. Interleukin (IL)-8 concentrations in culture medium after endothelial cells incubated with various glutamine (GLN) concentrations and stimulation with or without arsenic. Data are presented as the mean ± SD of triplicate measurements. *Significant different from the arsenic groups with different GLN concentrations. +Significant different from corresponding group at the same GLN concentration.

Fig. 2. Arsenic-stimulated migration of polymorphonuclear neutrophils (PMNs) across human umbilical vein endothelial cells (HUVECs) cultured in a human fibronectin-coated culture with the addition of different GLN concentrations. Results are presented as the mean ± SD of triplicate measurements. * PMNs across endothelial cells with 300 μM GLN were significant higher than those at 0, 600, and 1000 μM GLN in the arsenic groups. +Significant different from values with 0 and 300 uM GLN in the arsenic groups.

Table 1

Composition of the semipurified diet (g/kg)

Ingredient	GLN-supplemented group	Control group
Casein	165	220
GLN	45	--
Total nitrogen	34.4	34.4
Corn starch	667	657
Soybean oil	44	44
Vitamin mixture*	10	10
Salt mixture†	35	35
Methyl-cellulose	30	30
Choline chloride	1	1
DL-methionine	3	3

*The vitamin mixture contained the following (mg/g): thiamin hydrochloride 0.6, riboflavin 0.6, pyridoxine hydrochloride 0.7, nicotinic acid 3, calcium pantothenate 1.6, D-biotin 0.02, cyanocobalamin 0.001, retinyl palmitate 1.6, DL-α-tocopherol acetate 20, cholecalciferol 0.25, and menaquinone 0.005.

†The salt mixture contained the following (mg/g): calcium phosphate diabasic 500, sodium chloride 74, potassium sulphate 52, potassium citrate monohydrate 220, magnesium oxide 24, manganese carbonate 3.5, ferric citrate 6, zinc carbonate 1.6, cupric carbonate 0.3, potassium iodate 0.01, sodium selenite 0.01, and chromium potassium sulphate 0.55.

Table 2
Body weight (BW) and plasma glutamine (GLN) concentrations of the groups

Group[a]	BW (g)	GLN (nmol/ml)
CC	29.7 ± 1.2	529.5 ± 54.7
CG	28.9 ± 0.8	536.3 ± 60.8
AC	23.5 ± 1.0*	410.9 ± 41.3+
AG	23.6 ± 0.5*	545.0 ± 31.5

Data are expressed as the mean ± standard deviation.

*Significantly different from the CC and CG groups at $p < 0.05$.

+Significantly different from the other groups at $p < 0.05$.

[a] CC, no arsenic or GLN administered; CG, no arsenic but GLN supplementation; AC, with arsenic but no GLN supplementation; AG, with arsenic and GLN supplementation.

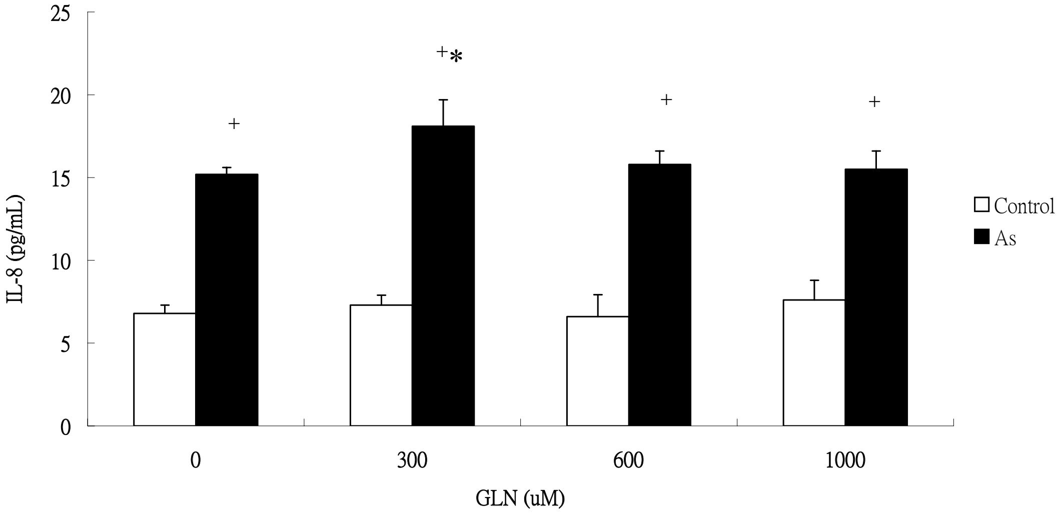
Table 3

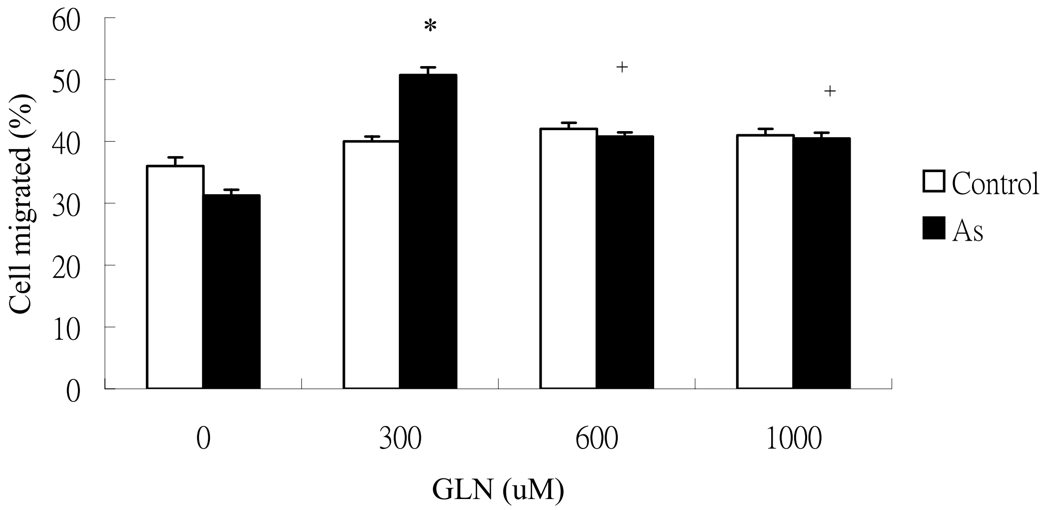
CD11b, and interleukin (IL)-8 receptor expressions on leukocytes, and ICAM-1, and VCAM-1 expressions on endothelial cells after incubation with various GLN concentrations and stimulation with or without arsenic

GLN	CD11b	IL-8 receptor	ICAM-1	VCAM-1
			%	
0 uM				
Control[a]	12.51±0.32	3.10±0.53	2.65±0.54	25.85±2.54
As	16.52 ± 4.03[*]	5.45 ± 0.54[*]	5.12 ± 0.83[*]	23.91 ± 0.92[*]
300 uM				
Control[a]	14.57±0.42	3.39±0.72	2.98±0.36	25.36±3.54
As	30.5 ± 0.63	10.72 ± 0.70	15.44 ± 0.22	56.72 ± 1.81
600 uM				
Control[a]	15.63±0.54	4.54±0.61	2.71±0.92	22.80±3.65
As	25.08 ± 0.55[+]	7.53 ± 0.51[+]	16.13 ± 0.52	30.37 ± 0.32[+]
1000 uM				
Control[a]	10.97±0.62	3.22±0.43	3.77±0.95	22.31±4.12
As	22.48 ± 0.47[‡]	8.58 ± 0.51[+]	16.21 ± 0.23	31.13 ± 0.55[+]

Data are presented as the mean ± SD of triplicate measurements.

[a]Significantly different from As group at the same GLN concentration except for VCAM-1 in 0 uM GLN. In the same column: [*]Significantly different from As groups with different GLN concentrations; [+]Significantly different from As group with 300 uM GLN; [‡] Significantly different from As groups with 300 and 600 uM GLN.


25
20
15
10
5
0
IL-8 (pg/mL)
+
+*
+
+
Control
As
0
300
600
1000
GLN (uM)


60
50
40
30
20
10
0
Cell migrated (%)
*
+
+
Control
As
0
300
600
1000
GLN (uM)