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

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Abstract

This study evaluated whether glutamine (GLN) concentration was related to endothelial surface molecule expression and the migration of polymorphonuclear neutrophils (PMNs) through endothelial cells (ECs) stimulated by arsenic. 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 μM arsenic, and PMNs were allowed to transmigrate to 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 cell adhesion molecule (CAM) and integrin expressions in arsenic groups were higher than in those without arsenic. Among the arsenic groups, the expression of CAMs on ECs and CD11b, and IL-8 receptor on PMNs was lowest with 0 μM compared with the other GLN concentrations. Vascular CAM-1 on ECs and CD11b on PMN expression were higher with 300 μM than with 600 and 1000 μM GLN. IL-8 secretions from ECs and PMNs were higher with 300 μM than with 600 and 1000 μM GLN, and this was consistent with the expression of the IL-8 receptor on PMNs. Polymorphonuclear neutrophil 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. Cell adhesion molecule expressions on ECs and PMNs were suppressed in the absence of GLN. A low GLN concentration comparable to catabolic conditions resulted in higher adhesion molecule expression and greater transendothelial migration of neutrophils. Glutamine administration at levels similar to or higher than physiological 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 an 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 the 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]. Cell adhesion molecules are important in the adhesion of monocytes, lymphocytes and neutrophils to

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activate 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. Glutamine provides a substrate for protein synthesis and precursors for nucleic acid biosynthesis [10]. Glutamine 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]. Glutamine 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]. 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 physiological concentrations should decrease CAM expression in HUVECs stimulated by arsenic and thus reduce the immigration of PMNs.

2. Materials and methods

2.1. HUVEC isolation and culture

HUVECs were isolated from the umbilical cord vein according to the method of Jaffe et al. [21]. 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 μg/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 two to three times for the experimental assay.

2.2. Polymorphonuclear neutrophils

Venous blood from a 25-year-old healthy woman was drawn into heparinized tubes. A buffy coat, diluted threefold with PBS, was applied on a Ficoll-Hypaque gradient and centrifuged for 30 min at 300×*g* at 4°C. Polymorphonuclear neutrophils 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.

2.3. 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-Dickinson) 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 μM 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 h. Polymorphonuclear neutrophils migrating and falling into the lower chamber were quantified by a microscopic counter in a hemocytometer.

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

Table 1
CD11b and 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 μM				
Control[a]	12.51±0.32	3.10±0.53	2.65±0.54	25.85±2.54
As	16.52±4.03[b]	5.45±0.54[b]	5.12±0.83[b]	23.91±0.92[b]
300 μM				
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 μM				
Control[a]	15.63±0.54	4.54±0.61	2.71±0.92	22.80±3.65
As	25.08±0.55[c]	7.53±0.51[c]	16.13±0.52	30.37±0.32[c]
1000 μM				
Control[a]	10.97±0.62	3.22±0.43	3.77±0.95	22.31±4.12
As	22.48±0.47[d]	8.58±0.51[c]	16.21±0.23	31.13±0.55[c]

Data are presented as the mean±S.D. of triplicate measurements.

[a] Significantly different from the As group at the same GLN concentration except for VCAM-1 in 0 μM GLN.

[b] Significantly different from the As groups with different GLN concentrations in the same column.

[c] Significantly different from the As group with 300 μM GLN in the same column.

[d] Significantly different from the As groups with 300 and 600 μM GLN in the same column.

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°C 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). 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 1×10^5 viable cells and analyzed by flow cytometry (Coulter).

2.5. Measurements of IL-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).

2.6. Statistical analysis

Data are expressed as mean±S.D. of triplicate measurements. Two-way ANOVA using Duncan's test was performed to determine whether GLN and arsenic affect the outcome. A P value of $<.05$ was considered statistically significant.

3. Results

3.1. 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 μM GLN than those incubated 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 1).

3.2. 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

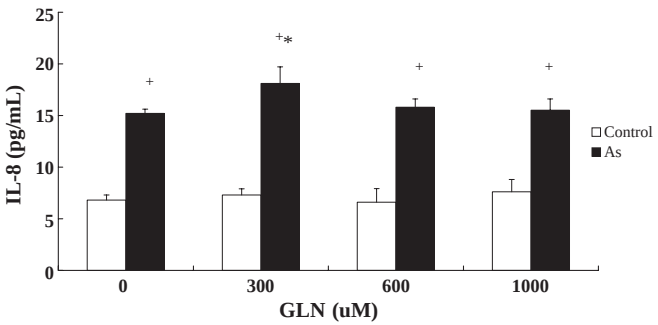

Fig. 1. Interleukin-8 concentrations in culture medium after endothelial cells incubated with various GLN concentrations and stimulation with or without arsenic. Data are presented as the mean±S.D. of triplicate measurements. *Significantly different from the arsenic groups with different GLN concentrations.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

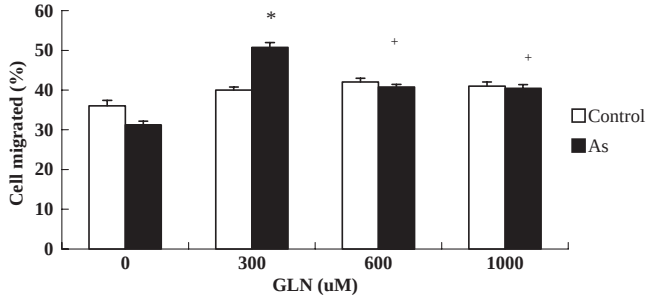

Fig. 2. Arsenic-stimulated migration of PMNs across HUVECs cultured in a human fibronectin-coated culture with the addition of different GLN concentrations. Results are presented as the mean±S.D. of triplicate measurements. *PMNs across endothelial cells with 300 μM GLN were significantly higher than those with 0, 600 and 1000 μM GLN in the arsenic groups. +Significantly different from values with 0 and 300 μM GLN in the arsenic groups.

expression was even lower with 1000 μM than with 600 μM GLN, whereas there were no differences in IL-8 receptor expressions between 600 and 1000 μM GLN (Table 1).

3.3. 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 were significantly lower with 600 and 1000 μM 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).

3.4. 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

In order to understand whether GLN concentrations may have an effect on CAM expression in arsenic exposure, we treated ECs and PMNs with different GLN concentrations including 0, 300, 600 and 1000 μM GLN in an in vitro study under the stimulation of arsenic. Three hundred micromolars of GLN is considered low level and may be observed in patients with catabolic conditions, whereas 600 μM is approximate to physiological levels in human plasma. Previous studies reported that plasma total arsenic levels were less than 1 μM in subjects with chronic arsenic exposure [22]. We used 0.5 μM 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 [22,23]. 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 μM) than with GLN administration. Arsenic group with 300 μM GLN had higher VCAM-1 and CD11b expressions than did the corresponding groups with 0, 600 and 1000 μM GLN. Glucose is the primary fuel for leukocytes and rapidly proliferating cells; however, GLN is the preferred energy source for the cells [24,25]. Previous reports also showed that a decrease in the GLN concentrations in culture medium decreased lymphocyte proliferation and macrophage function, and there was a close relationship between GLN concentrations and cell functions [12,18,26]. It is possible that adhesion molecules are expressed in accordance with GLN availability, but physiological levels of GLN down-regulate the expression of CAMs and have effects on attenuating inflammatory responses induced by arsenic. Previous studies have shown that nuclear factor (NF)-kB is involved in the regulation of many cytokines and adhesion molecules [27,28]. Whether NF-kB is responsible for decreasing GLN-mediated CAM expression is under investigation in our laboratory.

Interleukin-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 [29]. An in vitro study by Huang et al. [30] 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 PMN IL-8 receptor expression. These results suggest that low GLN concentrations comparable to catabolic conditions resulted in higher IL-8 expression, while nearly normal or higher than physiological GLN concentration reduces IL-8 production stimulated by arsenic. Coeffier et al. [31] reported that a high GLN concentration had inhibitory effects on IL-8 mRNA and protein expression. Huang et al. [30] 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. [32] 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 physiological and higher levels of GLN than with relatively 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. [33] also found that GLN can protect the liver from damage possibly mediated via GSH synthesis. Glutamine was found to be rate limiting for GSH synthesis, and the availability of GLN is critical in generating GSH stores [34]. Studies have shown that organic arsenicals inhibit GSH reductase activity, resulting in depletion of cellular GSH concentrations and a decreased ability of cells to protect against oxidants [2,35]. It is possible that the antioxidant property of GLN may be implicated in reducing arsenic-induced oxidative stress and has a favourable effect on decreasing inflammatory-related CAM expression.

In summary, this in vitro study showed that ECs and PMNs were activated after stimulation with arsenic. Cell

adhesion molecule expressions on ECs and PMNs were suppressed in the absence of GLN. Low GLN concentrations comparable to catabolic conditions resulted in higher CAM expressions and greater transendothelial migration of neutrophils. Glutamine 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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