Endothelial Dysfunction and Cellular Adhesion Molecule Activation in Preterm Infants with Hypoxic Ischemic Encephalopathy

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Abstract

Purpose: *This study aimed to study changes in endothelial function and the levels of adhesion molecules in preterm infants with hypoxic ischemic encephalopathy (HIE).* ***Methods****: The newborns were placed into either the control (22 infants) or the HIE group (112 infants).The concentrations of endothelin-1 (ET-1), soluble intracellular adhesion molecule-1 (sICAM-1), soluble vascular cellular adhesion molecule-1 (sVCAM-1) and nitric oxide (NO) were examined in peripheral blood samples of infants.* ***Results****: In infants with HIE sICAM-1 and sVCAM-1 levels were significantly increased on the 7th day of life compared with the 1st day of life ($p<0.05$). However, ET-1 and NO concentrations tended to decline over this period, and there were statistically significant decreases in nitrate levels during the first week of life ($p<0.05$).* ***Conclusion****: Increased sICAM-1 levels were associated with decreased ET-1 and NO levels, potentially indicating the positive role of activated leukocyte adhesion in the mitigation of endothelial dysfunction.*

Keywords: hypoxia; ischemia; leukocyte adhesion; brain injury; endothelial function

Introduction

Neonatal hypoxic ischemic encephalopathy (HIE) is a common and serious health condition that causes significant mortality and long-term morbidity (1).

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Following the initial hypoxic ischemic insult, the resultant brain injury develops over days or even weeks (2). Endothelial function plays an important role in the development of the resultant neuronal injury and in HIE pathogenesis (3). Hypoxic-ischemic injury leads to the production of pro-inflammatory cytokines (4,5) and chemotactic molecules (6) by the vascular endothelium. The endothelial cells become activated during the reperfusion that follows the initial cerebral ischemic event. This activation is followed by the recruitment of leukocytes to the area of injury (7). Numerous bidirectional signaling events occur between leukocytes and the vascular endothelium and these interactions are fundamental components of the inflammatory response to ischemia (8). Leukocyte-endothelial interactions are mediated by biologically active molecules, such as endothelins and nitric oxide (NO), which play an important role in the regulation of vascular tone (9,10). Different groups of cell adhesion molecules are also involved in these interactions (11). Adhesion molecules are expressed on endothelial cells, and the interactions between them are required for the firm adhesion of leukocytes to the vessel wall. Intercellular adhesion molecule-1 (ICAM-1; also known as CD54) and ICAM-2 (also known as CD102), as well as vascular cell adhesion molecule-1 (VCAM-1; also known as CD106), are involved in endothelium-leukocyte interactions. ICAM-1 and -2 and VCAM-1 have also been demonstrated to contribute to the inflammatory response following cerebral ischemia (12).

The present investigation was undertaken with the following objectives: 1) to identify changes in endothelial function and in the levels of soluble adhesion molecules in early adaptation days of preterm infants with HIE and 2) to determine the correlation between soluble adhesion molecule activation and endothelial function in these infants.

We estimated the degree of endothelial function via NO and ET-1 detection in the peripheral blood samples of infants. The state of the adhesion system was determined based on sICAM-1 and sVCAM-1 concentrations in the peripheral blood.

Materials and methods

Patients. This research is a component of a non-randomized prospective study that was designed to determine the role of perinatal factors in the neurologic and somatic health of preterm infants. The study protocol was reviewed and approved by the Problem Commission on Pediatric Research at Azerbaijan Medical University in June 2011. Newborns with gestational ages of 28-36 weeks and birth weights of 980-2600 g, who were delivered at the Sh. Aleskerova Clinical Maternity Hospital between January and November 2011, were recruited to participate in this study.

Newborns were excluded from the study if there was clinical or laboratory evidence of congenital infection, neonatal sepsis, or congenital malformation. The gestational age was assessed based on the date of the most recent menstrual period and an ultrasonogram. The gestational age was also confirmed using the scale described by Ballard et al. (13). The newborns were placed into either the control or the HIE group. The control group consisted of 22 preterm newborn infants who presented no neurological manifestations, who had normal cranial ultrasounds, and who did not require medication during the neonatal period. The HIE group consisted of 112 preterm infants, and the diagnosis of HIE was based on the Levene classification (14). The neurological assessment was performed according to the Dubowitz/Ballard scale (13).

Blood collection. Venous blood was collected on the 1st and the 7th day of life. No venous punctures were performed for the sole purpose of study-related analysis. The blood samples were collected in EDTA tubes and were centrifuged for 15-20 minutes. The plasma samples were frozen at -70ºC. Grossly hemolyzed samples were not included in the analysis.

Determination of ET-1, sICAM-1, and sVCAM-1 concentrations in peripheral blood by ELISA. The ET-1 levels were determined using a commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to a standard enzyme immunoassay procedure. The sICAM-1 and sVCAM-1 concentrations were determined using USCN kits (Life Science Inc., Wuhan, China).

Determination of NO concentration in peripheral blood. The NO concentration was quantified using a commercial kit (Thermo Scientific, Pierce Biotechnology, Rockford, USA). This test is based on the conversion of nitrate to nitrite via the action of the nitrate reductase enzyme. The nitrite levels are then detected using the Griess reaction, which absorbs visible light with a wavelength of 540 nm.

The interaction of NO in a given sample was measured by determining both the nitrate and nitrite concentrations in the sample. The samples were ultrafiltered through a 10,000 molecular weight cut-off filter and assayed directly. The nitrite concentrations were determined using the nitrite standard curve. The nitrate concentrations were calculated by subtracting the sample's initial nitrite concentration from the measured nitrite concentration following the enzymatic conversion of nitrate.

Statistical analysis. Analyses were performed using SPSS version 17 and Stata version 11. In all instances, significance was established at $p < 0.05$. Student's *t*-test and the Mann-Whitney test were used to compare parametric and non-parametric parameters. Categorical variables were analyzed using χ^2 or Fisher's exact test as appropriate. Pearson correlation was used for normally distributed variables and Spearman correlation for nonnormally distributed variables, correlation coefficients between variables were tested using the Fisher's Z transformation.

Results

The subjects' characteristics are presented in Table 1. The gestational age, birth weight, sex and maternal age did not differ between the study groups. Low Apgar scores at the 1st and 5th minutes, as well as intrauterine growth restriction and maternal preeclampsia, were observed at high frequencies in the HIE group.

The sICAM-1, sVCAM-1, ET-1 and NO concentrations are listed by study group in Table 2. The concentrations of the endothelial and adhesion system markers on the 1st and the 7th day were higher in infants with HIE than in the control infants, but sICAM-1 levels did not differ significantly from that of the control group on the 1st day. Compared with the levels that were observed on the 1st day, sICAM-1 and sVCAM-1 levels increased significantly by the 7th day of life ($p < 0.05$); however, the ET-1 and NO concentrations tended to decline, and nitrate levels decreased significantly during the first week of life ($p < 0.05$).

Correlation analysis showed that NO was correlated with sICAM-1 ($r= -0.55$; $p < 0.05$), but not with sVCAM-1 and ET-1 ($r= 0.02$ and $r= 0.05$, respectively). Similarly, ET-1 was not correlated with sICAM-1 and sVCAM-1 ($r= -0.02$ and $r= -0.07$, respectively). A statistically significant positive correlation was observed between the sICAM-1 and sVCAM-1 concentrations ($r= 0.43$; $p < 0.05$).

Discussion

We examined the levels of soluble adhesion molecules and endothelial dysfunction markers in the peripheral blood of preterm infants with HIE. Intercellular adhesion molecules are constitutively expressed at low levels on the cell membrane of endothelial cells. Upon cytokine stimulation, which occurs following hypoxia-ischemia, the expression of these molecules increases. There are a number of publications regarding the expression of the ICAM-1 homodimer on the membrane of cerebral endothelial cells in inflammatory conditions. The levels of this dimer are reported to increase following transient cerebral ischemia, reaching peak levels between 12 and 24 h post-injury (15,16). We did not selectively assay for the levels of this homodimer; however, in agreement with these previous studies, we observed an increase in the concentration of the soluble form of adhesion molecules in infants with HIE than in control healthy infants. It is difficult to explain the increase in soluble adhesion molecule concentration as a result of central nervous system pathology. The observed changes in the blood could easily be derived from vascular beds other than those in the central nervous system, particularly given that peripheral blood vessels constitute a much greater proportion of the total vessels than does the cerebral vasculature. Therefore, it is not possible to determine whether direct adhesion system activation occurs as a result of encephalopathy. However, these findings highlight the critical role of leukocyte adhesion in exacerbating inflammatory injuries in infants following cerebral ischemia.

We observed a significant increase in sICAM-1 levels through the end of the early neonatal period in the HIE group, although this parameter level did not differ from that of the control group on the 1st day. However, sVCAM-1 concentrations were higher beginning on the 1st day and remained at high levels until the end of early neonatal adaptation. High levels of sVCAM-1 in HIE infants beginning on the first day of life may indicate the intrauterine secretion of sVCAM-1, potentially resulting from intrauterine hypoxia. However, the role of VCAM-1 in inflammatory injury is not well elucidated. The results of previous studies are questionable. For instance, some researchers reported that the inhibition of VCAM-1 expression and leukocyte adhesion was neuroprotective in a model of transient global cerebral ischemia (17,18).

In contrast, the inhibition of VCAM-1 was not neuroprotective in a focal cerebral ischemia model (19,20). The observed significant increases in the concentration of the soluble form of VCAM-1 beginning in the first hours of life in our study provide a basis for the use of this parameter as a more sensitive and early marker of endothelial inflammation. Changes in sICAM-1 and sVCAM-1 levels during the first week of life indicate that vascular endothelial reactions occur in the early stages of endothelial inflammation, whereas leukocyte adhesion occurs in the later stages.

Nitrate products were detected in high concentrations in infants with HIE; however, these levels decreased significantly through the 7^{th} day of life. Compared with NO, we did not observe a statistically significant change in ET-1 levels during the first week of life; however, the ET-1 concentration was higher in infants with HIE compared to the levels that were observed in the control infants. High ET-1 and NO levels in infants with HIE may be connected with 1) the centralization of circulation and 2) compensation to protect against the effects of ischemia. These data are in agreement with the literature, which supports the role of ET-1 in endothelial dysfunction in infants (21,22). Among recently performed studies, we found a large amount of information regarding the role of ET-1 in pulmonary hypertension and in cardio-respiratory adaptation reactions to both extra-uterine life and post-ischemic endothelin-mediated vasoconstriction (23,24). In the present study, we observed decreased NO and ET-1 production during adhesion system activation in infants with HIE, and significant negative correlation was determined between nitrate and intercellular adhesion molecule synthesis. The conclusions that were reached in the present study require confirmation by more comprehensive research given that there are insufficient data in the literature regarding how changes in endothelial dysfunction markers affects the adhesion system in infants.

Our study has certain limitations. First, we were unable to analyze the concentrations of adhesion molecules, ET-1 and NO relative to the gestational age and intrauterine growth of the infants. This limitation was due to the limited number of infants in the analyzed subgroups. Our study examined peripheral blood parameters in preterm infants, and the primary purpose of the study was to identify relationships between adhesion system activation and endothelial function. To this end, studies of changes in the levels of adhesion molecules and endothelial markers in brain tissue would be useful.

In summary, increased plasma concentrations of sICAM-1, sVCAM-1, ET-1 and NO were observed in hypoxia-affected preterm infants. However, the direction of these changes differed. Moreover, we found that 1) VCAM-1 is a stable indicator of endothelial dysfunction during the early neonatal period and 2) that leukocyte adhesion reactions are activated after the activation of the vascular component of the adhesion system. Increases in sICAM-1 and sVCAM-1 levels were associated with decreases in ET-1 and NO concentrations during the early neonatal period. The activation of either ET-1 or NO is an indicator of severe inflammation and the acute stage of endothelial dysfunction. Therefore, the increase in the levels of adhesion molecules in the context of decreases in ET-1 and NO levels may indicate the positive role of activated leukocyte adhesion in mitigating neuronal disturbances. To confirm these results, further investigations and experimental studies regarding the role of vascular and leukocyte adhesion in endothelial dysfunction will be important.

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Table 1. Neonatal characteristics of the examined infants

	HIE group	Control group
Subjects (n)	112	22
Birth weight (g)	1881.4±40.69	1897.4±94.6
GA (wk)	33.1±0.26	33.6±0.7
Maternal age (yr)	25.46±0.51	24.8±0.9
Apgar score at 1 min	5.09±0.16*	7.2±0.21
Apgar score at 5 min	6.23±0.14*	7.6±0.31
Male	56 (50)	10 (45.45)
Female	56 (50)	12 (54.54)
Intrauterine growth restriction	20 (17.85)*	1 (4.54)
Maternal preeclampsia	30 (26.78)*	1 (4.54)

The data are expressed as the mean ± SEM and as n (% of total).
* $p < 0.05$ compared with control infants.

Table 2. sICAM-1, sVCAM-1, ET-1, and nitrate concentrations in the peripheral blood of infants in both groups

	HIE group (n=112)		Control group (n=22)	
	1st day	7th day	1st day	7th day
sVCAM-1, ng/mL	4146±292.3*	5517±458.9*†	2345±546.4	2458±646.5
sICAM-1, ng/mL	560.4±77.4	869.3±101.1*†	430±90.5	480±100.2
ET-1, pg/ml	5.44±0.47*	4.5±0.39	2.83±0.1	1.89±0.06
Nitrate, µM/L	41.96±4.84*	27.65±4.24*†	18.32±3.2	14.6±2.4

The data are shown as mean ± SEM.
* $p < 0.05$ compared with controls.
† $p < 0.05$ compared with levels on the 1st day.