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Original Article

Cytokine arrays analysis of plasma from acute mountain sickness susceptible and resistant individuals

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Abstract: Extensive studies have been done on mechanisms of acute mountain sickness (AMS), but AMS biomarkers are still little known. In the present study, we used cytokine arrays analysis to identify novel AMS-associated biomarkers in human plasma. Peripheral blood samples from 23 healthy volunteers were collected and AMS symptoms diagnosed using the Lake Louise Questionnaire (LLQ) after exposure to a high altitude of 3800 m. Plasma samples were pooled at two time points, low altitude (LA, altitude of 400 m) and 9-hour high-altitude exposure (HA, altitude of 3800 m). Protein composition of the samples was tested by a cytokine arrays analysis. After 9 hours of hypobaric hypoxia, a total of 30 cytokines were founded differentially expressed due to high-altitude stressor (fold change > 1.5, $P < 0.05$), among which 21 cytokines were up-regulated, and 9 cytokines were down-regulated; a total of 164 differentially expressed cytokines were observed (fold change > 1.5, $P < 0.05$), among which 144 cytokines were up-regulated and 20 cytokines were down-regulated. There are more inflammatory cytokines responding to high altitude in AMS- individuals than AMS+ individuals. Our results suggested that AMS- individuals had more ability to anti-inflammation damage than AMS+ individuals.

Keywords: Cytokines, biomarkers, acute mountain sickness, Chinese

Introduction

Millions of non-altitude acclimatized individuals are exposed to risky environments causing acute mountain sickness (AMS) annually [1], which usually occur within 6-12 h after rapid ascent to high altitudes above 2500 m [2]. Genetic and proteomic studies showed that certain biomarkers might be involved in AMS development. The levels of these biomarkers are different in AMS+ individuals and AMS- individuals [3, 4]. Recent studies suggested that inflammation process and cytokines closely related to AMS [5]. Therefore, it is helpful to explore the AMS-associated cytokines for predicting AMS. Cytokines array analysis can be used to analyze plasma samples from AMS susceptible (AMS+) and AMS resistance (AMS-) individuals to find the AMS-associated cytokines. Our results suggested that AMS- individuals had more ability to anti-inflammation damage than AMS+ individuals.

Materials and methods

Ethics statement

This study has been approved by the Institutional Review Board (IRB) of Lanzhou General Hospital of Lanzhou Military Command (Lanzhou, China). The use of human plasma samples for research purpose was authorized by the IRB of Lanzhou General Hospital of Lanzhou Military Command. All the volunteers agreed to participate in this study with signed consent document.

Subjects

We recruited 23 local Chinese volunteers aged 25 to 35 years. They primarily resided at an elevation of 400 m or lower in the area of Xi'an, China. Recruitment, screening, and exclusion criteria used in this study were as described previously [3, 6]. Briefly, screening procedures to assess general health status and to determine eligibility prior to participation included medical history check, physical and neurologi-

cal exam, standard blood and urine analysis, and a maximal exercise test. We excluded those who 1) had an abnormal complete blood count, chemistry panel, or liver function results; 2) were pregnant or intended to become pregnant within the near future; 3) history of migraine, headache, seizure, or head injury with loss of consciousness; 6) with mechanical limitations or metal implants that prohibited exercise; 6) were smoker; 7) were regular on prescription medications; inability to reach a workload of least 200 W during the incremental exercise test; 8) or altitude exposure greater than 2500 m within three months of study.

Study design and ascent profile

Peripheral venous blood samples were collected and AMS symptoms were evaluated at low altitude (400 m; Xi'an, China) 24 hours in advance. To acutely expose volunteers to high altitude, all subjects were transported to the altitude of 3800 m (Yushu, China) from Xi'an (400 m) in 3 hours by flight. AMS symptoms of subjects were evaluated immediately when landed. This is time point 0 h. The following studies were conducted in Yushu Bayi Hospital, which has a phase I clinical trial qualification and can treat high-altitude illness. Two hours after exposure to the altitude of 3800 m, all subjects completed three 5-minute sub-maximal exercise bouts on a cycle with 15 minutes rest in between in the ward. This is to increase the likelihood of developing AMS [7]. The whole process was monitored by one doctor and two nurses.

Evaluation of AMS status

To evaluate AMS status, subjects completed self-reported sections of the Lake Louise Questionnaire (LLQ) prior to exposure to the 3800 m high altitude (BL and 0 hour) as well as after 3, 6, 9 and 12 hours of hypobaric hypoxia. Subjects were asked to quantify their degree of headache, gastrointestinal upset, fatigue, and dizziness on a four-point scale (no symptoms = 0, mild = 1, moderate = 2, severe = 3). Subjects with a cumulative LLQ score equal or greater than 3 with headache after ascent to high altitude within 6 to 12 hours were defined as AMS+ individuals. Those with the sum of LLQ score equal or less than 2 or without headache after exposure to hypobaric hypoxia were considered as AMS- individuals [8].

Plasma collection and sample preparation

Plasma collection and sample preparation was performed as described previously [3]. Blood samples were collected in a semi-recumbent position from an antecubital vein by an indwelling intravenous cannula, and then placed in EDTA-coated blood collection tubes. The plasma was separated from blood cells by centrifugation and stored in 0.2 mL aliquots at -80°C until analysis.

Cytokine array analysis of patient serum

The set of 23 subjects was ranked according to the LLQ scores after exposure to 3800 m in 6 to 12 hours. The top 7 individuals with the highest LLQ scores were defined as the subset of AMS- group. The bottom 7 individuals with the lowest LLQ scores were regarded as the AMS+ group. Plasma samples collected from the 7 AMS+ subjects and 7 AMS- subjects were pooled at each time point respectively. This resulted in four pooled samples for analysis (two at low altitude for AMS+ and AMS- groups, named AMS+LA and AMS-LA separately; two at high altitude for AMS+ and AMS- groups, named AMS+HA and AMS-LA separately). The solution was afterwards centrifuged at 3000 g at 4°C, and an aliquot of the supernatant was taken for determination of protein concentration by Bradford protein assay method [9, 10]. The remaining supernatant was kept at -80°C for further analysis.

These four groups plasma samples (AMS+ LA, AMS+HA, AMS-LA, AMS-HA) from subjects were analyzed using a human cytokine antibody array (RayBio®, Wayen Biotechnologies, Shanghai, China) which including 508 cytokines, according to the manufacturer's instructions. Briefly, loading each 100 μl sample (20 μl plasma plus 80 μl 1×PBS, Ph = 8) into a separate dialysis tube. Place beaker on a stir plate and dialyze, stirring buffer gently for at least 3 hours at 4°C. Then exchange the 1× PBS buffer and repeat dialysis for at least 3 hours at 4°C. Dialyzed samples were labeled by biotin and prepared to be analyzed by cytokine array. Blocking buffer (400 μl per well) was added into each well of the microarray glass slides for 30 min. Samples (400 μl per well) were added and incubated overnight at 4°C. The slides were washed in washing buffer and incubated with a diluted Streptavidin-Fluor

Table 1. Changed levels of cytokines in AMS+ group pooled plasma sample and in AMS- group pooled plasma sample after short-term high altitude exposure

Cytokines	AMS+HA/ AMS+LA	AMS-HA/ AMS-LA
6 Ckine		2.72
Activin C		3.12
Activin RIB/ALK-4		2.52
Activin RII A/B		3.28
AgRP	38.50	
Angiopoietin-4		2.47
Angiopoietin-like 2		2.24
AR (Amphiregulin)		3.60
Artemin		2.91
Axl		2.18
BLC/BCA-1/CXCL13		2.96
BMP-5		3.36
BMP-6		2.76
BMP-7		2.40
BTC		4.14
Cardiotrophin-1/CT-1		2.23
CCL28/VIC		4.03
CCR5		2.93
Cerberus 1		2.71
CLC		0.50
CRIM 1		3.46
Cripto-1		5.25
Cryptic		3.78
CTGF/CCN2		3.54
CXCR3		2.66
CXCR5/BLR-1	2.02	3.33
CXCR6		2.11
D6		2.03
DAN		2.87
DANCE		4.15
Decorin		3.17
Dkk-3		2.22
DR3/TNFRSF25	0.21	
Dtk		2.36
EDA-A2		3.63
EDAR		2.13
EGF		60.22
EMAP-II		2.07
ENA-78		6.07
Endoglin/CD105		3.98
Endostatin		2.12
Endothelin		2.22
ErbB4		2.36
E-Selectin		3.53
FADD		2.40

at room temperature for 2 h. Fluorescent signal was detected using a array scanner (Gene Pix 4000B, Axon Instruments, USA) and analyzed using the GenePixPro6.0 (Axon Inxtruments, USA).

Statistics

Data are presented as 'mean ± standard deviations' and analyzed using the 'Student's t test' where indicated.

Results

Characteristics of subjects

Age (28.0 ± 3.3 years *vs.* 27.4 ± 1.5 years, P = 0.682), weight (69.0 ± 12.5 kg *vs.* 61.4 ± 13.2 kg, P = 0.291), height (170.3 ± 7.8 cm *vs.* 167.4 ± 9.0 cm, P = 0.536) and body mass index (BMI) (23.6 ± 2.7 kg/m^2 *vs.* 21.7 ± 2.3 kg/m^2, P = 0.168) were similar between AMS- and AMS+ groups. LLQ scores were lower in the AMS- group than the AMS+ group at 6 hours (1.00 ± 0.82 *vs.* 3.00 ± 1.00, P = 0.0015 < 0.05), 9 hours (0.43 ± 0.53 *vs.*6.29 ± 1.80, P = 2.72×10^{-6} < 0.05) and 12 hours (1.14 ± 1.22 *vs.* 4.29 ± 2.06, P = 0.0046 < 0.05) of exposure to the altitude of 3800 m.

AMS+ group global cytokine responses to acute high-altitude exposure

To investigate the global cytokine changes in plasma in AMS+ subjects after acute exposure to high altitude, cytokine arrays analysis was used to identify cytokine profiles of plasma samples of AMS+LA and AMS+HA groups. A total of 30 cytokines were founded differentially expressed due to high-altitude stressor (fold change > 1.5, P < 0.05), among which 21 cytokines were up-regulated, and 9 cytokines were down-regulated (**Table 1**). In the sections of discussion, we will discuss the potential relevance of these findings for human response to hypobaric hypoxia and AMS.

AMS- group global cytokine responses to acute high-altitude exposure

To probe the effects of short-term high altitude (3800 m) exposure on cytokine level changes in the plasma of AMS- individuals,

FAM3B		2.74
FGF R5		2.51
FGF-11		2.34
FGF-12		2.97
FGF-20		2.11
FGF-21		5.02
FGF-4		3.65
FGF-5		4.16
FGF-6		2.58
FGF-7/KGF		3.05
FGF-8	2.54	0.33
FGF-BP		5.22
FLRG		2.28
Follistatin	0.50	
GASP-2/WFIKKN		2.02
GCP-2/CXCL6		2.08
G-CSF R/CD 114		3.37
GDF11		2.56
GDF9		3.36
GFR alpha-4		2.76
Glut1		2.30
Glut3	0.49	
GM-CSF		3.07
GRO		3.19
GRO-a		3.01
Growth Hormone R (GHR)		0.36
HB-EGF	0.44	0.31
HCC-4/CCL16	27.83	3.21
HCR/CRAM-A/B		2.70
IFN-beta		2.03
IFN-gamma R1		2.46
IGFBP-1		3.06
IGFBP-2		2.27
IGFBP-6	0.39	
IGF-I SR		2.55
IGF-II		2.21
IL-1 beta		2.95
IL-1 F10/IL-1HY2		2.07
IL-1 sRII		2.76
IL-1 R5		3.05
IL-2 R gamma		3.51
IL-3		4.83
IL-3 R alpha		2.86
IL-5 R alpha		2.10
IL-6		2.99
IL-6 R		2.07
IL-9	0.45	
IP-10		2.23
IL-10 R beta	2.08	

we used cytokine arrays analysis to identify protein profiles of AMS+LA and AMS+HA groups. A total of 164 differentially expressed cytokines were observed (fold change > 1.5, $P < 0.05$), among which 144 cytokines were up-regulated and 20 cytokines were down-regulated (**Table 1**). The inflammatory factors were dramatically increased in AMS- group after short time hypoxia exposure. In the part of discussion, we will discuss the potential relevance of these results for human response to high altitude and AMS in details.

Discussion

Acclimation of human to hypoxia is required in some physiological or pathological conditions. Identifying the cytokines involved in physiological and pathological processes associated with hypoxia would shed light on the mechanisms of AMS. One recent study of AMS in a hypoxic chamber showed that AMS closely related to inflammatory process [5]. Our present plasma cytokines array study in plateau of the Han Chinese volunteers gives new data to address the potential AMS correlative cytokines. In the present study, we test the plasma cytokines of AMS+ and AMS- individuals in low altitude and short-term (9 hours) high-altitude exposure status. We found that cytokines response to acute hypobaric hypoxic exposure in AMS+ individuals and AMS- individuals were different using cytokines array analysis. These different cytokines may help to address the pathology of AMS.

Pathophysiology of AMS

Despite the fact that many theories explaining the development of AMS have been proposed during the past decades, the basic pathogenic mechanism of AMS is still fairly unclear. The blood brain barrier (BBB) theory, one of those hypotheses, suggests that hypoxia-induced hypoxemia will initiate an inflammatory response with the release of inflammatory mediators that contribute to an increase of the capillary pressure by over perfusion and vasodilatation, and elevate the permeability of the BBB by disrupting the BBB. This increases the potential for capillary

IL-12 p40		2.38
IL-12 p70		3.38
IL-13		2.30
IL-13 R alpha 1		2.56
IL-13 R alpha 2		2.91
IL-17B		3.21
IL-17R		2.26
IL-17RD		0.41
IL-20 R alpha		4.02
IL-22 BP		2.17
IL-24		2.84
IL-28A	2.19	5.76
IL-31		2.32
Kininostatin/kininogen	2.87	
Kremen-1		2.72
Lipocalin-2		0.35
L-Selectin (CD62L)		3.01
Lymphotactin/XCL1		2.03
MCP-3		2.53
MDC		2.30
MICA		0.42
MIP-1b	2.21	2.60
MIP-3 alpha		0.44
MMP-3		34.58
MMP-7	3.02	2.43
MMP-9	2.39	
MMP-10		2.18
MMP-12		5.01
MMP-15		2.85
MMP-19		2.22
MMP-25	0.31	5.26
NCAM-1/CD56		0.28
Nidgen-1		2.17
NOV/CCN3		2.12
NT-4		0.34
PARC/CCL18		2.07
PDGF R beta		2.44
PDGF-AA		0.48
PDGF-C	2.27	2.76
PDGF-D		0.49
Persephin		0.49
PlGF		3.19
Prolactin		3.20
P-selectin	37.09	
S100A10	3.15	
SCF	3.15	
SCF R/CD117		2.23
Siglec-5/CD170		3.27
Smad 1	2.07	0.40

leak and cerebral edema, which in turn causes the traction of the meninges and blood vessels, and high-altitude headache [11-13]. According to present evidences, inflammation response may play important roles in developing AMS. In our present study, we found that ILs of AMS- individuals significantly increased after stimulated by hypobaric hypoxia, but not in AMS+ individuals. In contrast to AMS+ individuals, inflammatory cytokines IL-1β, IL-1 F10, IL-1 sRII, IL-1 R5, IL-2 Rγ, IL-3, IL-3 Rα, IL-5 Rα, IL-6, IL-6 R, IL-12 p40, IL-12 p70, IL-13, IL-13 Rα1, IL-13 Rα2, IL-17B, IL-17R, IL-20 Rα, IL-22 BP, IL-24, and IL-31 were induced by short-term hypoxic stressor in AMS- individuals. This suggested that AMS- individuals might have more ability to response to hypoxia via inflammatory process than AMS+ individuals.

Immune system response to high altitude

The human immune system is sensitive to several of extrinsic factors including high altitude environmental stress. Cytokines involve in cell-to-cell signaling, which help regulate the intensity and duration of the immune response. Recently, more and more studies have focused on the influence of hypoxic on inflammatory cytokines, especially IL-6. In our present study, it appears that the environmental stress of altitude is sufficient to induce an elevation in plasma IL-6 and IL-6R levels in AMS- individuals, but not in AMS+ individuals. Further in-depth studies are needed to confirm functions of IL-6 in developing AMS.

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs)

After acute exposure to hypobaric hypoxia, amount of MMP-3, MMP-7, MMP-10, MMP-12, MMP-15, MMP-19, and MMP-25 in plasma significantly increased in AMS- individuals. In contrast, only MMP-7 and MMP-9 increased and MMP-25 decreased in AMS+ individuals. TIMP-1 and TIMP-3 were up-regulated and TIMP-2 was down-regulated after short-term high altitude exposure in AMS- individuals. In contrast, only TIMP-1 was test decline in AMS+ indi-

Smad 4		2.01
Soggy-1		2.08
Sonic Hedgehog (Shh N-terminal)		2.75
SPARC		0.19
Tarc		3.30
TECK/CCL25		2.72
TFPI		0.37
TGF-beta RII		2.56
TGF-beta RIII	3.25	
Thrombospondin-1		0.15
Thrombospondin-4	0.05	29.71
Thymopoietin		2.20
Tie-1		2.86
Tie-2		2.17
TIMP-1	0.04	3.12
TIMP-2		0.26
TIMP-3		2.82
TL1A/TNFSF15	15.31	12.17
TLR3	5.10	
TLR4		17.04
TNF-alpha		3.38
TNFRF18		2.71
TNFRSF10A		2.04
TNFRSF10D		7.30
TNFRSF11A	14.38	
TNFRSF11B		2.51
TNFRSF3	3.94	
TNFRSF6B		3.39
TNFRSF8		3.99
TNFSF10		35.06
TNFSF15		12.17
TNFSF4		2.49
TNFSF5		0.48
TSG-6		31.65
VCAM-1 (CD106)		27.27
VEGF	2.55	
VEGF-B		2.14
XEDAR		0.49

viduals. The MMPs are inhibited by specific endogenous tissue inhibitor of metalloproteinases (TIMPs). Dis-regulation of the balance between MMPs and TIMPs is also a characteristic of acute and chronic cardiovascular diseases. However, functions of MMPs and TIMPs in the development of AMS are still little known.

VEGF

It was noting that the vascular endothelial growth factor (VEGF) was induced in the AMS+ group after acute hypoxic exposure, but not in the AMS- group. Many studies have showed that VEGF seems likely to be a biomarker of AMS. VEGF is a critical angiogenic factor and hypoxia-induced protein [14, 15] that can increase vascular permeability [16, 17]. Blocking VEGF can prevent hypoxic brain edema [18]. In the present study, we observed that VEGF concentration in plasma increased 2.55 times after exposure to hypoxia than low altitude in AMS- individuals, but not in AMS- individuals. In AMS- individuals, we found that VEGF-B and PlGF increased after exposure to high altitude, while the relationship these two cytokines and AMS were not detected. Coupled with previous studies, we assume that VEGF may play an important role in developing AMS.

Study limitations

Here, we should address some limitations of our present study. First of all, the sample is from the peripheral blood. As mentioned in the recent study, plasma may not accurately represent the cerebral environment, but peripherally circulating proteins affect cerebral endothelial permeability [19], suggesting cytokines in plasma associated with AMS. In the present study, we looked for AMS biomarkers using the peripheral blood sample for the safety of the volunteers. Secondly, we analyzed sample pooling by cytokines array analysis. Performing proteomic studies on pooled samples compared with individual samples is supported by evidence that sample pooling reduces biologic variance between specimens in proteomic and microarray analyses [20-23] and, consequently, facilitates the identification of the most robust differences between groups [20, 24]. A comprehensive evaluation of the effect of sample pooling for proteomic analysis demonstrated that, for the majority of proteins, data obtained from pooled samples accurately represented the mean protein levels of individual samples that composed the pool rather than being skewed by one or two samples [20]. However, the samples pooling analysis methods effect on the results need further in-depth studies. Finally, although our work has provided some important clues, we do not make a further verification of our cytokines

array analysis results on animals or cells model. This primary reason is the subjective symptoms of AMS. Coupled with the elusive mechanisms of AMS, especially of high altitude headache, we cannot model the AMS in animals. To further test the cytokines array analysis results in human we need huge sample but face ethical difficulties.

Conclusions

Our present study found that acute exposure to high altitude dramatically affected inflammatory cytokines in AMS- individuals, but not AMS+ individuals. This suggested that AMS- individuals had more ability to anti-inflammation damage than AMS+ individuals. Coupled with our cytokine arrays results and prior evidence, it is hypothesized that increased inflammation is pathological and predominantly related to developing AMS.

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Disclosure of conflict of interest

None.

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